Biochemical processes for Balsamic-styled vinegar engineering

by

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DECLARATION

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

Signed **Date** **23 December 2019**

ABSTRACT

The South African wine industry is constantly facing several challenges which affect the quality of wine, the local/global demand and consequently the revenue generated. These challenges include the ongoing drought, bush fires, climate change and several liquor amendment bills aimed at reducing alcohol consumption and alcohol outlets in South Africa. It is therefore critical for the wine industry to expand and find alternative ways in which sub-standard or surplus wine grapes can be used to prevent income losses and increase employment opportunities. Traditional Balsamic Vinegar (TBV) is a geographically and legislative protected product produced only in a small region in Italy. However, the methodology can be used to produce similar vinegars in other regions. Balsamic-styled vinegar (BSV), as defined in this thesis, is a vinegar produced by partially following the methods of TBV while applying process augmentation techniques. Balsamic-styled vinegar is proposed to be a suitable product of sub-standard quality or surplus wine grapes in South Africa. However, the production of BSV necessitates the use of cooked (high sugar) grape must which is a less favourable environment to the microorganisms used during fermentation. Factors that negatively affect the survival of the microorganisms include low water activity due to the cooking, high osmotic pressure and high acidity. To counteract these effects, methods to improve the survival of the non-*Saccharomyces* yeasts and acetic acid bacteria used are essential.

The primary aim of this study was to investigate several BSV process augmentation techniques such as, aeration, agitation, cell immobilization, immobilized cell reusability and oxygen mass transfer kinetics in order to improve the performance of the microbial consortium used during BSV production.

The work for this study was divided into four (4) phases. For all the phases a microbial consortium consisting of non-*Saccharomyces* yeasts (n=5) and acetic acid bacteria (n=5) was used. Inoculation of the yeast and bacteria occurred simultaneously. The 1st phase of the study entailed evaluating the effect of cells immobilized by gel entrapment in Ca-alginate beads alongside with free-floating cells (FFC) during the production of BSV. Two Ca-alginate bead sizes were tested i.e. small (4.5 mm) and large (8.5 mm) beads to evaluate the effects of surface area or bead size on the overall acetification rates. Ca-alginate beads and FFC fermentations were also evaluated under static and agitated (135 rpm) conditions. The 2nd phase of the study involved studying the cell adsorption technique for cell immobilization which was carried-out using corncobs (CC) and oak wood chips (OWC), while comparing to FFC fermentations. At this phase of the study, other vinegar bioreactor parameters such as agitation and aeration were studied in contrast to static fermentations. One agitation setting (135 rpm) and two aeration settings were tested i.e. high (0.3 vvm min^{-1}) and low (0.15 vvm min^{-1}) aeration conditions. Furthermore, to assess the variations in cell adsorption capabilities among individual yeast

and AAB cells, the quantification of cells adsorbed on CC and OWC prior- and post-fermentation was conducted using the dry cell weight method.

The 3rd phase of the study entailed evaluating the reusability abilities of all the matrices (small Ca-alginate beads, CC and OWC) for successive fermentations. The immobilized cells were evaluated for reusability on two cycles of fermentation under static conditions. Furthermore, the matrices used for cell immobilization were further analysed for structure integrity by scanning electron microscopy (SEM) before and after the 1st cycle of fermentations. The 3rd phase of the study also involved the sensorial (aroma and taste) evaluations of the BSV's obtained from the 1st cycle of fermentation in order to understand the sensorial effects of the Ca-alginate beads, CC and OWC on the final BSV. The 4th phase of the study investigated oxygen mass transfer kinetics during non-aerated and aerated BSV fermentation. The dynamic method was used to generate several dissolved oxygen profiles at different stages of the fermentation. Consequently, the data obtained from the dynamic method was used to compute several oxygen mass transfer parameters, these include oxygen uptake rate (r_{O_2}), the stoichiometric coefficient of oxygen consumption vs acid yield ($Y_{O/A}$), the oxygen transfer rate (N_{O_2}), and the volumetric mass transfer coefficients ($K_L a$). During all the phases of the study samples were extracted on weekly intervals to evaluate pH, sugar, salinity, alcohol and total acidity using several analytical instruments. The 4th phase of the study involved additional analytical tools, i.e. an oxygen µsensor to evaluate dissolved oxygen and the 'Speedy breedy' to measure the respiratory activity of the microbial consortium used during fermentation.

The data obtained from the 1st phase of the study demonstrated that smaller Ca-alginate beads resulted in higher (4.0 g L^{-1} day^{-1}) acetification rates compared to larger (3.0 g L^{-1} day^{-1}) beads, while freely suspended cells resulted in the lowest (0.6 g L^{-1} day^{-1}) acetification rates. The results showed that the surface area of the beads had a substantial impact on the acetification rates when gel entrapped cells were used for BSV fermentation. The 2nd phase results showed high acetification rates (2.7 g L^{-1} day^{-1}) for cells immobilized on CC in contrast to cells immobilized on OWC and FFC, which resulted in similar and lower acetification rates. Agitated fermentations were unsuccessful for all the treatments (CC, OWC and FFC) studied. Agitation was therefore assumed to have promoted cell shear stress causing insufficient acetification during fermentations. Low aerated fermentations resulted in better acetification rates between 1.45–1.56 g L^{-1} day^{-1} for CC, OWC and FFC. At a higher aeration setting, only free-floating cells were able to complete fermentations with an acetification rate of 1.2 g L^{-1} day^{-1}. Furthermore, the adsorption competence data showed successful adsorption on CC and OWC for both yeasts and AAB with variations in adsorption efficiencies, whereby OWC displayed a lower cell adsorption capability compared to CC. On the other hand, OWC were less efficient adsorbents due to their smooth surface, while the rough surface and porosity of CC led to improved adsorption and, therefore, enhanced acetification rates.

The 3rd phase results showed a substantial decline in acetification rates on the 2nd cycle of fermentations when cells immobilized on CC and OWC were reused. While cells entrapped in Ca-alginate beads were able to complete the 2nd cycle of fermentations at reduced acetification rates compared to the 1st cycle of fermentations. The sensory results showed positive ratings for BSV's produced using cells immobilized in Ca-alginate beads and CC. However, BSV's produced using OWC treatments were neither 'liked nor disliked' by the judges. The SEM imaging results further showed a substantial loss of structural integrity for Ca-alginate beads after the 1st cycle fermentations, with minor changes in structural integrity of CC being observed after the 1st cycle fermentations. OWC displayed the same morphological structure before and after the 1st cycle fermentations which was attributed to their robustness. Although Ca-alginate beads showed a loss in structural integrity, it was still assumed that Ca-alginate beads provided better protection against the harsh environmental conditions in contrast to CC and OWC adsorbents due to the acetification rates obtained on both cycles. The 4th phase data obtained from the computations showed that non-aerated fermentations had a higher $Y_{O/A}$, r_{O_2}, N_{O_2} and a higher $K_L a$. It was clear that aerated fermentations had a lower aeration capacity due to an inappropriate aeration system design and an inappropriate fermentor. Consequently, aeration led to several detrimental biochemical changes in the fermentation medium thus affecting $K_L a$ and several oxygen mass transfer parameters which serve as a driving force.

Overall, it was concluded that the best method for BSV production is the use of cells entrapped in small alginate beads or cells adsorbed on CC under static and non-aerated fermentations. This conclusion was based on several factors such as cell affinity/cell protection, acetification rates, fermentation period and sensorial contributions. However, cells entrapped in Ca-alginate beads had the highest acetification rates. The oxygen mass transfer computations demonstrated a high $K_L a$ when Ca-alginate beads were used under static-non-aerated conditions compared to fermentations treated with CC. Therefore, a fermentor with a high aeration capacity needs to be designed to best suit the two BSV production systems (Ca-alginate beads and CC). It is also crucial to develop methods which can increase the robustness of Ca-alginate beads in order to improve cell retention and reduce the loss of structural integrity for subsequent cycles of fermentation. Studies to define parameters used for upscaling the BSV production process for large scale productions are also crucial.

Keywords: Aeration, Acetification rates, Adsorbed cells, Agitation, Balsamic styled vinegar (BSV), Ca-alginate beads, Cell immobilization, Corncobs (CC), Entrapped cells, Oak Wood Chips (OWC), Oxygen mass transfer.

DEDICATION

I dedicate this thesis to my parents who have in many ways defined what it means to love, to be patient and to be supportive.

“Pain is temporary. Eventually it will subside. If I quit, however, the surrender stays with me.”

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It is with immense gratitude that I acknowledge the support of the following institutions and people for their contribution towards the completion of my Doctor of Engineering degree:

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U.F Hutchinson

December. 2019

RESEARCH OUTPUTS

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

Published

Hutchinson, U.F.; Ntwampe, S.K.O.; Ngongang, M.M.; Du Plessis, H.W.; Chidi, B.S.; Saulse, C.; and Jolly, N.P, 2018. Cell immobilization by Gel Entrapment in Ca-alginate Beads for Balsamic-styled Vinegar Production. In Proceedings of the 10th International Conference on Advances in Science, Engineering, Technology & Healthcare (ASETH-18), Cape Town, South Africa, 19–20 November 2018. ISBN - 978-81-938365-2-1, https://doi.org/10.17758/EARES4.EAP1118214

Hutchinson, U.F., Jolly, N.P., Chidi, B.S., Ngongang, M.M. and Ntwampe, S.K.O., 2019. Vinegar Engineering: a Bioprocess Perspective. *Food Engineering Reviews*, pp.1-16. (Impact Factor: 4.217)

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Drafted for publication

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Hutchinson, U.F., Jolly, N.P., Chidi, B.S., Mewa Ngongang, M., du Plessis, H.W. and Ntwampe, S.K.O., 2020. Oxygen mass transfer kinetics during balsamic styled vinegar production.

LAYOUT OF THESIS

The research work presented in this thesis was conducted at the Agricultural Research Council, Infruitec-Nietvoorbij (Fruit, Wine and Vine Institute) Stellenbosch, Western Cape, South Africa. The thesis is presented as a compilation of 9 (nine) chapters, with each chapter being introduced separately. The CPUT Harvard method of referencing was used and all references are listed at the end of the thesis.

The thesis chapters are as follows:

- **Chapter 1**: This chapter covers the general introduction, which includes the background of the research topic, the problem statement, the motivation for conducting the study, the hypothesis, research questions and the delineation of the study.
- **Chapter 2**: This chapter is a review of biochemical vinegar engineering methods that can be applied in several vinegar production systems. This chapter also highlights the gaps in vinegar engineering applications.
- **Chapter 3**: This chapter reports on the materials and experimental methods which were employed in order to achieve the objectives of this study.
- **Chapter 4**: This chapter focuses on the effects of using cells immobilized by the entrapment technique in Ca-alginate beads for BSV production.
- **Chapter 5**: This chapter reports on the effects of using the adsorption technique for BSV production by using corncobs and oak wood chips. This chapter also covers the effects of agitation and aeration during BSV production.
- **Chapter 6**: This chapter reports on the reusability capabilities of cells immobilized using Ca-alginate beads, corncobs and oak wood chips for successive cycles of fermentation. The effects of the cell immobilization matrices on the sensorial attributes of the BSV are also given
- **Chapter 7**: This chapter is centred around the oxygen mass transfer kinetics during BSV production.
- **Chapter 8**: An overall summary of the study and future research recommendations are found in this chapter.
- **Chapter 9**: This chapter provides a list of all literature consulted in this study.

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$$r_s = \frac{dS}{dt} \quad (3.1)$$

$$r_p = \frac{dP}{dt} \quad (3.2)$$

$$\text{Absolute difference} = \text{Amount of increase} - \text{Reference amount} \quad (3.3)$$

$$\text{Relative difference} = \frac{\text{Absolute difference}}{\text{Reference amount}} \times 100 \quad (3.4)$$

$$C_6H_{12}O_6 \quad \rightarrow \quad C_2H_5OH \quad \rightarrow \quad CH_3COOH \quad (7.1)$$

$$C_6H_{12}O_6 \quad \rightarrow \quad C_2H_5OH + 2\,CO_2 \quad (7.2)$$

$$C_2H_5OH + O_2 \quad \rightarrow \quad CH_3COOH + 2H_2O \quad (7.3)$$

$$r_{O_2} = \frac{dC_L}{dt} \quad (7.4)$$

$$Y_{O/A} = \frac{r_{O_2}}{r_{acid}} \quad (7.5)$$

$$r_{acid} = \frac{d_{[acid]}}{dt} \quad (7.6)$$

$$\text{OTR} = K_L a(C^* - C_L) \quad (7.7)$$

$$\text{OTR} = \frac{dC_L}{dt} = K_L a\,.(C^* - C) \quad (7.8)$$

$$\text{OUR} = K_L a(C^* - C_L) \quad (7.9)$$

$$K_L a = \frac{OUR}{C^* - C_L} \quad (7.10)$$

$$\frac{\boldsymbol{dC_L}}{\boldsymbol{dt}} = K_L a\,.(C^* - \boldsymbol{C_L}) - q_{O_2}\,.C_X \quad (7.11)$$

GLOSSARY

Abbreviations/Symbols	Definition (units)
°C	Degree Celsius
°B	Degree brix
A	Area (cm^2)
AAB	Acetic Acid Bacteria
AcOH	Acetic acid
ARC	Agricultural Research Council
Atm	Standard atmosphere
BSV	Balsamic-Styled Vinegar
CC	Corncobs
Ca	Calcium
C_L	Dissolved oxygen in liquids ($g\ L^{-1}$)
Cm	Centimetre
C^*	Dissolved oxygen in gaseous phase ($g\ L^{-1}\ h^{-1}$)
D	Resulting diameter (cm)
EtOH	Ethanol
G	Grams
H	Height (cm)
Hr(s)	Hour(s)
HA	High aeration
IC	Immobilized cells
$K_L a$	Volumetric mass transfer coefficient
L	Litre
LA	Low aeration
Min	Minutes
mL	Millilitres
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
Nd	No date
OTR	Oxygen transfer rate ($g\ L^{-1}\ h^{-1}$)
OUR	Oxygen uptake rate ($g\ L^{-1}\ h^{-1}$)
OWC	Oak wood chips
Po_2	Oxygen partial pressure

r_{acid}	Rate of acetic acid formation (g L^{-1} h^{-1})
r_{O_2}	Rate of oxygen consumption (g L^{-1} h^{-1})
r_{sugar}	Rate of sugar consumption (g L^{-1} h^{-1})
R	Radius (cm)
Rpm	Revolutions per minute (rev/min)
r_p	Product formation (g L^{-1} day^{-1})
r_s	Substrate utilisation rate (g L^{-1} day^{-1})
SAWIS	South African Wine Industry Information and Systems
SEM	Scanning Electron Microscope
T	Time
T	Temperature (ºC)
TBV	Traditional Balsamic Vinegar
V	Volume
VVM	Vessel Volume per Minute
W	Weight (g)
YPD	Yeast/Peptone/Dextrose
$Y_{O/A}$	ratio of oxygen consumption vs acid yield

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

1.1 Introduction

South Africa is currently cultivating approximately 93 021 hectares of land for the production of wine grapes, with Chenin blanc being the most cultivated grape cultivar (Fig. 1.1) (SAWIS, 2018). Roughly 81% of the harvested wine grapes are exclusively used for wine production by the South African wine industries (SAWIS, 2018), consequently, these wine grapes are expected to meet strict quality standards required for wine production. However, the South African wine industry faces several challenges such as drought, bush fires (Basson, 2016) and climate change (Vink et al., 2009) which are significantly reducing the quality of wines produced and lowering the revenue. Bush fires not only damage property and vineyards, but the smoke affects vineyards in close proximity during the fire leading to an off odour in the resultant wine known as 'smoke taint'- a major concern for wine producers (Marston, 2016; De Vries et al., 2016). Climate change with the accompanying higher temperatures can lead to high sugar concentration in wine grapes thus causing high ethanol content in the wine (Anonymous, nd(a)). Higher ethanol content is a negative attribute in wines, and it is not desired by consumers. Furthermore, several anti-alcohol regulations are currently being considered to reduce alcohol consumption due to the high level of alcohol abuse in South Africa. These include the liquor amendment bill, which intends to increase the legal drinking age from 18 to 21, and other legislations aimed at reducing the number of alcohol outlets (Andersen, 2018; Anonymous, 2018). Accordingly, wine producers might have to seek alternative revenue generating outlets for the sub-standard quality wine grapes and surplus grapes. Therefore, the production of Balsamic-styled vinegar (BSV) has been deemed fit for this purpose. This could also be an opportunity to empower small business entrepreneurs (less expensive equipment is needed to produce vinegar than wine) while improving the economy of the country.

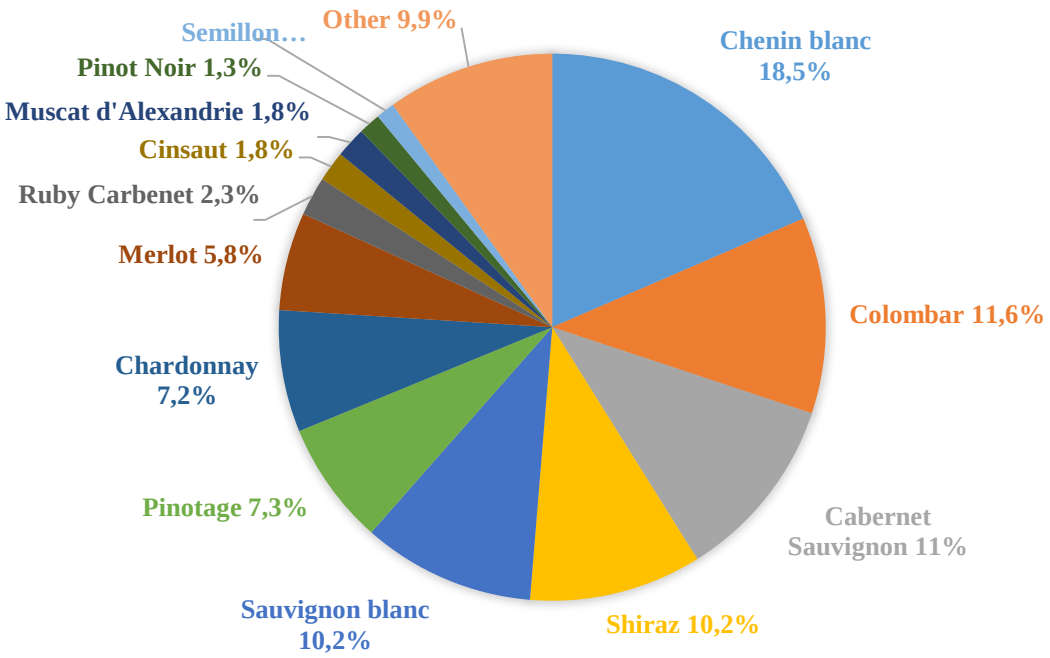

Figure 1.1 Percentage (%) of white and red wine grape vineyards in South Africa (SAWIS, 2018)

According to the Italian *Denominazione di Origine Protetta* (DOP) and the European Union's Protected Designation of Origin legislations, TBV is a geographically and legislative protected vinegar product which can only be produced in delineated areas, namely, the provinces of Reggio Emilia and Modena, Italy (Mattia, 2004; Solieri et al., 2006; Wheeler, 2014). This type of vinegar can be produced in other regions, but not called TBV, or Balsamic Vinegar of Modena. Consequently, the name Balsamic-styled vinegar (BSV) was proposed for a type of balsamic vinegar developed for the purpose of this study which is produced by partially following the methods of TBV.

The production of most types of traditional balsamic vinegars generally follows a simple approach without the necessity for high technology equipment. Briefly, the process commonly begins with the cooking of grape must until a high sugar concentration is achieved. Subsequent inoculation is often performed with an undefined culture of yeast and acetic acid bacteria (mother culture from a previous fermentation). A double fermentation process occurs, which begins with the conversion of sugar to alcohol by yeasts followed by the oxidation of alcohol to acetic acid by AAB (Oulton & Randal. 2002; Wheeler, 2014; Giudici et al., 2015). After fermentation is complete, the TBV is matured for a minimum of 12 years in a series of wooden barrels (Solieri et al., 2006; Giudici, & Rinaldi, 2007; Solieri & Giudici, 2008; Chinnici et al., 2016). Other balsamic vinegars e.g. Balsamic Vinegar of Modena can be sold after shorter periods of ageing, but these are manufactured by combining wine vinegar, cooked grape must and caramel (for colouring) (Wheeler, 2014; Giudici et al., 2015; May, 2016). All balsamic vinegars are comparatively high priced in contrast to other vinegars due to their quality, complexity and integrity (Wheeler, 2014; May, 2016).

A previous study (Hutchinson et al., 2019a), showed that the fermentation process for BSV was slower than what was anticipated leading to increased production times. Longer production times leads to unwanted increases in production costs, hence it was crucial to formulate an effective method for rapid fermentations. Consequently, this study was aimed at investigating several techniques to enhance the vinegar fermentation process with prospects of upscaling.

1.2 Problem statement.

Balsamic-styled vinegar is a new type of balsamic vinegar produced using white grape cultivars and could potentially penetrate the South African food markets. Previous studies showed that the BSV fermentation process was lengthy (>40 days) when cooked Chenin blanc grape must was used as the base material. The slow fermentation rates were attributed to the fastidious nature of AAB which require favourable environmental conditions for optimum microbial activity to be achieved. However, the problem in the present study is that authentic balsamic vinegar essentially necessitates the use of cooked grape must as a fermentation medium. Cooked grape must is associated with several environmental obstacles for the microorganisms used, which include high sugar concentration due to the cooking, accordingly accompanied by high osmotic pressure, low water activity and moderately high salinity. Furthermore, during BSV fermentation, the increase in acetic acid concentrations and decrease in pH also has antagonistic effects on the microbial consortium used. All these factors affect the microbial growth/activity, microbial survival, dissolved oxygen (DO) availability and could easily render the microbial consortium non-viable or could result in the entry of the microorganisms in the viable but non-culturable (VBNC) state. Consequently, it was necessary to employ several bioreactor modifications for BSV production, as a result, several questions needed to be answered since a study of this nature has not been conducted before.

1.3 Hypothesis

It was hypothesized that cell immobilization techniques will improve microbial activity and shorten the fermentation period. It was also hypothesized that the cell immobilization matrices used will have a positive contribution on the sensorial attributes of the final BSV.

1.4 Research questions

- Will immobilized cells lead to faster fermentation rates compared to free floating cells?
- Which immobilization matrix is most suitable for BSV production with regards to cell affinity, cell retention and acetification rates?
- Which immobilization technique (entrapment vs adsorption) is effective for BSV fermentation

- Are the immobilized cells reusable/recyclable for subsequent fermentations and can the immobilized cells retain adequate microbial activity and fermentation rates?
- Are static or agitated more effective for BSV fermentation?
- How will aeration affect the BSV fermentation in contrast to non-aerated fermentations?
- What aeration rate will be most suitable?
- what are the volumetric mass transfer coefficients of the studied BSV fermentations systems?
- What are the differences in oxygen uptake rates and oxygen transfer rates for immobilized cells under both aerated and non-aerated fermentations?
- What are the differences in the ratios of oxygen consumption vs acid yield for immobilized cells?
- How do the matrices used for cell immobilization affect the sensory characteristics of the BSV and consumer perception?

1.5 Aims and objectives

The aims and objectives of the study were:

Aim 1, Phase 1: To evaluate the effectiveness of the entrapment technique for cell immobilization for BSV fermentations.

Objective 1: Conducting BSV fermentations using two different sizes of Ca-alginate beads (small vs large) in order to evaluate to effect of bead size and surface area.

Objective 2: Conducting fermentations under static and agitated conditions for Ca-alginate bead fermentations and FFC treatments.

Aim 2, Phase 2: To investigate the effect of the adsorption technique and other bioreactor process augmentation techniques for BSV fermentation.

Objective 1: Comparing the adsorption matrices (CC and OWC) against FFC fermentations.

Objective 2: Testing the effects of aeration and agitation for adsorbed cells and FFC fermentation treatments.

Objective 3: Evaluating the variations in cell adsorption capabilities on CC and OWC for individual yeasts and AAB species.

Aim 3, Phase 3: To test the effectiveness of immobilized cells when they are reused for subsequent cycles of fermentation.

Objective 1: Evaluating the number of cycles the that recycled immobilized cells can complete at desired acetification rates.

Objective 2: Evaluating the structural integrity of cell immobilization matrices after being reused using the scanning electron microscope.

Objective 3: Evaluating the final BSV's for sensorial attributes.

Aim 4, Phase 4: To understand the oxygen mass transfer kinetics during BSV fermentation under aerated and non-aerated conditions

Objective 1: Evaluating the dissolved oxygen profiles during aerated and non-aerated BSV fermentations.

Objective 2: Using the dynamic method to determine and compute oxygen mass transfer parameters during BSV fermentation.

1.6 Delineation of the study

This study did not investigate the following:

- The fermentation of BSV using different grape cultivars.
- BSV fermentation in wooden barrels.
- The individual contributions of the selected microorganisms during mixed fermentations.
- Maturation and aging of the BSV.

1.7 Significance of the research

The study contributed to the following

- Broadening our understanding of cell immobilization and the differences in the matrices used for BSV production.
- Understanding the impacts of different cell immobilization matrices and how they contribute to the organoleptic properties of BSV.
- Understanding the reusability capabilities of the cell immobilization matrices for successive BSV fermentations.
- Understanding oxygen mass transfer kinetics during BSV production.
- A laboratory method for BSV production that has the potential of being upscaled for industrial applications.

1.8 Outcomes, results and contributions to research

In this study, valuable information regarding the contributions of cell immobilization, agitation and aeration were generated for BSV production. Acceptable BSV's with regards to taste and aroma were obtained. One conference proceeding, one review article and one full length article were published. Furthermore, two manuscripts have been drafted for publication in peer reviewed journals. This

research also contributes as a prerequisite for the completion of a Doctor of Engineering degree in Chemical Engineering.

CHAPTER 2

LITERATURE REVIEW

Published as: Hutchinson, U.F., Jolly, N.P., Chidi, B.S., Ngongang, M.M. and Ntwampe, S.K.O., 2019. Vinegar Engineering: A Bioprocess Perspective. *Food Engineering Reviews*, pp.1-16.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Vinegar is generally defined as a sour or acidic liquid obtained from a two-step fermentation process (Ebner et al., 2000; Ho et al., 2017; De Leonardis et al., 2018). The fermentation process utilises yeast for the anaerobic fermentation of sugar to ethanol and acetic acid bacteria (AAB) for the aerobic oxidation of ethanol to acetic acid (Fig. 2.1) (Solieri & Giudici, 2009; Ho et al., 2017; De Leonardis et al., 2018). As a key metabolite, acetic acid is an important ingredient in vinegar and its concentration defines the organoleptic characteristics of vinegars. Typically, vinegar is not generally classified as food, but as a food-flavouring agent (Solieri & Giudici, 2009; Nie et al., 2013), an important feedstock in the food industry (Nie et al., 2013) and a food preservative (Johnston & Gaas, 2006; Ghatak & Sen, 2016).

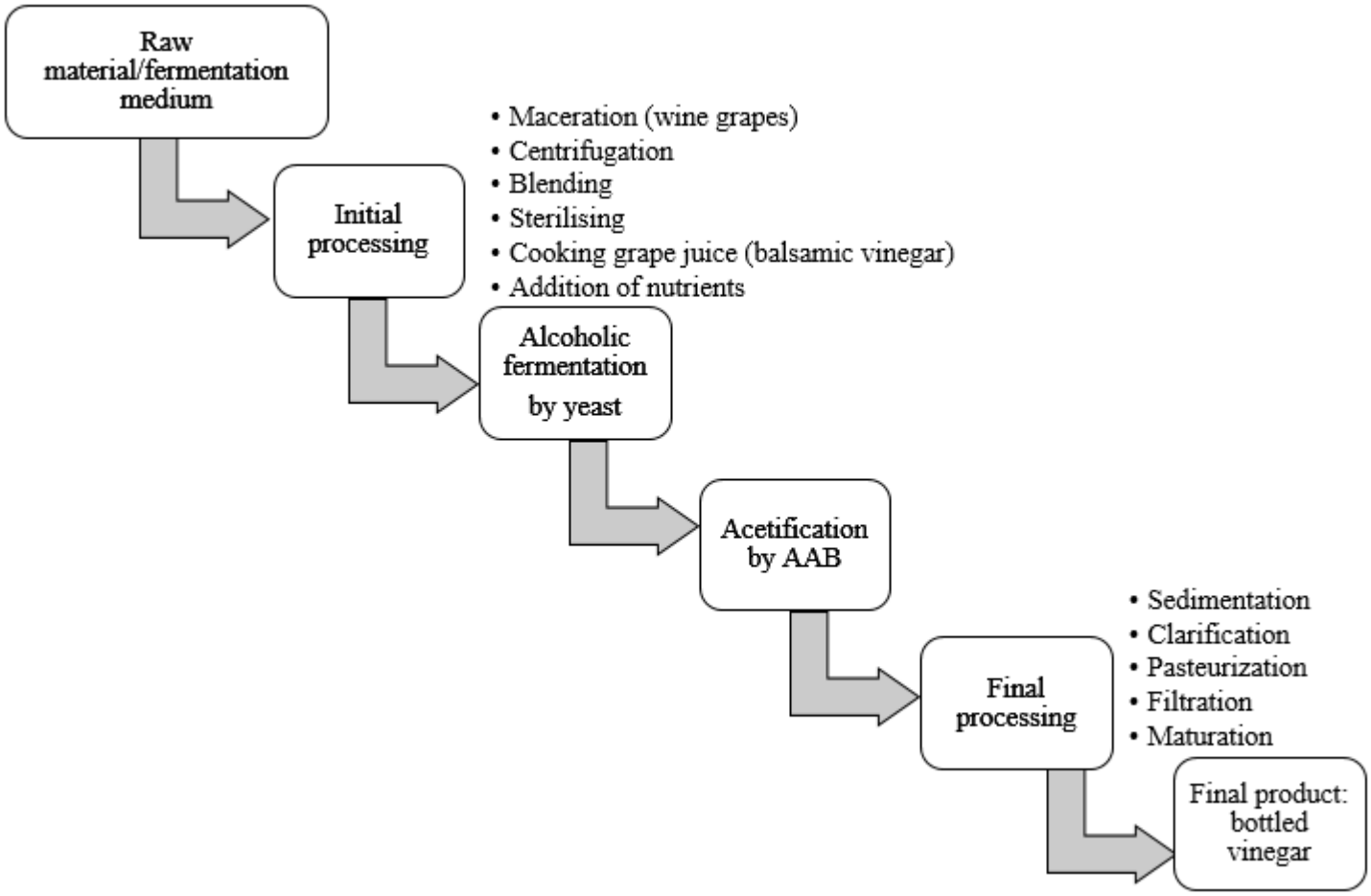

Figure 2.1 An illustration of the basic steps involved in vinegar production NB: not all steps are involved for all vinegar engineering systems.

Vinegar is made from numerous carbohydrate sources or food products that contain fermentable (reducible) sugar for yeast to metabolise (Solieri & Giudici, 2009; De Leonardis et al., 2018). Currently, there are numerous types of vinegars produced globally and most of these are produced from cheap raw materials, which is why most vinegars are lowly-priced. These raw materials can include by-products obtained from food processing, low quality fruit, agricultural surpluses and fruit waste (Solieri & Giudici, 2009; Mazza & Murooka, 2009, Tesfaye et al., 2002; De Leonardis et al., 2018). Some vinegars are obtained from non-fermentation processes, such as distilling alcohol, which is subsequently oxidised to acetic acid (Solieri & Giudici, 2009).

There are two distinct production methods for fermentation-based vinegars, namely the traditional and submerged methods. The traditional method relies on surface culture fermentations, whereby oxygen is obtained from the air. In simpler terms, this method applies low technological inputs and as a result, the fermentation period is longer and the vinegars are therefore expensive (Tesfaye et al., 2002; Fernández-Pérez et al., 2010). These expensive vinegars are usually those made in certain areas with regional and seasonal input of raw materials. Examples include oxos vinegar from Greece, sherry vinegar from Spain and the Traditional Balsamic Vinegar (TBV) from the provinces of Reggio Emilia and Modena, Italy (Solieri & Giudici, 2009). The second method is the submerged tank method, which entails the use of technologically advanced systems such as the use of spargers, coolers, antifoams, stainless steel fermentors and automated control systems (Tesfaye et al., 2002; Fernández-Pérez et al., 2010). The submerged method is typically used by large producers for the production of commercial vinegars, which are in high demand (Tesfaye et al., 2002). An example of a typical process distinction can be made between traditional wine vinegar fermentation processes that takes up to 2 months to achieve the required final product quality concentrations, and the industrial wine vinegar fermentation using the Frings acetator (submerged method) that only takes up to 20-24 hrs (Budak & Guzel-Seydim, 2010).

For these reasons, the application of biochemical engineering principles in vinegar production is important for large-scale producers. Not only because vinegar is a food flavouring agent found in virtually every household (Davis et al., 2015) but also due to its widespread use in the food industry (Branen et al., 2001; Hyuseinov & Ludwig, 2016; Nair et al, 2017, Wiley, 2017). In the context of this review, vinegar engineering refers to food, biochemical, bioprocess and chemical reaction engineering technology in the production of the vinegar. This will bring about an understanding of vinegar engineering and address some challenges, which customary microbiological and chemistry methods do not resolve. These parameters include bioreactor selection (design), dissolved oxygen mass transfer efficiency, effect of fluid mechanics on microorganisms and kinetic modelling of co-, by- and final products. In this review, some concepts in vinegar engineering system analysis are introduced. However, the introduction of new technologies can lead to authentication doubts, as some vinegars are produced following well-defined traditional approaches. Therefore, these new approaches are more fitting to vinegar production methods, which are not protected by legislation. There is a growing interest

in vinegar production utilising a variety of fruit, agricultural waste or raw materials. Some unusual vinegars include those produced from onion (Horiuchi et al., 2000a; 2004; Cheun et al., 2005; González-Sáiz et al., 2008a), hawthorn (Wenye et al., 2003; Zheng et al., 2010) and wood (Baimark & Niamsa, 2009; Mun & Ku 2010; Wititsiri 2011).

According to statistical data published by the Vinegar Institute (Vinegar Institute, 2005), global shares of various vinegars were balsamic vinegar (34%), red wine vinegar (17%), cider vinegar (7%), rice vinegar (4%), white vinegar (2%), while other vinegars were 36% (Fig. 2.2). Furthermore, in the United States, white distilled vinegars have the highest unit shares (68%), while in China, brewed and white fruit vinegars are the most popular.

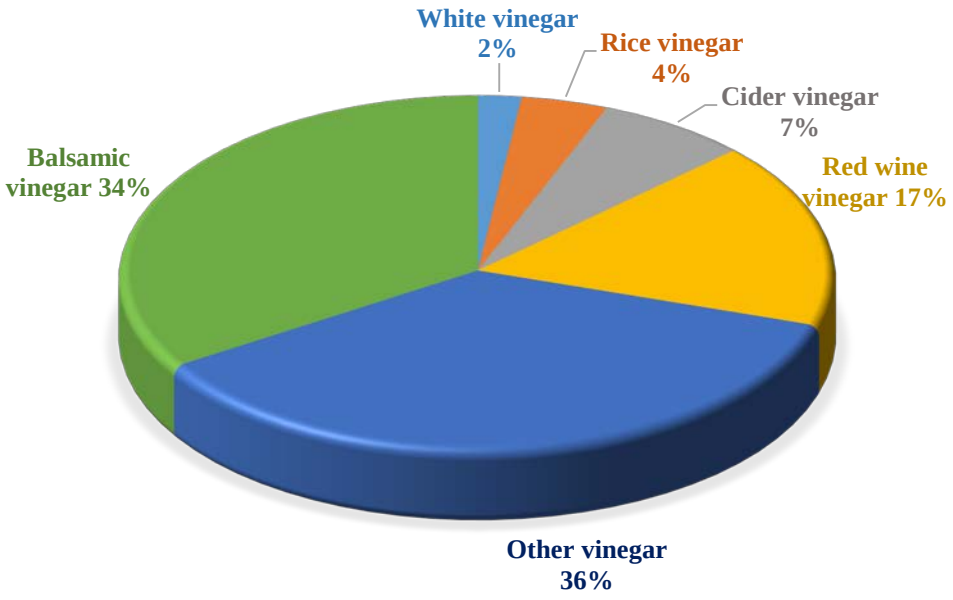

Figure 2.2 Global shares of various vinegars published by the Vinegar Institute in 2005. Adapted from https://versatilevinegar.org/market-trends/.

2.2 Vinegar engineering: patents and future outlooks

Patents are fundamental to vinegar engineering research. As a result, a substantial amount of vinegar patents has been filed during the 19th and 20th century (summarised in Table 2.1). Nevertheless, a brief outline of the history of vinegar engineering patents includes inventions that are primarily associated with bioreactor/acetifier design and configurations. One of the earliest examples of inventions in the 20th century was made by Heinrich et al. (1932 & 1937). These inventions entailed the cooling of the infusion mash/wort in order to prevent thermal disturbances that arise due to large generators used in

the acetifiers. Other inventions entailed the addition of temperature responsive elements in the bioreactor with the aim of regulating temperature and flow of the wort according to the required conditions of various vinegar products.

Another critical invention was aimed at preventing yield losses through evaporation by employing vats made from earthenware, wood and non-corrosive metals in rare cases (Brun, 1945). The latter was achieved by altering bioreactor designs to include cooling devices on the gas outlets aimed at cooling and condensing vinegar vapours exiting the exhaust outlets. Further improvements by Ernst (1961) were made in the submerged methods for vinegar production (continuous system) whereby continuous aeration using an air dispersing device was used. Bioreactors also contained cooling and heating coils for temperature control and a rotor for efficient liquid circulation within the system. The super-oxygenated atmosphere was also created in another invention by controlling oxygen demand in the production system in the second phase of fermentation according to the system's oxygen requirements. Foam accumulation during vinegar fermentations was controlled using rotational mechanical devices (Wittler & Frings, 1991). This particular invention allowed the accumulated foam to be broken down into gas and liquid vinegars that are subsequently transferred back into the vinegar broth inside the bioreactor.

Overall, these aforementioned inventions are evidence that researchers have mostly paid special attention/focused on bioreactor design-based parameters. Moreover, in vinegar engineering, there are several areas that can be explored in this regard. Some important inventions and patents not discussed in this section are also listed in Table 2.1.

Table 2.1: Vinegar production patents

Patent name & number	**Inventor(s)**
Method for the production of vinegar acids by oxidative fermentation of alcohols (US2707683A)	Otto & Heinz (1955)
Process for acetic acid fermentation (US3445245A)	Ebner & Frings (1969)
Two stage process for the production of vinegar with high acetic acid concentration (US4076844A)	Ebner et al. (1978)
Process for the production of vinegar with more than 12 g/100 mL acetic acid (US4503078A)	Ebner & Frings (1985)
Control arrangement for a vinegar fermentation process (US4773315A)	Enenkel & Frings (1988)

Patent name & number	Inventor(s)
Automated method for a semi-solid fermentation used in the production of ancient quality rice vinegar and/or rice wine (US4808419A)	Hsu (1989)

2.3 Vinegar engineering and bioreactors

2.3.1 Bioreactors: importance and general overview

Bioreactor studies have been conducted for various vinegar production systems using a diverse range of bioreactors (Table 2.2). Industrial vinegars generally employ the acetators/bioreactors (submerged approach) for vinegar production, due to the high yields obtained in such systems (Gullo et al., 2014). The use of bioreactors normally offers a controlled environment, which leads to faster production, reduced product losses and possibilities for optimisation (Boehme et al., 2014; Yan et al., 2014; van Noort, 2016). Most bioreactors are normally operated using optimised conditions and parameters. These include pH, temperature (Dendooven et al., 2000; Gullo et al., 2014), substrate/nutrients concentrations (Kim et al., 2005; Gullo et al., 2014), airflow rates (Mazutti et al., 2010), agitation and pressure (Garcia-Ochoa & Gomez, 2009). Optimization of the aforementioned parameters has been extensively studied for some fermentation systems. However, there is insubstantial research on the optimization of process parameters for bioreactors used in vinegar production. As bioreactors come in various designs to suit various biological systems in order to meet the demands of the process (Van't Riet & Tramper, 1991), they are generally operated using different operational modes, which include batch, fed batch, resting/immobilized cells and continuous systems (Garcia-Ochoa & Gomez, 2009).

2.4 Desired features in bioreactors

The most notable mutual and desired feature in most of the bioreactors used for vinegar production is the ability to permit high oxygen transfer and sufficient agitation. Agitation should sufficiently homogenise bioreactor contents and prevent low-oxygen areas, thus preventing dead zones and stuck fermentations. Additionally, there should be minimal oxygen transfer interruption, as this can slow down the process or render the AAB non-viable (Schlepütz et al., 2013; Gullo et al., 2014). To serve this purpose, the most commonly used bioreactor for commercial vinegar production was designed by Heinrich Frings 'Frings acetator' (García-García et al., 2009) and it achieves high product yields (95%) (Fregapan**e** et al, 1999**;** Qi et al., 2014). It is recommended because of low energy requirements (400 W/L) compared to other bioreactors and also contains a Frings alkolograph that quantifies alcohol concentration during fermentation (Qi et al., 2014). Importantly, the acetator also has a self-aspirating system that replaces system oxygenation by compressed air (García-García et al., 2009). The homogenous dispersion of very fine air bubbles is also another desired feature in industrial bioreactors;

hence, it is common to find bioreactors equipped with special turbines designed for this purpose (Schlepütz et al., 2013; Schlepütz & Büchs, 2013).

2.4.1 Bioreactor design and configurations

The design and configurations of a bioreactor play a critical role in the performance of the vinegar fermentation process. Bioreactor configurations include height to diameter ratio (aspect ratio), the surface area to volume ratio (Halladj et al., 2016), shape of vessel (de Ory et al., 2004; Halladj et al., 2016), stainless steel features (de Ory et al., 2004), impeller configurations (size, number of blades, location) (González-Sáiz et al., 2008b), turbines and gas inlets and outlets (González-Sáiz et al., 2009a). In order to emphasise the importance of design and configuration, a graphical depiction of how height to diameter ratio influences gaseous (O_2 and CO_2) exchange is demonstrated in Figure 2.3. In this figure, a small perforation diameter (D_{min}) and larger height to liquid-air interface area (H/S_{max}) favours alcoholic fermentation, while a larger perforation diameter (D_{max}) and smaller height to liquid-air interface area (H/S_{min}) favours the acetification process.

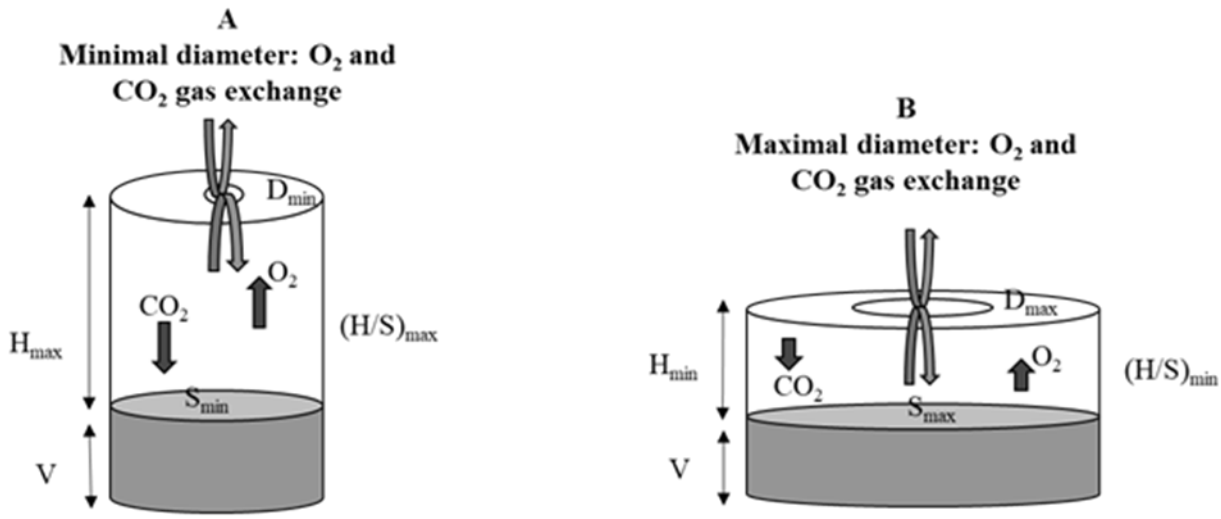

Figure 2.3 An illustration of the influence of geometic characterisitics on gaseous exchange. (A) favours alcoholic fermentation, (B) favours acetification, diffusion of gases is indicated by arrows, H/S ratio of container headspace (i.e. H= height to liquid-air interface area (S)), D = perforation diameter of the reactor lid (Halladj et al., 2016)

These are important factors to consider when designing a bioreactor for vinegar production. In addition, vinegar production systems which separate the alcoholic and acetification process can employ both geometric designs to suit the needs of each fermentation process. Previously, a 10L bioreactor equipped with a sparger located 2 cm from the bottom of the bioreactor for air diffusion was reported as a suitable design for vinegar production (González-Sáiz et al., 2009a). The bioreactor had two impeller flat blade Rushton turbines with a gap of 1.45 cm between the sparger and the first impeller, with each impeller having six blades (Fig. 2.4). Proximity placement of the sparger and impeller is not advisable, as this

would cause flooding and bubble coalescence. Similarly, if they are too far apart, this would impair foam mitigation. Temperature control can be maintained with the use of a water jacket and an oxygen probe must be installed to monitor dissolved oxygen.

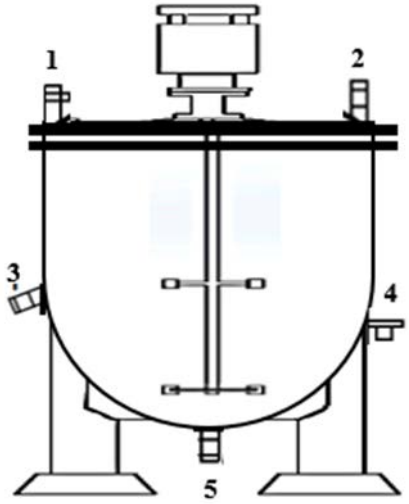

Figure 2.4 Reproduced scaled-up fermentor design. (1) air outlet and pressure valve; (2) inlet port; (3) sampling port; (4) air inlet; (5) outlet port (González-Sáiz et al., 2009a).

Mehaia and Cheryan (1991) used a membrane recycle bioreactor for the production of date extract vinegar. The design of this type of bioreactor included a hollow-fibre module with a polysulphane membrane, through which sterilized air was sparged at a rate of 0.4 L min^{-1}, achieving a high acetic acid production rate of 10.8 g L^{-1} h^{-1} (Table 2.2). Huang et al. (1998) studied vinegar production with fructose as a substrate, while using a fibrous-bed bioreactor (Table 2.2). The bioreactor was constructed using a glass column and contained a spiral-wound terry cloth. The quality and acetic acid concentration of vinegars are strain, bioreactor- and fermentation type-dependent. Acetic acid production rates for different types of bioreactors (fed batch and continous systems), including free-floating and immobilized cells are shown in Table 2.2.

Wine vinegar production, on the other hand, was studied using a non-commercial 100L bubble column reactor (Table 2.2) (Fregapane et al., 1999). The bioreactor was equipped with a novel gas-liquid dynamic sparger, which allowed the distribution of fine bubble sizes. This bioreactor showed increased acetification rates compared to other industrial bioreactors. An acetic acid production rate of 1.8 g L^{-1} h^{-1} coupled with a 94% ($^{v}/_{v}$) yield was achieved. Horiuchi et al. (2000a & b) studied a charcoal pellet bioreactor (Fig. 2.5) for acetic acid production from ethanol and onion alcohol, respectively, which was later improved for onion vinegar production (Fig. 2.6). The onion vinegar bioreactor was serially connected to an ethanol jar fermentor with the onion alcohol being seperately produced in the jar fermentor while being continuously fed into the charcoal pellet bioreactor whereby acetification took place (Fig. 2.5), achieving a high acetic acid production rate (Table 2.2).

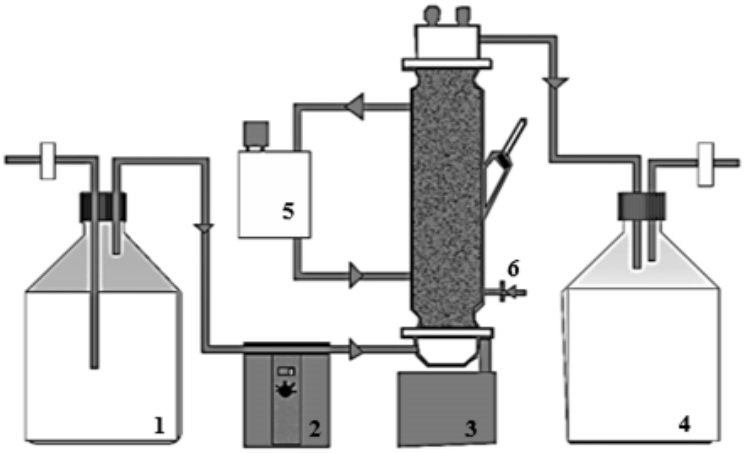

Figure 2.5 Reprodced charcoal pellet bioreactor (1) medium resevior; (2) peristaltic pump; (3) packed bed bioreactor; (4) broth resevior; (5) water bath (Horiuchi et al., 2000b).

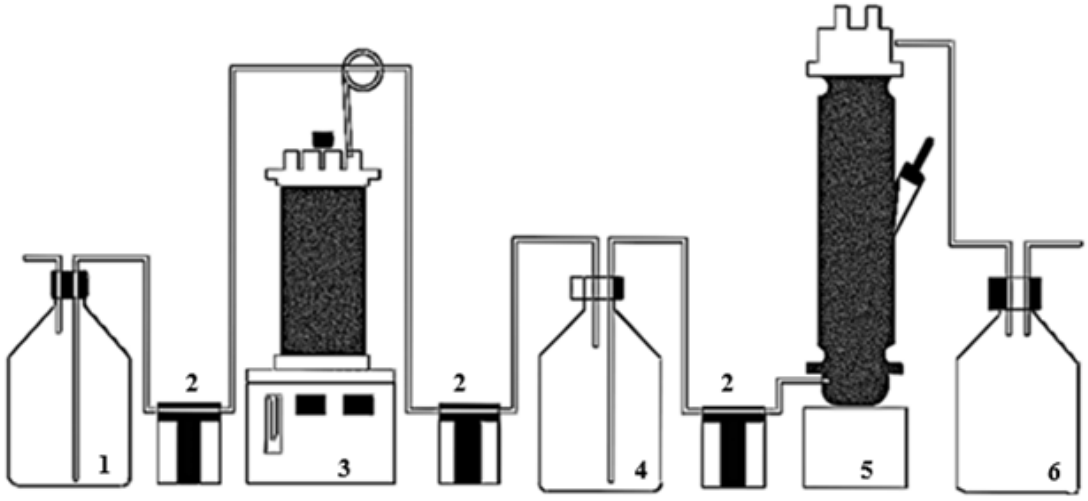

Figure 2.6 Reproduced continuous onion vinegar bioreactor design, (1) medium reservoir; (2) peristaltic pump; (3) jar fermentor; (4) onion alcohol broth reservoir; (5) charcoal pellet bioreactor ;(6) vinegar broth reservoir (Horiuchi et al., 2000a).

Initially, de Ory et al. (1999) designed a bioreactor using a cylinder-shaped stainless-steel reactor (Fig. 2.7) with geometric characteristics including an internal diameter of 0.47 m and a height of 1.4 m for a 225 L working volume. Temperature control was conducted by an interior heat exchanger which was attached to a thermostatic bath. Under this configuration, the reactor re-circulated the outlet gas streams through the bottom of the fermentation vessel with the use of an air pump and stainless-steel diffusers. In this design, the stainless-steel diffusers were also responsible for pneumatic stirring and maximum oxygen distribution. Furthermore, an industrial oxygen cylinder was connected to the reactor by means of an electro-valve (refer to Fig. 2.7), which can be switched off to maintain optimum dissolved oxygen (2 mg L^{-1}).

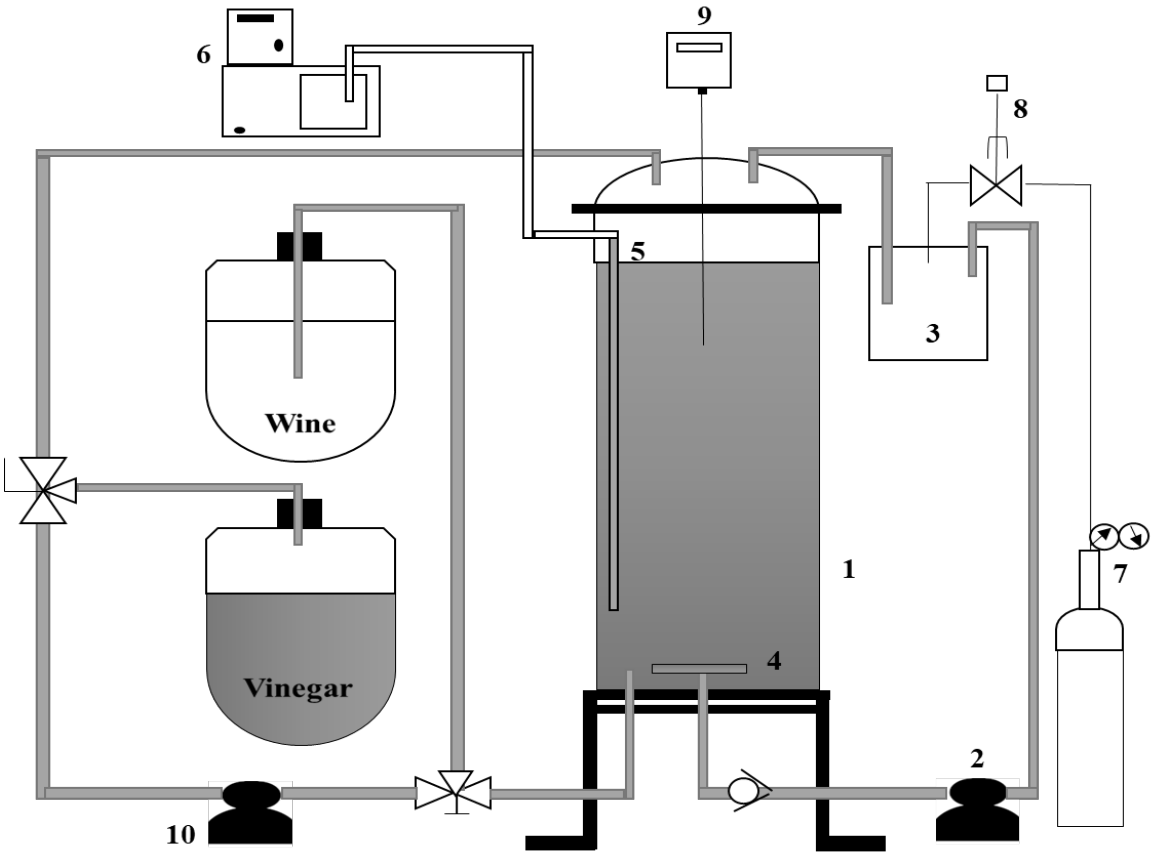

Figure 2.7 Reproduced industrial acetifier (1) reactor, (2) gas recycling pump, (3) expansion tank, (4) air diffusors, (5) heat exchanger, (6) thermostatic bath, (7) oxygen cylinder, (8) electro valve, (9) dissolved oxygen sensor, (10) feed inlet and effluent outlet pump. de Ory et al., (1999) as reported in de Ory et al., (2004).

Furthermore, an aerated-stirred pilot bioreactor was proposed to be an improvement of existing fermenters by González-Sáiz et al. (2009a). The study proposed the replacement of plate tops with dish tops in industrial fermenters while achieving an acetification rate of 13.2 and 11.1 g L^{-1} h^{-1} for the batch and continuous system, respectively.

Table 2.2: Bioreactor application studies in vinegar production

Product	Type of bioreactor	Bacteria used	Batch or continuous	Acetic acid production rate (g L^{-1} h^{-1})	Reference
Acetic acid from ethanol	Three-phase fluidized-bed bioreactor using immobilized cells	*Acetobacter aceti*	Continuous	0.051-0.138	Sun & Furusaki (1990)

Product	Type of bioreactor	Bacteria used	Batch or continuous	Acetic acid production rate (g L^{-1} h^{-1})	Reference
Date extracts vinegar	Membrane recycle bioreactor	*Acetobacter aceti*	Batch Continuous	0.5 10.8	Mehaia & Cheryan (1991)
Acetic acid production from fructose	Fibrous-bed bioreactor using immobilized cells	*Clostridium formicoaceticum*	Fed-batch Continuous Fed-batch Continuous	0.12 3.2 0.95 1.3	Huang et al. (1998)
Wine vinegar	Bubble column reactor equipped with a novel type of gas–liquid dynamic sparger	*Acetobacter pasteurianus*	Batch Continuous	1.25 1.8	Fregapane et al. (1999)
Acetic acid from ethanol	Packed-bed charcoal pellet bioreactor	*Acetobacter pasteurianus*	Continuous	3.9-6.5	Horiuchi et al. (2000a)
Onion vinegar	Charcoal pellet bioreactor	*Acetobacter pasteurianus*	Batch Continuous	8.0 3.3	Horiuchi et al. (2000b)
Industrial vinegar	Aerated-stirred fermenter	*Acetobacter aceti*	Batch Continuous	13.2 11.13	González-Sáiz et al. (2009a)
Hawthorn vinegar	Fed batch bioreactor	*Acetobacter pasteurianus*	Fed-batch	1.1	Zheng et al. (2010)
Corn vinegar	Air-lift acetifier	*Acetobacter aceti* WK	Semi-continuous	0.18-0.26	Krusong & Vichitraka (2011)

Product	Type of bioreactor	Bacteria used	Batch or continuous	Acetic acid production rate (g L^{-1} h^{-1})	Reference
Acetic acid from waste cheese whey	Multistage membrane-integrated hybrid process	*Acetobactor aceti* (NCIM-2116)	Continuous	4.06	Nayak & Pal (2013)
Acetic acid from ethanol	9 L Frings pilot acetator	*Acetobacter pasteurianus CICIM B7003-02*	Semi-continuous		Qi et al. (2014)
Rice vinegar	100 L Internal Venturi injector bioreactor	*Acetobacter aceti* WK	Semi-continuous	0.38 (high initial acetic acid) 0.40 (high initial acetic acid)	Krusong et al. (2015)
Acetic acid from cheese whey	Multi-stage Membrane-Integrated Bioreactor	*Acetobacter. aceti* (NCIM-2116)	Continuous	4.1	Nayak et al. (2015)
Fruit vinegar	6 L scaled bioreactor (INFORS, France)	*Acetobacter pasteurianus* KU710511	Batch	0.27 (apple vinegar) 0.22 (date vinegar)	Mounir et al. (2016)

2.4.2 Overall remarks on bioreactor design for vinegar production

Despite the large number of vinegars produced worldwide, bioreactors have only been studied for a few vinegars, and it is evident that there are several designs (geometric characteristics) of bioreactors depending on the type of vinegar produced and the distinct needs of the fermentation process. However, some basic conditions need to be defined, such as the bacteria used, optimum stirring speed and dissolved oxygen transfer rates.

2.5 Immobilized *versus* free-floating cells for vinegar fermentation

2.5.1 The importance of immobilized cell systems

Acetic acid bacteria are sensitive bacteria and it appears that the immobilization of AAB cells improves their efficiency (Kocher et al., 2006; Krusong & Vichitraka, 2011; Kumar et al., 2016; Zur et al., 2016). Generally, the immobilization of cells refers to restricting the motion of cells during fermentation. This can be done by using several techniques, which include entrapping the cells in a carrier, adsorbing the cells on a solid surface and mechanical containment behind a barrier (de Ory et al., 2004). The most commonly used method in fermentation systems is the adsorption of cells onto a solid surface, which can be performed using the static, dynamic batch, reactor loading and electrode positioning process (Anonymous, nd (b)). Cell immobilization offers defence against harsh environmental conditions such as low pH, osmotic stress and temperature including shearing due to agitation, which consequently increases biomass concentration (Sun & Furusaki, 1990; Huang et al., 1998; Kocher et al., 2006; Krusong & Vichitraka, 2011). Furthermore, cell immobilization provides the advantage of cellular longevity; however, it is also crucial to choose an inexpensive material to use for cell immobilization in order to minimise production costs (Sun & Furusaki, 1990; Kocher et al., 2006). This technique can be classified as a bioengineering technique which augments bioreactor design, while improving the total biomass surface area to carry-out biochemical reactions. It is an appealing approach that would benefit vinegar producers if implemented due to the rapid acetification rates obtained with immobilized cells (Kennedy et al., 1980; Talabardon et al., 2000; Kocher et al., 2006).

2.5.2 The choice of a support material: important factors to consider

According to the literature reviewed, there are always several reasons for selecting a specific support material for cell immobilization. The selection criteria include availability, benignity, influence of material on product quality, cost, physical properties and oxygen transfer efficiency in cell entrapping gels taking into account the radius and total surface area of the materials (Kennedy et al., 1980; Sun & Furusaki 1990; Horiuchi et al., 2000; Kocher et al., 2006; Krusong & Vichitraka, 2011; Krusong & Tantratian, 2014). Figure 2.8 depicts cell immobilization materials that are usually preferred during vinegar production. For example, the fibrous bed (Fig. 2.8A) was reported to promote high cell density (Talabardon et al., 2000), with the charcoal pellets (Fig. 2.8D) being selected for their high surface area and their microbial affinity (Horiuchi et al., 2000). The loofa sponge (Fig. 2.8C) was described as an edible and fibrous agricultural material with no safety concerns (Krusong & Vichitraka, 2011). Other carriers might be oak and/wood shavings (Fig. 2.8E) (Thiripurasundari & Usharani, 2011) which might impart certain flavours into the final product (Kyraleou et al., 2015; Laqui-Estaña et al., 2019) and alginate beads – Fig. 2.8F (Kocher et al., 2006; Hutchinson et al., 2018).

Figure 2.8 Cell immobilization materials used in vinegar studies. Fig. 2.8A cited from Krusong et al. (2010)

2.5.3 Cell immobilization: comparative analysis

As aforementioned, cell immobilization techniques vary in terms of which protocols to follow and materials to use. Table 2.3 lists several studies on the subject of AAB cell immobilization and acetification rates. Hydrous titanium (IV) oxide and hydrous titanium (IV) chelated cellulose were used to immobilize two *Acetobacter* strains for the conversion of ethanol (produced by fermenting wort) into acetic acid (Kennedy et al., 1980). Improved conversion rates were obtained; however, the performance of the two bacteria strains varied when using titanium oxide. This was attributed to the fact that one specie formed a slime (extracellular polysaccharide substances) while the other strain did not form a slime. The slime forming bacteria resulted in a 67% and a 24% relative difference increase in acetification rates when immobilized in hydrous titanium (IV) oxide using a small (2.5L) and large (8.0L) bioreactors, respectively. The non-slime forming bacteria performed better when the cells were freely floating in contrast to immobilized cells when using the smaller fermenter. In the larger bioreactors, titanium cellulose chelate was added for the immobilization of cells, and there was a relative increase in acetification rates compared to free floating cells. Kennedy et al., (1980) showed that immobilized cells and the aggregation of cells is an efficient strategy compared to free-floating cells for vinegar production. However, producers interested in employing this method would have to optimise

the concentration of titanium salts required and evaluate the residual salts in the final product for quality and gustatory perception purposes.

Fibrous bed matrices are widely employed for cell immobilization in the vinegar industry due to their robustness. Additionally, fibrous beds allow efficient mass transfer and do not undergo productivity reduction when immobilized cells are used repeatedly. Non-active immobilized cells can also be scraped off from fibrous beds and replaced with active cells instead of re-starting the immobilization process (Huang et al., 2002). Huang et al., (1998) immobilized *Clostridium formicoaceticum* cells for the production of acetic acid using fructose. In this study, the cells were adsorbed in the fibrous matrix by pumping 25 $mL.min^{-1}$ of the fermentation broth into the fibrous bed. The cells were immobilized after 36-48 h of continuous broth pumping. The immobilized cell fermentations were compared to free-floating cell fermentations in batch, fed-batch and continuous systems and all acetification rates are shown in Table 2.3. Nonetheless, the highest acetic acid concentration achieved in free cell and immobilized cell fermentation was 46.4 and 78.2 g L^{-1}, respectively. Here, it was concluded that cell immobilization by adsorption is one of the most employed cell immobilization techniques due to its simplicity and it is often cheaper, depending on the material used. Talabardon et al., (2000) also immobilized AAB cells by adsorption onto a fibrous bed matrix. This was also a comparative study between free-floating and immobilized cell fermentations for the production of acetic acid from lactose and milk permeate using *Clostridium thermolacticum* and *Moorella thermoautotrophica*. Free-floating cells resulted in acetification rates of 0.06 and 0.08 g L^{-1} h^{-1} using lactose and milk permeate, respectively. As for the immobilized cells, acetic acid production was increased to 0.54 and 0.30 g L^{-1} h^{-1} using lactose and milk permeates respectively. The studies used the adsorption of cells into a fibrous bed technique for the immobilization of cells, resulting in improved acetification rates.

The cell immobilization by entrapment technique is generally classified as expensive due to the cost of alginate and the cost of special equipment required to make gel beads (Saudagar et al., 2008). Nevertheless, this technique was evaluated and was found to compare well with the adsorption techniques during the production of sugar cane vinegar (Kocher et al., 2006). *Acetobacter aceti* cells were entrapped using a 4% ($^{w}/_{v}$) sodium alginate solution to form a cell paste, followed by expulsion through a syringe into a 0.2M $CaCl_2$ solution, consequently resulting in the formation of calcium alginate beads. Cell adsorption can also be employed using three different materials, i.e., corncobs (Fig. 2.8A), bagasse (Fig 2.8B) and wood shavings (Fig 2.8E). Comparative analysis of the gel entrapment and adsorption method for vinegar production is crucial because, apart from broadening the vinegar engineering knowledge, it also allows producers to make an informed decision concerning which technique to employ. Nonetheless, the alginate gel-entrapped and free cell fermentations showed relatively lower acetification rates compared to adsorbed cell fermentations (Table 2.3), an outcome that has been reported to occur in various studies (de Ory et al., 2004). In these studies, it was concluded that the lower acetification rates in entrapped cells were due to the lower surface area available for cell

immobilization; and as a result, dissolved oxygen and substrate mass transfer across the beads was limited (Kocher et al., 2006). Lower acetification rates when using the entrapment technique could also be attributed to the growth of cells near the gel surface or the gel radius being too large. For high acetification rates, the recommended gel radius is 1.0-1.4 mm with dissolved oxygen concentration of 0.031-0.14 mg L^{-1} (Sun & Furusaki, 1990). Interestingly, positive results were observed with the gel entrapment technique for Balsamic-styled vinegar production using beads sized 4.5-8.5 mm (Table 2.3) (Hutchinson et al., 2018). Their observations could be attributed to the microbial consortium of acetic acid bacteria used, the inoculum size, temperature treatments and several other factors.

In a different study, a loofa sponge (*Luffa cylindrica*) was used to immobilize cells for the adsorption of *Acetobacter aceti* WK cells for corn vinegar production (Krusong & Vichitraka, 2011). The sponge was cut into small pieces of 1-inch thickness, washed with water and then sterilized by submerging into 4% ($^{v}/_{v}$) acetic acid for 24 hours prior to being used as a cell support material. The loofa sponge is an environmentally benign porously structured material composed of lignin, cellulose and hemicellulose (Demir et al., 2008), which allows for high dissolved oxygen diffusion that eventually makes it an ideal material for cell immobilization (Krusong & Vichitraka, 2011). For this particular reason, it is widely grown in the subtropical regions of Korea, China, Brazil, Japan and some areas of Central and South America (Demir et al., 2008; Tanobe et al., 2014).

Table 2.3: Cell immobilization studies for vinegar production

Product	Immobilization material	Bacteria used	Acetification rate (g L^{-1} h^{-1})	Authors
Acetic acid from wort	Hydrous titanium (iv) oxide	*Acetobacter* strains	4.38 (2.5 L fermenter)	Kennedy et al. (1980)
			4.99 (8 L fermenter)	
Acetic acid from ethanol	Large-surface-area ceramic support	*Acetobacter aceti*	10.4 adsorbed cells (continuous)	Ghommidh et al. (1982)
Acetic acid from glucose	Calcium alginate gel particles	*Acetobacter aceti*	0.051-0.138 for entrapped and free cell fermentation	Sun & Furusaki (1990)
Acetic acid from fructose	Fibrous bed/matrix	*Clostridium formicoaceticum*	0.148-0.225 free cells (batch)	Huang et al. (1998)
			0.824-1.144 adsorbed (batch)	
			0.12 free cells (fed-batch)	
			0.95 adsorbed (fed-batch)	
			1.3-3.2 adsorbed (continuous)	
Acetic acid from lactose and milk permeate	Fibrous-Bed/matrix	*Clostridium thermolacticum* and *Moorella thermoautotrophica*	0.06 free cells (Lactose)	Talabardon et al. (2000)
			0.08 free cells (milk permeate)	
			0.54 immobilized (Lactose)	
			0.30 immobilized (milk permeate)	
Onion vinegar	Charcoal pellets	*Acetobacter pasteurianus*	3.3 adsorbed (repeated batch process)	Horiuchi et al. (2000)
Cashew apple juice vinegar	Polyurethane foam	Mixed culture	0.2 adsorbed cells	de Ory et al. (2004)
Sugar cane vinegar	Calcium alginate beads	*Acetobacter aceti*	0.06 free cells	Kocher et al. (2006)
			0.07 gel entrapped cells	
			0.09-0.10 adsorbed cells	
Tea vinegar	Sugarcane bagasse Corncobs	*Acetobacter aceti*	0.13 Sugarcane bagasse cells (Semi-continuous) 0.12 Corncobs cells (Semi-continuous)	Kaur et al. (2011)
Cashew apple juice vinegar	Bagasse, corn cobs and wood shavings	*Acetobacter aceti MTCC – 2975*	~ 0.01 for all materials used	Thiripurasundari & Usharani (2011)

Product	**Immobilization material**	**Bacteria used**	**Acetification rate (g L^{-1} h^{-1})**	**Authors**
Corn vinegar	Loofa sponge	*Acetobacter aceti* WK	0.18 -0.26 adsorbed cells	Krusong & Vichitraka, (2011)
Sugarcane Vinegar	Wood shavings	*Acetobacter aceti* AC1	0.24-0.95 adsorbed cells	Kocher & Dhillon, 2013
Rice wine vinegar	Loofa sponge	*Acetobacter aceti* WK	0.07-0.10 adsorbed cells	Krusong & Tantratian (2014)
Barley malt vinegar	Calcium alginate beads and agar beads	*Gluconobacter oxydans* NBRC 3432	0.17 calcium alginate beads 0.11 agar beads	Kaushal & Phutela (2017)
Balsamic-styled vinegar	Calcium alginate beads	*Microbial consortium*	0.16 Small calcium alginate beads 0.13 Large calcium alginate beads 0.025 Free floating cells	Hutchinson et al. (2018)

2.5.4 Overall remarks on cell immobilization

Overall, it appears that there are no optimal procedures for the immobilization of AAB cells. However, it is evident that cell immobilization using adsorption is the most common method. The gel entrapment method is also common; albeit at a laboratory scale. Additionally, recent studies focusing on cell immobilization for vinegar engineering are gravely lacking. Based on the reviewed studies, the immobilization of AAB cells by both entrapment and adsorption improves production rate compared to free-floating cells, and in most instances, the selection of the materials to use in a fermentation is important. However, according to most of the reviewed studies, the adsorption of AAB cells to a surface is more effective compared to gel entrapment. Although the materials or methods for adsorption could vary, the type of the vinegar being produced and bacteria used is of paramount importance. Employing cell immobilization for vinegar production also requires an appropriate bioreactor design since immobilization materials such as alginate beads have a low mechanical strength and can easily be damaged by impellers and high agitation rates; therefore, this design parameter would have to be taken into consideration. Ideally, materials used for cell immobilization must be able to resist operating conditions and mechanical influences. An example is fibrous bed matrices, which have been widely studied and are well understood in the process engineering industry. Hence, it is reasonable to conclude that fibrous beds offer a convenient cell immobilization approach.

2.6 Aeration during vinegar production

2.6.1 Importance of aeration during vinegar production

Aeration is reasonably recognised and well understood as an important bioreactor performance parameter for industrial vinegar production. However, this knowledge is frequently directed to a handful of spirit vinegars and not other varieties. Nonetheless, several studies have reported that oxygen transfer is the rate-limiting step for vinegar production (Ghommidh et al., 1982; Tesfaye et al., 2002; Qi et al., 2013; Gullo et al., 2014). This is attributed to AAB being strictly aerobic and requiring high dissolved oxygen levels to grow optimally and carry-out all the essential activities (Gullo et al., 2014). The stoichiometry behind the use of oxygen by AAB is that, one mole of oxygen is required for the oxidation of ethanol to produce one mole of acetic acid (Ghommidh et al., 1982; Qi et al., 2013). This means that, in most vinegar production processes, a continuous supply of oxygen is a necessity (Garcia-Ochoa & Gomez, 2009; Garcia-Ochoa et al., 2010). García-García et al., (2009) reported that vinegar fermentation exhibits an extremely high demand for oxygen to the extent that a 25 m^3 vessel at 20°C with an acetification rate of 2 g L^{-1} h^{-1} requires 20 m^3 of gaseous oxygen transfer per hour. If the oxygen concentration is very low, it can slow down the acetification process. Consequently, the oxygen uptake rate for the AAB used must be known, as this will allow proper control of oxygen in the fermentor. The primary challenge is the low solubility of oxygen in the fermentation medium during aerobic system operations such as those used for vinegar fermentation; therefore, it is critical to design a fermentation

system that can improve oxygen mass transfer between gaseous and liquid phases (Ghommidh et al., 1982).

It was also reported that a momentary interruption of oxygen transfer during fermentation could cause the inhibition of the acetification process. However, at higher acetic acid concentrations above 5%, the interruption of oxygen transfer was reported to be less detrimental (Gullo et al., 2014), which effectively highlights the need for adequate oxygen at the initiation of the vinegar engineering process. Schlepütz et al., (2013) proposed the use of a respiration activity monitoring system (RAMOS) which can assist in preventing the interruption of oxygen supply. The transfer of oxygen during a fermentation process requires hydrostatic pressure monitoring and reduced mechanical influences such as stirring speed on the cells. Other factors to consider when transferring oxygen for vinegar production are the microorganisms used and their oxygen uptake rate (Schlepütz & Büchs, 2013; Gullo et al., 2014).

2.6.2 Influence of volumetric mass transfer coefficient (k_La)

The volumetric mass transfer coefficient (k_La) is an important factor that is generally used to assess the competency of a bioreactor. Furthermore, k_La is used as an important tool when scaling up for vinegar production or any aerobic fermentation system (González-Sáiz et al., 2009a). The determination of k_La depends on several factors such as the geometric parameters of the bioreactor, hydrodynamics, airflow rates, media properties, morphology of microorganisms used and properties (Moutafchieva et al., 2013). Several methods are used to determine k_La, these include among others the sodium sulphite oxidation method and the gassing in method (Sobotka et al., 1982). k_La in shake flasks is determined by the inclusion of shaking parameters (shaking frequency, shaking diameter) as well as the flask size and working volume (Seletzky et al., 2007). This means that scaling up from conventional shake flasks to fermenters is possible. However, several laboratory scale studies for vinegar production often neglect this factor, subsequently results from these studies are not very useful for industrial scale fermentations because of the inconsistency of findings when scaling up.

A high value of k_La is one of the primary goals when designing a bioreactor because it is proportional to the reactor productivity. The aeration capacity of a bioreactor is fundamentally dependent on k_La. For this reason, several studies have investigated the relationship between airflow rates and k_La during vinegar production, with Fregapane et al. (1999) reporting improved k_La when airflow rates were increased for all vessel geometric parameters studied. The highest k_La values of 75-170 h^{-1} were obtained when airflow rates of 0.06-0.25 vvm min^{-1} were employed. Similar correlations were reported by Krusong et al. (2015), with an airflow rate of 1.1 min^{-1} in a 25 L bioreactor resulting in a k_La of 106 h^{-1}, while an airflow rate of 3.1 min^{-1} in a 75 L bioreactor resulting in a k_La of 93 h^{-1}. These studies accentuate the importance of sufficient airflow rates for high k_La or vice versa, notwithstanding the

geometric characteristics of the bioreactor. Furthermore, k_La for industrial fermenters such as the Frings acetator has been reported to range between 100 to 900 h^{-1} (García-García et al., 2009).

2.6.3 Aeration: comparative studies

Several oxygen transfer studies were conducted for vinegar production (Table 2.4 and 2.5). These studies paid a special attention towards oxygen pressure, uptake and acetification rates.

These include improving oxygen transfer during vinegar production by using a fixed-bed bioreactor with pulse flow due to its high oxygen transfer rate (Ghommidh et al., 1982). Fluctuating oxygen partial pressure (Po_2) during the process was achieved by mixing nitrogen and oxygen. The overall gas-liquid mass transfer coefficient *kLa* was determined when the cells were at a steady state with the oxygen uptake rate being determined using the mass balance method; whereby dissolved oxygen is measured at the liquid and gas phases of the reactor. However, the mass balance method on its own can be inaccurate; therefore, using stoichiometry for computations can be a solution, since it uses acetic acid productivity rate to calculate the oxygen uptake rate. Furthermore, since oxygen was termed as the rate-limiting step, consequently increasing inlet oxygen partial pressure (Po_2) can lead to increased dissolved oxygen uptake rate and increased acetic acid production rate (-see Table 2.4) (Ghommidh et al., 1982).

Table 2.4: Effect of varying Po_2 on acetification rate (adapted from Ghommidh et al., 1982)

Inlet oxygen partial pressure Po_2in (atm)	Oxygen uptake rate Qo_2 (g L^{-1} h^{-1})	Acetification rate *A* x *D* [1] g L^{-1} h^{-1}	Outlet oxygen partial pressure Po_2out (atm)
0.115	1.44	2.7	0.098
0.21	2.45	4.6	0.185
0.34	3.09	5.8	0.315
0.49	4.11	7.7	0.46
0.64	4.51	8.45	0.618
1	5.55	10.4	1

[1] *A x D* = Acetification rate x Dilution rate, Dilution rate= 0.513 h^{-1}

The optimum oxygen concentrations were defined for different functions by Park et al. (1989), which are 3-7 mg L^{-1} for effective oxygen consumption, and 2-15 mg L^{-1} for essential enzymes, such as alcohol dehydrogenase and acetaldehyde dehydrogenase. For acetification, the optimum oxygen was reported

to be 1-3 mg L^{-1}, and it was concluded that the electron transfer step during ethanol oxidation was the most sensitive to oxygen during the acetification process.

In a different study, the influence of oxygen partial pressures on wine vinegar production was investigated (Rubio-Fernández et al., 2004). Fermentations were conducted with varying air compositions ranging from 21 to 63% enriched oxygen content. The aeration rate was 0.06 vvm min^{-1}, with the results showing higher acetification rates when oxygen rich air, containing 30% oxygen was used. Fermentation time decreased from 65 hours to 35 hours with an overall acetic acid yield of 96–99%. It is important to note that, excessive aeration rates can result in excessive foam formation and loss of medium even when an antifoam system is used. Furthermore, excessive aeration also leads to the loss of ethanol due to evaporation which eventually leads to reduced yields (Krusong et al., 2015). Some other studies reporting on oxygen transfer include that of Qi et al. (2013) who introduced a model describing the ratio of oxygen consumption versus acetic acid yield, which could be used to evaluate fermentation efficiency. Overall, all of the aforementioned studies show the significance of efficient oxygen transfer during vinegar production, a key parameter that cannot be achieved without effective bioreactor design and configuration considerations. Table 2.5 shows several other studies which investigated aeration during vinegar production, with evidence that adequate aeration ranges from 0.05-1.00 vvm min^{-1}.

Table 2.5: Impact of aeration in vinegar production: comparative analysis

Product	**Air or oxygen**	**Aeration rate min^{-1} vvm**	**Acetification rate g L^{-1} h^{-1}**	**Authors**
Acetic acid	Oxygen rich air	0.10-0.13	4.60	Park et al. (1989)
Wine vinegar	Air	0.06	0.75	Fregapane et al. (1999)
		0.25	3.67	
Wine vinegar	Air	0.06	0.72	Rubio-Fernández et al. (2004)
	Oxygen rich air		1.35	
Spirit vinegar	Air	1.00		Schlepütz et al. (2013)
Industrial vinegar	Oxygen	0.13	1.81	Qi et al. (2013)

2.7 Mathematical computations used for assessing vinegar engineering

Mathematical models and computations are predominant in bioprocess engineering systems; they normally include the use of equations and computer software for adequate process evaluation and optimisation. Mathematical applications offer several advantages, such as process optimisation,

measuring efficiency, risk assessment, data interpretation, quality assessment and kinetic modelling or simulation. Table 2.6 lists some mathematical applications in 12 vinegar engineering studies. Virtually every element of the fermentation system requires the use of mathematics to be properly understood, and this includes mass transfer (González-Sáiz et al., 2008b, 2009b; Qi et al., 2013), hydrodynamic effects (González-Sáiz et al., 2009a), chemical developments (Ghosh et al., 2012), microbial growth (Sun & Furusaki, 1990; González-Sáiz et al., 2008b; Ghosh et al., 2012; Nayak et al., 2015), distillation (Bakar, 2014), cell immobilization (Sun & Furusaki, 1990), continuous or batch system efficiency analyses and bioreactor design and configuration. Furthermore, kinetic modelling of a fermentation process is one of the most common and extensively studied mathematical applications which can be applied in vinegar engineering; it assists in understanding the behaviour of a fermentation process. The kinetic models normally simulate and describe the relationship between microbial growth kinetics and chemical developments in the fermentation vessel over a certain period (Mitchell et al., 2004; González-Sáiz et al., 2009a).

Modelling microbial growth, substrate consumption and product formation has been done by several researchers (Sun & Furusaki, 1990; Garrido-Vidal et al., 2003; Pochat-Bohatier et al., 2003; Dobre et al., 2007; González-Sáiz et al., 2008b; Ghosh et al., 2012; Nayak et al., 2015). However; the models differ to some degree. For instance, Garrido-Vidal et al., (2003) used quadratic models which included the stoichiometric rates of oxidation and yield factors for more accurate and realistic process prediction results. González-Sáiz et al. (2009a) also included stoichiometric coefficients when calculating yield factors during fermentation. Ghosh et al. (2012) modelled palm juice vinegar using common mathematical equations such as the Monods and Luedeking-piret models to describe microbial proliferation during the fermentation process. Some other models include those proposed by Monteagudo et al. (1997) to model substrate utilization rates. These models are reliable, and they can easily be rearranged or manipulated to best describe any system, even a continuous fermentation system. In continuous systems, growth rates, substrate consumption and product formation are modelled or calculated with the inclusion of dilution rates, substrate feeding and effluent rates (Park et al, 1989; Sun & Furusaki, 1990; Garrido-Vidal et al., 2003). Additionally, some models on microbial growth include those developed by Pochat-Bohatier et al. (2003) which can be used to demonstrate mechanical shearing influences on microbial behaviour.

During industrial vinegar fermentation, stirring is one of the most important mechanical inputs; however, it can have a positive or detrimental effect. Stirring can affect the microorganisms, while influencing oxygen transfer during the acetification process. This effect can be studied and demonstrated, mathematically. This crucial part of the fermentation process has been understudied in vinegar engineering and the stirring speed (rpm) for vinegar production has not been properly defined. Thus far, a handful of studies were performed in which mathematical computations were used to demonstrate the effect of agitation during the fermentation process. González-Sáiz et al. (2009a &

2009b) reported on a range of stirring speeds and their effect. The agitation range was between 200 to 1000 rpm. The models showed that maximum microbial growth was achieved at a high agitation speed of 800 rpm. Additionally, it was reported that agitation higher than 800 rpm caused an inhibitory shearing effect and cell damage. Heat transferred into the system was calculated while taking into account the microbial heat generated during ethanol oxidation. It was concluded that the models proposed in the study could be applied in various aerobic processes with varying agitation systems. González-Sáiz et al. (2009b) further reported that conditions with very low agitation resulted in failed fermentations for both experimental and predicted results. In another investigation, Garrido-Vidal et al. (2003) studied agitation at a much lower range (50 rpm) in comparison to 1000 rpm; quadratic models, which include agitation, were used and these models showed that agitation, aeration rate and overpressure have an effect on oxygen transfer. It is also important to note that aeration has been reported to be more important in increasing oxygen transfer compared to agitation. Additionally, sparging and very high agitation speeds result in very high economical costs due to high energy inputs (González-Sáiz et al., 2008b).

Modelling of fermentation processes is normally done using laboratory-scale experiments; however, it is also crucial to study the scaling-up of the models for large-scale industrial applications. González-Sáiz et al. (2009a) studied models in a pilot fermenter and the scaling-up of the models for an industrial fermentation process. The response surfaces generated by the models led to the conclusion that superficial air velocity, aerated mechanical power input, hydrostatic pressure, temperature and concentrations of compounds must be similar to the pilot fermentor. Garcia-Ochoa and Gomez (2009) reported that oxygen transfer rate is the most important parameter for the scaling-up of bioreactors. It is crucial to ensure homogenous oxygen concentration in the fermentation medium for adequate growth and acetification rates. Therefore, agitation and aeration are also important parameters in ensuring homogenous oxygen concentration.

Fortunately, several studies have mathematically studied oxygen mass transfer for vinegar engineering (Table 2.6). These studies include one by González-Sáiz et al. (2008a) which modelled oxygen transfer in an oxygen saturated fermentation medium for vinegar production. They described oxygen concentration using the balance method, which is the balance between the oxygen transferred and the oxygen uptake rate. Dobre et al. (2007) proposed two models, the first which presumes that acetic acid formation is influenced by oxygen transfer from the gas to liquid phase, while the other model was similar to the Monods model, which presumed that acetic acid formation is influenced by substrate consumption. This study concluded that both models should be used simultaneously. Some other studies modelled the influence of oxygen transfer on specific growth rate of AAB (Garrido-Vidal et al., 2003).

Another important process parameter to model during vinegar production is the purification of the product. In most vinegar production processes, pure acetic acid must be obtained at the end of the

process. A comprehensive study conducted by Nayak et al., (2015) involved microfiltration and nanofiltration membranes to purify the product. The models used in this study included the influence of the filtration methods. Furthermore, the models also incorporated some other important parameters such as cross flow rates, dilution rates, pH and recycling of materials in correlation with substrate-product inhibition. It was concluded that the proposed models accurately predicted the performance of the process.

A unique study conducted by Bakar (2014) entailed the application of mathematical equations to demonstrate the effect of purifying the fermentation product, using batch distillation columns. The proposed equations included the calculation of boil-up rate during batch distillation, the average composition of the total material distilled and component balance for the hold-up tank.

Table 2.6: Studies entailing mathematical computations in vinegar engineering

Mathematical and computational assessments undertaken in vinegar engineering	**References**
Biomass growth Substrate consumption Product formation Yield factors	Sun & Furusaki (1990); Garrido-Vidal et al. (2003); Pochat-Bohatier et al. (2003); Dobre et al. (2007); González-Sáiz et al. (2008); Ghosh et al. (2012); Nayak et al. (2015)
Response surface methodology Design of experiment (DOE) software (Matlab 7.3.0.267 R2006b-MathWorks Inc.)	Garrido-Vidal et al. (2003); González-Sáiz et al. (2008a); González-Sáiz et al. (2009a); Ghosh et al. (2012); Bakar (2014)
Oxygen consumption Correlation between gas-liquid transfer and volumetric transfer coefficient Modelling oxygen concentration in an oxygen saturated fermentation medium Modelling volumetric oxygen transfer coefficient Stoichiometric coefficient of oxygen consumption versus acid yield.	Sun & Furusaki (1990); Dobre et al. (2007); González-Sáiz et al. (2008a & 2008b); Qi et al. (2013)
Mechanical equations Acid negative influence on bacterial growth	Pochat-Bohatier et al. (2003)
Oxygen flow rate towards wood surface Acetic acid mass balance in packed bed bioreactor Acetic acid mass balance taking into account recycling vessel Acetic acid mass balance without oxygen in liquid phase	Dobre et al. (2007)

Mathematical and computational assessments undertaken in vinegar engineering	References
Kinetic models for industrial vinegar production Dissolved oxygen concentration Thermal conductivity of fermentation medium Total energy consumption, Power of air blower Desirability function	González-Sáiz et al. (2009a)
Modelling ethyl acetate, ethanol, acetoin, acetic acid and water	González-Sáiz et al. (2009b)
Boil up rate of batch distillation Latent heat of vaporisation Differential material balance Average composition of total distilled material Bottoms holdup Component balance for holdup tank Total mass balance for reboiler Component mass balance	Bakar (2014)
Product inhibition Cross-flow rates Dilution rates Recycling of material Permeate flux output from microfiltration unit Change in substrate concentration after nanofiltration in permeate stream Change in product concentration after nanofiltration in permeate stream Modelling substrate and product concentrations at steady state Uncharged acetic acid concentration at final stage of nanofiltration Overall volumetric flux of acetic acid Rejection by nanofiltration membranes Diffusion and electro migration Acetic acid flux for acetate ions	Nayak et al. (2015)

2.8 Summary

Overall, based on the reviewed studies, the following can be extrapolated. (1) The descriptions regarding the design and configurations of a bioreactor have rather flexible design parameters; hence, the diversity with respect to bioreactors used for vinegar production. (2) Oxygen transfer and agitation seem to be major factors to be taken into consideration when designing a bioreactor for improved production. (3) Cell immobilization studies have been conducted for a variety of vinegars at laboratory scale. However, information on cell immobilization techniques is lacking for industrial scale vinegar production. (4) Some parameters still need to be optimised such as oxygen transfer rates, stirring speed, efficient cell immobilization methods and materials. (5) Although there may be no one size fits all, these parameters can be defined for selected microorganisms (6) Mathematical computations have mostly been done on commercial-industrial vinegars and these applications are gravely lacking in traditional vinegars. Several laboratory scale studies have not investigated scaling up procedures. (7) Studies

demonstrating the effect of hydrodynamics and mechanical influences/fermentation vessel design during vinegar production are scanty; therefore, these topics require more attention. Moreover, there is a necessity for studies, which will define a standard method for the production of different types of vinegar.

CHAPTER 3

MATERIALS AND METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Experimental methods for all phases

3.1.1 Preparation of fermentation medium

Frozen Chenin blanc grape must (22°Brix) obtained from the Agricultural Research Council (Nietvoorbij, Stellenbosch, SA) was thawed at 28°C. After thawing the grape must boiled using a double jacketed steam pot (S.W.18, Aluminium Plant and Vessel Co. Ltd., London, UK) until a sugar concentration of 30 °Brix was obtained. The grape must was aliquoted into Erlenmeyer flasks (Table 3.1), covered with cotton wool stoppers, subsequent to autoclaving at 120 °C for 20 min. After autoclaving, the grape must was analyzed chemically for sugar (°B), pH, alcohol (% *v*/*v*) and total acidity (g L^{-1}) using a density meter (Density meter DMA 35, Anton Paar, Graz, Austria), pH meter (Metrohm pH meter 632, Herisau, Switzerland), alcolyzer (Anton Paar, Graz, Austria) and minititrator (Hanna instruments minititrator HI 84502, Johannesburg, South Africa); respectively.

Table 3.1 Fermentation working volumes used for each phase of the study.

	Volumes (L)	Erlenmeyer sizes (L)
Phase 1	0.35	0.5
Phase 2	3.00	3.0
Phase 3	0.35	0.5
Phase 4	1.50	2.0

3.1.2 Pre-culture preparation

Cryopreserved non-*Saccharomyces* yeast (n=5) and acetic acid bacteria (AAB, n=5) (Table 3.2 & 3.3) cultures were obtained from the ARC Infruitec-Nietvoorbij culture collection. The yeast and bacteria cultures were individually grown in YPD (Merck, Modderfontein, South Africa) and in GM broth (glucose 0.8%, mannitol 1.7%, peptone 0.3%, yeast extract 0.5%) (Merck, Modderfontein, South Africa), respectively. Volumes of YPD and GM broth used for each phase of the study are shown in Table 3.4. The yeast and bacteria inoculums were incubated at 28 °C for 48 and 72 h respectively, prior to inoculation.

The non-*Saccharomyces* yeasts were selected based on a previous screening investigation. The yeast used in this study were selected due to high acid formation on calcium carbonate agar plates, desired

aroma of the final fermented product, osmophilic characteristics and the final concentration of the alcohol produced.

Similarly, acetic acid bacteria were obtained from previous isolation procedures on various sources; namely, grape pomace, healthy grapes and Shiraz wine. The bacteria used for this study were selected based on their ethanol oxidation rate when inoculated in diluted wine (6 % *v*/*v*) or their sugar utilization abilities in autoclaved grape juice.

Table 3.2. Non-*Saccharomyces* yeast used in the study.

Non-*Saccharomyces* Yeast		
Identity	**ARC Accession Numbers**	**Origin**
Candida pulcherrima	Y0839	Chardonnay grapes
Candida zemplinina	Y1020	Chardonnay grapes
Hanseniaspora guilliermondii	Y0558	Cabernet Sauvignon grapes
Kloeckera apiculata	C48V19	Chardonnay grapes
Zygosaccharomyces bailii	C45V69	Chardonnay grapes

ARC: Agricultural Research Council of South Africa.

Table 3.3. Acetic acid bacteria used in the study.

Acetic Acid Bacteria			
Identity	**ARC Accession Numbers**	**NCBI Accession Numbers**	**Origin**
Acetobacter pasteurianus	171/19	CP 021922.1	Healthy grapes
Acetobacter malorum	172/36	KU 686765.1	Shiraz wine
Kozakia baliensis	179/48	CP 014681.1	Grape pomace
Gluconobacter cerinus	126/34	KX 578017.1	Shiraz wine
Gluconobacter oxydans	172/36	LN 884063.1	Grape pomace

NCBI: National Center for Biotechnology Information.

Table 3.4. YPD and GM broth volumes used for inoculum preperation for each phase of the study

	YPD and GM Volumes (mL)
Phase 1	100
Phase 2	500
Phase 3	100 mL for Ca-alginate beads fermentations 200 mL for Corncobs and Oak chips fermentations
Phase 4	250

3.2 Phase 1-Chapter 4 methods: Cell immobilization by Gel Entrapment in Ca-alginate Beads for Balsamic-styled Vinegar Production

3.2.1 Calcium alginate infused YPD and GM preparation

A volume of 500 mL YPD and GM broths were prepared in a beaker. This was followed by preparing a 3% (w/v) alginate solution by dissolving 15 g of sodium alginic salt (Biolab, Merck, South Africa) into the YPD and GM broth solutions, respectively. The alginate infused YPD and GM broth solutions were autoclaved at 120°C for 20 min.

3.2.2 Cell immobilization: Small vs Large beads

Yeast and AAB cells were harvested by centrifugation of the liquid pre-cultures. The yeast and AAB cells were transferred to 250 mL YPD-alginate and GM-alginate solutions, respectively, and thoroughly mixed. Small (4.5 mm) beads (Fig. 3.1B) were prepared by extruding the alginate+broth solutions infused with cells through a 10 mL syringe. The beads were formed when a drop of the solution came into contact with a 0.9% (w/v) sterile calcium chloride solution (Biolab, Merck, South Africa). The larger (8.5 mm) beads (Fig. 3.1A) were prepared by extruding the alginate solutions infused with cells through a large 10 mL plastic pipette tip, which was obtained by cutting off the edge of the tip. Both small and large beads were allowed to properly solidify for an hour in the calcium chloride solution (Kocher et al., 2006; Kaushal & Phutela, 2017).

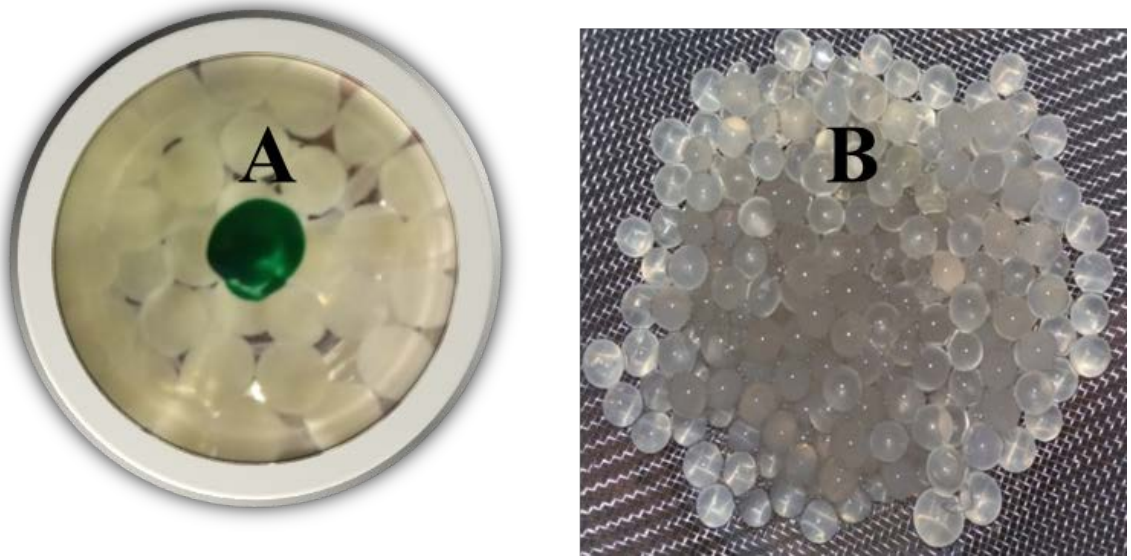

Figure 3.1 (A) Large beads (8.5 mm) in calcium chloride solution, (**B**) Small beads (4.5 mm) on a sterile strainer.

3.2.3 Inoculation

An inoculum size of 2% (v/v) was used for both immobilized and free-floating cell fermentations. Immobilized cell fermentations were inoculated by dropping 20 mL of yeast and AAB beads, respectively into the fermentation containers. The YPD and GM alginate solutions contained ~30% cells and therefore 20 mL of beads in 350 mL fermentation medium, making ~2% inoculum (Fig. 3.2A

& B). The free-floating cell fermentations were inoculated with 7 mL (2% v/v) of the yeast and bacterial consortium (Fig. 3.2C).

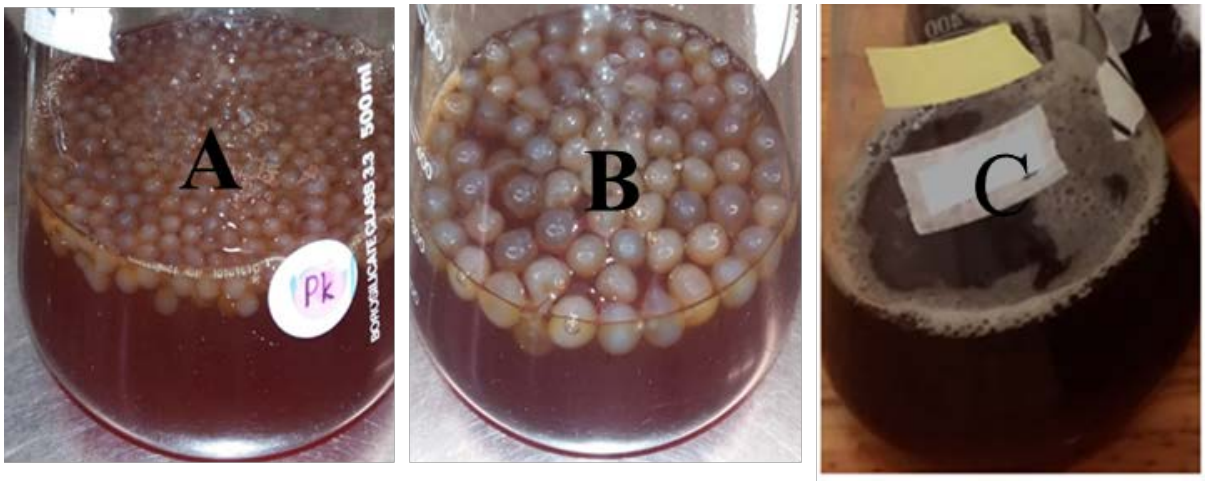

Figure 3.2 Balsamic-styled vinegar fermentations using cooked Chenin blanc grape must. (A) Small and (B) large calcium alginate beads with entrapped yeast and AAB cells, (C) free-floating cell fermentations.

3.3 Phase 2-Chapter 5 methods: Aeration, Agitation and Cell Immobilization on Corncobs and Oak Wood Chips Effects on Balsamic-Styled Vinegar Production

3.3.1 Sterilization of Corncobs and Oak Wood Chips

Corncobs (CC) were dried in the oven prior to cutting into smaller pieces using a cutting tool (sizes are shown in Table 3.5 and Figure 3.3). French oak wood chips (OWC) were used for this study. The OWC did not require any drying. Subsequently, the CC and OWC were separately transferred into 4 × 3 L Erlenmeyer flasks prior to autoclaving at 121 °C for 20 min. Autoclaving was repeated (n = 2) to maximize sterility.

Table 3.5. Size of corncobs and oak wood chips used in the study.

Adsorbents	Length (cm)	Width/Diameter (cm)	Circumference/Perimeter (cm)	Surface Area of One Piece (cm^2)	Surface Area of all Adsorbents Used (cm^2)
Corncobs	6.00 ± 1.05	4.00 ± 0.66	12.57	100.53	402 (4 pieces)
Oak woodchips	2.90 ± 0.65	1.80 ± 0.53	9.60	19.64	392 (20 chips)

Length and width results are the average of repeats ± standard deviations.

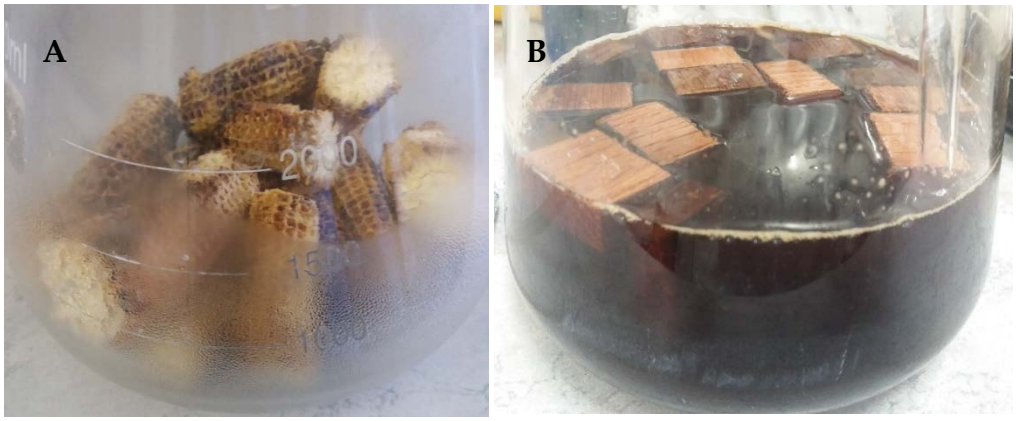

Figure 3.3 Corncobs and oak wood chips used in the study, (**A**) corncobs after autoclaving (**B**) oak wood chips in cooked grape must.

Yeast and bacteria were immobilized separately due to their difference in cell size. Furthermore, the differences in turbidity of the broth after yeast and bacteria cell growth was assumed to be a factor that might affect cell adsorption between the yeasts and bacteria.

3.3.2 Cell Immobilization on Corncobs and Oak Wood Chips

After yeast and bacteria were fully-grown, the inoculums were mixed resulting in a 2 L consortium of yeast and bacteria (Tables 3.2 and 3.3) (NB: yeast and bacteria consortiums were initially in separate flasks). Subsequently, 1 L of the yeast consortium was transferred into 3 L Erlenmeyer flasks, one containing CC and another one containing OWC. The same procedure was performed using the bacterial consortium. The yeast and bacterial cells were allowed to adsorb onto the surface of CC and OWC (serving as a solid support surface/bed) overnight at 28 °C.

3.3.3 Quantification of Cells Adsorbed on Corncobs and Oak Wood Chips Prior- and Post-Fermentation

The number of cells adsorbed on the CC and OWC for individual yeasts and bacteria were quantified using the dry cell weight method adapted from Stone et al. (1992) and Nguyen et al. (2009), with minor modifications. Prior to this, the yeast and bacterial cell concentration (Tables 3.2 and 3.3) in liquid suspension were individually quantified following the procedure described in Hutchinson et al. (2019a). Furthermore, the yeast and bacteria were individually studied to assess the variations in cell adsorption capabilities. Yeast and bacteria were grown individually following the procedure described in Section 2.2. Subsequently, the yeast and bacteria cells were individually adsorbed onto the CC and OWC following the procedure described in Section 2.4. CC and OWC were removed from the broth and dried in an oven set at 40 °C. The adsorbents were weighed daily until a stationery weight was reached.

To assess the quantity of cells adsorbed post fermentation, the CC and OWC were transferred into grape must and allowed to ferment for 20 days. Subsequently, the adsorbents were extracted and dried at 40

°C until a stationery weight was reached. To determine the number of cells adsorbed for the procedures, the difference of the weight of the adsorbents prior and post adsorption was computed.

3.3.4 Inoculation

After the adsorption process, the OWC and CC were extracted from the broth and allowed to dry for 4 h. A 3 L fermentation volume was used for this study. Four pieces of corncobs (2 pieces' yeast, 2 pieces Acetic Acid Bacteria) (Table 3.5) were used for inoculation in one flask. Twenty pieces of oak wood chips (Fig. 3.3B) were used for inoculation in another flask (10 pieces' yeast, 10 pieces AAB) (Table 3.5).

3.3.4.1 Static vs. Agitated Fermentations

Static and agitated fermentations were both incubated at 28 °C with agitated fermentations at 135 rpm. Agitated fermentations were conducted using an orbital shaker (FMH 200, FMH Instruments, Cape Town, South Africa). Under both static and agitated conditions, CC, OWC and FFC fermentations were conducted in triplicate.

3.3.5 Effect of Aeration

Air pumps (Resun® AC 9906, Longgang, Shenzhen, China) were used to sparge air into the Erlenmeyer flasks at different airflow rates, i.e., low aeration (LA = 0.15 vvm min^{-1}) and high aeration (HA = 0.30 vvm min^{-1}). Erlenmeyer flasks were covered with loose cotton wool stoppers and fermentations were conducted in triplicate.

3.4 Phase 3-Chapter 6 methods: Reusability and sensory effects of immobilized cells for subsequent balsamic-styled vinegar fermentations

3.4.1 Calcium alginate infused YPD and GM preparation

Calcium alginate infused YPD and GM solutions were prepared following the protocol described in 3.2.1.

3.4.2 Cell immobilization: gel entrapment

The cell entrapment technique was also conducted using a modified method adapted from Hutchinson et al. (2018). Yeast and AAB cells were harvested by centrifugation and transferred into a YPD-alginate and GM-alginate solutions (250 mL), respectively, prior to mixing thoroughly. The YPD and GM alginate solutions infused with cells were mixed to form a single homogenous suspension. Subsequently, the Ca-alginate beads were formed by extruding the alginate solution infused with cells through a syringe into a cold 0.9% ($^{w}/_{v}$.) sodium chloride ($NaCl_2$) solution (Biolab, Merck, South Africa). Each bead contained both yeast and AAB cells in contrast to the original method described in 3.2.2, whereby yeast and AAB cells were entrapped in separate Ca-alginate beads. The Ca-alginate

beads were allowed to solidify for an hour in the $NaCl_2$ solution (Kocher et al., 2006; Kaushal & Phutela, 2017).

3.4.3 Cell adsorption on corn cobs and oak wood chips

The cutting (CC only) and sterilization of CC and OWC was conducted using the method described in 3.3.1. However, due to working volume modifications, CC were cut into a length of 3 cm each (Table 3.6). Furthermore, in this phase of the study, yeasts and AAB inoculums were mixed (200 mL per inoculum, i.e. $200\ mL \times 10 = 2L$) and the adsorbents (CC & OWC) were separately submerged into 1L mixture of the consortium of yeast and AAB cells. The microbial consortium was allowed to adsorb onto the surface of the adsorbent overnight in a 28 °C incubation room.

3.4.4 Inoculation procedures

3.4.4.1 Ca-alginate beads inoculation

Since the working volume of the current phase of the study was equivalent to the working volume used in phase 1 for Ca-alginate beads fermentations, the inoculation process for the Ca-alginate beads was conducted using a similar protocol as described in 3.2.3.

3.4.4.2 Corncobs and oak wood chips inoculation

The inoculation of CC and OWC was also conducted by following the protocol described in 3.3.4. However, since the working volume was lower in the current phase, the quantity of adsorbents used to inoculate the fermentations was reduced (refer to Table 3.6).

Table 3.6: Details of the quantity of matrices used per 350 mL of fermentation medium

Adsorption					
Adsorbents	**Length (cm)**	**Width/Diameter (cm)**	**Circumference/Perimeter (cm)**	**Surface Area of One Piece (cm^2)**	**Surface Area of all Adsorbents Used (cm^2)**
Corncobs	3.0	3.9	12.25	54.29	108.58 (2 pieces)
Oak wood chips	2.90	1.80	9.60	19.64	117 (6 chips)
Entrapment					
	Volume of alginate infused with cells (mL)			**Size of beads (mm)**	
Ca-alginate beads	20			4.5	

3.4.5 Second cycle fermentations

3.4.5.1 Ca-alginate beads

After the completion of the 1st fermentation cycle, Ca-alginate beads were removed from the fermentation flasks using a sterile strainer and carefully rinsed with sterile distilled water. Subsequently, the Ca-alginate beads were inoculated into 3 x 350 mL cooked grape must (30°Brix) and incubated at 28°C for the second cycle of fermentations.

3.4.5.2 Corncobs and oak wood chips

Similarly, CC and OWC were removed from the fermentation flasks using a sterile strainer. The adsorbents were allowed to dry off any residual liquids from the 1st cycle of fermentation. The adsorbents were then transferred into 3 x 350 mL cooked grape must (30°Brix) and incubated at 28°C for the second cycle of fermentations.

3.4.6 Evaluation of the integrity of the immobilization matrices

Before and after the 1st cycle of fermentations, all the matrices were evaluated using the scanning electron microscope (SEM) (Thermo Fisher Nova NanoSEM 230 with a field emission gun (FEG), Eindhoven, Netherlands,). Ca-alginate beads were transferred into 1.5 mL Eppendorf microtubes containing 2.5 % glutaraldehyde and kept in a fridge for approximately 8 hrs. Thereafter, glutaraldehyde was discarded, and the samples underwent a dehydration procedure by submerging the beads through a series of alcohol concentrations starting from 30%, 40%, 50%, 60%, 70%, 90%, 95% to 100 % ($^{v}/_{v}$.). The samples were placed in the alcohol for 5 minutes for each step. Subsequently, samples were air dried for a few hours before SEM analyses. While CC and OWC were allowed to dry for 24 hrs before SEM analyses. Ca-alginate beads, CC and OWC were mounted on top of 10 mm aluminium pin stubs (Agar Scientific, Essex, United Kingdom) coated with carbon glue (Agar Scientific). All samples were sputter coated with carbon (Agar Scientific) using a vacuum evaporator for 90 seconds. The samples were then scanned under the SEM using several magnifications at an acceleration voltage of 20 kV. Additionally, the size of beads was measured using a light microscope (Leica Wild M400, Wetzlar, Germany) to evaluate bead size reduction.

3.4.7 Sensory evaluations

At the end of the 1st cycle fermentations, all cell immobilization matrices were removed from the BSV products, subsequent to centrifuging the vinegars to remove residual debris. Vinegars were kept at ambient temperature before organoleptic evaluations were conducted. The vinegars were evaluated by sixteen judges (male, female, 20-56 years old) from the Agricultural Research Council (South Africa) who were trained using commercial balsamic vinegars. The commercial vinegars were predominantly purchased from the major supermarket chains in Stellenbosch (Spar, Checkers, Woolworths, Pick ‘n Pay) and they include the Balsamic Vinegar of Modena. The “Check All That Applies” (CATA)

(Adams et al., 2007; Pramudya & Seo, 2018) method was used to evaluate the vinegars. Evaluated sensory descriptors were Appearance-Colour; Appearance-Viscosity; Appearance-Clarity; Overall aroma intensity; Aroma & Flavour description; Taste-sweet/acid; Acid-sugar balance; After taste; After taste persistence and the Liking rating with 49 sub-categories for all main categories. The data obtained was compared with 30 commercial balsamic vinegars (identities not disclosed) which were also evaluated by the same judges (NB: six vinegars were evaluated per day). The aim of using this method was to compare the produced BSV to commercially available balsamic vinegars in South Africa.

3.5 Phase 4-Chapter 7 methods: Oxygen mass transfer kinetics during Balsamic-styled vinegar production production

3.5.1 Bioreactor description and fermentation set up

Fermentations were conducted in triplicate using 2 L batch bioreactors with a working volume of 1.5 L. Both non-aerated and aerated fermentations were carried-out at 28°C. Aerated fermentations were aerated using air pumps (Resun® AC 9906, Longgang, Shenzhen, China) at an airflow rate of 0.15 vvm min^{-1}. Non-aerated bioreactors were covered with loose cotton wool stoppers to partially mimic surface culture methods. Only cells entrapped in Ca-alginate beads and cells adsorbed on CC were studied in this chapter due to high acetification rates observed in previous phases. Due to the working volume used in the current study, inoculum sizes were therefore adjusted accordingly.

3.5.2 Dissolved oxygen and microbial respiration rate quantification

To quantify DO, an oxygen microsensor (Unisense µsensor Opto F1 UniAmp, Aarhus, Denmark) was used at 5-day intervals for both aerated and non-aerated fermentations. The dynamic method was used to measure the respiratory activity/ oxygen uptake rate (OUR) of the microbial consortium of yeasts and AAB (Garcia-Ochoa et al., 2010). This method was performed by stopping the gaseous oxygen flow in the inlets of the batch bioreactors for 5 minutes (Tribe et al., 1995; Singh 1996; Garcia-Ochoa et al., 2010). During that period, the DO was measured using an oxygen microsensor. At this point, the OUR was determined based on the reduction of oxygen after the oxygen was interrupted (Singh 1996; Bandyopadhyay et al., 2009, Garcia-Ochoa et al., 2010). Subsequently, the oxygen was reintroduced into the system while maintaining the same OTR. This method was used with 2 assumptions: (1) the microbial consortium was not affected during the interruption and maintained the same respiratory activity, (2) there was no exchange of oxygen between the liquid and gas phase during the interruption (Garcia-Ochoa et al., 2010).

Furthermore, non-aerated fermentations were also evaluated for respiratory activity with the assumption that low quantities of dissolved oxygen are constantly introduced into the bioreactor through the loose cotton wool stoppers. The oxygen probe was inserted into the bioreactor for 5-10 minutes for each bioreactor to monitor the dissolved oxygen profile/kinetics during several fermentation stages.

3.5.3 Quantifying microbial respiration using the Speedy Breedy

The Speedy Breedy (BACTEST Ltd, Colchester, United Kingdom) was used as an extra tool to compute the respiration activity of the microbial consortium used during BSV fermentation. The Speedy Breedy is a respirometer that measures respiratory activity by recording the changes in pressure as a result of gaseous exchanges caused by microbial respiration in a closed (aerobic or anaerobic) culture chamber (Miller, 2019). To monitor the respiratory activity in the current study, free floating cells (FFC) of yeast and AAB were inoculated into cooked grape must (2 x 1.5 L) using the methods described by Hutchinson et al. (2018) & (2019b). During fermentation, samples were withdrawn and aliquoted into the Speedy Breedy chambers (aerobic) for evaluations. The Speedy Breedy was set at conditions similar to the CC and OWC fermentations. i.e. 28°C and static. Since the Speedy Breedy can only measure respiratory activity for a maximum of 5 days. Samples were therefore withdrawn from the FFC fermentations bioreactors at the following stages: the 1st set was evaluated from day 0 to 4, the 2nd set was day 5 to 10 and the 3rd set was day 11 to 15. This was done in order to evaluate the respiratory activity at different fermentation stages.

3.6 Phase 1, 2, 3 & 4

3.6.1 Batch fermentation setup and sampling

All fermentation treatments (in triplicate) were incubated at 28°C for all phases of the study. Samples were analysed on a weekly basis (5-7 days intervals) for chemical developments, these include sugar consumption (°Brix), alcohol fluctuations (% $^{v}/_{v}$.), total acid formation (g L^{-1}) and pH using a density meter (Density meter DMA 35, Anton Paar, Graz, Austria), Alcolyzer (Alcolyzer Wine M, Anton Paar, Graz, Austria), minititrator (Hanna instruments minititrator HI 84502, Johannesburg, South Africa) and pH meter (Metrohm pH meter 632, Herisau, Switzerland), respectively. All concentrations were converted to g L^{-1} for consistency in reporting where applicable.

3.6.2 Data Handling

Data was analyzed and computed using Microsoft Excel v2016 (Microsoft, Redmond, Washington, DC, USA). Substrate (*S*) (sugar) consumption rates and product (*P*) (acetic acid) formation rates were calculated using Equations (3.1) and (3.2) respectively, with an assumption that the initial reaction rate determines the overall fermentation rate. NB: change in time (*t*) was expressed as *dt* for both equations. Furthermore, relative differences as employed in other studies (Hutchinson et al., 2019a; Saltini & Akkerman, 2012) were calculated using Equations (3.3) and (3.4) in order to show the significance of the differences observed under the different conditions studied. The data from the CATA questions (phase 3) were analysed by correspondence analysis (CA) to produce a bi-dimensional representation (biplot) of the vinegar samples and the relationship between samples and attributes of the CATA questions. The CA was performed using XLSTAT (Version 2015.1.03.15485, Addinsoft, Paris). All

results are an average of three biological repeats accounting for standard deviations which were calculated using Microsoft Excel v2016.

$$r_s = \frac{dS}{dt} \qquad \text{Eq.3.1}$$

$$r_p = \frac{dP}{dt} \qquad \text{Eq. 3.2}$$

$$\text{Absolute difference} = \text{Amount of increase} - \text{Reference amount} \qquad \text{Eq.3.3}$$

$$\text{Relative difference} = \frac{\text{Absolute difference}}{\text{Reference amount}} \times 100 \qquad \text{Eq. 3.4}$$

CHAPTER 4

Cell immobilization by Gel Entrapment in Ca-alginate Beads for Balsamic-styled Vinegar Production

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CHAPTER 4

Cell immobilization by Gel Entrapment in Ca-alginate Beads for Balsamic-styled Vinegar Production

4.1 Introduction

Traditional Balsamic Vinegar (TBV) is produced by fermenting cooked (concentrated) grape must of the grape varieties of Trebbiano or Lambrusco (Solieri et al., 2006; Giudici & Rinaldi, 2007) in barrels. The traditional method involves the use of an undefined/spontaneous inoculum by means of surface culture methods and the vinegar is matured for a minimum of 12 years after fermentation (Solieri et al., 2006; Giudici & Rinaldi, 2007; Solieri & Giudici, 2008; Chinnici et al., 2016). TBV can only be produced in Italy, in the provinces of Modena and Reggio Emilia. Therefore, any balsamic vinegar produced outside the demarcated areas cannot be called TBV. Thus, the term, Balsamic-styled vinegar (BSV) was adopted for the vinegars in this investigation, where submerged methods such as agitation and cell immobilization by gel entrapment are investigated. Cell immobilization refers to the physical confinement or restriction of cells from being mobile or free-floating in the fermentation vessel (De Ory et al., 2004). Several vinegar studies have reported that cell immobilization reduces microbial sensitivity to harsh environmental conditions i.e. high acid, low pH, high sugar, low water activity and osmotic stress in the fermentation vessel, thus improving acetification rates (Kocher et al., 2006; Krusong & Vichitraka, 2011; Kumar et al., 2016; Żur et al., 2016). Cell immobilization is performed using various techniques, which include entrapment in a gel/within a porous matrix, flocculation (natural flocculation/aggregation, artificial flocculation/cross-linking), immobilization on a solid surface (adsorption on a surface, electrostatic binding on a surface or covalent binding on a surface), and mechanical containment behind a barrier (microencapsulation, interfacial microencapsulation, containment between microporous membranes) (Kourkoutas et al., 2004). However, encapsulation within a gel and adsorption to a surface have been the most studied methods in vinegar fermentation systems (De Ory et al., 2004). Regardless of the techniques, cell immobilization has never been studied for any Balsamic vinegar fermentation. Thus, the aim of this study was to immobilize cells by gel entrapment and to evaluate the effect of bead size/surface area and agitation on acetification.

4.2 Objectives

- To investigate two different sizes of Ca-alginate beads (small vs large) for BSV fermentation in order to understand the effect of bead size and surface area.
- To conducting a comparative analysis between static and agitated conditions for Ca-alginate fermentations and FFC treatments.

4.3 Materials and methods

All materials and methods are described in Chapter 3.

4.4 Results and Discussion

4.4.1 Still vs agitated fermentation kinetics

Traditional Balsamic Vinegar (TBV) fermentation is conducted under non-agitated conditions and not submerged in contrast to most industrial vinegars. Submerged methods include the use of technologically advanced systems making use of spargers, stirrers, coolers, antifoams, stainless steel fermentors, including automated control systems (Fernández-Pérez et al., 2010). Therefore, the present study introduced submerged techniques in Balsamic-styled vinegar (BSV) production such as agitation and cell immobilization. Sugar consumption followed a similar trend for both small (4.5 mm) and large (8.5 mm) sized bead fermentations (Fig. 4.1A, D). The consumed sugar was calculated at the initial stages of the fermentation (day 0 to 3). It ranged between 161 to 176 g L^{-1} for all treatments under agitated conditions, while the sugar consumed for all non-agitated fermentations ranged between 31 to 54 g L^{-1} (Fig. 4.1A, D). This indicates that the activity of the yeast consortium used was enhanced by agitation. Alcohol is an intermediary product/substrate in this context and the alcohol kinetics are difficult to explain, due to formation and consumption occurring simultaneously. Figure 4.1B & E shows that alcohol production was higher under agitated conditions compared to still conditions. This phenomenon was also supported by the sugar consumption kinetics (Fig. 4.1A), which is directly proportional to alcohol formation. Alcohol production during the initial stages (day 0 to 3), for agitated conditions was between 88 to 112 g L^{-1}, while the alcohol produced for still fermentations was between 7.3 to 7.6 g L^{-1} for all treatments.

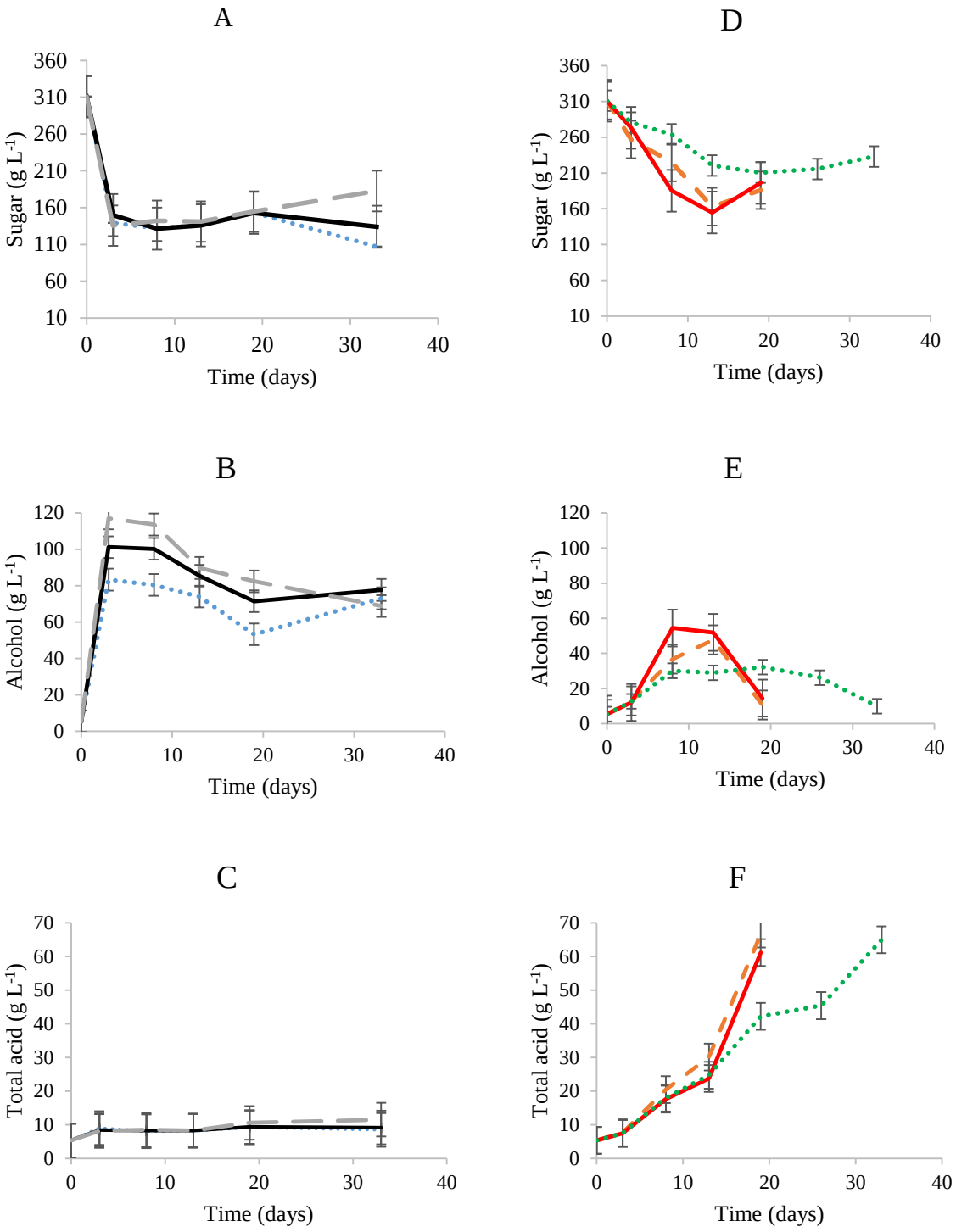
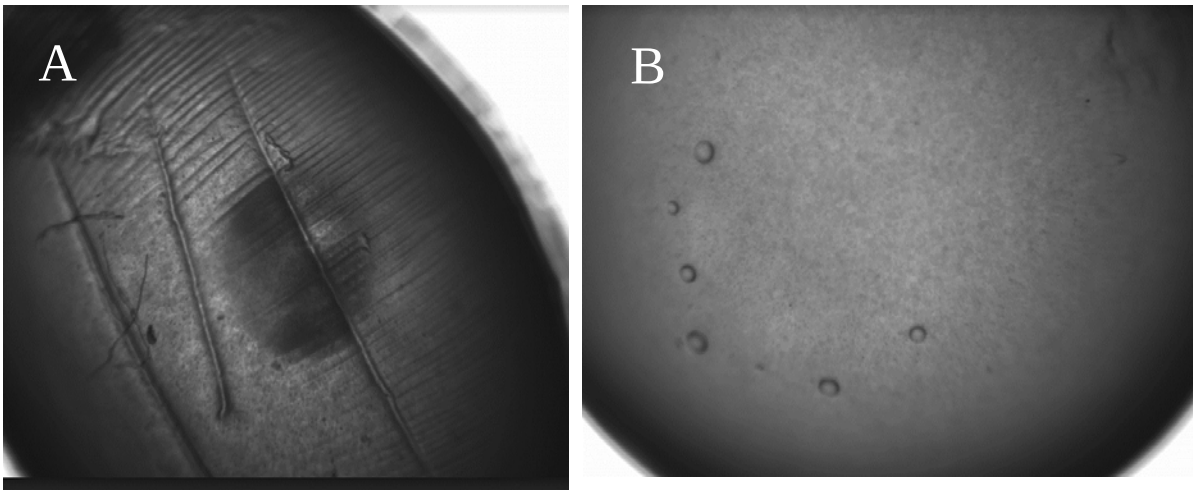


Figure 4.1 Sugar consumption, alcohol and total acid production during the BSV fermentation process. Values are the means of three fermentations.

The acetification rate is the key step in BSV fermentation, as the strength of vinegar is gauged by assessing the final acetic acid concentration. In balsamic vinegar, only 6% (60 g L^{-1}) of acetic acid is required in the final product. Based on the acetification rates observed (Fig. 4.1C, F), it appears that agitation does not favour acetification but rather promotes alcoholic fermentation. The results showed

that agitation negatively affected the acetification process (Fig. 4.1C) and it is possible that the high agitation speed (135 rpm) could have contributed to the reduced acetification profiles. Overall, acetification rates for both agitated (Fig. 4.1C) and still fermentations (Fig. 4.1F) displayed a rather constant and/or exponential acid production pattern throughout the fermentation. The acetification rates for agitated fermentations ranged between 0.1 to 0.2 g L^{-1} day^{-1}, while the rates were 0.6 to 4.0 g L^{-1} day^{-1} for still fermentations. Acetification was negatively affected by agitation (Fig. 4.1C), possibly due to shear stress and therefore requiring still/static conditions for optimum activity. Another possibility is that the high rate of alcohol production might have affected the survival/activity of AAB, as they may have been unable to tolerate high alcohol concentrations. However, Du Toit & Pretorius (2002) reported that AAB are viable at an ethanol concentration of 10 and 14% ($^{v}/_{v}$). Gullo & Giudici (2008) found high strain variability among AAB strains with regard to ethanol tolerance and some AAB strains grew on a 5% ($^{v}/_{v}$) ethanol medium, while others grew on 10% ($^{v}/_{v}$). Agitation may not have been appropriate, using the gel-entrapment immobilization technique, as it causes damage to the beads and perhaps resulting in cell leakage, although cell leakage can also occur in non-agitated fermentations (Tsen et al. 2004). Photographs of the bead surface also support this hypothesis (Fig. 5.4). This damage (Fig. 4.2A, C) prevented the re-usage of gel beads for subsequent fermentations. Non-agitated beads (Fig. 4.2B) appeared smoother with microscopically visible porosity (Fig. 4.2D), than those that were agitated (Fig. 4.2A, C).

Agitated beads **Non-agitated beads**



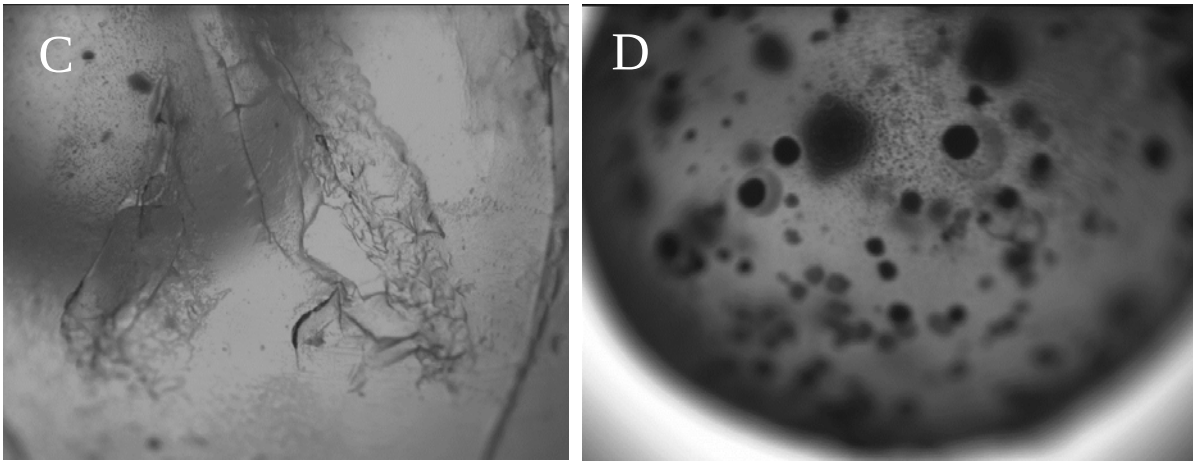


Figure 4.2 External surface photographs of agitated and non-agitated Ca-alginate beads after fermentation. (**A**) Agitated bead at 100x magnification (**B**), non-agitated bead at 100x magnification, (**C**), agitated bead at 400x magnification (**D**), non-agitated bead at 400x magnification.

4.4.2 Immobilized vs free floating cell fermentation kinetics

Cell immobilization by gel entrapment for BSV fermentations was investigated simultaneously with free-floating cell fermentations. Based on the results of fermentation kinetics, cell immobilization improved acetification rates under still conditions (Fig. 4.1F). All the fermentation kinetics were negatively affected by agitation (Fig. 4.1C) and are therefore not discussed in this section. Sugar consumption (Fig. 4.1D) followed a similar trend for both small (4.5 mm) and large (8.5 mm) sized bead fermentations, with less sugar being consumed during free-floating cell fermentations. Sugar consumption (Fig. 4.1D) was directly proportional to the alcohol produced (Fig. 4.1E). Improved total acid formation was achieved using immobilized cell fermentations compared to free-floating cells (Fig. 4.1F) while small beads were more efficient (4.0 g L^{-1} day^{-1}) than large sized beads (3.0 g L^{-1} day^{-1}) and free-floating cells (0.6 g L^{-1} day^{-1}). Furthermore, 60 g L^{-1} of acetic acid was achieved after 16, 19 and 31 days for small sized, large sized beads and free-floating fermentations, respectively. Kocher et al. (2006) investigated adsorption on corncobs, bagasse and wood shavings and cell entrapment in 4% (w/v) sodium alginate for sugar cane vinegar and found that adsorbed cells resulted in relatively higher acetification rates (0.09-0.10 g L^{-1} h^{-1}) compared to entrapped cells (0.07 g L^{-1} h^{-1}) and free-floating cells (0.06 g L^{-1} h^{-1}). These authors suggested that lower acetification rates by the entrapped cells may be caused by a low surface area, which leads to inadequate substrate and dissolved oxygen transfer rates.

4.5 Summary

Based on the current results, agitation of BSV fermentations is disadvantageous for the process system and optimisation of the agitation settings is necessary. Non-agitated (static) fermentations were effective, with entrapped cells resulting in higher acetification rates. The surface area of the beads also

affects the acetification rates. Therefore, immobilized cells using small beads without agitation are recommended for BSV production. More research work on the effect of air and oxygen transfer rate during BSV fermentations is recommended.

CHAPTER 5

Aeration, Agitation and Cell Immobilization on Corncobs and Oak Wood Chips Effects on Balsamic-Styled Vinegar Production

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CHAPTER 5

Aeration, Agitation and Cell Immobilization on Corncobs and Oak Wood Chips Effects on Balsamic-Styled Vinegar Production

5.1 Introduction

Similar to other vinegars, balsamic vinegar is a food flavouring agent or condiment, which contains acetic acid as its main ingredient (Solieri & Giudici, 2009). Balsamic vinegar is characterized by its sour and sweet taste (Giudici et al., 2009) and can be used as a salad dressing, and for soothing sore throats among other uses (Truta et al., 2009). Balsamic-styled vinegar (BSV) is a term adopted from Traditional Balsamic vinegar (TBV) of Modena and Reggio-Emilia Italy. BSV is a new type of balsamic vinegar which has potential for being produced in South Africa using Chenin blanc wine grapes, since they are the most cultivated grape cultivar in South Africa (SAWIS, 2018).

Cell immobilization generally refers to a technique, which is performed to prevent cells from being freely suspended in the fermentation medium (De Ory et al., 2004; Kumar et al., 2016; Żur et al., 2016). Cell immobilization improves biomass growth, increases cell stability, protects cells from the toxic environment, and provides a reusability option (Kocher et al., 2006). Most fermentations generally employ the free-floating cell (FFC) method, which is effective in less stressful fermentation systems. However, during balsamic vinegar fermentation, there are multiple factors that have antagonistic effects on the microorganisms involved in the fermentation. For instance, the fermentation medium (cooked grape must) used for Balsamic-styled vinegar has a very high sugar content, low water activity and high osmotic pressure (Solieri & Giudici, 2008; Gullo & Giudici, 2008; Tofalo et al., 2009). Furthermore, the high concentrations of acetic acid during fermentations can also have a negative impact on the microbial consortium used (Sousa et al., 2012; Giudici et al., 2015). These environmental stresses can cause the microbial consortia to enter the viable but non-culturable state, resulting in reduced microbial activity (Oliver, 2000; Mamlouk & Gullo, 2013). Consequently, to counteract these effects, cell immobilization is an ideal approach, which can minimize the impact of stressful conditions on the microorganisms used.

Various methods are used to perform cell immobilization. These include entrapment in a gel matrix, immobilization on a solid surface and mechanical containment behind a barrier (Kourkoutas et al., 2004); however, to date, numerous studies focus on cell immobilization via gel entrapment or immobilization on a solid surface (adsorption) (De Ory et al., 2004). In Balsamic-styled vinegar fermentation, cell immobilization has previously been studied using the gel entrapment technique (Hutchinson et al., 2018), which is generally an expensive technique. However, cell immobilization by

adsorption in combination with aeration and agitation effects has never been tested for balsamic vinegar production. Therefore, in this current study, cell immobilization on corncobs and oak wood chips was investigated using the adsorption technique, which is commonly classified as a simple and often cost-effective method depending on the support material used (Kocher et al., 2006).

Corncobs are an inexpensive and abundant agricultural by-product obtained from the corn-milling process. They are currently used as animal feed, with 80% of their dry matter being comprised of cellulose and hemicellulose (Inglett, 1970; Vaughan et al., 2001; Sun & Cheng., 2002; Ruengruglikit & Hang, 2003; Shen et al., 2008). These aforementioned characteristics impart unique attributes that brand corncobs as an ideal material for cell immobilization. On the other hand, oak wood chips are abundantly used as an alternative to wooden barrel aging in the wine industry for economic reasons. They are known to improve the sensorial qualities of the final product due to the flavours they impart to wines (Gutiérrez Afonso, 2002; Kyraleou et al., 2015; Laqui-Estaña et al., 2019). For this reason, the current study selected oak wood chips, based on both their cell immobilization properties and potential impartation of organoleptic characteristics to the BSV. Nevertheless, oak wood chips are usually imported to South Africa and they cost more than corncobs. Consequently, this study investigated readily available inert materials which can be obtained at varying cost for cell immobilization, in order to allow producers to make an informed decision in achieving the desired BSV quality. Furthermore, the study explored the capabilities of the selected materials for cell affinity to the adsorbents selected and fermenter performance in both aerated and agitated cultures systems.

5.2 Objectives

- To compare the influences of the adsorption matrices (CC and OWC) selected against FFC fermentations for cell affinity and acetification rates during BSV fermentation.
- To testing the effects of aeration and agitation on adsorbed cells and FFC during BSV fermentation.
- To evaluating the variations in cell adsorption capabilities on CC and OWC for individual yeasts and AAB species.

5.3 Materials and methods

All materials and methods are described in Chapter 3.

5.4 Results and discussion

5.4.1 Non-Aerated Fermentations

5.4.1.1 Static vs. Agitated Fermentations

TBV fermentation is normally carried-out under static conditions using bacteria. This process is slow and inexpensive (Giudici et al., 2009); however, a rapid fermentation period for TBV is not a

fundamental objective, since TBV is matured for a minimum of 12 years after fermentation (1), while BSV can be sold without ageing.

Rapid vinegar production is a major goal for industrial processes. BSV can also be produced rapidly if the conditions are ideal for the microorganisms used. For the purposes of this study, it was important to evaluate the effects that agitation could have on BSV production in contrast to stationary fermentations. Agitation is also recommended for spirit industrial vinegar production to generally shorten the fermentation period (González-Sáiz et al., 2009). In the current study, the sugar consumption rates for all treatments under agitated conditions ranged between 6.36 and 7.12 g L^{-1} day^{-1} whereas 2.68 and 6.10 g L^{-1} day^{-1} were observed under static conditions. The relative differences for sugar consumption between FFC and immobilized cells were calculated to be 14% (OWC and FFC) and 41% (CC and FFC) under static conditions, while the relative differences under agitated conditions were calculated to be 5% (OWC and FFC) and 9% (CC and FFC) under static conditions. The ethanol production step of the process was more effective under agitated conditions, as compared to static conditions (Fig. 5.1A, D). However, ethanol formation/consumption rates were not calculated, since alcohol is an intermediate product/substrate which is produced and consumed simultaneously. Furthermore, acetic acid production was unsatisfactory for all treatments under agitated conditions (Fig. 5.1F). Agitation resulted in acetification rates ranging from 0.11 to 0.13 g L^{-1} day^{-1}, with the highest acetic acid concentration of 10.7 g L^{-1} being achieved at day 35. However, for a successful BSV fermentation, an acetic acid concentration of at least 50 g L^{-1} is usually required (Gullo & Giudici, 2008). Hutchinson et al. (2018) made similar observations when using cell immobilization with calcium alginate beads for BSV production. It is unclear if these observations were species (acetic acid bacteria) dependent or due to the agitation settings used. In light of these observations, other vinegar studies have successfully applied agitation speeds between 100 (Talabardon et al., 2000) and 200 rpm (Horiuchi et al., 2000), with industrial spirit vinegars successfully employing maximum agitation up to 900 rpm (González-Sáiz et al., 2009; Park et al., 1989; Pochat-Bohatier et al., 2003). This suggested that the current agitation setting could not have negatively impacted the microbial activity in the fermentations. Furthermore, most vinegar production systems generally employ agitation and aeration simultaneously (Pochat-Bohatier et al., 2003; Schlepütz et al., 2013); therefore, it is important to consider that agitation might only be beneficial under such settings.

Fig. 5.1C shows successful acetification/alcohol oxidation profiles for all treatments under static fermentations. Total acid formation rates ranged from 1.46 and 2.70 g L^{-1} day^{-1} for all of the treatments studied. The relative differences for total acid formation between agitated and static fermentations were 93%, 92% and 95% for FFC, OWC and CC, respectively. Furthermore, the low substrate (sugar) consumption rates (2.68–6.10 g L^{-1} day^{-1}) resulted in much lower alcohol formation rates (Fig. 5.1B) but high total acid formation rates (Fig. 5.1C). These observations could mean that the AAB microbial

activity was ideal at low alcohol concentrations, suggesting that the agitation speed should be optimized with the aim of reducing alcohol formation rates, in order to benefit the AAB used. Furthermore, if agitation is not suitable for BSV fermentations, regardless of the speed, this might benefit producers, because agitation is energy intensive and the cost input might escalate, not only due to the energy usage but also due to the mechanical equipment required for agitation.

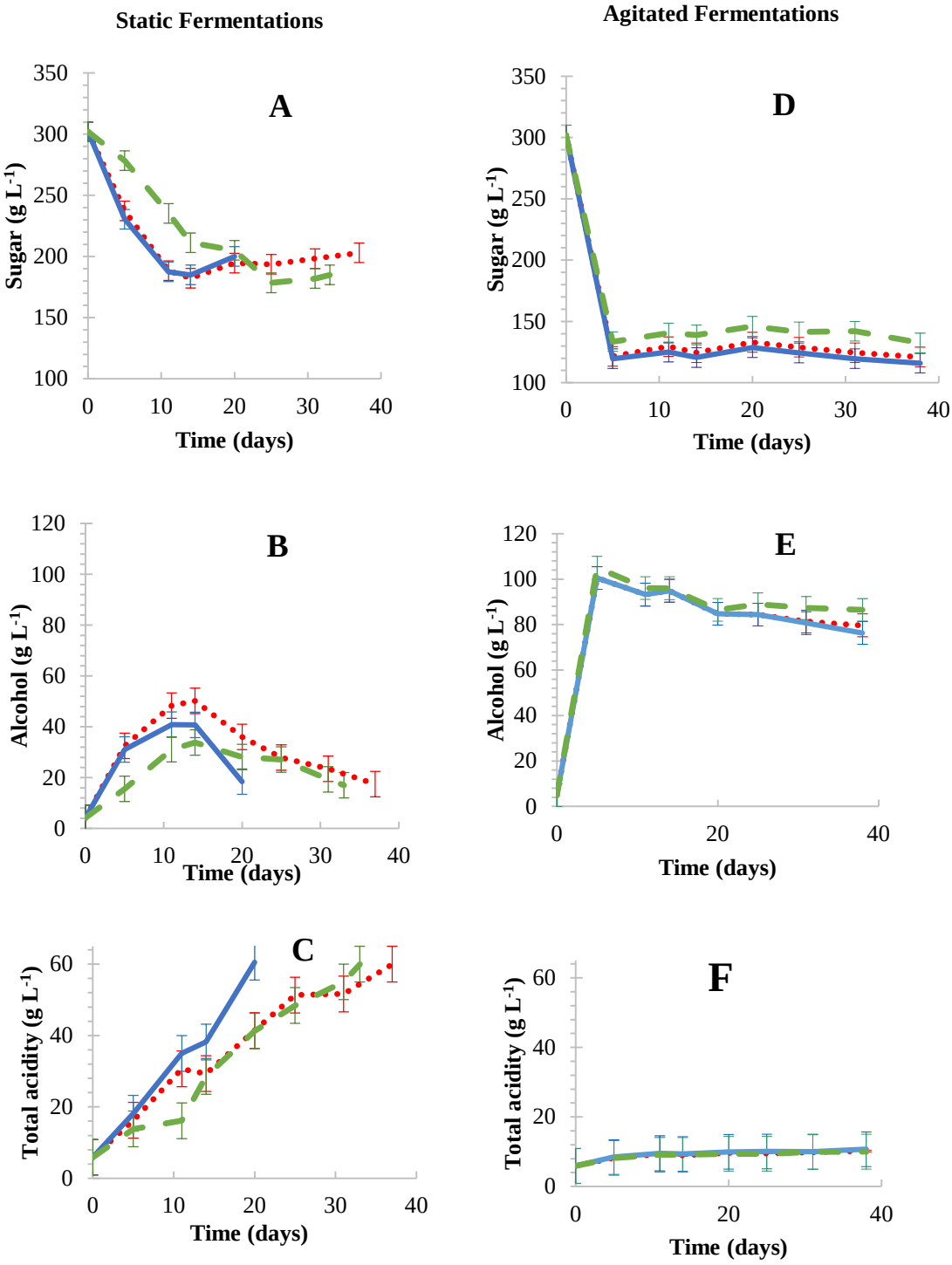


Figure 5.1 Chemical developments under static (**A**–**C**) and agitated (**D**–**F**) conditions. Sugar (**A**, **D**), alcohol (**B**, **E**) and total acid (**C** ,**F**) developments during fermentation. ····· Oak wood chips, ——— Corncobs, - - - Free-floating cells. Results are the average of three biological repeats accounting for standard deviation.

5.4.1.2 Effect of the Adsorbents Used

The adsorption technique for cell immobilization has been studied using several inert materials for various vinegar production systems (Talabardon et al., 2000; Horiuchi et al., 2000; Kennedy et al., 1980; Ghommidh et al., 1982). In the current study, CC were compared to OWC for cell affinity and

influence on BSV production. These materials differ in terms of their biological composition as they have different physical and structural attributes, as well as adsorption capacity and surface chemistry.

Since agitated fermentations did not meet the required performance for BSV production, i.e., achieving 60 g L^{-1} acetic acid, a more comprehensive discussion is provided in this section on the fermentations that were only conducted under static conditions. Additional reasons attributed to the unsuccessful agitated fermentations, which were characterized by low acetification rates (0.11 to 0.13 g L^{-1} day^{-1}) may be due to the interference of cell adsorption by agitation conditions, which eventually resulted in freely suspended cells.

Moderately higher sugar consumption rates of 8.14 and 5.14 g L^{-1} day^{-1} for OWC and CC, respectively, were observed at the initial stages (day 0–14) of the fermentation process (Fig. 5.1A). Alcohol formation rates were also moderately higher for CC and OWC fermentation than FFC fermentations (Fig. 5.1B). While, total acid formation for cells immobilized on CC was consistently higher throughout the fermentation process, with total acid formation rates of 2.7 g L^{-1} day^{-1} being achieved in the shortest fermentation period (20 days). Comparatively, FFC and OWC fermentations resulted in lower total acid formation rates of 1.64 and 1.46 g L^{-1} day^{-1} with a longer fermentation period of 33 and 37 days, respectively. Furthermore, the relative differences between FFC and immobilized cells were 11% (OWC and FFC) and 39% (CC and FFC). Therefore, it was deduced that cells adsorbed on CC fermentations led to the highest microbial activity because of the adsorbents' attributes, i.e., rough surface and porosity, which improved the adsorption of cells. As a result, the microbial consortium was able to form a highly effective community when adsorbed onto the CC. Contrarily, the OWC are relatively smoother than the porous CC, which consequently led to the poor adsorption of cells.

Cell immobilization on CC was also studied for the production of tea vinegar, with acetification rates of 2.88 g L^{-1} day^{-1} being obtained (Kaur et al., 2011). Several studies often use wood shavings for cell immobilization instead of OWC. For example, Thiripurasundari and Usharani (Thiripurasundari & Usharani, 2011) and Kocher and Dhillon (2013) reported acetification rates of 0.24 g L^{-1} day^{-1} and 5.76–22.8 g L^{-1} day^{-1} when using wood shavings for the production of cashew apple vinegar and sugar cane vinegar, respectively. It is not clear as to the reasons why the acetification rates for these two studies were different; however, other factors such as substrate availability, bacteria strain used and other fermentor conditions might have played a role. Furthermore, wood shavings and OWC may not be comparable materials due to their differentiated physical properties, and thus, differentiated adsorption capabilities.

Table 5.1 shows a comparison of other studies which investigated the effect of cell immobilization by adsorption in other vinegar production systems. The current work established a direct relationship

between cell immobilization and higher acetification rates. Similarly, other studies (Krusong & Vichitraka, 2011) also reported a proportional relationship between cell immobilization and acetification rates, with the fibrous bed being reported to show a conspicuous increase in acetification compared to FFC (Table 5.1). Furthermore, when a loofa sponge was used, it led to increased acetification rates (Krusong et al., 2014) which were similar to the rates observed in the current study at static conditions.

Table 5.1. A comparison of the performance of immobilized cells in other studies.

Product	Adsorbent	Agitation	Acetification Rate ($g\ L^{-1}\ day^{-1}$)	Reference
Balsamic-styled vinegar	Corncobs Oak wood chips	135 rpm	FFC: 0.11 CC: 0.13 OWC: 0.11	Current study
Acetic acid from lactose and milk permeate	Fibrous-Bed/matrix	100 rpm	FFC (lactose): 1.44 FFC (milk permeate): 1.92 IC (Lactose): 12.96 IC (milk permeate) 7.2	(Talabardon et al., 2000)
Rice wine vinegar	Loofa sponge	1 Hz reciprocating shaking rate = 60 rpm	IC: 1.68–2.4	(Krusong et al., 2014)

IC: immobilized cells; CC: Corncobs; FFC: free-floating cell; OWC: oak wood chips.

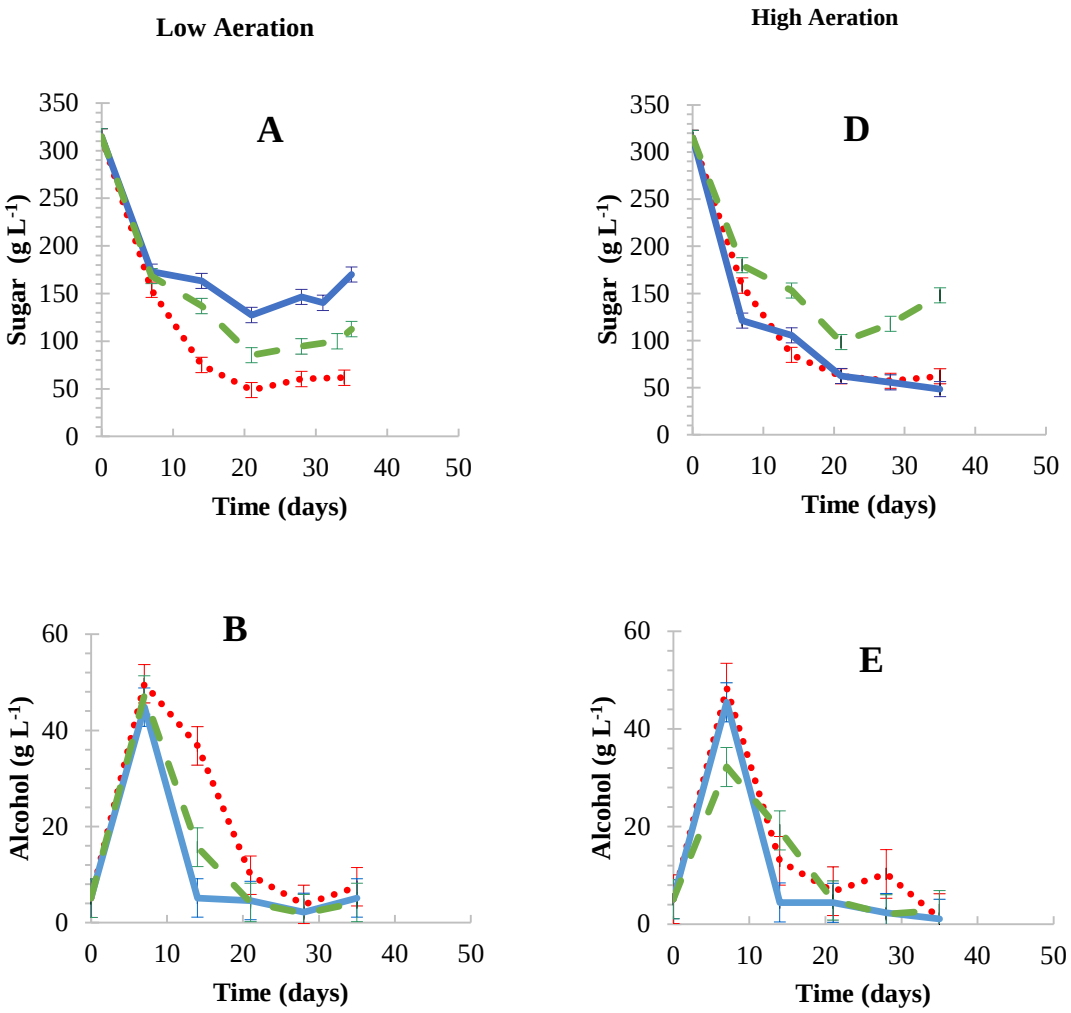
Table 5.1 also shows that agitation speeds of 100 rpm, including reciprocating shaking speed of 60 rpm were employed effectively in other studies. These findings illustrate that agitation may also be dependent on several other factors, such as the microorganisms used, the type of vinegar being produced and other fermentor conditions.

5.4.2 Aerated Fermentations

5.4.2.1 Effect of Aeration Rates

Aeration is generally a fundamental aspect in most vinegar fermentation systems (Fregapane et al., 1999; 2004). Aeration systems vary between transferring pure oxygen or air, with pure oxygen systems being noted as expensive due to the cost of technical grade oxygen (Ouyang et al., 2018) and deleterious effects such as hyperoxia which leads to cellular death. For this study, aeration with air only was investigated as it was deemed cost effective. It was observed that both high aeration (HA) and low aeration (LA) led to similar alcohol formation/consumption profiles (Fig. 5.2 B, E). The observations

were surprising since alcoholic fermentation is often performed under anaerobic conditions or low dissolved oxygen conditions (Bamforth, 2000; Hiralal et al., 2014). Consequently, yeast performance was expected to also be negatively affected by HA, albeit that yeasts are generally characterized as facultative anaerobes and some yeasts can still survive or grow under aerobic conditions (Watson et al., 1978; Bekatorou et al., 2006; Chatre et al., 2014; Boss & Day, 2016). Since only air (with 21% oxygen) was used, it was also plausible that different alcohol consumption profiles could have been observed if technical grade oxygen was tested—a condition that can exacerbate hyperoxia.



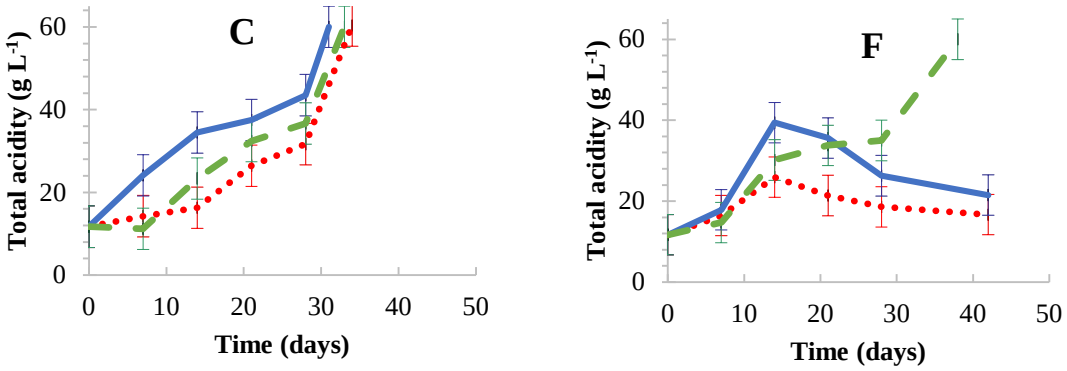


Figure 5.2 Chemical developments under low (**A**–**C**) and high (**D**–**F**) aeration. Sugar (**A**, **D**), alcohol (**B**, **E**) and total acid (**C**, **F**) developments during fermentation. ----- Oak wood chips, ——— Corncobs, – – – Free-floating cells. Results are the average of three biological repeats ± standard deviation.

Nevertheless, total acid formation, which is a key step in BSV production was higher (1.42–1.56 g L^{-1} day^{-1}) at LA (Fig. 5.2C) as compared to HA (0.14–1.2 g L^{-1} day^{-1}) (Fig. 5.2F) for cell immobilized treatments. However, FFC completed the fermentations under both aeration settings with acetification rates of 1.27 and 1.46 g L^{-1} day^{-1} for HA and LA, respectively. Additionally, over oxidation has been reported as a common challenge in most vinegar production systems (10). It seems unlikely that the excessive/increased oxidation rates may have affected the fermentations in this study; since the alcohol profiles under HA and LA conditions (Fig. 5.2B, E) were similar to some degree. Other studies reported higher acetification rates compared to the current study when aeration was employed. Rubio-Fernández et al. (2004) reported acetification rates of 17.24 and 32.4 g L^{-1} day^{-1} for wine vinegar production when air and oxygen rich air (0.06 vvm) were used respectively. Additionally, Qi et al. (2013) reported acetification rates of 43.44 g L^{-1} day^{-1} for industrial vinegar production when oxygen (0.13 vvm m^{-1}) was used. It is important to mention that the observations between the current study and other studies are influenced by several factors, which include media composition and the AAB strains used. The most obvious influential factor is that BSV fermentation involves high sugar concentration/high osmotic pressures with the simultaneous involvement of non-*Saccharomyces* yeasts and AAB. Furthermore, spirit vinegar production is generally faster than the production of other vinegars. It is unfortunate that there are no similar BSV studies with which to compare the current data, due to the traditional techniques used for balsamic vinegar production.

5.4.2.2 Performance of Adsorbents Used Under Aerated Conditions

When cells adsorbed on the different materials were evaluated at the initial stages of fermentation, sugar consumption showed similar profiles with sugar consumption rates between 20.15 to 27.2 g L^{-1} day^{-1} under both aeration (HA and LA) settings for all treatments (Fig. 5.2A, D). After 7 days, sugar consumption at LA was higher for OWC fermentations with rates between 3.32 g L^{-1} day^{-1} followed

by FFC and CC with a sugar consumption rate of 1.80 and 0.11 g L^{-1} day^{-1}respectively (Fig. 5.2A). On the other hand, HA, CC and OWC fermentations led to relatively similar results, as well as the highest sugar consumption rates (2.70–3.46 g L^{-1} day^{-1}) compared to FFC (1.28 g L^{-1} day^{-1}) (Fig. 5.2D). The lowest sugar consumption rates were observed in FFC fermentations under HA with trends that we cannot compare with any previously available literature. However, it is possible that the activity of freely suspended yeast cells under HA was reduced by AAB FFC that eventually led to a higher production of acetic acid. Overall, sugar consumption rates were 4.14, 5.78 and 7.4 g L^{-1} day^{-1} under LA and 7.62, 4.82 and 7.23 under HA for CC, FFC and OWC, respectively. The relative differences for sugar consumption between LA and HA fermentations were 2%, 17% and 46% for OWC, FFC and CC; respectively. Furthermore, alcoholic fermentation was successful on all immobilized and FFC fermentations for both aeration settings. Alcohol formation/consumption profiles were moderately similar for all treatments studied under both aeration settings.

Total acid formation provided an insight on the productivity of the process evaluated. It was evident, that CC fermentations had the highest total acid formation at LA, followed by FFC and then OWC fermentations. The differences observed were minor, since the total formation rates were 1.56, 1.46 and 1.42 g L^{-1} day^{-1} with a fermentation period of 31, 33 and 34 days for CC, FFC and OWC fermentations, respectively. Under HA, only FFC fermentations completed the fermentation with an acetification rate of 1.2 g L^{-1} day^{-1} in 38 days, while CC and OWC fermentations initiated by showing an increase in total acid subsequent to its decrease from day 14 to 42. It was not well understood as to how the FFC fermentations were able to reach the required levels of total acid concentration. A credible assumption could be that HA may have disrupted the adsorbed cells, thus causing and maintaining a low microbial activity. Overall, based on the results obtained, it was evident that agitation and HA did not favor the production of BSV, where cell immobilization by adsorption was used.

5.4.3 Variations in Cell Adsorption Capabilities among the Yeast/Bacterial Species Used

5.4.3.1 Individual Yeast Adsorption on Corncobs and Oak Wood Chips

The phenomenon of adsorption efficiency is critical to the current study. The adsorption by different microorganisms varied due to cell affinity differences to the adsorbents surface used. However, it is possible that microorganisms employed will adsorb differently when tested as a consortium. With regards to yeast adsorption on CC, it was observed that *C. zemplinina* had the lowest number of cells adsorbed prior and post fermentation. Z. *bailii* initially exhibited low cell adsorption before fermentation; however, a relatively higher cell adsorption (92.93%) after fermentation was observed. Yeasts species such as *C. pulcherrima, H. guilliermondii* and *K. apiculata* showed a noticeably high cell adsorption prior and post fermentation (Table 5.2).

Furthermore, yeast cell adsorption was different for both OWC and CC. Some yeast species such as *C. zemplinina* (59.54%) and *Z. bailii* (28%) showed a relative decrease in cell adsorption post fermentation (Table 5.2). The trends showed that *C. zemplinina* and *Z. bailii* might have low cell affinity to the smooth 'easy-to wash-off' OWC surface during the fermentation compared to CC surfaces. On the other hand, *C. pulcherrima* (87.78%), *H. guilliermondii* (3.00%) and *K. apiculata* (48.67%) showed a relative increase in cell adsorption capacity during the fermentation, with *H. guilliermondii* showing the lowest adsorption. Overall, the cells adsorbed ($g \cdot g^{-1}$) on CC and OWC were not comparable since they have different densities, including surface chemistry properties.

5.4.3.2 Individual Bacteria Adsorption on Corncobs and Oak Wood Chips

The adsorption of bacteria onto CC and OWC was also evaluated (Table 5.3). *A. pasteurianus* and *A. malorum* appeared to have the highest (0.1461 and 0.095 $g \cdot g^{-1}$, respectively) cell adsorption efficiency on CC before fermentation (Table 5.3). *K. baliensis, G. cerinus* and *G. oxydans* had relatively similar adsorption efficiency on CC before fermentation, with relatively low variations (97.76%, 99.26%, 96.12%, respectively) observed post fermentation. Similar decreases in adsorption efficiency patterns on OWC were observed between *A. malorum* and *K. baliensis* (Table 5.3). *A. pasteurianus* had the lowest initial cell adsorption (0.0020 $g \cdot g^{-1}$) before fermentation; however, it had the highest percentage cell increase (98.87%) after fermentation. *G. cerinus* and *G. oxydans* showed a similar pattern before and after fermentation, with 52.40% and 56.33% in cell adsorption after fermentation. Other studies have investigated the cell adsorption on CC and wood shavings; however, these studies did not report the number of cells adsorbed on the adsorbents used, which leads to the lack of comparative data.

Overall, these results showed a successful adsorption on CC and OWC for both yeasts and bacteria, but with varying adsorption efficiency. However, the data also displayed an unstable cell adsorption profile on OWC, since a decrease in cell adsorption was observed for some yeast and bacterial species. The data further suggested a more sustainable approach on the reusability of immobilized cells on CC and OWC treatments.

Table 5.2. Evaluation of yeast cells adsorbed on corncobs and oak wood chips.

		Yeast Cells Adsorbed on Corncobs			Yeast Cells Adsorbed on Oak Wood Chips		
Identity	**Cell Concentration YPD Broth (Cells·mL^{-1})**	**Before Fermentation (g·g^{-1})**	**After Fermentation (g·g^{-1})**	**Relative Difference (%)**	**Before Fermentation (g·g^{-1})**	**After Fermentation (g·g^{-1})**	**Relative Difference (%)**
Candida pulcherrima	2.34×10^5	0.0371	1.1580	96.80	0.0214	0.1751	87.78
Candida zemplinina	7.40×10^5	0.0006	0.0290	97.93	0.0903	0.0566	−37.32
Hanseniaspora guilliermondii	7.70×10^5	0.0110	0.7906	98.61	0.1001	0.1032	3.00
Kloeckera apiculata	1.95×10^6	0.0283	1.6955	98.33	0.0926	0.1804	48.67
Zygosaccharomyces bailii	3.97×10^5	0.0400	0.5661	92.93	0.1785	0.1393	−21.96

Table 5.3. Evaluation of bacterial cells adsorbed on corncobs and oak wood chips.

		Bacteria Cells Adsorbed on Corncobs			Bacteria Cells Adsorbed on Oak Wood Chips		
Identity	**Cell Concentration GM Broth (Cells·mL^{-1})**	**Before Fermentation (g·g^{-1})**	**After Fermentation (g·g^{-1})**	**Relative Difference (%)**	**Before Fermentation (g·g^{-1})**	**After Fermentation (g·g^{-1})**	**Relative Difference (%)**
Acetobacter pasteurianus	3.97×10^5	0.1461	1.1968	87.79	0.0020	0.1772	98.87
Acetobacter malorum	1.25×10^6	0.0948	1.0882	91.29	0.1851	0.1545	−16.53
Kozakia baliensis	3.13×10^5	0.0260	1.1589	97.76	0.1224	0.1122	−8.33
Gluconobacter cerinus	2.22×10^6	0.0116	1.5618	99.26	0.1498	0.3147	52.40
Gluconobacter oxydans	6.38×10^5	0.0426	1.0977	96.12	0.1008	0.2308	56.33

5.5 Summary

According to all the results obtained, it is evident that cell immobilization improves acetification rates during BSV fermentation. However, the highest acetification rates were obtained under static and non-aerated conditions. Corncobs were observed to be the most suitable material for cell immobilization presumably due to their physical structure. Consequently, the shortest fermentation period was 20 days when cells were immobilized on corncobs under static fermentation conditions. A study to investigate the concurrent optimization of agitation and aeration as well as the effect of corncobs and oak wood chips on organoleptic properties is recommended. The current study therefore, serves as a foundation for cell immobilization by adsorption on materials during Balsamic-styled vinegar production.

CHAPTER 6

Reusability and sensory effects of immobilized cells for subsequent balsamic-styled vinegar fermentations

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CHAPTER 6

Reusability and sensory effects of immobilized cells for subsequent balsamic-styled vinegar fermentations

6.1 Introduction

Balsamic-styled vinegar (BSV) is a derivative form of traditional balsamic vinegar (TBV), which was specifically developed for this study. The production of TBV follows strict regulations and generally employs conventional methods, which lead to substantially longer fermentations in contrast to submerged methods (Tesfaye *et al*., 2002; Fernández-Pérez *et al*., 2010). However, other generic balsamic vinegars do not have to adhere to such strict regulations and conventional methods do not apply. Therefore; BSV can be successfully produced using numerous techniques including process performance augmentation strategies such as cell immobilization as demonstrated by Hutchinson et al. (2018) & (2019b). Since TBV's are premium quality and highly priced products (Tesfaye *et al*., 2002; Fernández-Pérez *et al*., 2010) which most consumers cannot afford, other forms of balsamic vinegars for regular usage can be opted for. Therefore, BSV's can be produced at large scale in order to supply to a consumer base. Consequently, it is crucial to investigate methods that can improve the quality of BSV, while optimising the fermentation process for industrial scale operations.

Previously, the effects of cell immobilization using Ca-alginate beads, corncobs (CC) and oak wood chips (OWC) for BSV production were studied (Hutchinson et al., 2018 & 2019b). However, the reusability of the immobilized cells was not evaluated, which is the primary focus of the current study because optimal process performance at low cost input, which results in rapid fermentations, is fundamental in the commercialisation of BSV. Therefore, if recycled immobilized cells retain a high microbial activity, while retaining high product formation rates, this can reduce overall input costs with substantial monetary savings in large scale operations. Furthermore, it is probable that the reusability and the performance of immobilized cells could vary depending on the immobilization matrix or the technique that was used to immobilize the cells. In the current study, cells were immobilized using both adsorption on CC and OWC, and entrapment in Ca-alginate beads. These two cell immobilization techniques are different and therefore the resultant fermentations were expected to culminate in differentiated reusability outcomes; albeit, not previously reported for BSV fermentations.

Generally, the materials studied for cell adsorption are not only different with regards to their physical structure and origin, they could also affect the sensorial attributes of the final BSV. As such, investigating the organoleptic properties of the vinegars was deemed as a rational procedure.

Corncobs are an agricultural by-product obtained from the corn milling process, with 80% of their dry matter being composed of cellulose and hemicellulose (Vaughan et al., 2001; Sun & Cheng., 2002; Shen et al., 2008), while OWC are commonly used for flavour improvement in the wine industry to impart the woody attributes in wine (Kyraleou et al., 2015; Laqui-Estaña et al., 2019). Polymetric matrices such as Ca- and Na-alginate are made from brown algae extracts and are known for their viscosifying, gelling and stabilizing properties with diverse applications in the food, beverage, cosmetics, textile and pharmaceutical industries (Hecht & Srebnik, 2016). Therefore, the data generated from this study can allow large scale BSV producers to make informed decisions when choosing the immobilization matrix and technique to optimise production rates, organoleptic attributes and cell reusability while minimising operational costs.

For high cell retention and fermenter performance, cell immobilization has been frequently reported to have several advantages, with the option of reusing/recycling the immobilized cells being the prominent advantage (Park et al., 1999; Kocher et al., 2006; Rahman et al., 2006; Rao et al., 2008; Nigam et al., 2009, Shyamkumar et al., 2014). However, very limited studies have been done to support the reusability of immobilized cells, mostly for balsamic vinegar fermentation systems. Furthermore, most studies reporting on the recycling of immobilized cells are based on one step fermentation systems, whereby one species converts a single substrate directly into the final product (Park et al., 1999; Rahman et al., 2006; Rao et al., 2008; Nigam et al., 2009, Shyamkumar et al., 2014). However, BSV fermentation is a two-step process; whereby sugar serves as the initial substrate for non-*Saccharomyces* yeast to ferment to an intermediate product, i.e. alcohol, while the alcohol also serves as a substrate for the production of acetic acid by acetic acid bacteria (AAB). This means that both the non-*Saccharomyces* yeast and AAB immobilized cells must survive the initial cycle of fermentation in order to be reused in successive fermentations. For this specific reason, this study investigated the reusability of a consortium of immobilized non-*Saccharomyces* yeasts and AAB for BSV production. The recycled cells were entrapped in calcium (Ca)-alginate beads and with adsorption being on corncobs (CC) and oak wood chips (OWC).

6.2 Objectives

- To evaluate the reusability abilities of immobilized cells for subsequent cycles of fermentation.
- To study the structural integrity of cell immobilization matrices after being reused using the scanning electron microscope.
- To evaluate the BSV's for sensorial attributes.

6.3 Materials and methods

All materials and methods are described in Chapter 3.

6.4 Results and discussion

6.4.1 Comparative analyses of sugar consumption rates for 1st and 2nd cycle BSV fermentations

6.4.1.1 1st cycle fermentations

The 1st cycle of the fermentations was successfully completed (Fig. 6.1) with sugar consumption rates being relatively similar for all fermentations studied. These results are similar to reports of Hutchinson et al. (2018; 2019b) (Chapter 4 & 5), Fermentations carried-out using cells adsorbed onto OWC had the highest sugar consumption rates (4.97 g L^{-1} day^{-1}), followed by Ca-alginate beads and CC resulting in the lowest sugar consumption rates (Fig. 6.1).

6.4.1.2 2nd cycle fermentations

The 2nd cycle fermentations had considerably lower sugar consumption rates compared to the 1st fermentation cycle (Fig. 6.1). A decline of sugar consumption when immobilized cells were reused was calculated at relative differences of 94.7%, 56.2%, 31.7% for CC, OWC and Ca-alginate beads, respectively. Overall, the Ca-alginate beads had the lowest differentiation in sugar consumption rates when cells entrapped in the beads were reused (Fig. 6.1). Results indicate that Ca-alginate beads serve as a better matrix for the protection of consortium cells compared to the other matrices studied. A logical explanation behind this could also be due to the fact that when the entrapment technique is used, cells are essentially encapsulated and are far more protected from the surrounding environment in contrast to the CC and OWC. Although the adsorption technique for CC and OWC preparation, prevents cells from being freely suspended into the fermentation medium; the cells are still exposed to the harsh fermentation conditions, which in effect can make the cells ineffective in subsequent fermentations. It is important to also mention that the production of TBV, rice vinegar and some wine vinegars also use the back-slopping approach, which is similar to re-using microbial cells in the form of a “seed vinegar”. The seed vinegar, often called “mother vinegar” is basically an undefined starter culture extracted from the previous vinegar for consistency purposes and not to speed up the process (Gullo & Giudici, 2008; Bekatorou, 2019). Although cell immobilization has been described by several researchers as suitable for cell recyclability (Park et al., 1999; Rahman et al., 2006; Rao et al., 2008; Nigam et al., 2009, Shyamkumar et al., 2014), our results show that cell immobilization does not work as well for BSV production.

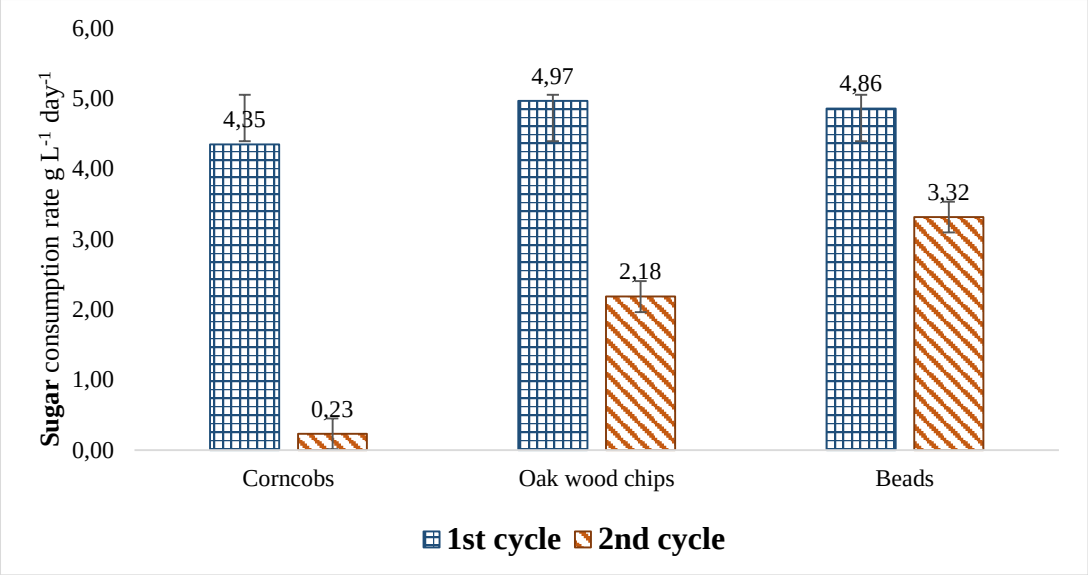


Figure 6.1: Sugar consumption rates for two cycles BSV fermentation using recycled immobilized cells

6.4.2 Comparative analyses of acetification rates for 1st and 2nd cycle BSV fermentations

The rate of acetic acid formation is by far the most important step in this process, since the rapidity and success of the process is determined by this step.

6.4.2.1 1st cycle fermentations

As expected, and in agreement with our previous work, the 1st cycle of fermentations completed successfully (Fig. 6.2). Cells entrapped in Ca-alginate beads had the highest acetification rates (2.51 g L^{-1} day^{-1}), followed by CC (2.27 g L^{-1} day^{-1}) with OWC (1.17 g L^{-1} day^{-1}) having the lowest acetification rates. However, the rates were slightly reduced compared to (chapter 4 & 5). This could be due to the cell immobilization process used in this study, whereby yeasts and bacteria cells were mixed prior to immobilization on the selected matrices, a strategy which was different in our previous work (Hutchinson *et al.*, 2018 & 2019b). Another reason could simply be due to the fact that biological systems such as fermentation may not always behave precisely the same due to biological and environmental factors.

6.4.2.2 2nd cycle fermentations

The 2nd cycle of fermentations had lower acetification rates for all treatments studied (Fig. 6.2). The reductions in acetification rates were calculated at relative differences of 86.8%, 94.0%, 64.5 % for CC, OWC and Ca-alginate beads, respectively. Ca-alginate beads had the lowest difference in acetification rates, indicating that Ca-alginate beads, provided better protection to yeast and the AAB. Furthermore, several researchers have investigated alginate beads for reusability instead of the adsorption method (Park et al., 1999; Rahman et al., 2006; Rao et al., 2008; Nigam et al., 2009, Shyamkumar et al., 2014).CC were initially anticipated to have high acetification rates when reused because of their structural attributes, in particular their porosity and the rough surfaces, which was adequately suited for

BSV production when compared to OWC, with results indicating enhanced acetification rates. However, an acetification rate of 0.3 g L^{-1} day^{-1} is low for the 2nd cycle fermentations.

Consequently, it is therefore important to calculate the losses and benefits that each cell immobilization matrix offers. The use of CC for the 1st cycle of fermentations is still beneficial and cheaper than using Ca-alginate beads. Consequently, a producer might have to reconsider repeating the adsorption process in order to conduct the second batch of fermentations. This would include the development of a preparation strategy for the CC such that it's attributes closely resembles that of Ca-alginate beads. Overall, the current study confirmed that the OWC were inefficient in achieving the aims (rapid fermentations) of this study for both cycles. In our previous work, OWC resulted in acetification rates which were similar to that of free-floating cells which defeats the aims of decreasing BSV fermentation periods by using immobilized cells. However, consideration was given on how OWC might improve the sensorial qualities of the vinegar and were therefore further tested in the current study. OWC had a lower cell affinity and some of the yeast and AAB were not adsorbed on the surface after the 1st batch of fermentations (Hutchinson *et al*., 2019b), which is in agreement with our previous studies.

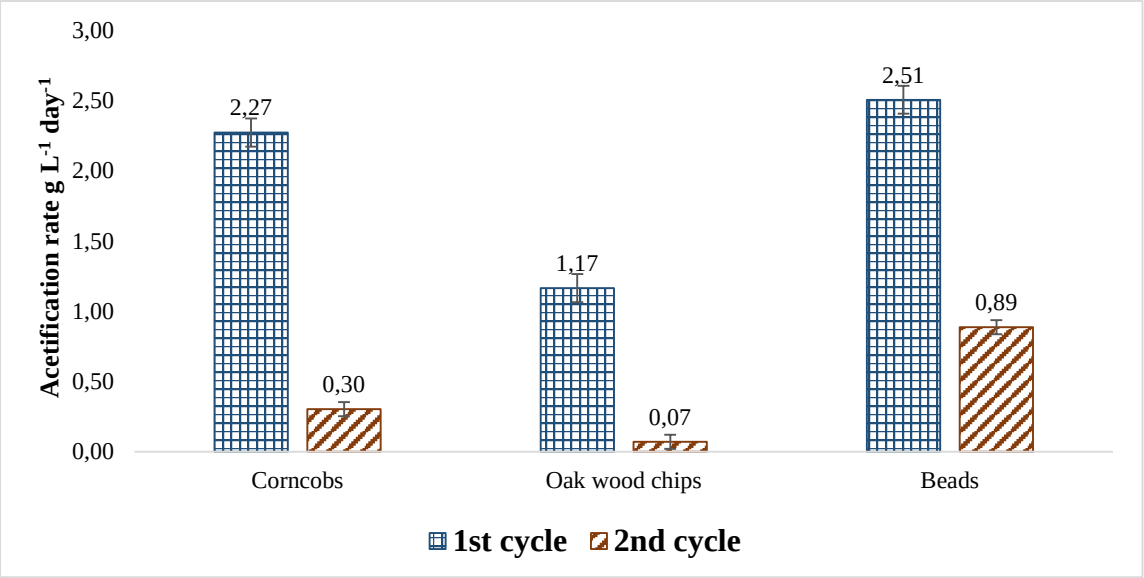


Figure 6. 2: Acetification rates for two cycles BSV fermentation using recycled immobilized cells

The acetification rates obtained when using Ca-alginate beads for the 2nd cycle could be acceptable depending on the manufacturers goals. However, since Ca-alginate is costly (Table 6.1), it might be beneficial to reuse the beads although the acetification rates may be reduced by up to 64.5% and the fermentation period may increase from 19 to 50 days (Table 6.2), which will ordinarily further increase operational costs. This means, if the operational cost of reusing the beads is less than the cost of not reusing the beads, then it could be advisable to reuse the beads to save on inoculum cost in large scale operations.

Table 6.1: Cell immobilization matrices used in the study and their sale price

Cell immobilization matrix	Price (Rands) per 250g	Amount used in the study for cell immobilization per 350 ml fermentation volume
Calcium alginate	1114	0,56 g (20 mL of alginate solution infused with cells)
Oakwood chips	105	(add weight) 108.58 cm^2
Corncobs	0.75 – 2 (generally sold in tons ~ R3000 to 8000 per ton)	(add weight) 117 cm^2

Another reason that could have affected the 2nd cycle of fermentations is the secondary adaptation process to the new conditions when the beads are transferred into the 2nd cycle matrix. Although cell immobilization offers protection for microorganisms, in a system such as BSV the yeast may not survive when the acetic acid concentration reaches 60 g L^{-1}. Furthermore, it might also be beneficial to consider reducing the fermentation working volume (grape) for the 2nd cycle of fermentations in order to account for the cells lost after the 1st cycle. Therefore, an alternate approach would be sequential inoculations rather than co-inoculations. Immobilized yeast cells can be utilized to perform the alcoholic fermentation until the desired alcohol concentration is achieved. Thereafter, immobilized AAB cells can be introduced into the system after transferring the yeast to the next batch. This method could retain the activity of both yeast and AAB immobilized cells. Other possible modifications that could be used to improve the reusability of the immobilized cells are the use of other methods of adsorbing the cells on the surface of the selected matrices. These methods include the dynamic batch, reactor loading and electrode positioning processes (Hutchinson et al., 2019c).

Table 6.2: fermentation period for both cycles of fermentation.

Cell immobilization matrix	1st cycle fermentation period (days)	2nd cycle fermentation period (days)
Corncobs	21	Incomplete
Oak wood chips	40	Incomplete
Ca-alginate beads	19	50

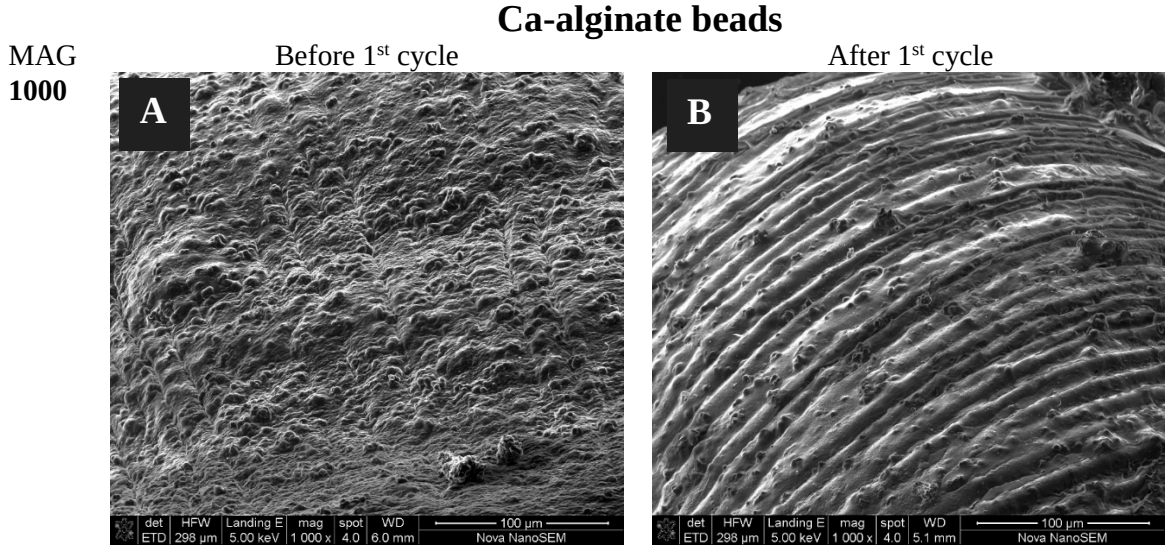
NB: Initial grape must before fermentation had 12.7 g L^{-1} of total acid and fermentations were monitored until an acetic acid concentration of 60 g L^{-1} was achieved

6.4.3 Structural morphology of cell immobilization matrices before and after BSV fermentation

6.4.3.1 Ca-alginate beads

The changes of the cell immobilization matrices in structural morphology after fermentation has a major impact on the efficacy of the matrices when they are reused. With regard to Ca-alginate beads, it is evident that the structure of the beads undergoes several alterations during the 1st cycle of fermentation (Fig. 6.3). Several textures were observed, which varied from rough with bumps, rock texture, smooth with undulated bumps and crusts including some ridge and furrow like structures. The changes in the bead structure was initially discovered by measuring the size of the beads whereby a 20% (4.5 to 3.6 mm) reduction in bead size was observed. The effects of size reduction of the beads was also observed

at a lower magnification (1000X) (Fig. 6.3B), whereby the surface texture of the beads showed some folds (furrow) in contrast to Fig. 6.3A. The reduction in bead size is possibly due to the loss of moisture and the high acid during BSV fermentation. At a higher magnification (5000X), a “rock like” texture is observed on the beads before fermentation with pores that are tightly connected (Fig. 6.3C), however, after fermentation (Fig. 6.3D), the beads appear to have a smooth surface with undulated bumps and ruptured pores. At 10000X magnification (Fig 6.3E & F), a rock like surface is still observed on the beads before fermentation, while the beads after fermentation show a relatively smooth surface with an undulated crust. It is unfortunate that a cross section analyses of the beads was not conducted, as it might have provided a proper representation of the internal structure and the presence of microbial cells. The changes in the bead structure and size affected the performance of beads during the 2nd cycle of fermentations. The loss of structural integrity of the beads possibly led to the reduction in cell retention and thus lower productivity rates. Rao et al. (2008) discovered that increasing the alginate concentration during the preparation of the beads reduces the loss of structural integrity and the loss of cells. A lower alginate concentration was attributed to larger pore sizes and thus a higher release of cells into the fermentation medium during fermentation. Consequently, increasing the alginate concentration and exploring other options that could increase the robustness of the beads is crucial for BSV fermentations.



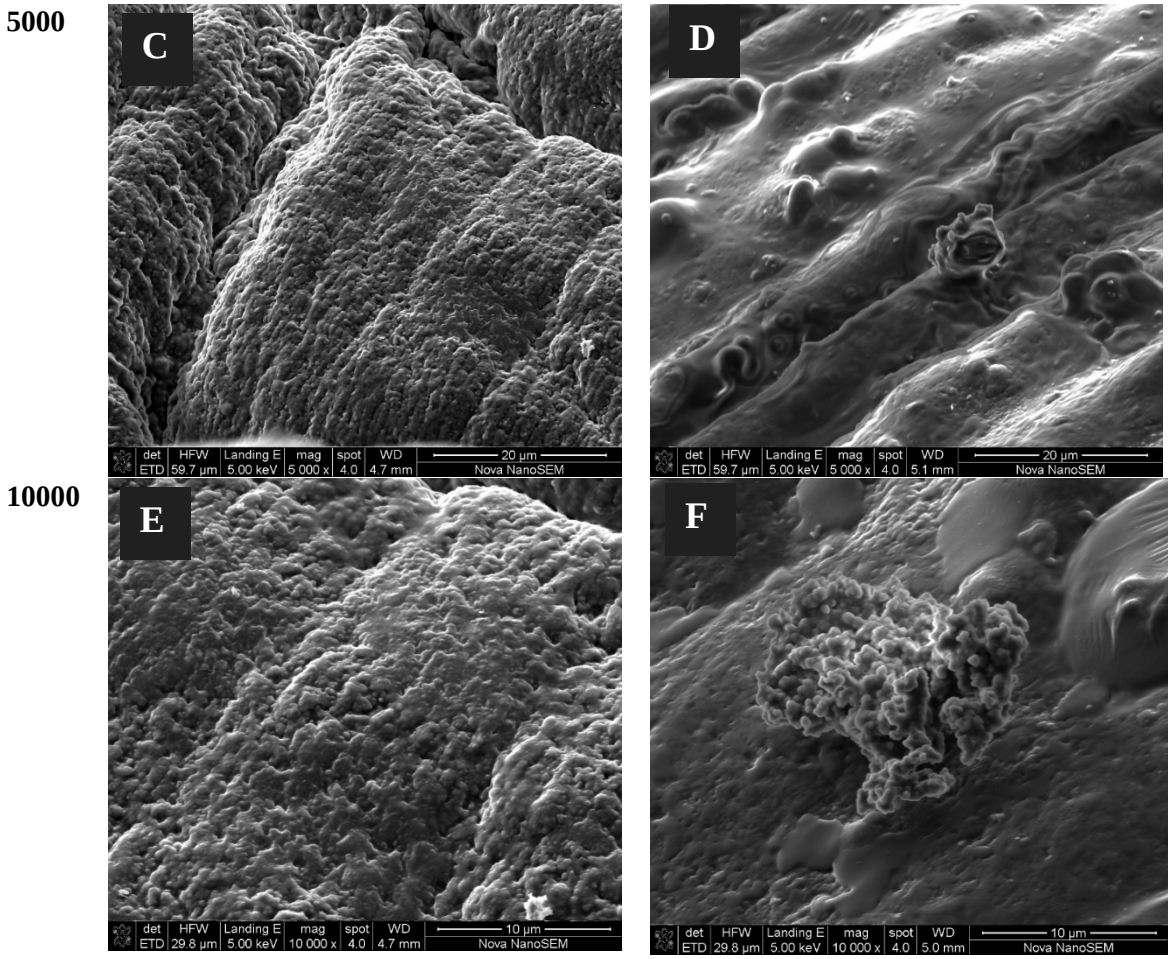


Figure 6.3: Scanning electron micrograph pictures of Ca-alginate beads before and after 1st cycle fermentations at several magnifications ie. 1000X (A, B), 5000X (C, D) and 10 000X (E, F).

6.4.3.2 Oak wood chips

It was observed that OWC were relatively similar before and after the 1st cycle of fermentations (Fig. 6.4), which was attributed to their robust nature. At a lower magnification (1000X), a typical plant material and wood like surface was observed (Fig. 6.4 A, B). Furthermore, microbial cells are visible on both pictures. While at a higher magnification (5000X), the structure of the OWC still appears similar before and after the 1st cycle of fermentations (Fig. 6.4 C, D). Furthermore, microbial cells which resemble yeast are unevenly present on the surface of the OWC. These observations were reasonable, because although OWC could not achieve high acetification rates, they were able to achieve sugar consumption for both cycles of fermentation (Fig. 6.1). This means yeast could have a higher cell adsorption capacity to the wood structure compared to AAB.

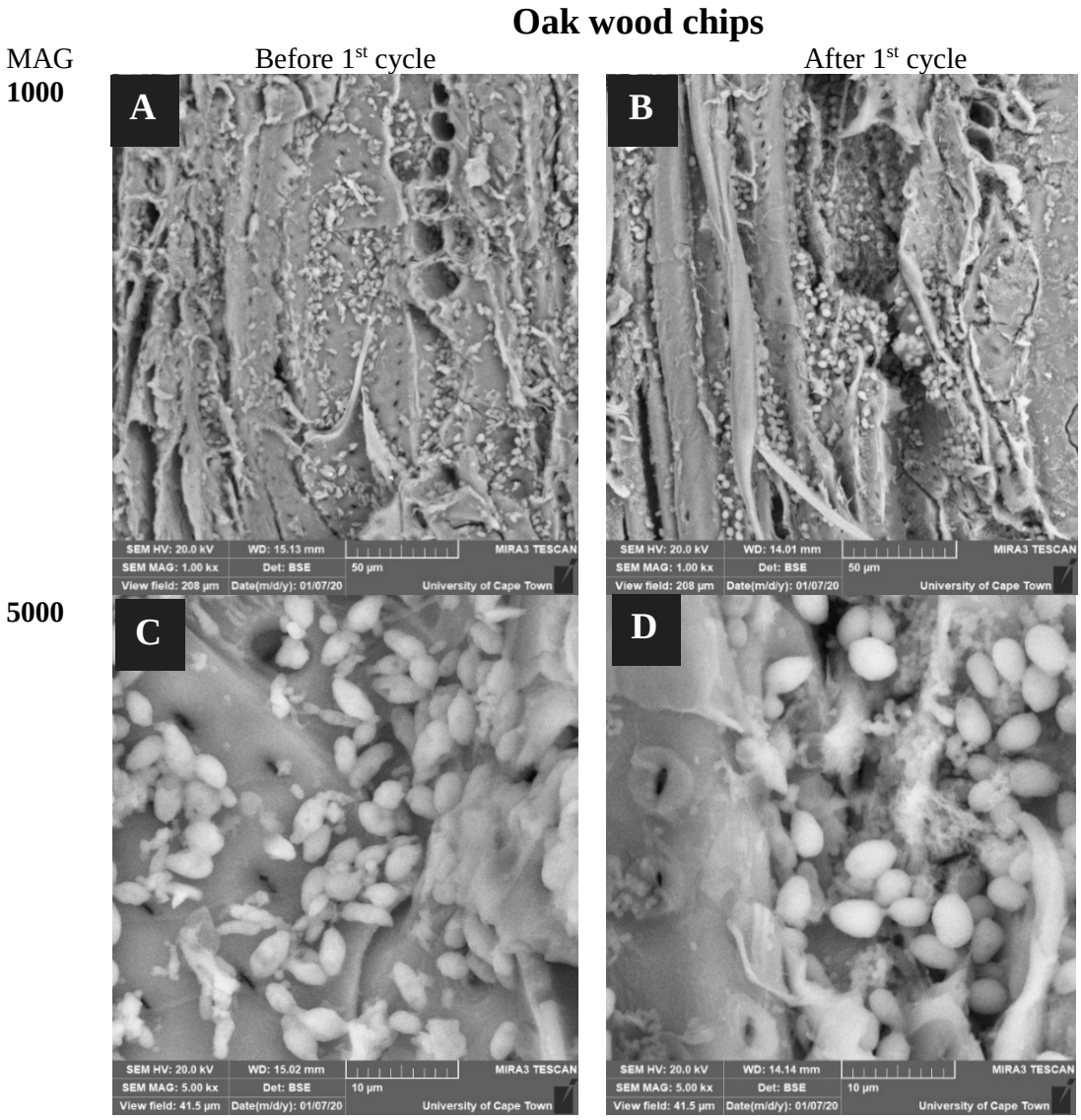


Figure 6.4: Scanning electron micrograph pictures of oak wood chips before and after 1st cycle fermentations at two magnifications ie. 1000X (A, B) and 5000X (C, D).

6.4.3.3 Corncobs

Corncobs generally have a rough and porous surface. The SEM images provided a clearer microscopic representation of the level of roughness and porosity of CC. At a lower magnification (1000X), an analysis of the chaff/beeswing portion (Fig. 6.5) of the CC showed that after the 1st cycle of fermentation (Fig. 6.6B), the CC have larger pores compared to the pores before the 1st cycle (Fig. 6.6A). The increase in pore size could be attributed to the high acid concentration which might have led to structural alterations. Furthermore, an increase in microbial cells was observed on all the images after the 1st cycle of fermentation (Fig. 6.6 B, D, F). However, the SEM images do not demonstrate the viability of the cells, therefore the increase in cells can be correlated to growth activity during the 1st cycle, however most of the cells might have been non-viable when transferred into the 2nd cycle.

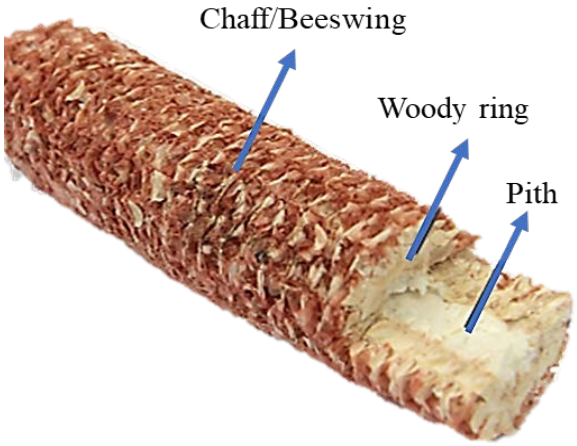


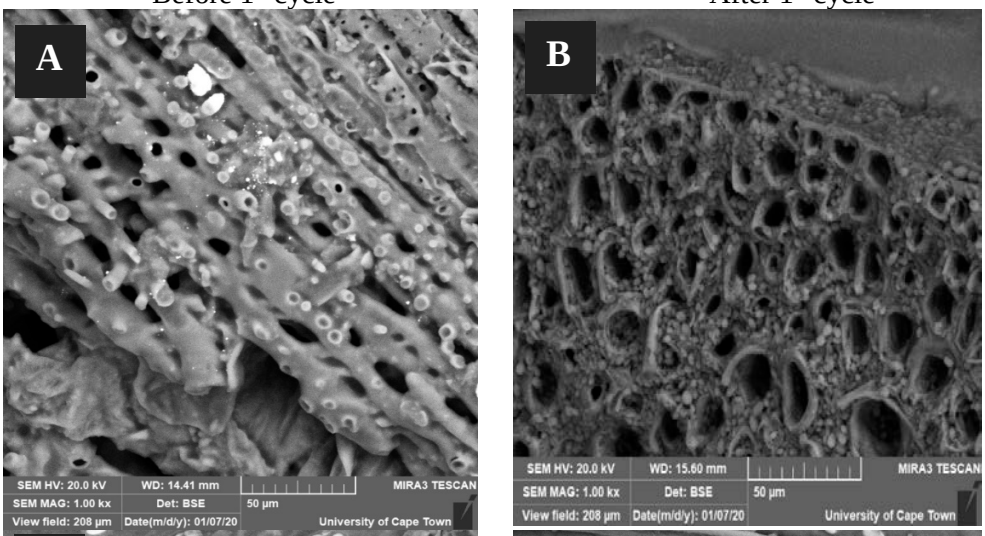
Figure 6.5: An image showing the structure of a corncob.

The analyses of the pith and woody ring section (Fig. 6.5) of the CC showed capsule like structures, which resemble bacteria (Fig. 6.6C, D). The capsule like structure are of a higher number and are more prominent after the 1st cycle of fermentations (Fig. 6.6D). If the capsule like structures are indeed bacteria cells, that means that the pith and wood ring section of the CC has a higher cell affinity for AAB cell adsorption. At a magnification of 1000X, the analyses of the chaff/beeswing portion (Fig. 6.5) of the CC further shows a higher number of microbial cells (predominantly yeasts cells) around the pores/layers of the beeswing after the 1st cycle of fermentation (Fig. 6.6F). Overall, all these images demonstrate that during BSV fermentation, the microbial cells multiply on the surface of CC. The SEM images also prove that CC (Fig. 6.6F) have a higher cell affinity than OWC (Fig. 6.4D), CC appear to have substantially higher number of microbial cells compared to OWC. Furthermore, the reduction of productivity rates on the 2nd cycle of fermentations could be attributed to the loss of viability of cells and not to the loss of cells on the immobilization matrix.

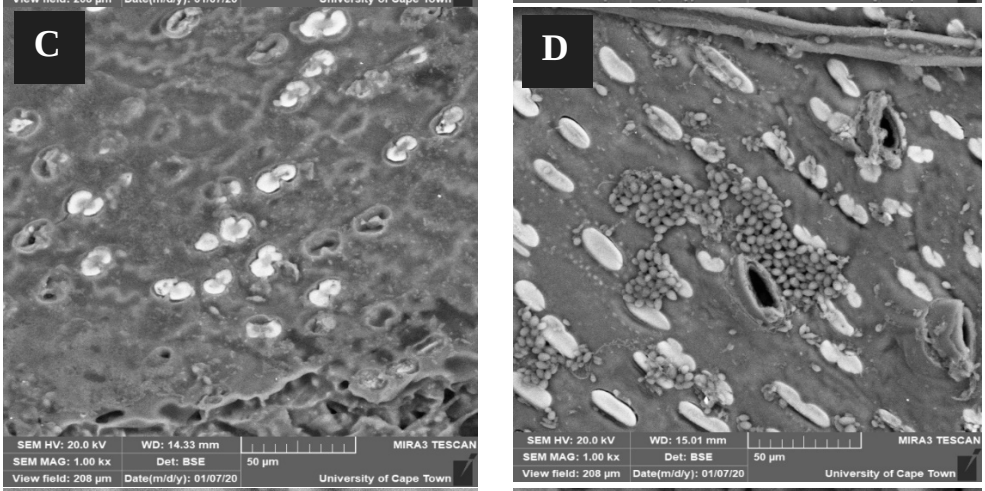
Corncobs

Before 1st cycle | After 1st cycle

1000



1000



5000

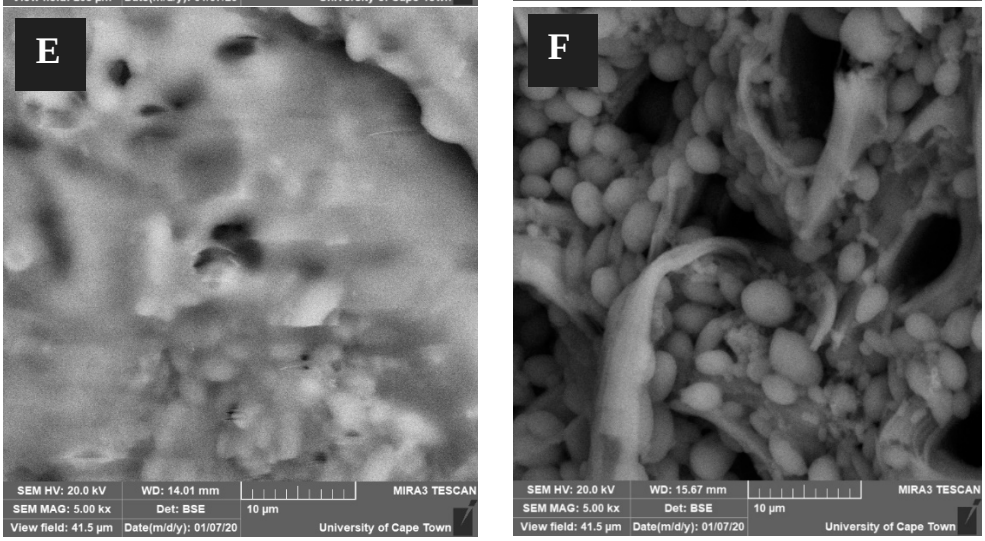


Figure 6.6: Scanning electron micrograph pictures of corncobs before and after 1st cycle fermentations at several magnifications.

6.4.4 Sensory attributes of the BSV's compared to commercial BSV's

Only the 1st cycle products were evaluated for sensorial characteristics. For the 2nd cycle fermentations, only fermentations treated with Ca-alginate beads produced a BSV, while the CC and OWC treatments did not produce usable samples. Therefore, it was decided not to do any sensory analyses on the 2nd cycle fermentation samples. The yeast consortium was selected based on the ability to produce acid, pleasant aroma, their osmophilic characteristics and percentage alcohol production under pre-determined conditions (data not published). The cell immobilization matrices were selected based on the knowledge that they are environmentally friendly and do not have any harmful effects on humans when present in food products. However, as previously mentioned, the studied matrices are different with regard to biological and structural composition, which makes it possible for the matrices to contribute positively or negatively to the sensorial qualities of the final BSV by imparting favourable or unfavourable compounds. It is also possible for the matrices to cause the secretion of off-flavour compounds by the microorganisms used. Generally, the sensorial qualities of any food product determines the success of the products research and development including the production process to be designed. Ideally, the general appearance of acceptable balsamic vinegar is described as dense and viscous; it resembles the texture of syrup and should be glossy and deep brown in colour. It is not harsh on the nose and palate such as spirit vinegars. Therefore, a required attribute is that balance is required between the sweetness and acidity (Giudici, Gullo, Solieri, & Falcone, 2009).

In the current study, a comparative analysis was conducted between BSV's and 29 commercial balsamic vinegars obtainable in local South Africa supermarkets. A wine vinegar was also added as an external reference. The visual appearance of the BSV's (Fig. 6.7A) were described as light brown with low viscosity (like water), similar to that of wine vinegar, while the majority of the commercial balsamic vinegars were described as dark brown or brown-black with medium viscosity. These observations concurred with what was expected because the BSV's were evaluated directly post-fermentation with no maturation, addition of caramel or thickening agents. With regard to flavour (Fig. 6.7B), all the BSV's were also grouped together and the judges described all the treatments to have a flavour of cooked fruit and some saltiness, while they were also described as slightly sweet and less intense. On the other hand, the commercial balsamic vinegars were described to have diverse attributes as shown on the PCA plot (CA correspondence analyses) (Fig. 6.7B).

The acid-sugar balance is one of the most important attributes for balsamic vinegar. Most of the commercial balsamic vinegars were described as balanced with only a few being described as unbalanced and highly acidic. The BSV's were described as slightly acidic for the CC and OWC treatments, while BSV's fermented with Ca-alginate beads were described as unbalanced and too acidic (Fig. 6.7C). The reason behind these descriptions is unclear because all the BSV products had similar acid concentrations. However, it is also possible that this could be due to a low residual sugar content in the Ca-alginate beads products, which caused the products to be perceived as highly acidic.

The consumer acceptance or “likeness rating” is also by far the most important rating. The BSV’s from the CC and Ca-alginate beads treatments were associated with ‘liked a lot’ by the judges, while the BSV’s obtained from the OWC treatments were ‘neither liked’ nor ‘disliked’. This was an interesting observation since most of the commercial balsamic vinegars covered the full spectrum from ‘dislike’ to ‘like a lot’.

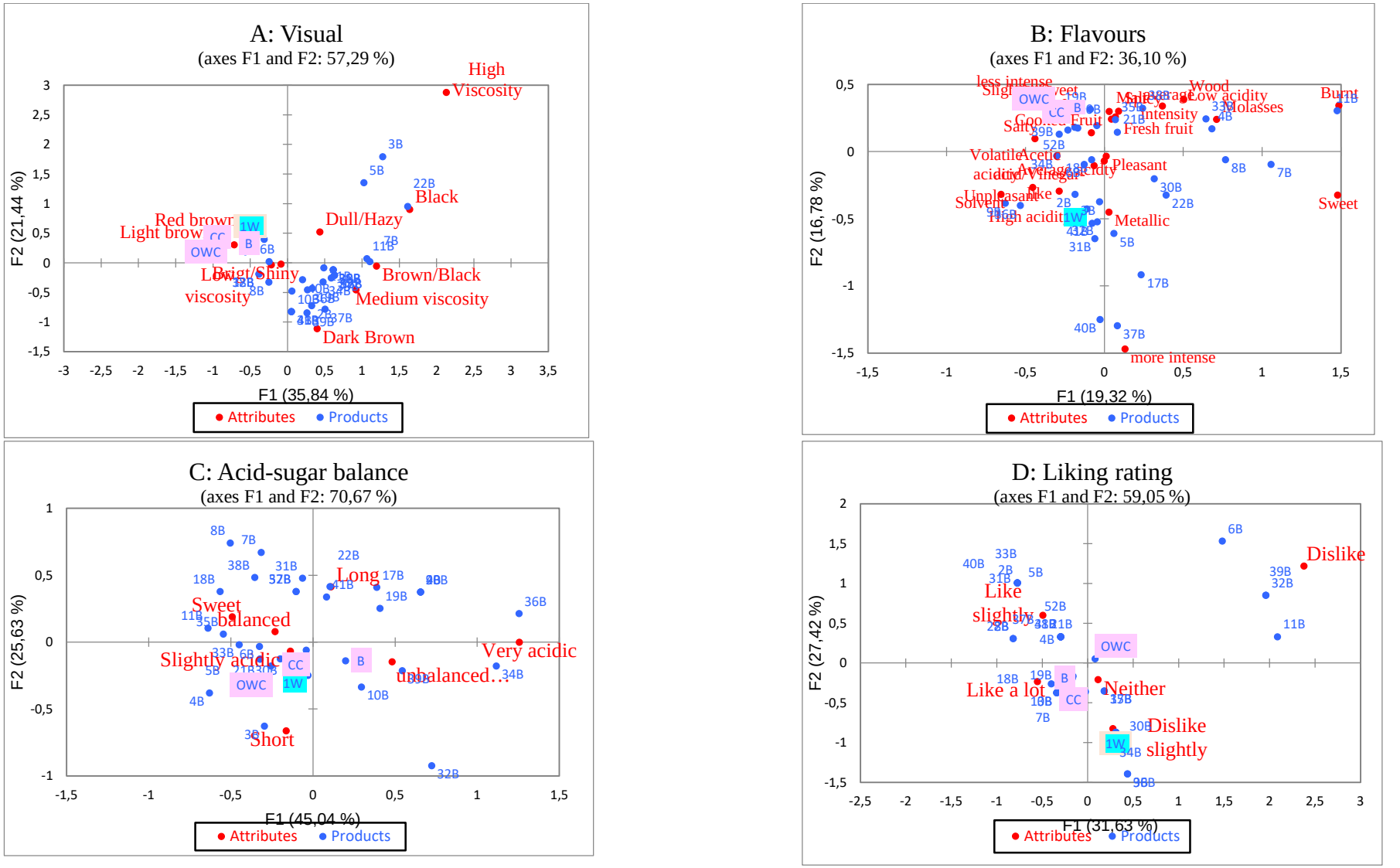


Figure 6.7: Sensory evaluation data for BSV and commercial balsamic vinegars. A) Visual, B) Flavours, C) Acid-sugar balance, D) consumer acceptance/liking rating. All BSV samples were shaded purple (CC: corncobs, OWC: oak wood chips, B: beads), with one wine vinegar being shaded light blue (1W: wine vinegar

6.5 Summary

Chemical developments and sensory data showed that CC and Ca-alginate beads are suitable immobilization matrices with regards to acetification rates and sensorial qualities for the 1st cycle of fermentations. The reusability of immobilized cells for BSV production led to much slower acetification rates on the 2nd cycle of fermentations compared to the 1st cycle. Cells immobilized using Ca-alginate beads resulted in higher acetification rates for the 2nd cycle compared to cells adsorbed on both CC and OWC. Therefore, for sustainable or quality BSV production it is recommended that a fresh inoculum is used for new cycles. SEM analyses showed that Ca-alginate beads undergo visible structural alterations after the 1st cycle of fermentations, while CC and OWC did not show drastic structural modifications due to their robustness. This data can be useful when choosing a matrix for cell immobilization to produce BSV. Methods to improve the robustness of alginate beads and a sequential inoculation strategy are therefore recommended for further stability testing of the immobilized cells when they are reused for subsequent fermentations.

CHAPTER 7

Oxygen mass transfer kinetics during Balsamic-styled vinegar production

CHAPTER 7

Oxygen mass transfer kinetics during Balsamic-styled vinegar production

7.1 Introduction

Generally, the design of a vinegar fermentor must consider high aeration capacity as a vital design feature in order to achieve ideal oxidation/acetification rates (Schlepütz et al., 2013; Gullo et al., 2014). This is due to the eminent fact that acetic acid bacteria (AAB) are strictly aerobic bacteria and thus require high oxygen concentrations for optimal microbial activity. Consequently, oxygen transfer is considered as the rate limiting step in several vinegar production systems (Ghommidh et al., 1982; Tesfaye et al., 2002; García-García et al., 2009; Qi et al., 2013; Gullo et al., 2014).

Numerous studies have been conducted for industrial spirit vinegar production systems to improve the oxygen transfer rate, aeration capacity and the aerobic oxidation reaction carried-out by AAB (Tesfaye et al., 2002; Qi et al., 2013). These include technical modifications such as the use of pure oxygen, elevated partial pressure, high broth agitation, and in most cases, the reconstruction of a fermentation vessel (de Ory et al., 2004; Halladj et al., 2016; Zhou et al., 2017). However, due to the diverse AAB species, their fragile nature and the diverse vinegar production systems, targeted researches to improve the productivity of the process are still continuing.

Spirit vinegars are predominantly produced using the semi-continuous fermentation system, which entails the periodic withdrawal of a portion of culture medium when the concentration of the residual alcohol is low and subsequently refilling the remaining medium with fresh alcoholic medium to obtain the required working volume (García-García et al., 2009; Qi et al., 2013 & 2014). However, BSV fermentation in contrast to industrial vinegars does not employ the use of alcoholic stocks, and the alcoholic fermentation and oxidation process occur simultaneously in the same vessel (Eq. 7.1) (Hutchinson et al., 2018, 2019a, b). Habitually, the alcoholic stocks used for industrial vinegar as well as several other vinegar production systems are obtained from fermentation vessels which are separate from the acetification vessels (de Ory et al., 2004; García-García et al., 2009; Qi et al., 2013 & 2014). Consequently, this means that a vinegar production process similar to that of BSV is more complex with regards to oxygen transfer dynamics due to the presence of yeast and AAB in the same vessel which happen to have paradoxical oxygen requirements. For this reason, aeration has not been a fundamental aspect in Traditional Balsamic Vinegar (TBV) production (Solieri and Giudici, 2009; Giudici et al., 2015). Therefore, this raises the question if submerged techniques such as aeration, support the anaerobic (alcoholic) (Eq. 7.2) and strictly aerobic oxidation (Eq. 7.3) process during BSV production?

$$\text{Glucose} \xrightarrow{\textbf{Yeast}} Ethanol \xrightarrow{\textbf{AAB}} Acetic\ acid$$

$$C_6H_{12}O_6 \rightarrow C_2H_5OH \rightarrow CH_3COOH \quad \text{Eq. 7.1}$$

$$\text{Glucose} \xrightarrow{\textbf{Yeast}} Ethanol + Carbon\ dioxide$$

$$C_6H_{12}O_6 \rightarrow C_2H_5OH + 2\,CO_2 \quad \text{Eq. 7.2}$$

$$Ethanol + Oxygen \xrightarrow{\textbf{AAB}} Acetic\ acid + Water$$

$$C_2H_5OH + O_2 \rightarrow CH_3COOH + 2H_2O \quad \text{Eq. 7.3}$$

Nevertheless, oxygen mass transfer is typically a complicated process, as it involves the transfer of dissolved oxygen (DO) from the gaseous phase through the gaseous-liquid interface to the liquid phase (García-García et al., 2009) (Fig. 7.1). Oxygen can easily dissolve in the air; however, the composition of the liquid phase can significantly influence the DO content (Pittoors et al., 2014). With regards to BSV production, the fermentation medium (cooked grape must) has several environmental factors that could significantly affect the transfer of DO between the gaseous and liquid phase, these include the temperature of the fermentation medium, relatively high salinity, high osmotic pressure and low pH (Atkinson et al., 2007; Hanmante et al., 2014; Irvine, 2017; Braga et al., 2018). AAB are also known to form extracellular polysaccharides during fermentation which also negatively affects the oxygen mass transfer kinetics (Garcia-Ochoa et al., 2010; Sengun, 2017; Lynch et al., 2019). Another challenging factor is that the yeasts, which are part of the BSV microbial consortium produce CO_2 (Eq. 7.2) during alcoholic fermentation which could also affect the DO content in the fermentation medium (Cheng, 2001).

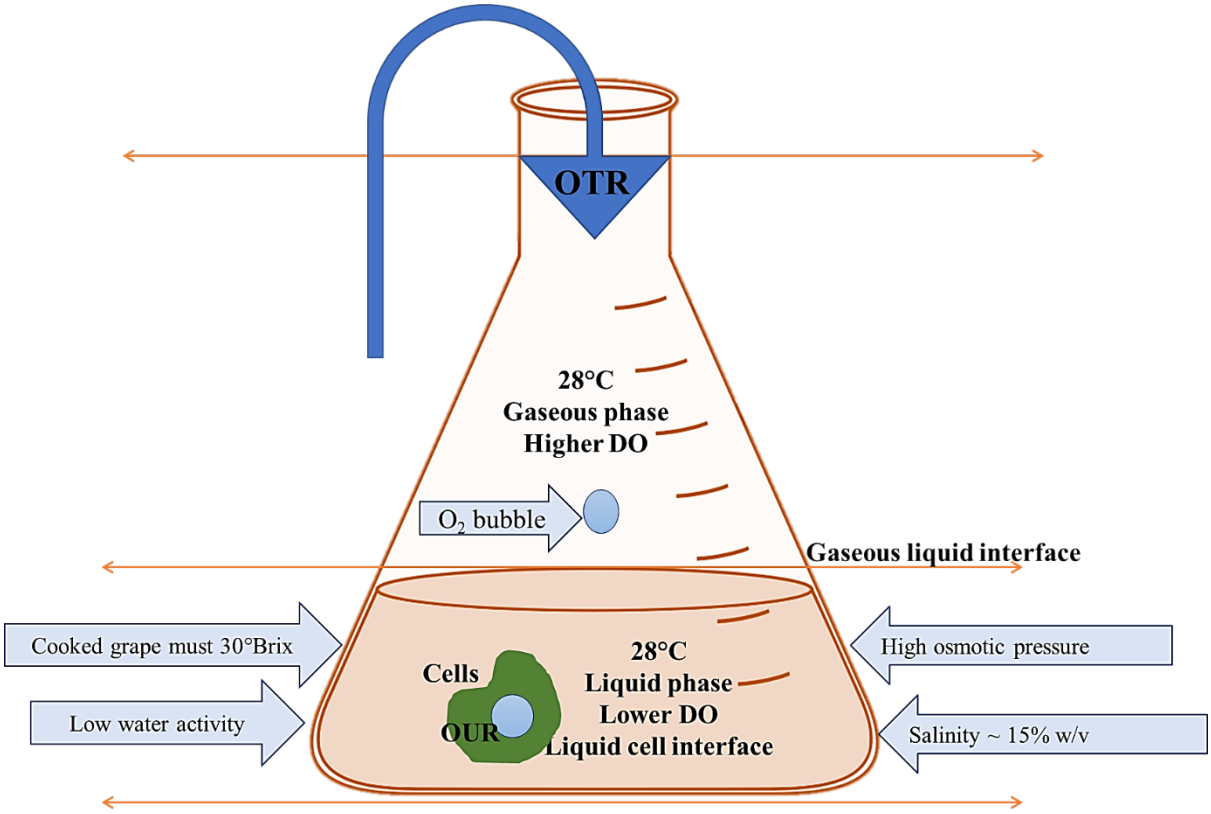


Figure 7.1: A diagrammatic description of the oxygen mass transfer process between the gaseous to liquid phase during BSV production

As a result, this study was conducted with the primary aim of understanding the oxygen mass transfer dynamics during BSV production, while employing mathematical computations to provide a more profound understanding of the effects of aeration and surface culture methods for BSV production process.

7.2 Objectives

The objectives of this part of the work were to:

- To compare the oxygen mass transfer kinetics between aerated and non-aerated fermentations for BSV production.
- To apply mathematical computations in order to describe the BSV production process.

7.3 Materials and methods

Methods for preparation of fermentation medium, inoculum preparation, cell immobilization, sampling procedure, analytical methods are described in chapter 3. Only mathematical computations are described in chapter.

7.3.1 Determination of oxygen mass transfer parameters and mathematical computations

Several valuable oxygen mass transfer parameters were evaluated using the data obtained from the dynamic method. These include the oxygen uptake rate (OUR also expressed as r_{O_2}), the stoichiometric coefficient of oxygen consumption vs acid yield ($Y_{O/A}$), the oxygen transfer rate (OTR also expressed as N_{O_2}), the volumetric mass transfer coefficients ($K_L a$).

7.3.1.1 Determining the oxygen uptake rate

To determine the oxygen uptake rate (OUR), the data generated from the dynamic method was used to compute r_{O_2} by using the DO concentrations profiles generated during biotransformation while aeration was interrupted. Eq. 7.4 was used to compute r_{O_2} where dC_L represents the change in DO concentration in the BSV fermentation medium, while *dt* represents the change in time (Zhou et al., 2017).

$$r_{O_2} = \frac{dC_L}{dt} \qquad \text{Eq. 7.4}$$

7.3.1.2 Determining the stoichiometric coefficient of oxygen consumption vs acid yield

To determine the ratio of oxygen consumption vs acid yield, Eq. 7.5 was used. Where r_{O_2} (g O_2 L^{-1} hr^{-1}) represented the oxygen consumption rate. While r_{acid} (g AcOH L^{-1} h^{-1}) represents the acetification rate determined at different stages of the BSV fermentation, which is calculated by using Eq. 7.6 (Qi et al., 2013 & 2014).

$$Y_{O/A} = \frac{r_{O_2}}{r_{acid}} \qquad \text{Eq. 7.5}$$

$$r_{acid} = \frac{d_{[acid]}}{dt} \qquad \text{Eq. 7.6}$$

7.3.1.3 Determining the oxygen mass transfer coefficient and oxygen transfer rate

Since biomass concentration was not quantified during BSV fermentation, the oxygen mass transfer parameters were determined using the dynamic method with the exclusion of biomass concentration while assuming 'steady state' conditions during the interruption ($\therefore OTR = OUR$). In most batch systems, OUR changes rapidly over time due to biomass growth and substrate depletion. However, in a process as slow as BSV fermentation, relatively steady state conditions were assumed due to the short time interval for aeration interruption and long fermentation period. OUR can be considered constant, thus leading to the combination of both kinetics in a reactor, i.e., $OTR = OUR$. Consequently, $K_L a$ was determined using a biological system equation with the inclusion of OUR. Thus $K_L a$ (hr^{-1}) was determined using the estimated OUR from the DO concentrations profile generated during aeration interruption. Therefore Eq. 7.7 was used to determine $K_L a$, Where C* is the DO concentration at the gaseous phase, while C_L is the DO concentration at the liquid phase (Sudjarwadi, 2019).

$$\text{OTR} = K_L a(C^* - C_L) \quad \text{Eq. 7.7}$$

However, OTR can also be expressed as

$$\text{OTR} = \frac{dC_L}{dt} = K_L a\,.(C^* - C) \quad \text{Eq. 7.8}$$

Since $OTR = OUR$ under steady state conditions, Eq. 7.4 and Eq. 7.8 result in Eq. 7.9 written as:

$$\text{OUR} = K_L a(C^* - C_L) \quad \text{Eq. 7.9}$$

∴ Eq. 7.9 can be written as Eq. 7.10 to calculate $K_L a$

$$K_L a = \frac{OUR}{C^* - C_L} \quad \text{Eq. 7.10}$$

With the use of Eq. 7.10, $K_L a$ was determined at different stages using OUR and $(C^* - C_L)$ values. From these data, the oxygen transfer rate was therefore calculated using Eq. 7.7 and the $K_L a$ values obtained from Eq. 7.10.

7.4 Results and Discussion

To properly understand the oxygen mass transfer kinetics during BSV production, several parameters were used to assess the correlation between oxygen mass transfer and chemical developments during fermentation. For consistency purposes when using mathematical computations, all units were converted to g L^{-1} and rates are represented in g L^{-1} h^{-1} and not g L^{-1} day^{-1} as compared to the other chapters.

7.4.1 Chemical developments during non-aerated and aerated BSV fermentations.

Chemical developments were extensively discussed in other chapters of this study, therefore, the chemical developments are only briefly discussed and used for correlation to oxygen mass transfer parameters. Nevertheless, sugar consumption profiles obtained from the data generated showed that aerated fermentations resulted in higher sugar consumption rates (r_{sugar}) at the initial stages of the fermentation process in contrast to non-aerated fermentations for both CC and Ca-alginate beads fermentations (Table 7.1 A, B; Fig. 7.2). While acetification rates (r_{acid}) were higher for both CC and Ca-alginate beads fermentations when non-aerated conditions were used compared to aerated conditions (Fig. 7.1 E, F). In previous studies (Hutchinson et al., 2019b; Chapter 5), similar sugar consumption and total acid production profiles were observed when aeration was studied on cells immobilized on CC. However, aeration was not studied on cells immobilized on Ca-alginate beads and dissimilar profiles were therefore anticipated.

Table 7.1: Substrate consumption and sugar consumption rates for aerated and non-aerated fermentations.

		Rate of sugar consumption (r_{sugar}) (g L^{-1} h^{-1})	Rate of acid formation (r_{acid}) (g L^{-1} h^{-1})
Non-aerated fermentations	Corncobs	0.33	0.08
	Ca-alginate beads	0.41	0.10
Aerated fermentations	Corncobs	0.82	0.05
	Ca-alginate beads	0.78	0.04

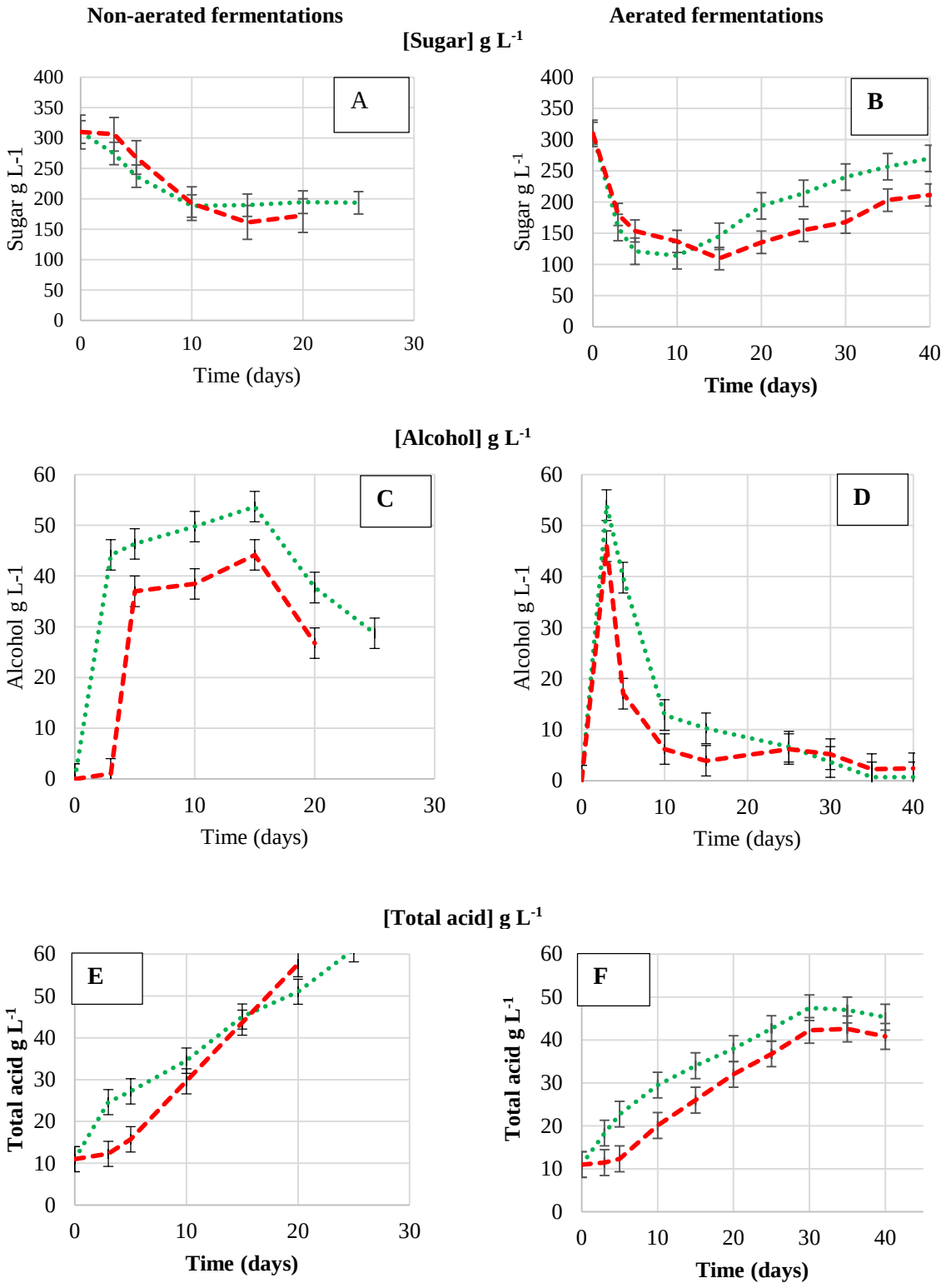


Figure 7.2: Sugar consumption and total acid formation during BSV fermentation under aerated and non-aerated conditions. ····· Corncobs, – – – Ca-alginate beads.

7.4.2 Oxygen mass transfer kinetics

7.4.2.1 Oxygen uptake rates

The oxygen uptake rates (r_{O_2}) were higher for non-aerated fermentations compared to aerated fermentations for both CC and Ca-alginate beads fermentation (Fig. 7.3 A, B). These observations are unexpected for a vinegar production system because oxygen is consumed for three functions, which are cell growth, maintenance and product formation (Calik et al., 2006). Applying aeration during vinegar production is therefore expected to result in higher product formation rates. The profiles observed in this study substantiate the fact that BSV fermentation is different from several vinegar production systems, where aeration often results in higher acetification rates and high r_{O_2} compared to non-aerated conditions. The r_{O_2} data (Fig. 7.3 A, B) also demonstrated that CC and Ca-alginate beads have higher r_{O_2} at different phases of the fermentation process. It was observed that CC have a higher r_{O_2} at the initial stages of the fermentation in contrast to Ca-alginate beads which have major r_{O_2} towards the end stages of the fermentations.

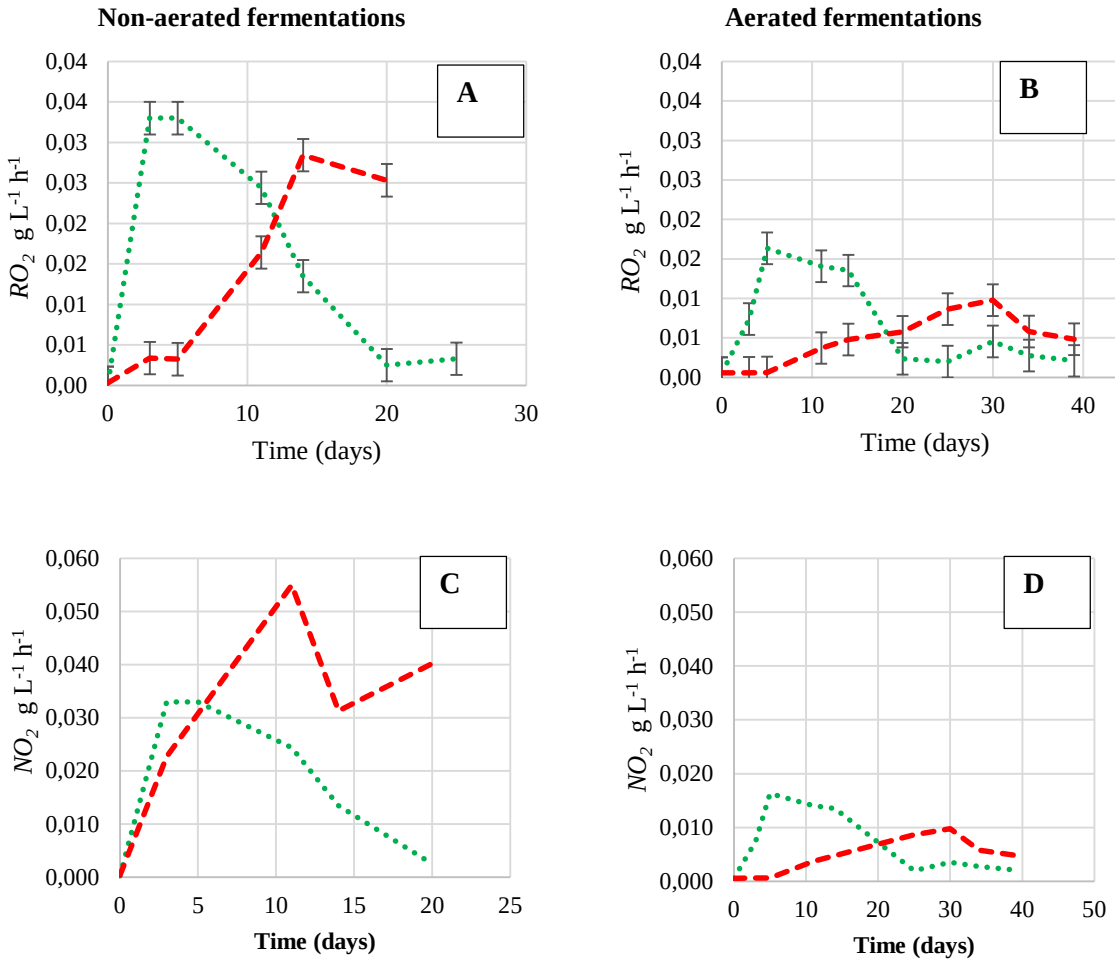


Figure 7.3: Oxygen uptake rate during BSV fermentation under aerated and non-aerated conditions. ····· Corncobs, – – – Ca-alginate beads.

The r_{O_2} profiles (Fig. 7.3A, B) also correlate with the chemical development profiles (Fig. 7.2A, B, C, D), which demonstrate that Ca-alginate beads have lower r_{sugar} and r_{acid} at the initial stages of the fermentation under both aerated and non-aerated conditions. As previously determined, that $OTR = OUR$, it was therefore not surprising that r_{O_2} profiles (Fig. 7.3 A, B) followed a similar pattern to the N_{O_2} profiles (Fig. 7.3 C, D).

7.4.2.2 The relationship between oxygen uptake rate and acetification rates during BSV production

Figure 7.4 provides a clear demonstration of the relationship between r_{O_2} and r_{acid}. These profiles (Fig. 7.4) show that there is a proportional relationship between r_{O_2} and r_{acid}. Fermentations treated with CC resulted in a high r_{O_2} and r_{acid} at ~day 3 to 10 and ~day 3 to 15 for non-aerated and aerated fermentations respectively. While fermentations treated with Ca-alginate beads showed a high r_{O_2} and r_{acid} between ~day 15 to 20 and ~day 10 to 30 for non-aerated and aerated fermentations respectively. The reason behind these r_{acid} and r_{O_2} profiles was assumed to be due to a longer lag phase when Ca-alginate beads/entrapped cells are used due to the minimal exposure to the surrounding nutrients. The porosity of the beads possibly increases as the fermentation process progresses and thus resulting in a higher nutrient exchange/metabolic activity. This could be the reason entrapped cells have been reported to cause low acetification rates when used for rapid vinegar fermentation process, the longer lag phase is not suitable for industrial sprit vinegar production where fermentations are completed in a few hours. However, adsorbed cells are exposed to the fermentation medium immediately after inoculation and are therefore quick to metabolize the available substrates. These data can assist in optimizing the BSV fermentation process by identifying fermentation stages in which bioreactor modifications can be made in order to maintain a high r_{O_2} and r_{acid} throughout the fermentation process, hence radically reducing the fermentation period.

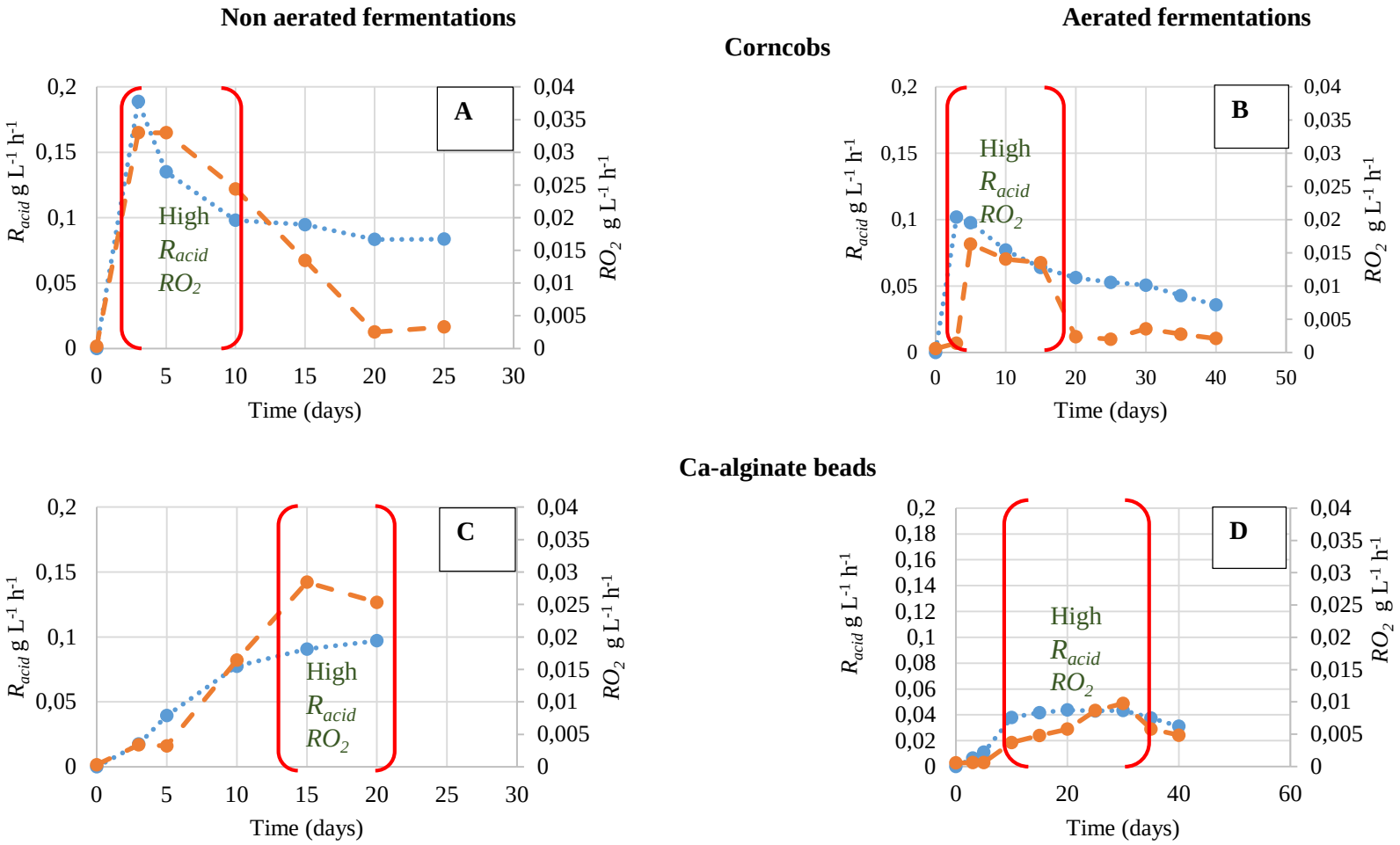


Figure 7.4: Acetification rates versus oxygen consumption rate during BSV production, r_{acid} ; r_{O_2}

7.4.2.3 Stoichiometric coefficient of oxygen consumption

Stoichiometric coefficients for oxygen consumption and stoichiometric yields for alcohol oxidation are important parameters to determine for vinegar production because they contribute in determining the competence of a fermentation process (Qi et al., 2013 & 2014). However, in BSV fermentation, alcohol serves as an intermediate product produced by yeasts and as a substrate for AAB (Eq. 7.1). Hence the determination of the stoichiometric yields for alcohol oxidation requires the consideration of the rates of alcohol production, consumption and evaporation in both non-aerated and aerated fermentations, which is laborious process and can lead to inaccurate data, therefore the stoichiometric yield for alcohol oxidation was not calculated. However, the general theoretic stoichiometry yield for alcohol oxidation is 1:1 (i.e. 1 g EtOH/ g AcOH yield) for vinegar fermentation (de Ory et al., 2004a; Qi et al., 2013). Nevertheless, to determine the stoichiometric coefficient ($Y_{O/A}$), r_{O_2} and r_{acid} values were graphically plotted on the *Y* and *X* axis respectively (Fig. 7.5), while the calculated slope was determined to be $Y_{O/A}$. Consequently, the average stoichiometric coefficients of oxygen uptake versus acetification rates were determined to be ~0.208, and ~0.17 g O_2/ g AcOH yield for CC fermentations when non-aerated and aerated conditions were used respectively (Fig. 7.5 A, B). While the $Y_{O/A}$ were ~0.2869 and ~0.1663 g O_2/g AcOH yield for Ca-alginate beads fermentations when non-aerated and aerated conditions were used respectively (Fig. 7.5 A, B). Overall, Ca-alginate beads (non-aerated) had the highest $Y_{O/A}$, with CC (non-aerated) in 2nd place, followed by CC (aerated) in 3rd place, while Ca-alginate beads (aerated) resulted in the lowest $Y_{O/A}$. A study done by Qi et al. (2013) for industrial vinegar production showed that $Y_{O/A}$ was 0.63 g O_2/g AcOH and 0.84 g O_2/g AcOH in two different semi-continuous operation systems. The $Y_{O/A}$ values obtained by Qi et al. (2013) were 3 times higher than the $Y_{O/A}$ obtained in the current study. Qi et al. (2013) also reported that a high biomass was obtained for the semi-continuous operation system that had the highest $Y_{O/A}$. Unfortunately, in the current study biomass concentration was not measured throughout the fermentation process since immobilized cells were used and it is difficult to quantify both yeast and AAB biomass changes when entrapped and adsorbed cells are used during fermentation. Nevertheless, it appears that industrial vinegar production requires high O_2 consumption for both biomass growth and AcOH production. The same cannot be expected for BSV production, since there are additional nutrients in the BSV fermentation medium compared to the alcoholic stocks used for industrial acetic acid production (Wood, 2012). As a result, biomass growth and thus r_{acid} do not rely heavily on oxygen availability as a primary substrate for cellular growth, maintenance and product formation during BSV fermentation.

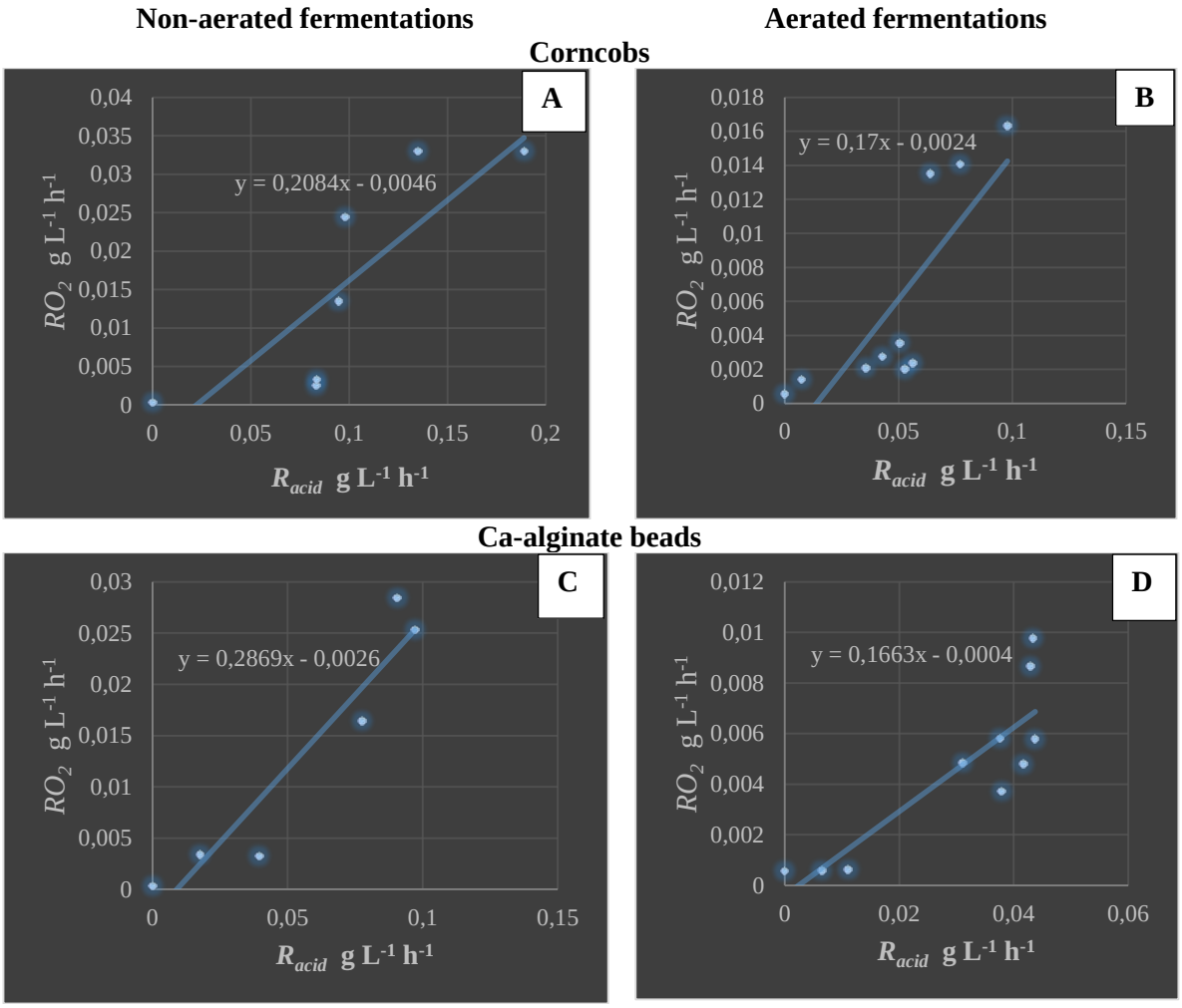


Figure 7.5: Graphical depiction of the determination of the stoichiometric coefficient of oxygen consumption vs acid yield ($\boldsymbol{Y_{O/A}}$) i.e. oxygen consumption rate over acetification rate for all fermentation treatments.

7.4.2.4 Volumetric mass transfer coefficient

The volumetric mass transfer coefficient ($K_L a$) was used to determine the aeration capacity of the bioreactors used in this study. Fluctuating values of $K_L a$ were obtained when Eq. 7.10 was used to calculate $K_L a$ at several stages of the BSV fermentation (Fig. 7.6). The $K_L a$ values obtained for all treatments studied show that the highest $K_L a$ is obtained when using Ca-alginate beads under non-aerated and aerated conditions (Fig. 7.6). However, the highest $K_L a$ (21.2 hr^{-1}) was obtained under non-aerated conditions when Ca-alginate beads were used (Fig. 7.6 A). The $K_L a$ values obtained in the current study were substantially lower compared to $K_L a$ in industrial vinegar production which has been reported to range between 100 to 900 hr^{-1} (Garcia-Ochoa et al., 2010). This means that the aeration capacity of the batch bioreactors used was low, which was expected, since a bioreactor was not constructed with the purposes of improving $K_L a$. It is important to note that, $K_L a$ is predominantly influenced by several bioreactor features which include fermentor design (aeration system design,

geometric configurations), chemical-physical factors (viscosity, liquid composition), biomass concentration, hydrodynamic attributes of the liquid phase (air bubble size, airflow rate) (AlAhmady, 2011; Pittoors et al., 2014). For this reason, properly designed bioreactors/ fermentors have a high $K_L a$. However, in this study, 2 L batch bioreactors were sparged with air without the use of other bioreactor features, while the non-aerated fermentations were covered with loose cotton wool stoppers. The data obtained shows that applying aeration on its own does not guarantee a high $K_L a$, because $K_L a$ was determined while considering C^*, C_L and r_{O_2}. While these parameters were also influenced by the design of the BSV aeration system, airflow rate, bubble size, BSV medium composition, medium viscosity and biomass concentration. Furthermore, it was observed that fermentations treated with CC resulted in low $K_L a$ under both aerated and non-aerated conditions. This means that, the presence of CC in the bioreactor has an influence on the aeration capacity of the bioreactor, probably due to added matter in the liquid medium. Therefore, if a fermentor with a high aeration capacity would be constructed for CC fermentations, the r_{acid} would also be increased substantially. Overall, these data demonstrate that aeration led to lower r_{acid} due o the low aeration capacity of the bioreactors.

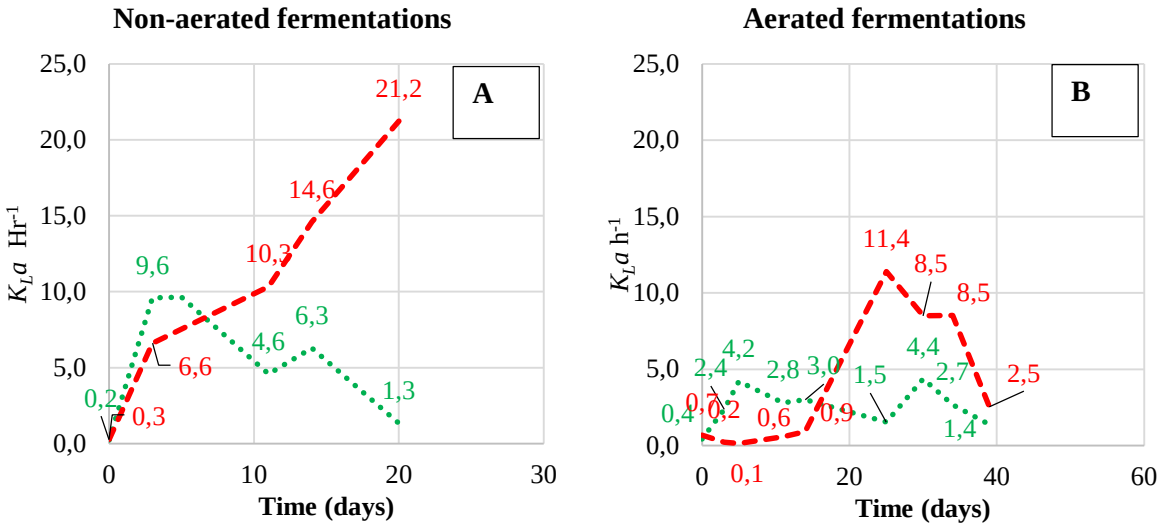


Figure 7.6: Volumetric mass transfer changes during BSV fermentation. ····· Corncobs, – – – Ca-alginate beads.

7.4.2.5 Dissolved oxygen and salinity changes during BSV fermentation

The relationship between dissolved oxygen (DO) and salinity during the BSV fermentation process was also assessed, since salinity is known to cause the reduction in the dissolving efficacy of O_2 in liquids. In the current study, there was no clear correlation regarding the influence of salinity in DO concentrations under non aerated conditions for both CC and Ca-alginate beads treatments (Fig. 7.7 A-D). The salinity content showed a sustained reduction during the BSV fermentation while DO concentration showed a reduction followed by a relatively steady increase for all treatments studied. However, a higher reduction in salinity was observed under aerated conditions which was attributed to be due to the aeration process. The DO profiles for both non-aerated and aerated fermentations generally followed a relatively similar pattern.

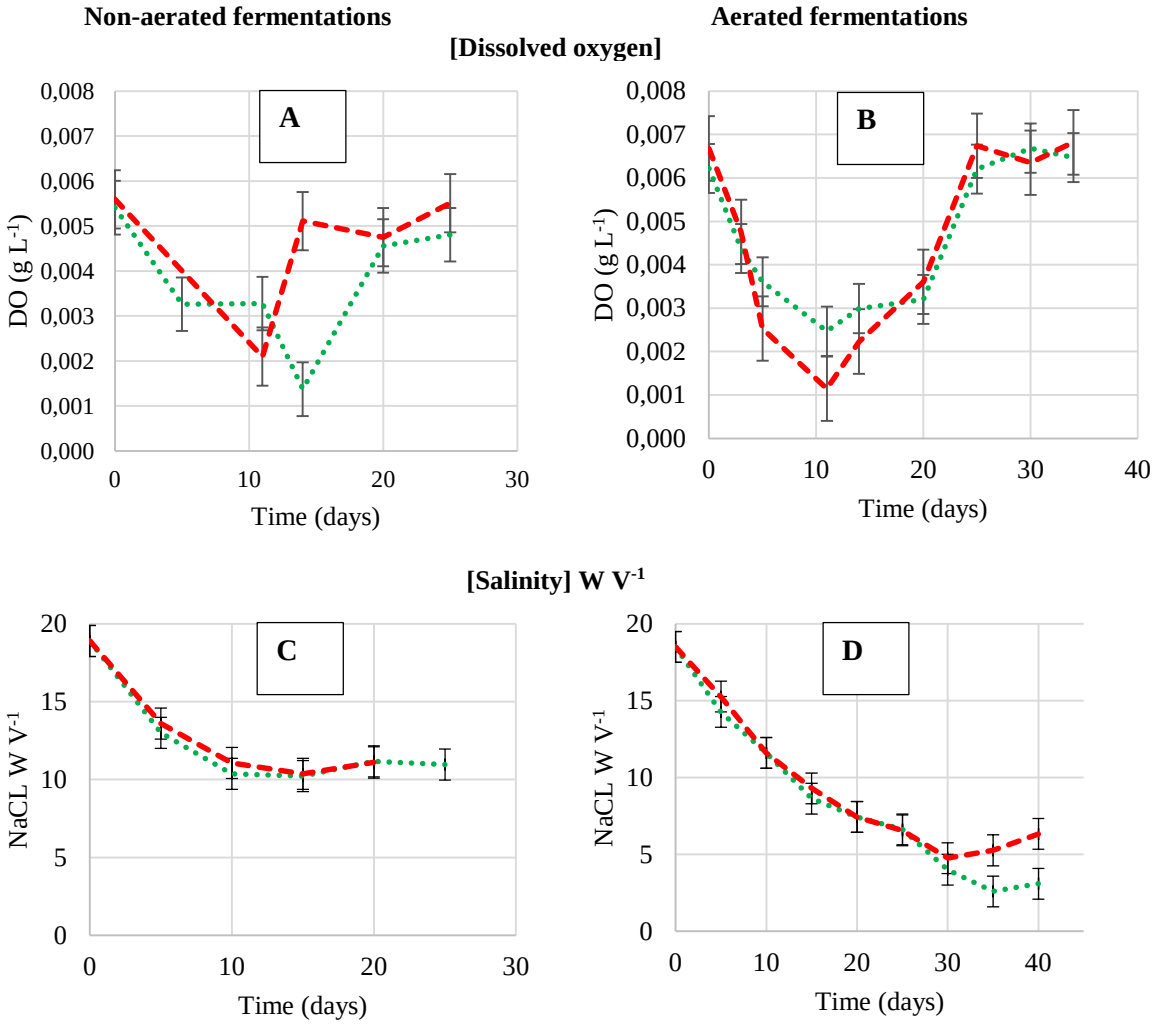


Figure 7.7: Dissolved oxygen and salinity changes during BSV fermentation under aerated and non-aerated conditions. ····· Corncobs, – – – Ca-alginate beads.

During non-aerated fermentation, the initial DO was lower than non-aerated fermentations which proves that the aeration process increased the DO in the fermentation medium. During the intermediate stages of the BSV fermentation, a decrease in DO was observed under both aerated and non-aerated conditions. This was initially attributed to be due to the aeration capacity changes during the intermediate stages of fermentation. However, the $K_L a$ profiles obtained in Fig. 7.6 do not demonstrate a lower aeration capacity at the intermediate stages of the BSV fermentation for all treatments studied.

7.4.2.6 Respiratory activity of free-floating cells during BSV fermentation

To assess the respiratory activity of the microbial consortium used, the Speedy Breedy was used to monitor the changes in pressure during BSV fermentation when using free-floating cells (FFC) of yeast and bacteria as a consortium. The FFC were used instead of cells adsorbed on CC or entrapped in Ca-alginate because the matrices cannot fit through the chambers used in the Speedy Breedy. The data obtained demonstrated that, at the 1st five days (Fig.) of BSV fermentation, there was a low pressure (mbar) increase between 0 min to 4000 min (2.7 days), however, a rapid increase in pressure was observed from ~5000 min (3.47 days), where ~1000 mbar increased to ~1600 mbar. These results show a low respiratory activity at the 1st three days of fermentation in both chambers, which was attributed to the lag phase of microbial growth.

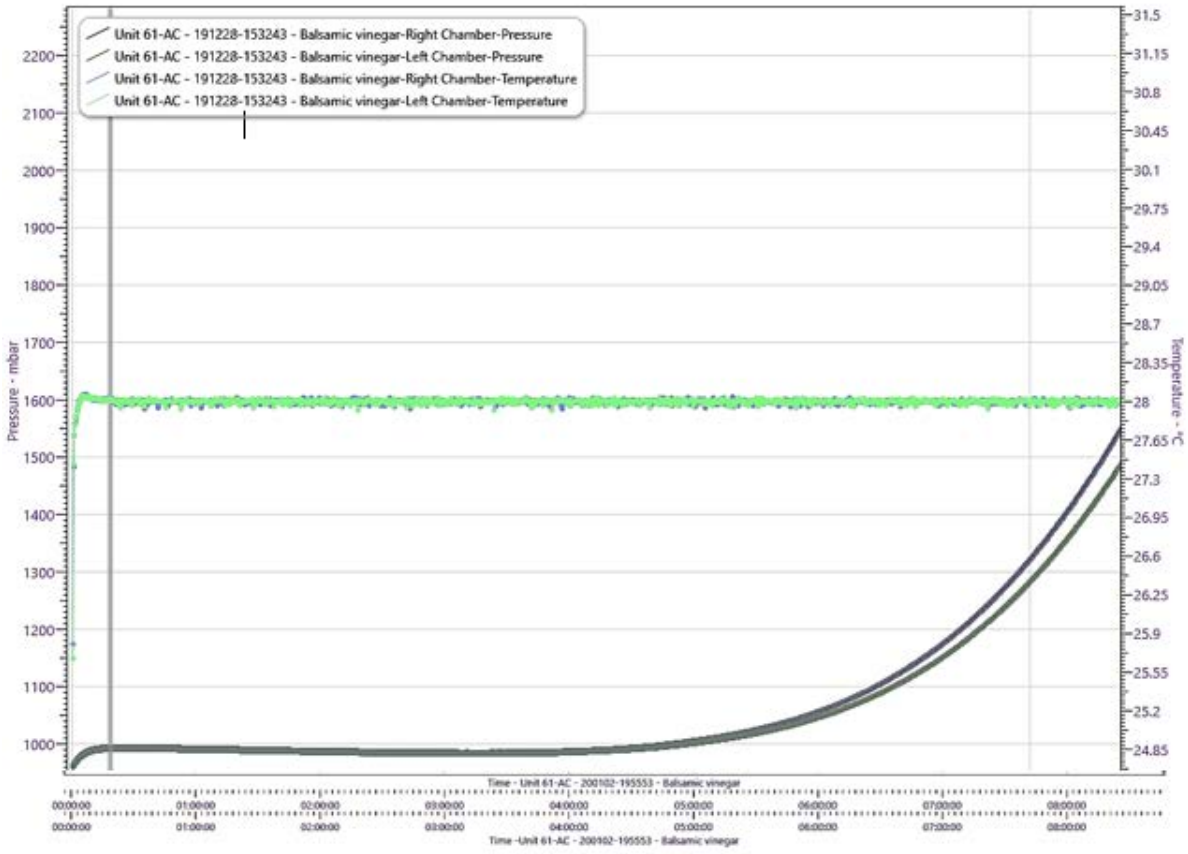


Figure 7.8: Respiratory activity between day 0 to 4 when using FFC for BSV production. BSV, Temperature

The results obtained between day 5 to 10 (Fig. 7.9) showed an exponential increase in pressure after 1000 min (0.69 days), the pressure increased from 1240 (at 1000 min) to ~1640 mbar. The exponential increase in pressure was attributed to the exponential growth phase of the microbial consortium when assuming that respiratory activity is proportional to biomass growth.

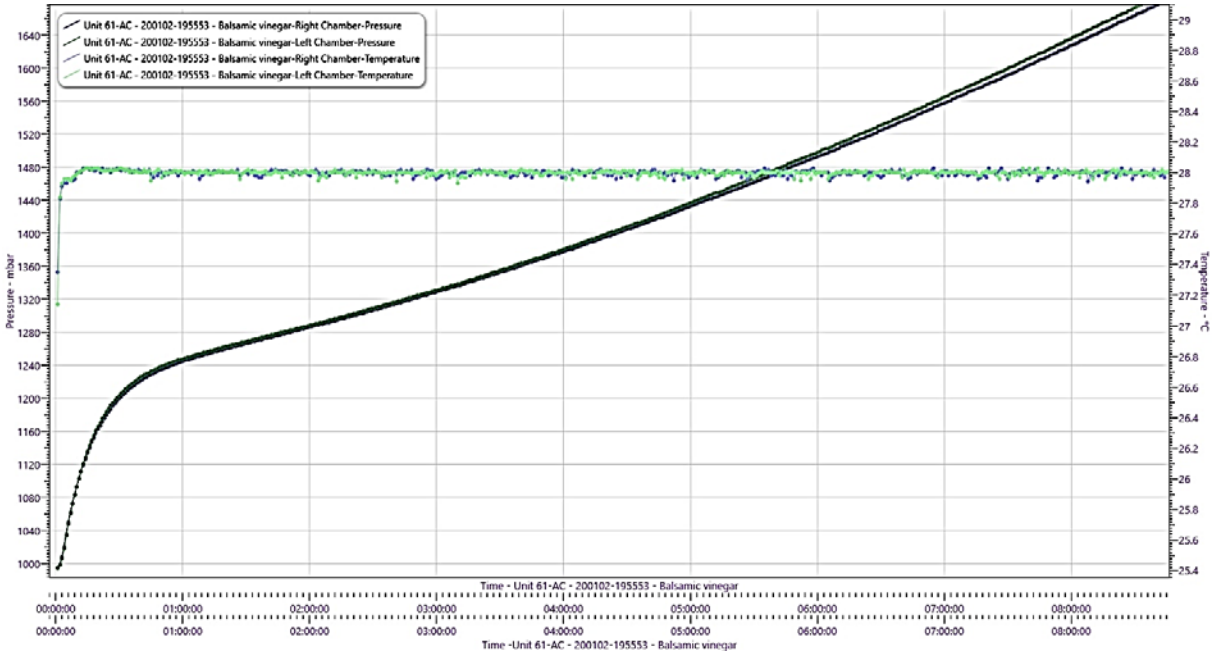


Figure 7.9: Respiratory activity between day 5 to 10 when using FFC for BSV production. . BSV, Temperature

During day 11 to 15 (Fig. 7.10), a substantial decrease in respiratory activity was observed. A sudden pressure decrease from ~980 mbar to ~800 mbar was observed. The data obtained in Fig. 7.8, 7.9 & 7.10 demonstrate that the respiratory activity obtained during this period was mainly due to yeast microbial activity when assuming that respiratory activity was proportional to product formation. The decline in respiratory activity in Figure 7.10 also demonstrates that the respiratory activity could have been yeast, because yeasts are probable to have a lower respiratory activity after the production of alcohol. Table 7.2 also provides details regarding the chemical developments of the Speedy Breedy samples, whereby yeasts produced high levels of alcohol in both chambers, while AAB produced minimal levels of total acid. These data demonstrate that it is difficult to measure the respiration of both yeasts and AAB when they are used as a mixed inoculum due to the different metabolic processes.

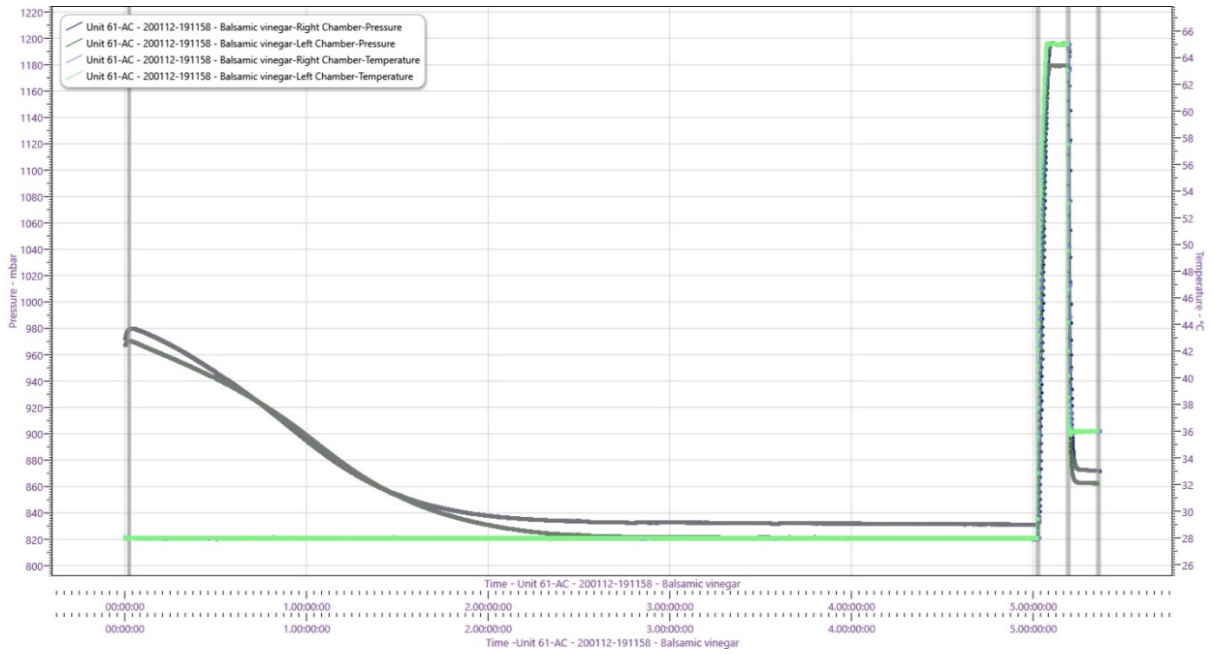


Figure 7.10: Respiratory activity between day 11 to 15 when using FFC for BSV production. . BSV, Temperature

Table 7.2: Alcohol and total acid changes after respiratory activity monitoring in the Speedy Breedy

Days	4	10	15
Chamber 1: [Alcohol] g L^{-1}	4.10	33.50	47.20
Chamber 2: [Alcohol] g L^{-1}	4.10	31.60	48.70
Chamber 1: [Total acid] g L^{-1}	12.40	14.40	15.22
Chamber 2: [Total acid] g L^{-1}	12.50	14.80	15.57

7.4.3 Theoretical reasons for the slow acetification rates during aerated BSV fermentations

This part of the study provided a reflective understanding concerning oxygen mass transfer kinetics during BSV production. However, there are several reasons that were assumed to cause the lower r_{acid} observed under aerated conditions. These reasons include but are not limited to EtOH evaporation, CO_2 production, loss of nutrients, loss of moisture and reduction in DO. The application of aeration during BSV production causes several complications. As observed in (Fig. 7.2 B, E). Aeration visibly led to the production of EtOH, while a high rate of EtOH disappearance was also observed in contrast to non-aerated fermentations. However, due to the low r_{acid}, it was therefore discernible that the disappearance of EtOH under aerated conditions was not due to the biological oxidation of EtOH by AAB but rather due to evaporation which was enhanced by the aeration process. The evaporation of EtOH also leads to low stoichiometric yields for alcohol oxidation, instead of the theoretical 100% (i.e, 1:1), the evaporation of alcohol substantially decreases the ratio in practical fermentations. Alcohol evaporation

generally occurs in both non-aerated and aerated conditions, however, the aeration process is known for causing higher evaporation rates (Qi et al., 2014; Schlepütz, & Büchs, 2014; Gullo et al., 2014), since the speed at which air flows above the surface or in liquid causes several molecules in the liquid to dissipate into the air (Moghiman & Joudat, 2007; He et al., 2019). For this reason, aeration also caused the loss of moisture and H_2O in the fermentation medium. BSV fermentation is generally lengthy compared to industrial vinegar fermentation. Therefore, when aeration was applied for >20 days, this resulted in a substantial reduction (± 40%) of the fermentation working volume and consequently the water activity was also reduced which is detrimental for AAB (Solieri & Giudici, 2008; Tofalo *et al*., 2009). It is also possible that aeration could have caused the reduction/evaporation of other nutrients that are chemically bonded to H_2O and are important for the survival and activity of AAB. Furthermore, aeration also caused a higher reduction in saline (Fig, 7.5D) compared to non-aerated fermentation (Fig. 7.5C). The reduction of saline could not have been due to evaporation, because although salt dissolves in liquid, it is not actually chemically bonded to the liquid, consequently, when H_2O evaporates, the salt remains in the residual liquid (Akridge, 2008).

The production of CO_2 during fermentation might have had detrimental effects on the AAB. According to the r_{sugar} profiles, yeasts are clearly not affected under both non-aerated and aerated conditions, thus a higher r_{sugar} and $r_{alcohol}$ is observed under aerated fermentations in contrast to non-aerated fermentations (Table 7.1, Fig. 7.2 C, D). The presence of high CO_2 might have lowered the metabolic activity, or had lethal effects on the AAB (Cheng, 2001), thus lowering r_{acid}. Furthermore, although the alcoholic and acetification process is separated during industrial vinegar production systems, the problem of CO_2 build up is also a common occurrence (García-García et al., 2009; Garcia-Ochoa et al., 2010). For this reason, it may be useful to employ special devices CO_2 extraction in the fermentation vessels.

Fundamentally, the long fermentation period during BSV fermentation could be the major reason for aeration being unsuitable for the process, because the longer the fermentation length, the better the chance of aeration to make alterations to the fermentation medium that are unsuitable for the microbial consortium. The r_{acid} were also calculated at the first 5 days of the fermentation period (Table 7.2), which served as initial r_{acid}. The data (Table: 7.2) obtained showed that CC treatments had relatively similar r_{acid} for both non-aerated and aerated conditions, while Ca-alginate beads also had relatively similar r_{acid} for both non-aerated and aerated conditions at the initial stages of the fermentation. However, when the fermentation progresses, the r_{acid} rates decrease due to the high evaporation of alcohol (Fig. 7.3 C, D), which resulted in very low concentrations of alcohol during the late stage of the fermentation, as a result, the overoxidation of AcOH to CO_2 and H_2O occurred.

Table 7.3: Acetification rates at the first 5 days of the BSV fermentation process

	Initial rate of acid formation (r_{acid}) (**g L^{-1} h^{-1}**)	Initial rate of acid formation (r_{acid}) (**g L^{-1} day^{-1}**)
Non-aerated corncobs	0.061	2.72
Aerated corncobs	0.097	2.34
Non-aerated Ca-alginate beads	0.11	0.25
Aerated Ca-alginate beads	0.01	0.26

Similar challenges are experienced with industrial spirit vinegar production, however the short fermentation period (20-24 hrs) (Budak & Guzel-Seydim, 2010) lessens the severity of the aeration effects. In other words, although AAB are strictly aerobic and require high DO concentrations for optimal activity, it seems that applying aeration during BSV fermentation is more detrimental than beneficial due to the aforementioned reasons.

7.5 Summary

Studying the oxygen mass transfer kinetics during BSV production provided a more reflective understanding of the BSV fermentation system used. Non-aerated fermentations had higher Stoichiometric coefficients for oxygen consumption vs acid yield, for both CC and Ca-alginate beads treatments. Ca-alginate beads have a longer lag phase in contrast to CC treatments for both aerated and non-aerated conditions. The volumetric mass transfer coefficients demonstrated that aerated fermentations had a low aeration capacity compared to non-aerated fermentations. Fermentations carried out with CC had a lower aeration on both non-aerated and aerated conditions. While Ca-alginate beads fermentation had the highest aeration capacity. This data demonstrates that the use of CC could result in considerably higher acetification rates if a suitable bioreactor that improves $K_L a$ could be constructed. Overall, the kinetics prove that aeration is not a necessary addition for BSV fermentation, unless extra technical modifications are made.

CHAPTER 8

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

CHAPTER 8

GENERAL DISCUSSION, SUMMARY AND CONCLUSION

8.1 General discussion, summary and conclusions

In the current study, process augmentation techniques such as cell immobilization, aeration and agitation were studied for the production of Balsamic-styled vinegar (BSV) at laboratory scale. A defined microbial consortium of non-*Saccharomyces* yeast (n=5) and acetic acid bacteria (AAB) (n=5) was used for BSV fermentation via a simultaneous co-inoculation strategy. The study commenced by investigating the effect of cells immobilized by the entrapment technique in Ca-alginate beads alongside free-floating cells (FFC), while also studying the effects of beads size (surface area) and agitation during BSV fermentation. The results obtained demonstrated that the surface area of the beads had an impact on acetification rates. Consequently, small beads resulted in higher acetification rates with a fermentation period of 16 days, in contrast to large beads which resulted in relatively lower acetification rates with a fermentation period of 19 days, while FFC cells resulted in a fermentation period of 31 days. Agitation led to substantially negligible acid developments and incomplete fermentations. This was therefore attributed to shear stress and bead damage when agitation was used, which was further assessed and confirmed via microscopic studies.

Although cell immobilization using the entrapment method yielded high acetification rates, it is, however, an expensive technique and it requires the use of high technology and specialized equipment for larger scale productions. Consequently, cell immobilization by the adsorption technique was also tested on corncobs (CC) and oak wood chips (OWC) in contrast to free-floating cell (FFC) fermentations under static, agitated, low aerated (LA) and high aerated (HA) conditions. Corncobs are less expensive compared to alginate and OWC, and a producer can choose a suitable matrix based on cost. Furthermore, the results obtained when adsorbed cells were assessed showed that static-non aerated conditions led to the highest acetification rates compared to agitated, low aerated and high aerated fermentations. Moreover, cells adsorbed on CC resulted in the highest acetification rates with a fermentation period of 20 days, compared to the other adsorbents studied which led to a fermentation period of >30 days. Furthermore, a LA setting resulted in higher acetification rates compared to the other treatments studied. The high acetification rates when CC were used was therefore attributed to high cell affinity due to the rough and porous structure of the corncobs. Agitation also led to incomplete fermentations when adsorbed cells and FFC were used. The variations in cell adsorption for the individual yeasts and AAB was also evaluated before and after fermentation. Consequently, it was

observed that there were variations in cell adsorption capabilities for yeast and AAB cells, however, a lower cell adsorption capability was observed on OWC.

The reusability of immobilized cells is one of the prominent advantages of cell immobilization. It was therefore rational to test the reusability of Ca-alginate entrapped cells and cells adsorbed on CC and OWC. However, the acetification rates obtained on the 2nd cycle fermentations were substantially lower than the 1st cycle. Only Ca-alginate beads were able to produce BSV during the 2nd cycle, while the adsorbed cells (CC and OWC) could not produce a BSV within an anticipated time frame. The scanning electron microscope was used to assess the structural integrity of the matrices used for cell immobilization before and after the 1st cycle. The images showed a visible loss of structural integrity for Ca-alginate beads after the 1st cycle of fermentation. While CC showed minimal structural changes and OWC chips retained their structure, these observations were attributed to the robustness of the OWC and CC. Taking all of aforementioned in consideration, it is therefore not recommended to reuse any of the immobilized cells without making modifications.

The BSV's obtained from the 1st cycle of fermentations were evaluated for sensory attributes using the 'check all that applies' (CATA) method. The sensory data showed that BSV's fermented with cells adsorbed on CC and cells entrapped in Ca-alginate were 'liked' by the judges, while BSV's produced using cells adsorbed on OWC were neither 'liked nor disliked'. Overall, the data obtained on the 1st three phases of the study showed consistency with regards to the matrices that yield the highest acetification rates. Ca-alginate beads and CC resulted in higher acetification rates on all phases of the study in contrast to OWC fermentation and free-floating cells. Furthermore, agitation and aeration resulted in lower acetification rates for all treatments studied during all the phases of this study. Therefore, to further understand and to provide a more profound comparative analysis of the effect of aeration in contrast to non-aeration, oxygen mass transfer studies were conducted.

Only CC and Ca-alginate beads were tested for oxygen mass transfer kinetic studies due to the sustainable high acetification rates when tested during the other phases of the study. Several oxygen mass transfer parameters were computed, which include oxygen uptake rate, the stoichiometric coefficient of oxygen consumption vs acid yield, the oxygen transfer rate and the volumetric mass transfer coefficients. The data generated from oxygen mass transfer computations showed that the volumetric mass transfer coefficient is undoubtedly the most important parameter to focus on in order to improve the BSV production system. The data demonstrated that the aeration capacity of the selected bioreactors was low under aerated conditions. This was assumed to be caused by the improper design of the bioreactor and the design of the aeration system used. The aeration system used had detrimental effects on the fermentation medium, oxygen uptake rate, biomass formation and consequently the aeration capacity was affected. Nonetheless, the primary aims of reducing the BSV fermentation period

from >40 days were achieved, and all the questions asked at the commencement of the study were answered. Furthermore, an effective and relatively basic protocol for BSV production using immobilized cells has been formulated at laboratory scale. Overall, based on the data obtained, cells adsorbed on CC or cells entrapped in Ca-alginate beads are recommended for BSV production under static and non-aerated conditions. This conclusion was made based on fermentation periods, acetification rates, cell affinity/cell protection and sensorial contributions. However, the best method with regards to cost is the use of CC instead of CA-alginate beads.

8.2 Recommendations for future work

It is recommended that further process augmentation techniques be investigated and implemented for BSV production. These separation of the alcoholic and acetous fermentation processes by using a sequential inoculation process could be investigated and implemented. This could ascertain the reusability of the immobilized cells for successive fermentations and probably for several cycles, thus lowering the production cost. The design of a suitable fermentation system is crucial, whereby the aeration capacity will be increased by including a fine bubble aeration system and suitable geometric configurations. However, these technical additions for BSV production are costly. Another important aspect is the further ageing of the vinegars. Here techniques to shorten the ageing should be investigated. This study focused on the production process and chemical changes that occurred during BSV production, but more sensory research is needed to improve the organoleptic properties of BSV.

CHAPTER 9

REFERENCES

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