Isolation and characterisation of the antimicrobial peptides produced by acetic acid bacteria

by

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

Wine quality is greatly influenced by the number of microorganisms, which occur throughout the winemaking process. Yeasts are responsible for the alcoholic fermentation, the lactic acid bacteria (LAB) are responsible for malolactic fermentation (MLF), while acetic acid bacteria (AAB) are responsible for converting ethanol to acetic acid. These microorganisms are present on the grapes and in the cellar and these consequently serve as gateways to the fermentation tanks where they will affect the wine quality. However, these microorganisms can be seen either as beneficial or as wine spoilage microorganisms, depending on the conditions that prevail throughout the winemaking process. It is thus very important to prevent any process that could lead to the lowering of the wine quality. In this regard, some of the factors that should always be evaluated include the quality of the grapes, winemaking techniques and quality control.

One of the measures that have been implemented during winemaking to ensure the microbial stability is the use of chemical preservatives. Sulphur dioxide (SO_2) has been, and is, used widely as primary preservative in winemaking. However, an ever-increasing consumer resistance against the use of chemical preservatives has developed as it poses possible health risks and decreases the sensorial quality of wine. An alternative approach to chemical preservation that has triggered numerous new investigations, is biological preservation or biopreservation. This is the use of the natural microbial flora and/or their antimicrobial products, such as bacteriocins, to inhibit or destroy the other sensitive microorganisms that are unwanted in the same environment.

Evidence in the wine industry has shown that bacterial spoilage still is a very common problem in many wineries. This bacterial spoilage can lead to, amongst other, two main problems, which are of great concern to winemakers. This include high levels of volatile acidity, resulting in the wine having a vinegary off-flavour, and sluggish/stuck fermentation, which is the result of compounds such as acetic- and other fatty acids that causes inhibition of the yeast's growth. With acetic acid being the common link in both cases, it became evident that investigations should be performed on the main producer of acetic acid, namely AAB. As a result, AAB turned out to be one of the main spoilage microorganisms associated with winemaking.

Most of the research on biopreservation in the food and beverage industry has been performed on the Gram-positive LAB. The fact that their spectrum of inhibition currently excludes most Gram-negative bacteria, specifically AAB, indicated that AAB should be screened in search of possible antimicrobial compounds that could be applied to control their cell numbers during winemaking. No evidence of antimicrobial action amongst AAB could be found in literature, therefore this work was considered novel.

The main objectives of this study were to screen wine isolates of AAB for the production of antimicrobial compounds. This was followed by the isolation and

preliminary characterisation of the antimicrobial substances produced. Various attempts to optimise the production of the antimicrobial compounds and isolation procedures, were also included. This study forms part of a larger research programme that has been initiated at the Institute for Wine Biotechnology at Stellenbosch University on the biopreservation in wine.

Our results indicated that possible antimicrobial compounds of proteinaceous nature, produced by AAB isolated from wine, do exist. It was found that two different species of AAB, namely *Acetobacter aceti* and *Gluconobacter frateurii*, produced antimicrobial compounds that inhibited other species of AAB. Preliminary results indicated that these compounds are heat sensitive and stable in a wide pH range. It was also shown that after the action of proteolytic enzymes, such as proteinase K and α-chemotrypsin, all inhibitory activity was lost. This study also revealed the existence of the species *Gluconobacter frateurii*, which have not yet been associated with the winemaking environment.

This study made a valuable contribution to the limited amount of information and understanding of AAB, not only in the wine environment, but also elsewhere. The results and findings of this research would serve as platform for further projects. This might soon lead to the development of antimicrobial substances or tailored wine-yeasts with antimicrobial abilities, which can be applied during winemaking to assist the winemaker in combatting high cell numbers and subsequent spoilage by AAB.

OPSOMMING

Wynkwaliteit word beïnvloed deur 'n verskeidenheid van mikroörganismes wat regdeur die wynmaakproses teenwoordig is. Die giste is vir die alkoholiese fermentasie, die melksuurbakterieë (MSB) vir die appelmelksuurgisting, terwyl die asynsuurbakterieë (ASB) vir die omskakeling van etanol na asynsuur verantwoordelik is. Al hierdie mikroörganismes is teenwoordig op die druiwe en in die kelder, en dit dien gevolglik as 'n weg waardeur hulle in die fermentasietenke kan kom om sodoende die wynkwaliteit te beïnvloed. Hierdie mikroörganismes kan egter gesien word as óf voordelig óf as wynbederfmikroörganismes, afhangende van die heersende kondisies gedurende die wynmaakproses. Dit is daarom baie belangrik om enige proses te voorkom wat tot 'n verlaging in wynkwaliteit kan lei. Wat laasgenoemde aanbetref, is daar sekere faktore wat altyd geëvalueer moet word, naamlik die druifkwaliteit, wynmaaktegnieke en kwaliteitsbeheer.

Een van die maatreëls wat geïmplementeer is om mikrobiologiese stabiliteit tydens die wynmaakproses te handhaaf, is die gebruik van chemiese preserveermiddels. Swaweldioksied (SO_2) word algemeen gebruik as primêre preserveermiddel tydens wynmaak. Daar is egter 'n toenemende verbruikersweerstand teen die gebruik van chemiese preserveermiddels, aangesien dit moontlike gesondheidsrisiko's kan inhou, asook tot 'n verlaging in sensoriese kwaliteit van die wyn kan lei. 'n Alternatiewe benadering vir chemiese preservering, wat reeds tot verskeie nuwe ondersoeke gelei het, is biologiese preservering of biopreservering. Dit is die gebruik van die natuurlike mikroflora en/of hulle antimikrobiese produkte, soos bv. bakteriosiene, om die sensitiewe mikroörganismes wat in dieselfde omgewing voorkom, se groei te inhibeer óf om hulle dood te maak.

Aanduidings vanuit die wynbedryf dui daarop dat bakteriese bederf steeds 'n algemene probleem is wat in baie kelders ondervind word. Hierdie bakteriese bederf kan onder andere twee hoofprobleme veroorsaak, wat 'n groot bekommernis vir verskeie wynmakers is. Dié probleme sluit in hoë vlakke van vlugtige suurheid, wat gevolglik die wyn 'n asyn-afgeur gee, en slepende/gestaakte fermentasies, wat die gevolg is van komponente soos asynsuur en ander vetsure, wat die gis se groei inhibeer. Die feit dat asynsuur die gemeenskaplike faktor in beide gevalle was, het daarop gedui dat 'n ondersoek rakende die hoofproduseerder van asynsuur, naamlik ASB, benodig word. ASB word gevolglik as een van die hoofbederforganismes wat met die wynmaakproses geassosieer word, beskou.

Die meeste navorsing oor biopreservering in die voedsel -en drank bedryf is op die Gram-positiewe MSB gedoen. Die spektrum van inhibisie van die bakteriosiene van MSB sluit egter die meeste Gram-negatiewe bakterieë uit, veral ASB, en dit dui daarop dat ASB gesif moet word in 'n soektog na antimikrobiese substanse wat moontlik gebruik kan word om hul getalle tydens die wynmaakproses te beheer. Geen bewyse kon tot dusver uit die literatuur gekry word met betrekking tot

antimikrobiese aktiwiteit teen ASB nie, daarom word hierdie navorsing dus as nuut beskou.

Hierdie studie se hoofdoelwitte was om die wyn-isolate van ASB vir die produksie van antimikrobiese peptiede te sif. Dit is gevolg deur die isolasie en voorlopige karakterisering van die geproduseerde antimikrobiese komponente. Daar is ook verskeie pogings aangewend om die produksie van die antimikrobiese substanse, asook die isolasieprosedures, te optimiseer. Hierdie studie vorm deel van 'n groter navorsingsprogram oor biopreservering van wyn wat deur die Instituut vir Wynbiotegnologie by die Universiteit van Stellenbosch geïnisieer is.

Die resultate het daarop gedui dat antimikrobiese substanse van proteïenagtige aard, afkomstig vanaf wyn-isolate van ASB, wel bestaan. Daar is gevind dat twee veskillende spesies, naamlik *Acetobacter aceti* en *Gluconobacter frateurii*, antimikrobiese peptiede produseer, wat ander spesies van ASB kan inhibeer. Voorlopige resultate het getoon dat hierdie substanse hitte-sensitief is en ook stabiel is oor 'n wye pH-reeks. Daar was ook aanduidings dat, ná die aksie van proteolitiese ensieme, soos bv. proteïnase K en α-chemotripsien, al die inhibitoriese aktiwiteit verlore gegaan het. Hierdie studie het ook die voorkoms van die spesies *Gluconobacter frateurii* aangedui, wat nog nie tot dusver met die wynmaakomgewing geassosieer is nie.

Hierdie studie maak 'n waardevolle bydrae tot die beperkte hoeveelheid inligting oor en begrip van ASB, nie net in die wynomgewing nie, maar ook in die algemeen in die natuur. Die bevindinge en resultate van hierdie navorsing sal as basis dien vir verdere projekte wat sal volg. Dit kan moontlik binnekort lei tot die ontwikkeling van antimikrobiese substanse, en ook pasgemaakte wyngiste met antimikrobiese vermoëns, wat tydens die wynmaakproses gebruik kan word om sodoende die wynmaker in staat te stel om die hoë bakteriese getalle en die gevolglike bederf deur ASB, te beheer.

This thesis is dedicated to my family, especially my parents, Rudie and Julie.

Hierdie tesis is aan my familie opgedra, veral aan my ouers, Rudie en Julie.

BIOGRAPHICAL SKETCH

Adriaan Oelofse was born in Wynberg, Cape Town on the 10th of January 1977. He attended Vredenburg High School and matriculated in 1994. Adriaan enrolled at Stellenbosch University in 1995 and obtained a BSc degree in 1998, majoring in Biochemistry and Microbiology. In 2000, Adriaan enrolled at the Institute for Wine Biotechnology and obtained an Honours degree in Wine Biotechnology in December of that year. He enrolled for his Master's degree in Wine Biotechnology at the same Institute in 2001.

ACKNOWLEDGEMENTS

PREFACE

This thesis is presented as a compilation of five chapters, with each of the chapters being introduced separately. Chapter 3 is written according to the style of the journal *International Journal of Food Microbiology*.

CONTENTS

CHAPTER 1

Introduction
&
Project Aims

1. GENERAL INTRODUCTION AND PROJECT AIMS

1.1 INTRODUCTION

One of the oldest preservation technologies, which also contributes to the wholesomeness, flavour, aroma and texture of the end product, is fermentation. All these attributes are associated with the microflora present in the raw material or are characteristics of the selected starter culture. New ideas for preservation or, more specifically, biopreservation currently are under investigation within the food and beverage industries (Cunningham et al., 1991; Leistner, 1992; Fleet, 1998; Du Toit and Pretorius, 2000; Soomro et al., 2002).

The fermentation process in winemaking is one of the oldest and has spawned considerable interest over the centuries (Ross et al., 2002). Winemaking is not only an ancient art, but also a great example of where the natural complexity of a product is a direct reflection of its quality. With the involvement of microorganisms in this complex ecology, numerous investigations are currently underway in an attempt to further understand the significance of these microorganisms in winemaking. Some of the different microorganisms involved in this process include the yeasts that are responsible for the alcoholic fermentation, lactic acid bacteria (LAB), which are responsible for the malolactic fermentation (MLF), and acetic acid bacteria (AAB), which mainly are associated with spoilage (Fleet, 1998; Ribéreau-Gayon et al., 2000). The alcoholic fermentation is the most important biochemical process during winemaking and therefore any interference or inhibitory action exerted on the yeasts directly affects this process and may result in sluggish/stuck fermentation.

Many investigations regarding the causes of sluggish/stuck fermentation have been carried out (Henschke, 1997). These studies include the effect of nutrient deficiency, ethanol toxicity (D'Amore and Stewart, 1987), temperature extremes (Sharf and Margalith, 1983), oenological practices (Bisson, 1999) and inhibition by acetic acid and other fatty acids (Rasmussen et al., 1995; Du Toit, 2000).

It has been indicated that acetic acid can affect both the chemical composition of the must and wine and the metabolism of the yeast (Grossman and Becker, 1984; Joyeux, 1984b; Du Toit and Pretorius, 2002). Acetic acid occurs naturally in wine, as it is produced by yeasts during alcoholic fermentation and by LAB during MLF. However, it is mainly the action of the AAB, which produce this acid up to undesirable levels in the wine, that leads to acetification of the wine (Drysdale and Fleet, 1988). The spoilage capabilities of AAB are additionally of concern due to the fact that they can also cause surface films, turbidity, colour change and loss of alcohol, as AAB use the latter to produce acetic acid (Drysdale and Fleet, 1988; Fugelsang, 1997).

In order to control unwanted bacterial cell numbers and the consequent spoilage of foods and beverages, chemical preservatives, such as sorbic acid, benzoic acid and sulphur dioxide (SO_2) are applied (Du Toit and Pretorius, 2000). SO_2 especially is used during winemaking and is one of the oldest known chemicals with antioxidative and antimicrobial properties. These properties, however, are dependent on the pH of the aqueous environment. It has been indicated that the antimicrobial activity of SO_2 is only exerted when it is in the molecular form (Fugelsang, 1997). At

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wine pH values (3.0-4.0), only 5% of SO_2 is believed to be in the active molecular form. The other 95% is in the form of bisulphite ions (Romano and Suzzi, 1993).

The efficiency of this chemical preservative in the wine environment is further questioned, as the must and wine contain different SO_2-binding substances, such as acetaldehyde, anthocyanins, keto-acids and dihydroxyacetone (Romano and Suzzi, 1993; Fugelsang, 1997). These compounds are produced as metabolites by wild yeasts (*Candida* spp., *Saccharomycodes ludwigii*), fungi (*Botrytis cinerea*), LAB (*Lactobacillus* spp., *Leuconostoc mesenteroides*) and AAB (*Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconobacter oxydans*) (Fleet, 1992; Sponholz, 1993; Fugelsang, 1997; Du Toit and Pretorius, 2002). With the focus staying on AAB, previous studies have reported that high concentrations of SO_2 (> 100 mg/l) were necessary to fully inhibit the growth of some *Acetobacter* spp. (Joyeux et al., 1984a). It has also been revealed that the SO_2 sensitivity of AAB is strain dependent (Du Toit, 2000).

The above factors suggest the possible usage of SO_2 at undesired levels to ensure complete inhibition of AAB or, more generally speaking, to obtain microbial stability. The use of high levels of SO_2 will detract from the organoleptic quality of wine and, together with increasing consumer concerns regarding the health benefits of chemical preservatives (can cause severe asthma attacks and headaches), it has inspired the research community to explore alternative methods of preservation (Du Toit and Pretorius, 2000). The alternative, which has already triggered a new dimension of preservation, is the use of biological preservatives.

Biopreservation is the use of antimicrobial compounds from plants, animals and microorganisms to preserve products via a more natural process in an attempt to generate products with a longer shelf-life, of higher quality, that are less heavily preserved and that are considered to be healthier. Biopreservation has been applied in the food industry with the use of bacteriocins, especially nisin leading the frontier (Soomro et al., 2002). Bacteriocins, which commonly are produced by certain Gram-positive LAB, are ribosomally-synthesised antimicrobial peptides that are secreted by the bacterial producer cells to inhibit other (usually but not always) closely related bacteria (Ennahar et al., 1999, Du Toit and Pretorius, 2000; Chin et al., 2001). The use of bacteriocins as biopreservatives in winemaking has been evaluated, as their producer organism, LAB, is a natural inhabitant of the wine environment (Daeschel, 1989; Radler, 1990; Strasser de Saad et al., 1995; Du Toit, 2002). Other biological substances that also have been investigated for use during winemaking are the zymocins or killer toxins produced by yeasts as antifungal compounds (Boone et al., 1990), and bacteriolytic enzymes such as lysozyme (Gerbaux et al., 1997, 1999).

As AAB are capable of causing spoilage during winemaking, biological compounds that could effectively combat these Gram-negative bacteria would prove very useful to answer consumer demands for reducing the levels of chemical preservative in wine. The known spectrum of bacteria on which the antimicrobial activities of Gram-positive and Gram-negative bacteria have proved noteworthy, seem to exclude AAB. Therefore, an investigation to find bacteriocin-like compounds

produced by AAB that inhibit closely related species could well contribute to broadening the future of biopreservation, especially in the wine industry.

1.2 PROJECT AIMS

This study forms an integral part of a larger research project on the biopreservation of wine that is being conducted at the Institute for Wine Biotechnology. The use of novel antimicrobial peptides produced by AAB would be useful for the biopreservation of wine spoilage bacteria. With advances in recombinant DNA technology, the bacterial-encoding genes for antimicrobial activity by AAB could, similarly to that of LAB (Schoeman et al., 1999), be transferred into the wine yeast *Saccharomyces cerevisiae.* This genetically engineered *S. cerevisiae* secretes the biologically active bacteriocin and this will provide the winemaker with a yeast that can combat spoilage LAB while performing its primary function, and possibly also bring about a concomitant reduction in the levels of chemical preservatives used. This will contribute to the production of the healthy, high quality products demanded by consumers. The specific aims of this study were as follows:

(i)   to screen various isolates of acetic acid bacteria isolated from grapes, must and wine for the production of antimicrobial peptides;

(ii)   to isolate these peptides, when found;

(iii)   to identify the producer strains by means of biochemical tests and PCR-RFLP analysis of the 16S rDNA;

(iv)   to determine the spectrum of inhibition against other wine-associated microorganisms, such as yeasts, lactic acid bacteria and acetic acid bacteria;

(v)   to treat the peptides with proteolytic enzymes to verify their proteinaceous nature;

(vi)   to determine the functionality of these peptitides in a pH and temperature range;

(vii)   to determine the mode of action of the antimicrobial peptides; and

(viii)   to determine the size of the peptide.

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CHAPTER 2

Literature review
The significance of acetic acid bacteria: Friend or foe?
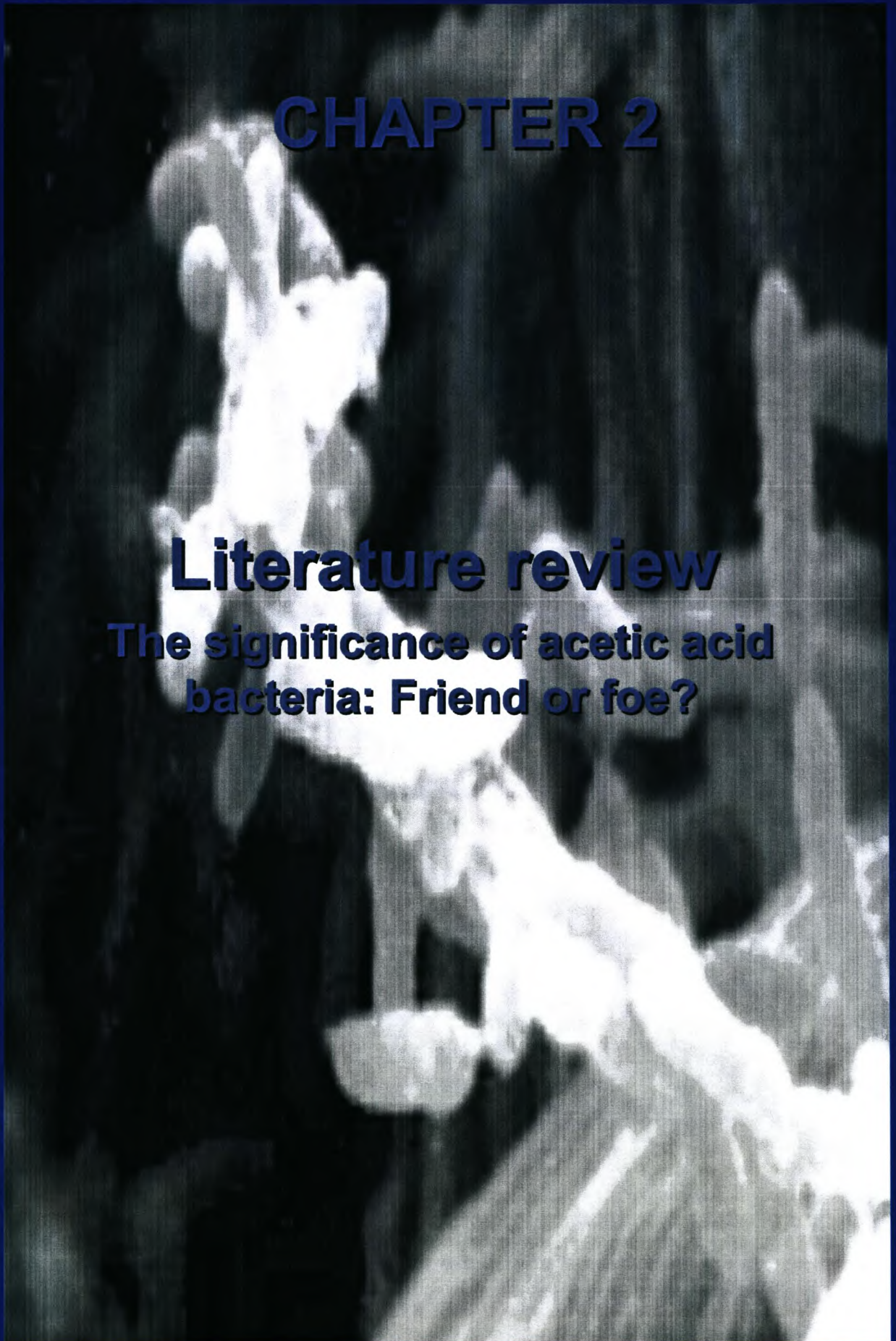
2. THE SIGNIFICANCE OF ACETIC ACID BACTERIA: FRIEND OR FOE?

2.1 INTRODUCTION

The involvement of microorganisms in the fermentation of alcoholic beverages has been a subject of interest for centuries. In the mid-1800s, Louis Pasteur observed the conversion of grape juice into wine by the action of yeast (Drysdale and Fleet, 1988). He also noticed the presence of bacteria that were capable of causing spoilage. It was the acidification, or more specifically the acetification, of wine that motivated the early microbiologists. The formation of a pellicle on the wine after exposure to air was associated with acetification, hence the spoilage organism involved was designated acetic acid bacteria (AAB).

Acetic acid bacteria are ubiquitous in nature and are found on flowers and in association with deteriorating fruit and vegetables (Fugelsang, 1997; Gupta et al., 2001). With regard to fermented beverages from grapes, the contribution of these bacteria was of less significance than that of yeast and lactic acid bacteria (LAB). It was thought that their participation during the alcoholic fermentation was limited, as the winemaking process is anaerobic and AAB are classified as aerobic microorganisms. It was believed that sound winemaking practices, such as minimising the contact of the grape juice and wine with oxygen, could easily control the negative influence of AAB on wine quality. In recent years, however, the significance of AAB has become more prominent.

An increasing number of wineries started to experience the problem of sluggish or stuck fermentation during the late 1980s. Many causes of sluggish or stuck fermentation have been given, ranging from nutrient deficiency, ethanol toxicity and inhibitory compounds, such as acetic acid and other fatty acids (Bisson, 1999). Acetic acid is a natural component of wines, as it is produced by yeasts during the alcoholic fermentation and by LAB during malolactic fermentation (Drysdale and Fleet, 1988). However, the action of spoilage organisms such as AAB can increase the levels of acetic acid to undesirable levels. As acetic acid constitutes more than 90% of the volatile acidity (VA) of wine, and because sluggish or stuck fermentations often are associated with wines that show high levels of VA, the significance of AAB has become a subject of renewed interest. Research benefited when it was revealed that these organisms, which previously were believed to be strict aerobes, appeared to grow and survive under anaerobic conditions (Drysdale and Fleet, 1985) due to their ability to use other substances, such as quinones and reducible dyes, as final electron acceptors instead of oxygen (Adlercreutz and Mattiasson, 1984).

Today, AAB are known to affect both the chemical composition of the must and wine and the metabolism of the yeast. These bacteria can cause surface films, turbidity, colour change, loss of alcohol production and acidification in food and beverages. All of these contribute to the poor sensory quality of the final product. In

this review, some of the uses of AAB for industrial and pharmaceutical applications will be discussed. The research conducted on other commodities has made an invaluable contribution to a better understanding of AAB in a wine-related environment. A more detailed discussion of the influence of AAB on wine quality will follow, and this will include factors affecting their metabolism, their survival in anaerobic conditions and their interaction with other wine-related organisms.

2.2 USES OF ACETIC ACID BACTERIA

2.2.1 Vinegar production and acetate oxidation

Vinegar has been in use since historic times. It was considered to be the first antibiotic in the medical field, has been used for the preservation of foods and is also used as a cooking ingredient. However, it was more commonly used as the drink of slaves and soldiers during the era of the Roman Empire (Fugelsang, 1997).

Vinegar was originally obtained from ethanol-containing solutions that had undergone natural fermentations and the nature of the process was not understood for centuries. It was not until 1862 that Louis Pasteur described and recognised that the "mother of vinegar" was in fact a mass of living organisms that caused acetic acid fermentation (Saeki et al., 1997b). The involvement of AAB in vinegar and other oxidised fermentations remained unexplored for almost a century, until the early 1960s, when Nakayama indicated two novel cytochromes in AAB that catalysed the oxidation of alcohol and an aldehyde respectively. Nevertheless, it was believed that the action of cytosolic NAD(P)$^+$-dependent enzymes was responsible for the occurrence of vinegar production. It was only in the late 1970s when a membrane-bound alcohol-dehydrogenase was isolated from AAB. This was a revolutionary breakthrough in the understanding of vinegar production or, more specifically, ethanol oxidation.

Today it is known that vinegar is a product of the biological oxidation of ethanol carried out by AAB (**Fig. 2.1**). Matsushita and his co-workers (1994) have described the bioenergetics of AAB and current knowledge on ethanol oxidation very well. They have shown that, although the NAD(P)$^+$-dependent alcohol (ADH) and aldehyde dehydrogenases (ALDH) have been isolated and characterised from *Acetobacter*, vinegar production largely was the result of the action of the membrane-bound NAD(P)$^+$-independent ADH and ALDH quinoproteins containing the prosthetic pyrroloquinoline (PQQ) group. From **Fig. 2.2** it can be seen that these enzymes link together into a simple respiratory chain and generate the necessary proton motive force. This is the fundamental element of ethanol oxidation. What follows is a further look at the whole process of vinegar production.

$$C_2H_2OH \ + \ O_2 \longrightarrow CH_3COOH \ + \ H_2O$$

ethanol   oxygen   acetic acid   water
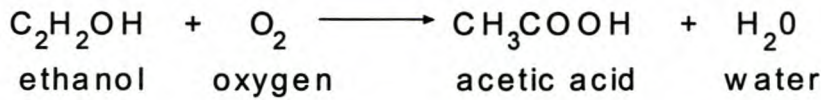
Fig. 2.1. The net enzymatic reaction for the formation of acetic acid from ethanol.
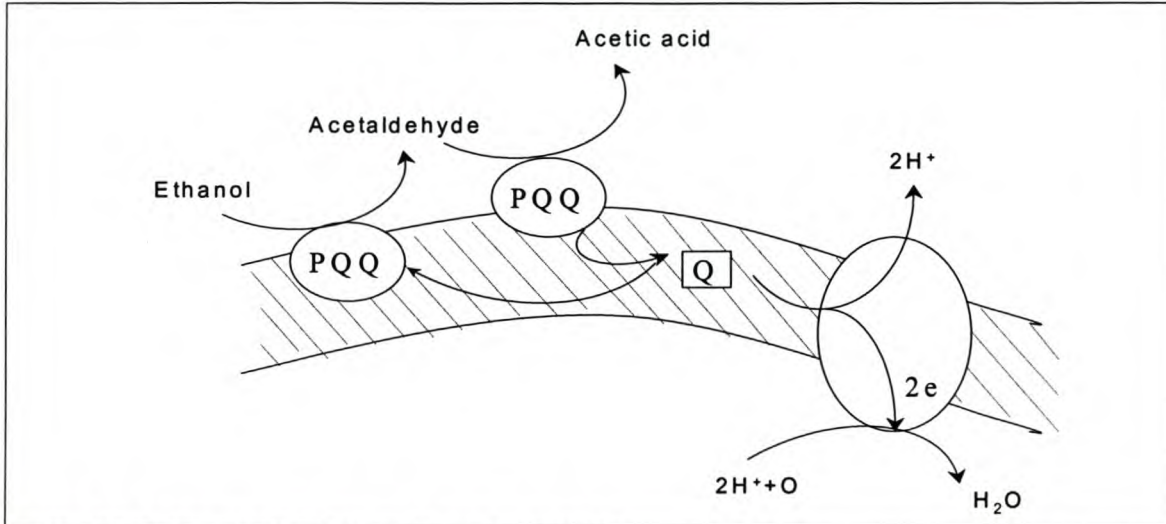
Fig. 2.2. Ethanol oxidation by *Acetobacter* (Adams, 1998).

A schematic representation of the entire process of vinegar production can be seen in **Fig. 2.3**. Industrially, there are two methods for the production of vinegar (Adams, 1998; Nanda et al., 2001). The first method is a slow, static surface fermentation. Although this method is time consuming, it renders a low-tech cost-effective product. This process is also regarded as the traditional method for vinegar production and lasts an average of 30 days. The second method includes an oxygenation process that makes a faster, submerged fermentation possible (Hitschmann and Stockinger, 1985). This method has evolved into the modern-day approach, which uses the so-called Frings-acetators (Frings, Bonn, Germany). This process technology delivers a high standard, as automation is possible and final acetic acid concentrations of up to 17% are achieved (Sokollek et al., 1998a).

The complex taxonomic history of AAB makes much of the interest in it purely academic. This is why the use of defined pure starter cultures of AAB in commercial fermentations is rarely employed (Adams, 1998; Nanda et al., 2001). Although it is not essential, the use of undefined cultures from semi-continuous batches can be controlled very well and satisfactorily without strict and costly sterility controls. Additionally, the use of pre-batches as starter cultures has been the method employed for centuries (Sokollek et al., 1998a). However, in the light of ever-increasing health concerns and public interest, the traditional vinegar production methods have come under scrutiny. Therefore, an increasing amount of research, especially regarding the use of defined starter cultures, has been conducted recently. Many problems that derive from strain isolation, cultivation and preservation of vinegar bacteria, have been mentioned in the literature (Kittelmann et al., 1989; Sievers et al., 1992; Sokollek and Hammes, 1997).

FRUIT   GRAIN, ROOT CROPS
      (STARCH)

$$(C_6H_{10}O_5)_nH_2O \xrightarrow[\text{(acid)}]{\text{enzymes}} xC_6H_{12}O_6 \quad + \quad yC_{12}H_{22}O_{11}$$
Starch   glucose   maltose

FERMENTABLE MONO- AND DISACCHARIDES

ALCOHOLIC FERMENTATION

$$C_6H_{12}O_6 \xrightarrow[\text{anaerobic}]{\text{yeast}} 2C_6H_5OH \quad + \quad 2CO_2$$
glucose or fructose   ethanol   carbon dioxide

ETHANOL

ACETIFICATION

$$C_2H_5OH + O_2 \xrightarrow[\text{air}]{\text{AAB}} CH_3CO_2H \quad + \quad H_2O$$
ethanol   acetic acid
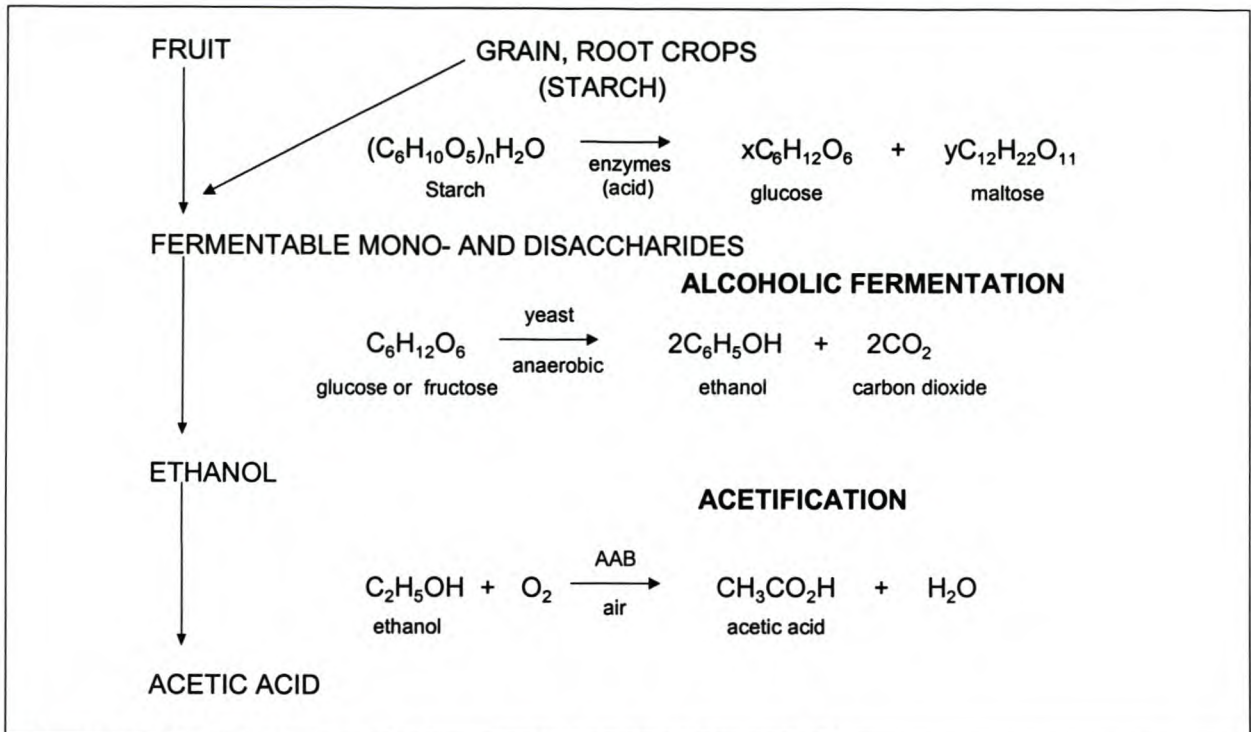
ACETIC ACID

Fig. 2.3. Schematic outline of vinegar production (Adams, 1998).

As an alternative, Sokollek and his co-workers (1998a, b) suggested the use of basal solutions, which were supplemented with glacial acetic acid and ethanol after autoclaving. The concentrations of these supplements are indicated by their volumes (in ml) per 100 ml solution. For example, AE-broth (2a/3e) means that 2 ml of acetic acid and 3 ml of ethanol are employed per 100 ml of solution. Isolates from spirit vinegar could be cultivated in AE-media and isolates from cider or wine vinegar could grow readily in RAE-media (reinforced AE) to high total cell counts (>10^9 cells/ml). Various a/e concentrations are used, depending on the strain of AAB involved (Sokollek et al., 1998a). The basal solution of RAE-media contains glucose (4% m/v), yeast extract (1% m/v), peptone (1% m/v), $Na_2HPO_4.2H_2O$ (0.338% m/v) and citric acid (0.15% v/v). The basal solution of modified AE-media consists of yeast extract (0.2% m/v), peptone (0.3% m/v), glucose (0.5% m/v) and Acetozym DS (0.15% v/v). Acetozym DS is distributed by Frings (Bonn, Germany) and consists of a mixture of nutrients and mineral salts. Additionally, in comparison to 90% cell recoveries from lyophilised preparations, an increase of up to a 100% was obtained by using 20% malt extract as cryo-protectant (Sokollek et al., 1998a).

Of the two genera of AAB popularly used for vinegar productions, *Gluconobacter* and *Acetobacter*, the former is preferred least. Species of *Gluconobacter* are less ethanol tolerant and therefore favour sugar-rich environments more than the *Acetobacter* species (Joyeux et al., 1984a). It has been reported that, although most strains of *Gluconobacter* can oxidise ethanol (at < 5% v/v) to acetic acid, these organisms are not capable of surviving in the alcoholic environment, even after aeration (Drysdale and Fleet, 1989b). As most industrial vinegar fermentations are

preceded by an alcoholic fermentation, the resulting lower sugar content, together with the presence of ethanol, makes this more favourable for *Acetobacter,* as reported by De Ley et al. (1984). Additionally, the inability of *Gluconobacter* to form surface films, which are necessary during static vinegar production, also makes them less adequate (Asai, 1968; Adams, 1998). Earlier strains isolated from vinegar productions belonged to the genus *Acetobacter* and included the species *Acetobacter pasteurianus, Acetobacter xylinus*, *Acetobacter hansenii* and *Acetobacter aceti* (Kittelmann et al., 1989; Swings, 1992). Some of these strains easily can produce between 50 and 150 g/l acetic acid during vinegar fermentations (Sievers et al., 1997; Lu et al., 1999). Recent studies indicated that *Acetobacter europaeus* from central Europe (Sievers et al., 1992), *Acetobacter oboediens* and *Acetobacter pomorum* (Sokollek et al., 1998b) also are involved in vinegar production. A study that focussed on the characterisation of AAB from traditional static fermentations of rice vinegar (Komesu) and unpolished rice vinegar (Kuroso) revealed that all stages of their fermentations were dominated primarily by *A. pasteurianus* (Nanda et al., 2001). In this instance, the latter species have established themselves into nearly pure cultures since they were started in 1907.

The use of specific species of AAB during vinegar production does not seem to be linked to the type of raw material used during experimental fermentations. From the literature, the most researched and used AAB appear to be *A. xylinus* and *A. europaeus* (Sokollek et al., 1998a, b). It is believed that vinegar can be made from any non-toxic raw material from which a juice containing fermentable sugars can be extracted. Vinegar is usually named after the substrate from which it is made. The range of this topic, however, is too broad for the purposes of this literature review. A list of the substrates used mostly can be seen in Adams (1998).

One of the advantages of vinegar production is that it can also put agricultural waste to good use. Horiuchi et al. (1999) investigated the use of low-quality onions as raw material for vinegar production. The juice of red onions (cultivar *Kurenai*) was more favourable than that of other onions, as it had the highest levels of sugar and required nutrients. By using the yeast *Saccharomyces cerevisiae,* followed by *A. aceti,* as batch cultures, a product of great quality was obtained. The total amino acid and total organic acid content of the red onion vinegar was 1.6 to 6.9 and 3.5 to 11.5 times higher respectively than that of conventional vinegars. These results make the under-investigated areas of pure starter cultures and experimental raw materials very promising in the development of new vinegar products.

The intensive consumption of acetic acid by AAB, often referred to as acetate "over-oxidation", is accompanied by a corresponding cell-biomass increase in the organisms (Saeki et al., 1997b). According to Bergey's Manual of Systematic Bacteriology (De Ley et al., 1984), all strains belonging to the genus *Acetobacter* oxidise acetic acid into carbon dioxide and water. This phenomenon of aerobic acetate anabolism by AAB is a serious problem in many countries, as further oxidation is highly unwanted. In traditional fermentations, the safest way to avoid

acetate oxidation was to terminate the vinegar fermentation while small amounts of ethanol were still present (Saeki et al., 1999). Nevertheless, alternative ways to overcome acetate oxidation are in use today, including the selection of microorganisms that show the least oxidative capability in vinegar fermentations and acetic acid accumulation of more than 5%. It has also been noted that thermotolerant strains of AAB (isolated in Thailand) oxidised more acetate than the non-thermotolerant strains (Saeki et al., 1997a).

It has been indicated that strains of the species *Acetobacter ranscens* subsp. *pasteurianus* and *Acidomonas methanolica* are well known for their ability to cause acetate oxidation (Saeki et al., 1997b). A characteristic of these acetate oxidators is a typical biphasic growth curve (**Fig. 2.4**). From **Fig. 2.4** it can be seen that the initial ethanol content in the vinegar mash is utilised rapidly, with a consequent increase in acetic acid until the first stationary phase after about 20 to 40 h. This stationary phase continues for a period of 100 h. Thereafter, a drastic exponential phase of bacterial growth is observed, with a rapid decrease in acetic acid. It is believed that, before the second growth phase reaches stationary mode, glucose levels are also depleted (glycerol levels have also been noted to decrease) (Saeki et al., 1997b).

The conversion of acetate to carbon dioxide and water, together with a corresponding increase in cell biomass, is dependent on the smooth functioning of the tricarboxylic acid (TCA) cycle. As enzymes make citrate with acetyl-CoA during the TCA cycle, enzymatic investigations on acetate oxidation also have become very important (Ameyama et al., 1985; Saeki et al., 1999). It has been shown that the activity of phosphoenolpyruvate carboxylase is stimulated by the addition of glycerol to the culture medium of *A. ranscens*. A small amount of acetyl-CoA produced during glycerol anabolism was believed to be the main stimulant and resulted in improved acetate oxidation, followed by an increase in cell biomass during the second growth phase. The enzymatic activities of acetyl-CoA synthetase and phosphotransacetylase also increased during acetate oxidation. This suggests the existence of a possible metabolic pathway during acetate oxidation that is linked to citrate formation via acetyl-CoA. More detailed research, however, still needs to be conducted, as the enzymes involved in acetate oxidation are of great significance.
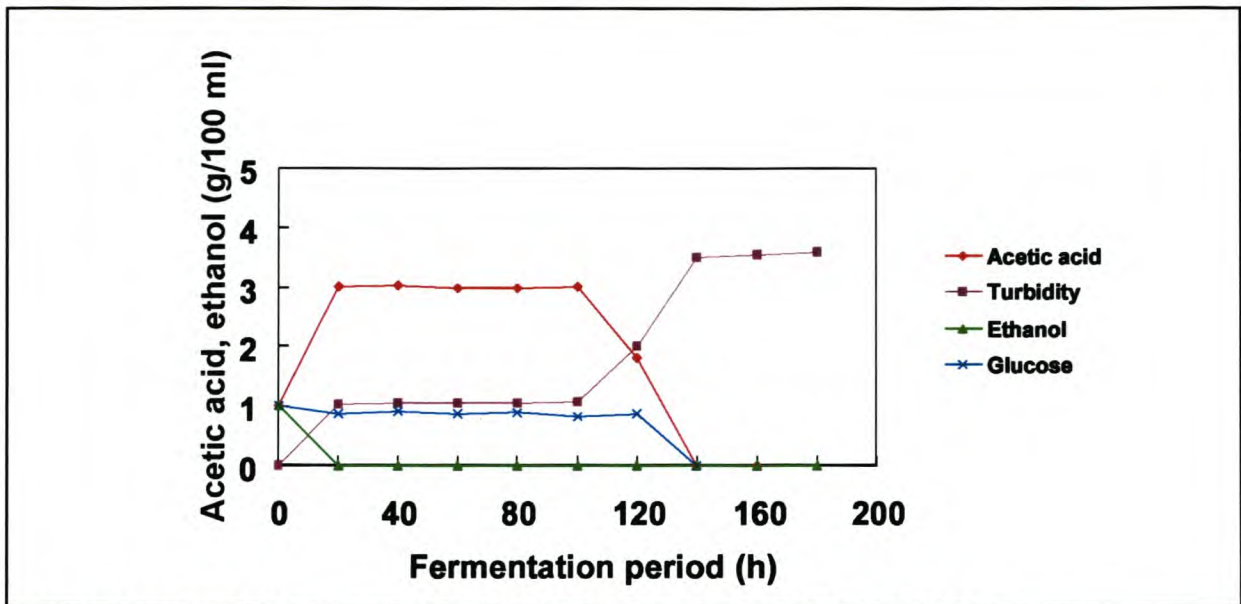
Fig. 2.4. Typical biphasic growth curve of *A. ranscens* (Saeki et al., 1997b).

2.2.2 Cellulose

Cellulose is one of the most abundant biological macromolecules on Earth. It forms the basic structural matrix of the cell walls of higher plants, fungi and seaweeds. Although cellulose popularly has been isolated from plants for a long time, alternative methods have been developed. These include enzymatic in vitro synthesis, chemosynthesis from glucose derivatives and biosynthesis by different types of microorganisms (**Fig. 2.5**) (Klemm et al., 2001). With the latter being the focal point, the development of systems for overproducing this polymer microbially have made a significant technological impact.

Bacterial cellulose exhibits a higher degree of polymerisation and crystallinity than the vegetal sources (Bertocchi et al., 1997). Although the molecular structure of plant and microbial celluloses are the same, the latter are preferred. The cellulose fibrils of bacteria are not embedded within lignin, pectin, hemicellulose and other waxy aromatic substances, as are plant-originated celluloses, thus making them extremely pure. Additionally, microbially synthesised celluloses differ from plant celluloses with respect to their ultra-fine network structure, mechanical strength in the wet state and high water absorption capacity. It is known that several bacteria produce cellulose as a byproduct of sugar metabolism (*Acetobacter, Rhizobium, Sarcina* and *Agrobacterium*) (Bertocchi et al., 1997; Jonas and Farah, 1997). Other bacteria known from the literature to do this include the genera *Achromobacter, Aerobacter, Alcaligenes, Pseudomonas* and *Zooglea* (Klemm et al., 2001). However, special attention has been given to strains of *Acetobacter*, especially *Acetobacter xylinum*.
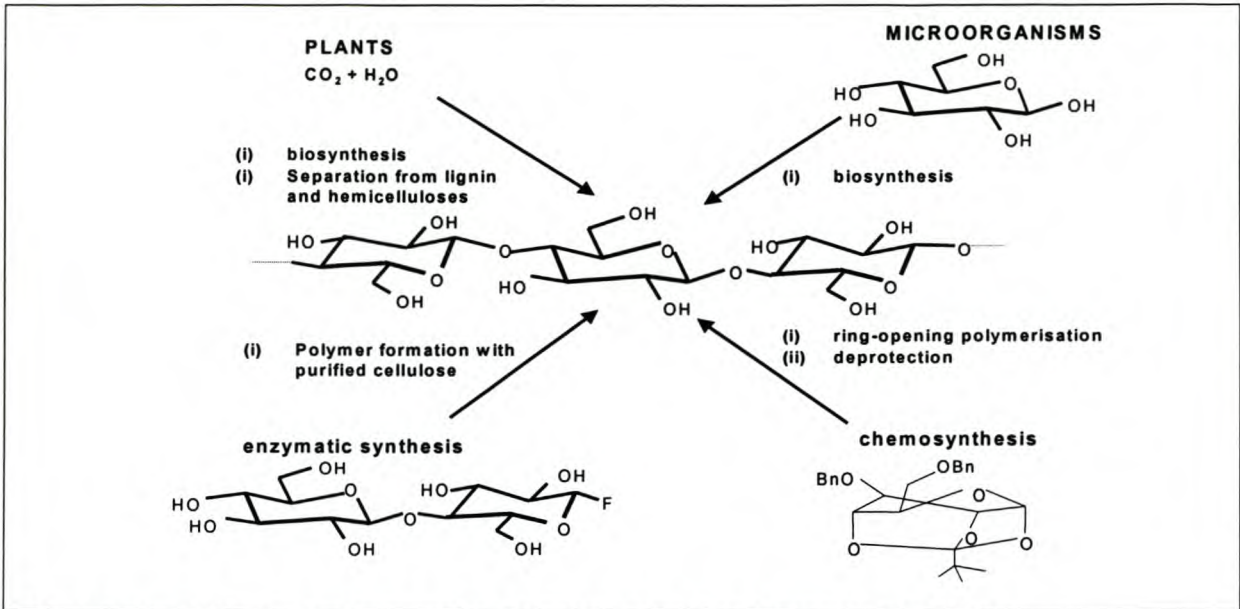
Fig. 2.5. The different pathways of cellulose production (Klemm et al., 2001).

The synthesis of cellulose in *A. xylinum* occurs between the outer and the cytoplasmic membrane by synthesising complexes or terminal complexes (TC) that are in association with pores at the surface of the bacterium (Jonas and Farah, 1997). Cellulose formation is initiated through the elongation of glucan chain aggregates from the synthesising complexes (**Fig. 2.6**). This forms the fibrils that assemble to form microfibrils, which, in turn, shape into a tightly assembled ribbon (Klemm et al., 2001). A matrix of interwoven ribbons constitutes the bacterial cellulose pellicle that is visible at the air/liquid interface of the culture medium. The proposed biochemical pathway for cellulose synthesis by *A. xylinum* from D-glucose can be seen in **Fig. 2.7**. Starting from a static culture, these non-pathogenic bacteria produce pure cellulose extracellularly within a few days. After an initial adaptation period of 1 to 2 days, glucose is utilised and cellulose formation commences. **Fig. 2.8** is a graphical representation of the utilisation of glucose with the corresponding cellulose formation within 15 days. This conversion of the monosaccharide D-glucose by the membrane-bound dehydrogenases into gluconic acid will be discussed later. Other sugar sources also have been investigated as alternative substrates for cellulose production (Jonas and Farah, 1997). All the sources were less active than glucose, except for arabitol and mannitol. These sugars produced 6.2 and 3.8 times more cellulose than glucose respectively. Jonas and Farah (1997) noted these values as the highest in comparison to other published results.

Research has also been done on the composites that formed when the AAB were incubated in the presence of cellulose derivatives (Astley et al., 2001). This was reported as very useful for the possible manipulation of the microstructure and the aggregation behaviour of microbial cellulose (Klemm et al., 2001). Such derivatives include water-soluble polymeric substances, such as carboxymethylcellulose (CMC) or hemicelluloses, chitosan and fluorescent dyes, as well as enzymes such as endoglucanase (Astley et al., 2001). In addition, thermodynamically more stable

crystalline cellulose can be induced by incubation at low temperatures, by incubation in a more viscous culture medium, or by a mutant strain of *Acetobacter*. In a study that aimed to create a cellulose over-producing strain, the wild-type *A. pasteurianus* strain was subjected to chemical mutagenesis (Bertocchi et al., 1997). In this instance, the putative mutation of an *A. pasteurianus* strain showed no effect on the physico-chemical properties of the cellulose (Bertocchi et al., 1997). However, double the amount of cellulose was produced by the mutagenised strain of *A. pasteurianus* in comparison to the wild-type strain. No direct comparison regarding the production of this microbial polysaccharide by various *Acetobacter* species is evident from the literature. All that is mentioned are indications to the possible use of other *Acetobacter* species rather than the more popular use of *A. xylinus*. The above-mentioned therefore is an area that should be investigated further.

The pure and crystalline form in which microbial cellulose can be synthesised is very advantageous. This, together with the prospect of cultivation on different growth supports, such as tubes and dishes, makes it possible to manufacture cellulose of the desired shape necessary for various applications. The industrial applications of cellulose include its use to maintain viscosity in food, for cosmetic support, filter membranes, non-woven fabrics for the repair of old documents etc. (**Table 2.1**) (Jonas and Farah, 1997). More recent applications, specifically in the medical field, include its use as temporary artificial skin coverings in the treatment of burns, ulcers and dental implants. These cellulose products are traded under the names Gengiflex® and Biofill® (Jonas and Farah, 1997; Klemm et al., 2001). One of the best applications of bacterially synthesised cellulose (BASYC®), currently undergoing trials, is its use as artificial blood vessels in microsurgery (Klemm et al., 2001). Its high mechanical strength in the wet state, enormous water retention capability and low roughness of the inner surface of BASYC make it ideal for this purpose.

The implementation of AAB in such vital procedures and fundamental applications serves as inspiration for greater exploration. From these instances it is evident that these microorganisms can clearly be beneficial, therefore the popular connotation of these microbes as "spoilage bacteria" should be re-thought.

Table 2.1
Applications of bacterial cellulose (Jonas and Farah, 1997).

Material	Application
Temporary artificial skin (Biofill®, Bioprocess® and Gengiflex®)	Therapy of burns, ulcers and dental implants
Non-woven paper or fabric	Improvement of latex and other binders Repair of old documents
Sensitive diaphragms	Stereo headphones
Cellulose	Immobilisation of proteins, chromatography
Edible cellulose	Addition to food
Cellulose	Stabiliser of emulsions in cosmetics, food
Cellulose	Coating compositions
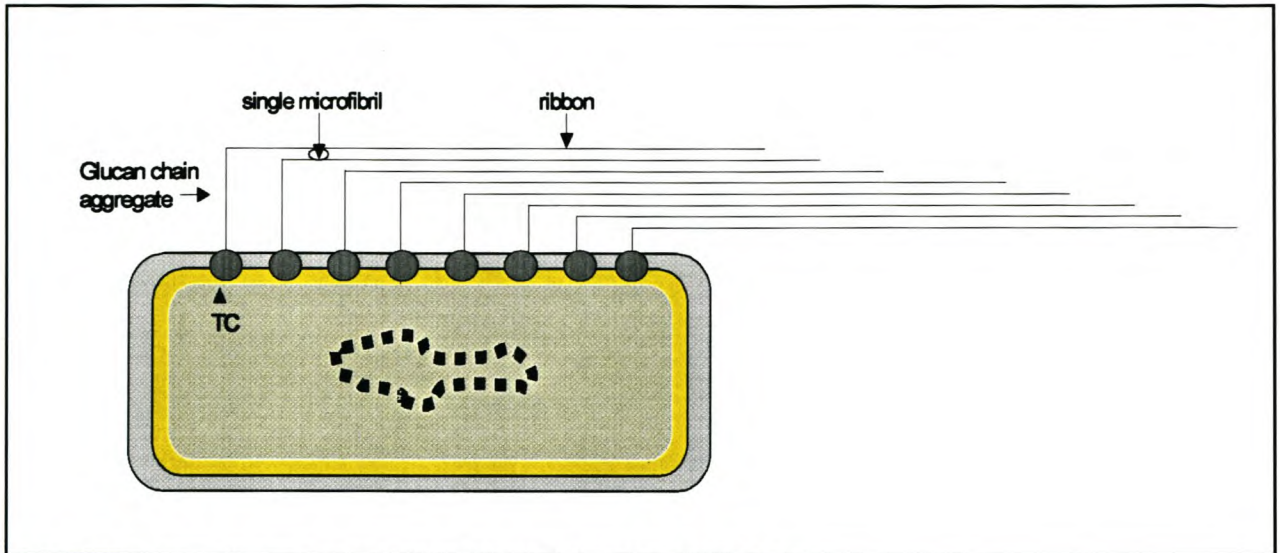
Fig. 2.6. Formation of bacterial cellulose (Klemm et al., 2001).
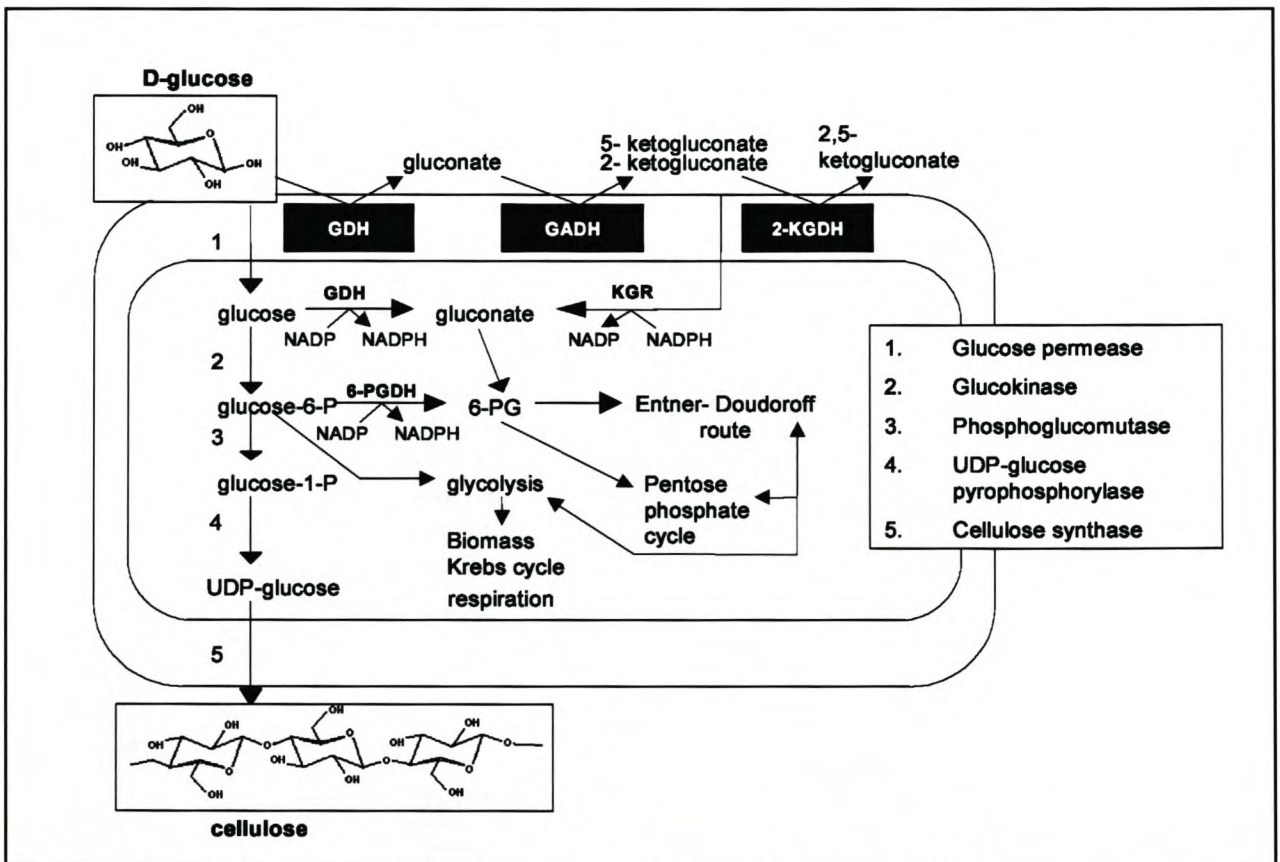
Fig. 2.7. Pathways of glucose metabolism by *A. xylinum* (Klemm et al., 2001).
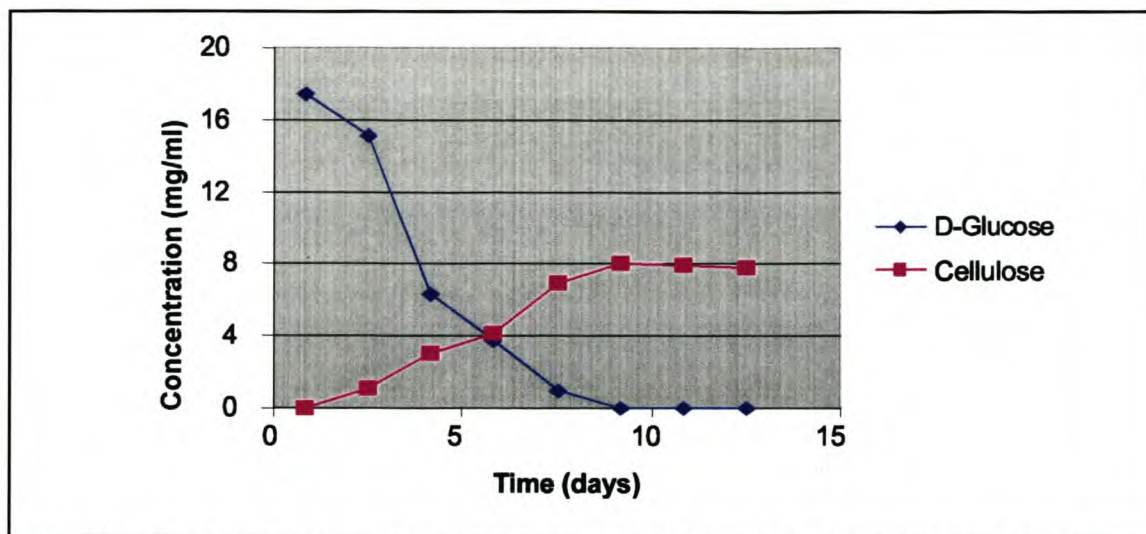
Fig. 2.8. Utilisation of D-glucose in the formation of cellulose (Klemm et al., 2001).

2.2.3 Dihydroxyacetone

AAB are commonly known for their spoilage action, primarily due to the high amounts of acetic acid produced in fruit-rot scenarios. However, the production of other metabolites, such as gluconate, ethyl acetate and dihydroxyacetone (DHA), contribute significantly to the culture environment. With recent technology enabling the specific purification of such metabolites, large-scale production with consequent applicability has become common practice. The process of ketogenesis (also refer to section 2.3.2.3), in which glycerol is strongly converted into DHA ($C_3H_6O_3$), is widely reported for AAB and has been in use industrially for decades (De Ley and Swings, 1984). Ketogenesis is one of the primary characteristics used for identification purposes, as all *Acetobacter* species, excluding *A. pasteurianus*, are capable of this glycerol bioconversion (Joyeux et al., 1984a, b), although to varying degrees. All species of *Gluconobacter* are renowned for strong ketogenesis (De Ley and Swings, 1984; Moonmangmee et al., 2000) and are used commercially for DHA production.

DHA is a sugar and is also known for its sweet/etheric sensory property. It must be mentioned that, from a winemaker's viewpoint, DHA formation is unwanted, as it not only binds SO_2 but also decreases glycerol levels, thus reducing smoothness and affecting the mouth feel of the wine (Fugelsang, 1997). The purification process of DHA involves natural solvents only; ethanol obtained from microbial fermentations is used mostly, but no acetone is used (http://www.soliance.com).

The application of DHA comes from its ability to react naturally with the amino acids of the human skin. The subsequent skin colouration to brown by the Maillard reaction makes it ideal for use in sun-tanning lotion (Asai, 1968; Swings, 1992). It currently is the most active self-tanning agent providing the possibility of a natural tan without UV exposure. DHA is used in products at concentrations of 2.0 to 5.0% under the chemical name of 1,3-dihydroxy-2-propanone (http://www.optimaspecialty.com).

2.2.4 L-ascorbic acid

L-ascorbic acid forms an essential part of our daily nutrition. It is used mainly for its antioxidant properties, but also in ointment to treat burns (pharmaceutical preparations), to protect flavour and aroma, and as a dietary supplement in aquaculture feeds (Hancock and Viola, 2002). This essential vitamin (vitamin C) or L-ascorbic acid is produced through the conversion of D-glucose via a key intermediate, 2-keto-L-gulonic acid (Reichstein et al., 1993). Although several approaches to the production of L-ascorbic acid have been reported (Boudrant, 1990), the most popular method of commercial production for many years has been the Reichstein method. This process involves five steps: (1) reduction of D-glucose, (2) oxidation of D-sorbitol by *Acetobacter* (*Gluconobacter*), (3) protection of carbonyl groups, (4) oxidation with permanganate, and (5) deprotection of acetonide. However, this method is reported to yield only 50% L-ascorbic acid from D-glucose (Boudrant, 1990).

In attempts to raise the process efficiency and reduce the capital costs involved, other investigations that could increase the yield of L-ascorbic acid have been carried out. These include the direct fermentation of 2-keto-L-gulonic acid (2-KLGA) in a recombinant *Gluconobacter oxydans* strain (Saito et al., 1998). Through classic microbiology combined with recombinant DNA technology, the native bacterial promoter was replaced with the *Escherichia coli* tufB promoter. This resulted in higher production levels of 2-KLGA, which promises improved methods for the mass production of vitamin C. Numerous other investigations are currently being performed in which the genes encoding the enzymes involved in the metabolic pathways for L-ascorbic production are expressed in alternative host organisms, such as *Pantoea citrea* and *Corynebacterium* spp. (Hancock and Viola, 2002). Significant yield improvements of 2-KLGA have been obtained from both L-sorbose (up to 81%) and L-sorbosone (up to 83%) in resting cells after the membrane-bound sorbosone dehydrogenase of *A. liquefasciens* was expressed in a *G. oxydans* strain (Shinjoh et al., 1990). The two most commercially advanced methods, according to Hancock and Viola (2002), are the oxidation of D-glucose via D-gluconate, 2-keto-D-gluconate and 2,5-diketo-D-gluconate (2,5-DKG pathway) to 2-keto-L-gulonate (2-KLG), and the oxidation of D-sorbitol or L-sorbose to 2-KLG via the intermediate L-sorbosone (sorbitol pathway, **Fig. 2.9**). Although a number of organisms have the ability to oxidise L-sorbose, including *Acetomonas, Escherichia, Pseudomonas, Bacillus* and *Klebsiella, Gluconobacter* and *Acetobacter* seem the most promising at this stage. The use of 2-KGL as sole carbon source has proved to be a more useful method for the selective screening of microorganisms with L-sorbose- and L-sorbosone dehydrogenases, and this currently is the suggested method (Lee and Pan, 1999; De Wulf, 2000).

The molecular approach to increase L-ascorbic acid (vitamin C) production, as opposed to the Reichstein method, will reduce the amount of solvents and reagents used, such as acetone, sulphuric acid and sodium hydroxide. In addition, the former

approach will result in a reduction in waste disposal costs, as stringent environmental control is necessary to recycle these compounds. This once again proves the significance of AAB, not only as great experimental organisms, but also as producers of invaluable products for pharmaceutical applications.

D-Glucose

Glucose dehydrogenase

Hydrogenation

D-Gluconic acid

D-Sorbitol

Gluconate dehydrogenase

D-Sorbitol dehydrogenase

2-Keto-D-gluconic acid

L-Sorbose

2-Diketo-D-gluconate dehydrogenase

L-Sorbose dehydrogenase

2,5-Diketo-D-gluconic acid

L-Sorbosone

2,5-Diketo-D-gluconate dehydrogenase

L-Sorbosone dehydrogenase

2-Keto-L-gulonic acid

2,5-Diketo-D-gluconic acid pathway

D-Sorbitol pathway

L-Ascorbic acid

Fig. 2.9. Microbial pathways for L-ascorbic acid synthesis (Hancock and Viola, 2002).

2.2.5 Other uses

As mentioned previously, AAB are involved in many traditional beverages. The high acetic acid-forming ability of AAB make them ideal for use in *Acetobacter*-fermented tea or Kombucha (Stadelmann, 1961). Kombucha or "tea fungus", as it is more commonly referred to in the literature, is the result of a symbiotic growth of bacteria (*A. xylinum, Acetobacter xylinoides*) and yeasts (*S. cerevisiae, Schizosaccharomyces pombe, Saccharomyces ludwidgii*, etc.) cultured in a sweetened tea solution (Sreeramulu et al., 2000). During the fermentation of this sucrose-enriched tea (black tea), the formation of a microbial mat (**Fig. 2.10**), consisting of the yeasts and bacteria involved, is evident. This is ascribed to the formation of cellulose by the *Acetobacter* strains (*A. xylinum*) (Greenwalt et al., 2000). Together with cellulose, gluconic acid is also synthesised by AAB from the glucose liberated from the added sucrose (refer to section 2.2.2). The liberated fructose is metabolised into ethanol and carbon dioxide by yeasts. The fermentation process of Kombucha usually lasts for 7 to10 days and a decrease in pH from 5 to 2.5 is characteristic of the acetic acid produced by the AAB.

cellulose fibre

bacterial cell
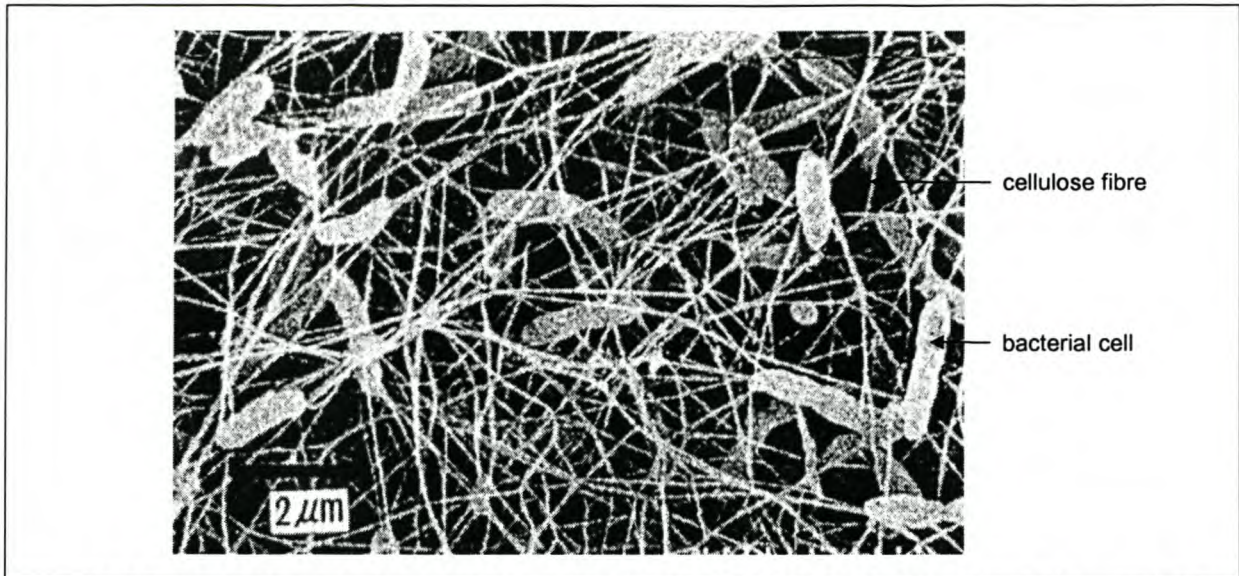
Fig. 2.10. Scanning electron micrograph of a microbial mat formed during cellulose production (Klemm et al., 2001).

It has long been believed that the consumption of Kombucha is beneficial to health, as it can reduce blood pressure, boost the immune system, relieve arthritis and possibly cure cancer. The use of Kombucha, "an ancient remedy", has been questioned, however, and numerous investigations regarding its actual health benefits are underway (Greenwalt et al., 2000; Sreeramulu et al., 2000).

AAB are also known for their role in cocoa fermentations (Schwan, 1998). Before raw cocoa (*Theobroma cacao*) can qualify as chocolate, it has to be processed and cured. The curing process involves fermentation, followed by drying and roasting. Species of *Gluconobacter* and *Acetobacter* are used in combination with yeasts (*S. cerevisiae*) and LAB (*Lactobacillus lactis, Lactobacillus plantarum*) for the fermentation of the mucilaginous pulp. AAB are responsible for converting the produced ethanol into acetic acid and then into carbon dioxide and water. Their microbial activity is known to be exothermic and elevated temperatures have been reported in the fermenting mass (Schwan, 1998). Further attributes of AAB that aid cocoa fermentation include the acidification of the cocoa bean preparation, the hydrolysis of proteins in the cotyledons and the production of volatile compounds that are believed to contribute to the chocolate flavour.

It is evident that the natural contributions by these microbes, which occur widely on various fruits and vegetables, raise questions about the negative impact (spoilage) with which they are commonly associated. The next section will focus on the occurrence of AAB in a medium that has received a considerable amount of interest in recent years, namely, the more negative association of these bacteria in the winemaking environment.

2.3 ACETIC ACID BACTERIA IN WINEMAKING

The importance of AAB in the winemaking environment has been highlighted as the result of a paradigm shift that occurred. In contrast to the previous belief in the aerobic nature of AAB (Joyeux et al., 1984a), the ability of these bacteria to survive and grow during anaerobic conditions has become increasingly evident (Drysdale and Fleet, 1985). This breakthrough has triggered a network of interest that has proved immensely valuable in clarifying the role in and influence of AAB on the quality of wine.

2.3.1 Different species in must and wine

2.3.1.1 Characteristics and species

AAB belong to the family *Acetobacteriaceae* and are Gram-negative, catalase-positive rods (De Ley et al., 1984; De Ley and Swings, 1984; Holt et al., 1994), although other parameters, such as Gram-variable and spherically shaped, are also known (Gosselé et al., 1984). Bergey's Manual of Systematic Bacteriology describes the appearance of mobility by peritrichous flagella, polar flagella and AAB that are non-motile (De Ley et al., 1984). Additional characteristics of AAB can be seen in **Table 2.2**. The taxonomy of AAB is relatively complex and has changed substantially in recent years (Swings, 1992; Yamada et al., 1997; Trček and Teuber, 2002). Currently, there are five genera into which AAB are divided: *Acetobacter (A.)*, *Gluconobacter (G.)*, *Gluconacetobacter (Ga.)*, *Acidomonas (Ac.)* and *Asaia (As.)*. Of these, *A. aceti, A. pasteurianus, Gluconacetobacter hansenii* (formerly *A. hansenii*), *Gluconacetobacter liquefasciens* (formerly *A. liquefasciens*) and *G. oxydans* have thus far been isolated from grapes and wine (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002). *G. oxydans* is associated predominantly with unspoiled grapes, due to its low ethanol tolerance and high sugar preference, and enters the must at cell numbers of 10^2-10^5 cells per ml (Joyeux et al., 1984a; Du Toit and Lambrechts, 2002). From the onset of fermentation, species of *Acetobacter* start to dominate *Gluconobacter*, with *A. pasteurianus* and *A. aceti* prevailing, up to cell numbers of 10^6-10^8 cells per ml (Drysdale and Fleet, 1989b; Du Toit and Lambrechts, 2002). Recent evidence suggests that *Ga. hansenii* and *Ga. liquefasciens* also occur at quite significant numbers during the middle and later stages of alcoholic fermentation (Poblet et al., 2000; Du Toit and Lambrechts, 2002).

2.3.1.2 Isolation and identification methods

Researchers have developed various ways for the isolation of AAB. The media most commonly used contain a suitable carbon source, vitamins, a source of nitrogen and other growth factors. The carbon sources include glucose, mannitol, ethanol and acetic acid (also refer to section 2.2.1). $CaCO_3$ and bromocresol-green are used as acid indicators (Swings and De Ley, 1981; De Ley et al., 1984; Drysdale and Fleet, 1988). As described by Du Toit and Pretorius (2002), the use of GYC, YPM and YPE is most supportive for AAB isolated from wine. GYC medium consists of glucose (5% m/v), yeast extract (1% m/v), $CaCO_3$ (from 1 to 3% m/v) and agar (1.5% m/v). YPM medium consists of mannitol (2.5% m/v), yeast extract (0.5% m/v), peptone (0.3% m/v) and agar (1.5% m/v), while YPE (pH 5.5) contains yeast extract (1% m/v), ethanol (2% v/v), peptone (0.5% m/v) and agar (1.5%). Additionally, the selected media contain pimaricin or cyclohexamide (50 mg/l) to eliminate yeast growth, and nisin (50 mg/l), penicillin or streptomycin (25 mg/l) to inhibit the growth of LAB (Drysdale and Fleet, 1988; Silva et al., 1995). Incubation at 30°C for 5 to10 days is advised before colony counts are done. This is because not all strains of AAB can form colonies after two-day incubation periods (Sanni et al., 1999). The survival rates have also been found to be best after storage at -80°C in 60% glycerol (Du Toit and Pretorius, 2002; Jordaan et al., 2002).

The phenotypic identification of AAB, especially at species level, is difficult. There are few reasons currently known that explain this difficulty. One such reason might be the structural alteration of crucial enzymes. It has been reported that the enzyme, alcohol dehydrogenase, can undergo transformation from an active to an inactive form and vice versa (Matsushita et al., 1994). Another reason is the occurrence of spontaneous mutations at high frequencies, which are attributed to the presence of insertion elements (Beppu, 1993; Takemura et al., 1993). Since AAB are regarded mainly as spoilage organisms, their identification in the food and beverage industry is of great significance. This is why improved species identification methods are constantly being developed, especially as the earlier methods are very time-consuming and inaccurate.

The identification methods used until recently consisted of biochemical and physiological tests (Holt et al., 1994). These tests include the use of various C-sources, such as glycerol, sodium acetate and dulcitol, to name but a few (**Table 2.2**). Due to unsatisfactory and non-reproducible results obtained with the biochemical tests, taxonomists have shifted to using more advanced molecular techniques. For the purpose of taxonomic classification, ubiquinone systems have been used as the distinguishing factor to reclassify *Gluconacetobacter* (Q-10 equipped; previously subgenus of *Acetobacter*) as a new genus, thus separating it from *Acetobacter* (Q-9 equipped) (Trček and Teuber, 2002). However, a more reliable method for identification and species differentiation is the use of restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified 16S rDNA (Poblet et al., 2000; Ruiz et al., 2000). This approach is currently

used as a rapid and reliable method, although alterations to it in an attempt at optimisation are continuously found in current publications. PCR-RFLP analysis of the 16S-23S rDNA intergenic spacer (ITS) region has been mentioned previously, although it was not found to be useful for identification up to species level (Ruiz et al., 2000). The 16S-23S rDNA ITS also contains conserved sequences with functional roles (Sievers et al., 1996), as do the 16S rDNA sequences (Sievers et al., 1994; Poblet et al., 2000). Among the enzymes tested for restriction analysis of the 16S rDNA PCR product of 1450 bp (as obtained by Ruiz et al., 2000), *Taq*I and *Rsa*I were the most discriminatory. Lately, an improved technique has been published that indicates an even more accurate molecular identification of AAB. The latest genetic and restriction analysis of the 16S-23S rDNA ITS regions of AAB (Trček and Teuber, 2002) has not only contributed to the development of a database of 16S-23S rDNA restriction profiles (http://ulises.umh.es/RISSC), but also suggests 12 restriction groups to which the most prevalent AAB can be assigned (**Table 2.3**). Sequence alignment of the 16S-23S ITS regions between the various AAB species is much lower in comparison to the 94.2-94.6% similarities of the 16S rDNA sequences (**Table 2.4**). According to Barry et al. (1991), this is ascribed to a higher existence of evolutionary configuration in spacer regions. The significance of the 16S-23S rDNA ITS regions derives from the discovery of two highly conserved sequences (96-100%). The latter were found in all spacers of AAB and encode for tRNA^{Ile} (77 bp) and tRNA^{Ala} (75 bp). Both these tRNAs also contained the 3'-end terminal sequence CCA, which normally is found in mature tRNA. Additionally, another highly conserved region of 17 nucleotides has also been found in the spacers of all AAB. In the near future, this may contribute greatly to the identification of these highly evolved microorganisms.

The illogical behaviour of AAB, which often complicates their classification, has made other previously exercised identification methods appear insufficient. The longer, more complicated phenotypic and genotypic methods that are suggested in the literature include numerical analysis of whole cell proteins by SDS-PAGE (De Ley et al., 1984; Gosselé et al., 1984; Pot et al., 1994, Du Toit, 2000), DNA/DNA hybridisation (Sievers et al., 1994) and DNA/rDNA hybridisation (Gillis and De Ley, 1980). Plasmid profile comparisons have also been suggested, as these are believed to be strain-specific characteristics of AAB (Mariette et al., 1991). Other, more recent, procedures that have been described for taxonomic grouping from vinegar research include the enterobacterial repetitive intergenic consensus (ERIC)-PCR method (Nanda et al., 2001) and random amplified polymorphic DNA (RAPD) fingerprinting (Trček et al., 1997).

Table 2.2
Characteristics differentiating genera and species of acetic acid bacteria isolated from wine[ø]

Characteristic	A. aceti	A. pasteurianus	Ga. hansenii	Ga. liquefasciens	G. oxydans
Growth on carbon sources:					
Ethanol	+	d	-	+	d
Sodium acetate	+	d	-	d	-
Dulcitol	-	-	d	-	d
Ketogenesis from:					
Glycerol	+	-	+	+	+
Sorbitol	+	-	+	+	
Mannitol	d	-	+	+	
Total oxidation of ethanol	+	+	+	+	-
Oxidation of D-, L- lactate	+	d	+	+	-
Formation of water-soluble brown pigments on GYC*	+	-	-	+	±
5-Ketogluconic acid from D-glucose	+	-	d	d	+

Ø	From Drysdale and Fleet (1988), latest classification used
d	strains strongly positive
*	GYC: D-glucose (50 g/l), yeast extract (10 g/l) and CaCO$_3$ (30 g/l)

Table 2.3
Different restriction groups of acetic acid bacteria as described by Trček et al. (2002)

Acetobacter	*Gluconacetobacter*	*Gluconobacter*
A. aceti	Ga. europaeus/ Ga. xylinus	G. oxydans
A. pasteurianus/ A. pomorum	Ga. intermedius/ Ga. oboediens	G. asai
	Ga. hansenii	G. cerinus/ G. frateurii
Acidomonas	Ga. liquefasciens	
Ac. methanolica	Ga. sacchari	
	Ga. diazotrophicus	

Table 2.4
Percentage of nucleotide similarity among the acetic acid bacteria based on 16S-23S rDNA ITS regions (Trček et al., 2002)

Species	Sequence similarity (%)					
	1	2	3	4	5	6
1. *A. aceti*						
2. *A. pasteurianus*	67.8					
3. *Ga. europaeus*	64.1	67.3				
4. *Ga. hansenii*	56.8	69.7	69.4			
5. *Ga. liquefasciens*	69.3	65.6	60.0	61.4		
6. *Ga. xylinus*	59.6	64.5	78.3	64.1	58.7	
7. *G. oxydans*	62.7	59.2	61.4	61.6	62.6	56.8

2.3.2 Metabolism

2.3.2.1 Carbohydrate metabolism

The ubiquitous distribution of AAB points to their ability to use different carbon sources. *Gluconobacter* strains metabolise a broad range of sugars and acid-derivatives of these sugars. This is concurrent with their domination on grapes and in must, where high sugar levels prevail (Joyeux et al., 1984b). *Acetobacter* and *Gluconacetobacter* (previously species of *Acetobacter*) are also capable of metabolising sugars, but can also oxidise ethanol. This explains the domination of *Gluconacetobacter* and *Acetobacter* species more after the onset of fermentation, as well as their use in vinegar production (Drysdale and Fleet, 1988; Du Toit, 2000). The main carbohydrates that are metabolised include D-glucose, D-fructose, D-sorbitol, D-mannitol, glycerol, D-gluconate and keto-D-gluconate. However, the ability to oxidise these substrates depends on the enzymes in the cells.

Most of the glucose conversion in AAB is performed by a membrane-bound $NAD(P)^+$-independent glucose dehydrogenase, but they also possess a membrane-bound $NAD(P)^+$-dependent glucose dehydrogenase (Du Toit, 2000). According to Qazi et al. (1991), glucose is oxidised to glucono-δ-lactone and from there to gluconic, 2-keto-D-gluconic and 2,5-diketo-D-gluconic acid respectively. The importance of glucose metabolism is of great interest, as this process yields metabolites that are of industrial importance, as discussed earlier (section 2.2.2). **Fig. 2.11** is a schematic representation of glucose-oxidising systems in AAB.

As mentioned previously, other carbohydrates, such as fructose, arabinose, galactose, mannitol, mannose, ribose, sorbitol and xylose, can also be utilised by AAB (De Ley et al., 1984). A membrane-bound fructose dehydrogenase is responsible for oxidising fructose to 5-oxofructose, which is then further metabolised by a reductase (Ameyama et al., 1985; Barbe et al., 2001). The conversion of D-mannitol and D-sorbitol to D-fructose and L-sorbose respectively has also been investigated (Moonmangmee et al., 2000). Some of the other enzymes that have been identified and characterised to be involved in these biochemical processes (**Fig. 2.12**) are quinohemoprotein D-mannitol dehydrogenase from *Gluconobacter melanogenus*, *Gluconacetobacter xylinus* (Oikawa et al., 1997) and *Gluconobacter suboxydans* (Adachi et al., 1999), different membrane-bound D-sorbitol dehydrogenases (SLDH) with pyrroloquinoline quinone (PQQ) functioning from *G. suboxydans* (Shinagawa et al., 1982), and membrane-bound D-ketogluconate dehydrogenases (Shinagawa et al., 1999).

The utilisation of glucose by *Acetobacter* species is done through the hexose monophosphate pathway, as well as through the Embden-Meyerhof-Parnas and Entner-Doudoroff patways (De Ley et al., 1984; Drysdale and Fleet, 1988; Attwood et al., 1991). The general metabolism of hexose and pentose sugars has been reported to be weak for *A. pasteurianus* and *A. aceti*. (Drysdale and Fleet, 1988; Du Toit, 2000). However, the utilisation of carbon sources is postulated to be highly

strain dependent and is also determined by environmental conditions (Du Toit and Lambrechts, 2002). De Ley (1961) found that not all strains of *Acetobacter* can utilise glucose effectively, as was later proven when it was found that some *A. pasteurianus* strains were also unable to grow on glucose as sole carbon source (De Ley et al., 1984; De Ley and Swings, 1984). *Gluconobacter* has also been reported to use the pentose phosphate pathway, although this route is believed to be pH dependent (Olijve and Kok, 1979). The produced metabolites are further oxidised via the tricarboxylic acid pathway (TCA) and, as previously mentioned, converted into carbon dioxide and water in the case of *Acetobacter* species (Drysdale and Fleet, 1988). It remains to be investigated which other substrates produced by other wine-related organisms, such as yeasts, LAB and fungi (*Botrytis*), can be utilised and what effect they have on AAB.
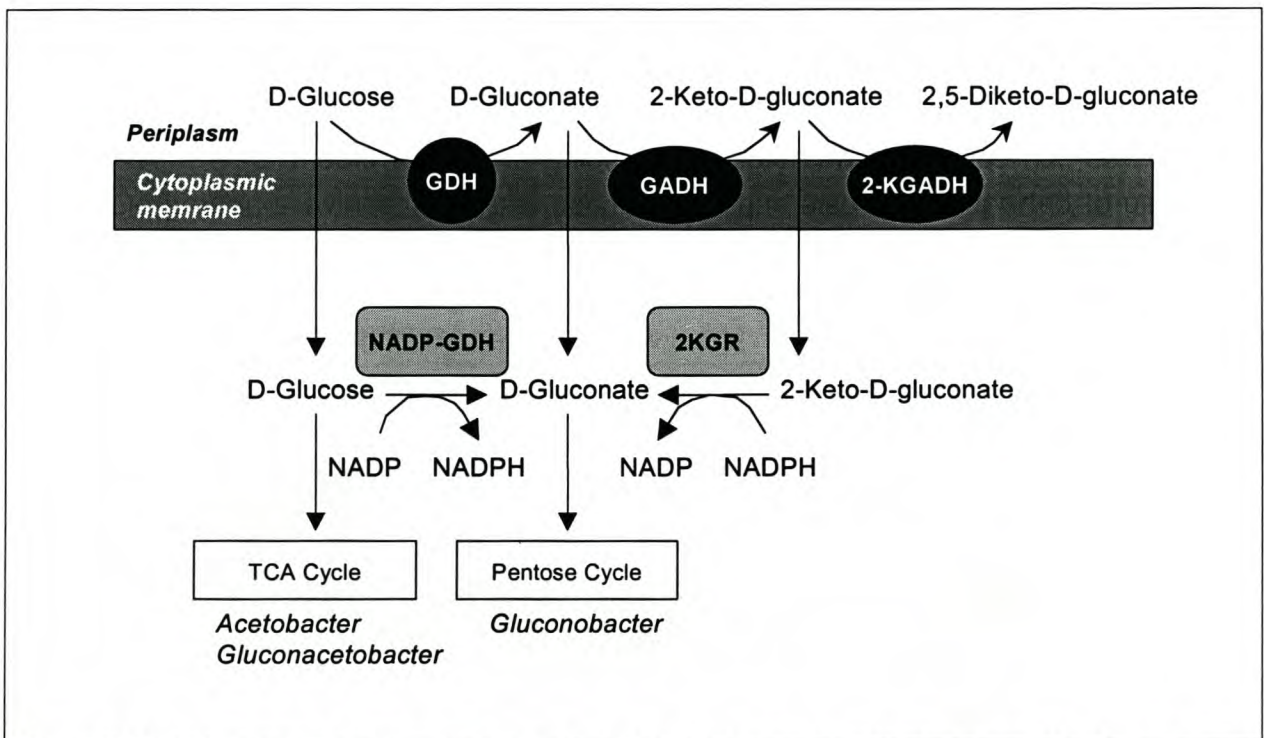
Fig. 2.11. Glucose-oxidising systems in AAB. Quinoprotein D-glucose dehydrogenase (GDH), and flavoproteins D-gluconate dehydrogenase (GADH) and 2-keto-D-gluconate dehydrogenase (2KGADH), are located on the outer surface of the cytoplasmic membrane. NADP-dependent D-glucose dehydrogenase (NADP-GDH) and NADP-dependent 2-keto-D-gluconate reductase (2KGR) are working in the cytoplasm (Matsushita et al., 1994).
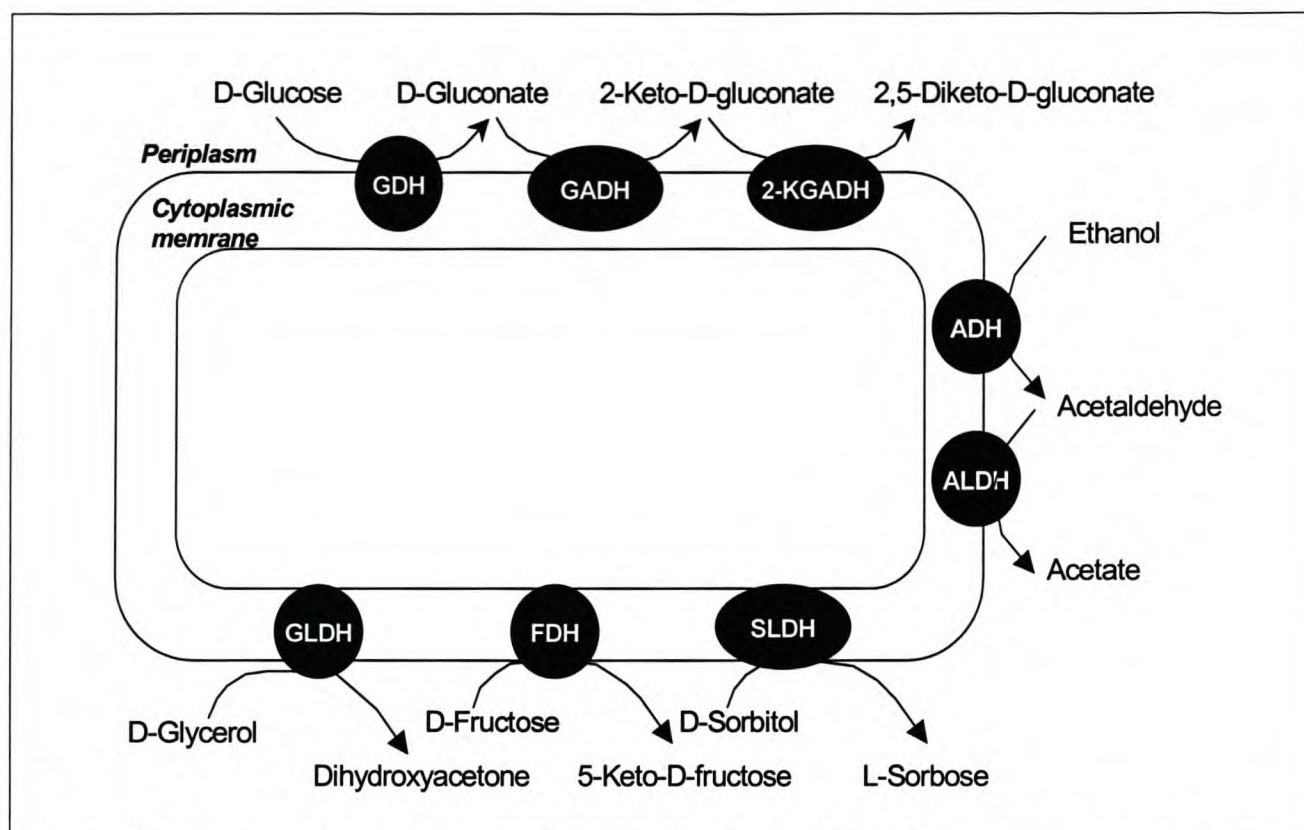
Fig. 2.12. Diagram showing primary dehydrogenases in the alcohol- and sugar-oxidising systems of AAB. Circles show quinoproteins, which include D-glucose dehydrogenase (GDH), alcohol dehydrogenase (ADH), aldehyde dehydrogenase (ALDH) and glycerol dehydogenase (GLDH). Ellipses show flavoproteins, including D-gluconate dehydrogenase (GADH), 2-keto-D-gluconate dehydrogenase (2-KGADH) and D-sorbitol dehydrogenase (SLDH) (Matsushita et al., 1994).

2.3.2.2 Ethanol oxidation and acetate production

The conversion of ethanol into acetic acid is the process by which the microorganisms involved were designated as AAB. As was mentioned in the section on vinegar production, this biochemical process is understood well and of the utmost industrial importance. To elaborate on the two pivotal $NAD(P)^+$-independent enzymes (mentioned previously) involved in ethanol oxidation, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), some of their other characteristics will be discussed. Firstly, ADH consists of two-type subunits (found in *Acetobacter polyoxgenes*) or three-type subunits (found in *A. aceti* and *A. pasteurianus*) (Saeki et al., 1997b). The latter is described as two large units, alcohol dehydrogenase (subunit I, 78 kDa) and cytochrome *c* (subunit II, 48 kDa), and a smaller protein (subunit III, 20 kDa) that supports the two larger units with their membrane association (Kondo and Horinouchi, 1997). The larger subunits play a role in the intramolecular electron transport from ADH to ubiquinone and then to terminal oxidase. This enzyme is inactivated through an insertion into the cytochrome *c* sequence, which is believed to be the result of a spontaneous mutation, followed by a consequent loss of ethanol oxidising capability (Kondo and Horinouchi, 1997). In

addition to this genetic instability in AAB, the occurrence of insertion elements causing spontaneous mutations at high frequencies has also been linked to deficiencies in acetic acid resistance (Ohmori et al., 1980) and cellulose formation. The enzyme activity of ADH is more stable in *Acetobacter* species than in *Gluconobacter*, with activity at pH values as low as 2.0 (Matsushita et al., 1994).

The second major enzyme, ALDH, further oxidises the product of ADH, namely acetaldehyde, into acetate to complete the conversion of ethanol to acetic acid. The ALDH from *G. suboxydans* (Adachi et al., 1980), *A. ranscens* (Hommel and Kleber, 1990), *A. polyoxgenes* (Fukaya et al., 1989), *A. aceti* (Muraoka et al., 1981) and *A. europaeus* (Thurner et al., 1997) has been purified and characterised. All these enzyme complexes contained two-type subunits, except for *A. aceti* and *A. europaeus*, which possess three subunits. The information on the subunit-encoding genes is still limited, although the study conducted on *A. europaeus* revealed molecular masses of 79, 46 and 17 kDa for the three subunits, with an indication of a cytochrome *c*, a heme B and a 2FE-2S cluster from the different spectrum (Thurner et al., 1997). The activity of ALDH is also maintained at lower pH values than its pH optimum of 4.0 to 5.0 (Adachi et al., 1980).

Although the thermostability of ALDH is higher than that of ADH, both these enzymes showed little difference in their thermostability between thermotolerant and mesophilic strains. Ethanol sensitivity has been reported for ALDH, which leaves the probability of acetaldehyde build-up in a fermentation environment (Muraoka et al., 1983). Increased acetaldehyde levels correlating with lower dissolved oxygen concentrations (Drysdale and Fleet, 1989b) have led to the belief that certain wine conditions are more tolerable for ADH and can contribute to the ineffective functioning of SO_2. As acetaldehyde also binds SO_2 very effectively, the lowered activity of ALDH, resulting in an acetaldehyde build-up, could explain some of the survival of AAB under these strenuous anaerobic conditions. This repression of the antimicrobial function of SO_2 should be considered and re-evaluated together with the occurrence of AAB at high cell numbers.

Contrary to the suggestion questioning the oxygen-dependent activity of the ALDH enzyme responsible for acetic acid production, it is quite evident that some AAB are capable of contributing high amounts of acetic acid to wines, especially aerated ones (Drysdale and Fleet, 1989a). This, in turn, questions the function of the $NAD(P)^+$-dependent enzymes (in comparison to the $NAD(P)^+$-independent enzymes), as their lower reported activity (Takemura et al., 1993; Matsushita et al., 1994) under specific conditions might be of greater significance.

The gathering of large quantities of acetic acid, as produced by AAB, leads to the formation of VA in the wine, which is directly disadvantageous to the product quality as it gives wine a vinegary/acetone-like aroma, which is highly objectionable at levels exceeding 1.2 g/l (the legal limit in South African wines) (Bisson, 1999). The increasing number of winemakers reporting elevated levels of acetic acid or VA has triggered numerous investigations (Eglinton and Henschke, 1999).

The VA of South African wines caused by AAB is largely the result of *Acetobacter* species, as *A. pasteurianus* and *Ga. hansenii,* on occasion, have caused VA of up to 4 g/l (Du Toit, 2000). In this instance, the *G. oxydans* strain tested produced very little VA (< 0.2 g/l), although the contrary has been reported (Drysdale and Fleet, 1989b). An interesting study revealed that an *Acetobacter* strain produced most of its acetic acid during the stationary and death phases (Kösebalan and Özilgen, 1992). This proposes the possibility of increased acetic acid concentrations during prolonged storage and should definitely be taken into consideration.

The further oxidation of acetic acid into carbon dioxide and water distinguishes *Acetobacter* from *Gluconobacter*. The latter strains are unable to do this due to the fact that they lack the two functional enzymes, α-ketoglutarate dehydrogenase and succinate dehydrogenase, which are necessary for a functional TCA cycle (Drysdale and Fleet, 1988). The over-oxidation of acetic acid, which is highly unwanted in the vinegar industry due to the loss of acetic acid, is most unfortunately unlikely in wine, as the other carbon sources, such as glucose and ethanol, gain preference over acetic acid (Saeki et al., 1997a). For more information regarding this, together with information on the acetic acid resistance of AAB, refer to the review by Du Toit and Pretorius (2002).

2.3.2.3 Ketogenesis or glycerol oxidation

The ability of AAB to perform ketogenesis from glycerol is of great industrial importance, as mentioned earlier. This conversion of glycerol to DHA, however, also suggests an alternative to the growth and survival of AAB in wine. This is supported by the use of electron acceptors other than oxygen, such as *p*-benzoquinone, for the utilisation of glycerol as carbon source during some wine conditions (Adlercreutz and Mattiasson, 1984). The enzyme responsible for ketogenesis is a membrane-bound NAD(P)$^+$-independent glycerol dehydrogenase (GLDH) and contains PQQ as a prosthetic group. This enzyme was first isolated from *Gluconobacter industrius* (today known as *G. oxydans* subsp. *industrius*) by Ameyama et al. (1985) and also catalyses the oxidation of other polyhydroxyl alcohols, such as *meso*-erythritol and D-arabitol, at similar rates (Matsushita et al., 1994). The process of ketogenesis is used as a biochemical method to distinguish *A. pasteurianus* from other *Acetobacter* species, as the former is unable to produce DHA from glycerol (Drysdale and Fleet, 1988). The formation of DHA is unwanted during winemaking, however, as it not only reduces the contribution of glycerol to the smoothness and mouthfeel of the wine, but it also binds SO_2, rendering its antimicrobial function less effective. Drysdale and Fleet (1989a) reported an *A. aceti* that, on occasion, reduced the wine's glycerol concentration (from 6.43 to 2.39 g/l) below the threshold value of 4.0 to 5.0 g/l. It has been mentioned that AAB utilise a minor proportion of glycerol for biomass and energy synthesis via the phosphorylating oxidative pathway (Švitel and Šturdik, 1994).

2.3.2.4 Organic acids

The significance of organic acids regarding their metabolism by AAB in wine needs more investigation. It is known that the TCA cycle is used for the oxidation of organic acids. These acids include acetic, lactic, malic, citric, succinic, tartaric, fumaric and pyruvic acids. *Gluconobacter* species are unable to oxidise acetic and most other organic acids, as they lack a functional TCA cycle (Drysdale and Fleet, 1988; Holt et al., 1994). It has been mentioned that the amount of succinic acid increased more in must in which *Gluconobacter* (70 mg/l) rather than *Acetobacter* (43 mg/l) had grown (Joyeux et al., 1984b). In the same study, noticeable decreases in malic and citric acid concentrations of the must by *Acetobacter* strains were observed (from 4.7 to 1.8 g/l and 230 to 147 mg/l respectively). Another acid formed by AAB, which can further influence wine quality, is propionic acid. Concentrations of between 10 to 30 mg/l of propionic acid can be formed by AAB, which may result in an objectionable aroma (Drysdale and Fleet, 1989a). An *Acetobacter* strain capable of degrading D-lactate four times faster than L-lactate was found by De Ley (1959). The same researcher also stated that another *Acetobacter* strain converted most of the lactate in the medium to acetoin. This also contributes to the negative impact of AAB on wine aroma, as acetoin causes an unwanted buttery characteristic and is considered as a spoilage indicator. The importance of organic acids has been investigated in greater detail in other wine-related organisms (yeasts, LAB). In order to make more comprehensive deductions, a greater interest must be shown in AAB and their role in organic acid metabolism in order to clarify their position and significance, as has been the case with other wine-related inhabitants.

2.3.3 Factors that influence the survival of acetic acid bacteria during fermentation and storage of wine

The number of publications that specify and describe the fundamental factors involved in the existence of AAB have increased drastically over the past few years. A noteworthy addition to the current literature on AAB (in winemaking) is a review by Du Toit and Pretorius (2002). However, a quick overview that highlights some of the crucial parameters that affect AAB has to be presented, as it is appropriate for the purposes of this thesis.

2.3.3.1 Oxygen

As mentioned previously, the oxygen requirement of AAB was the key parameter that led to renewed interest in these Gram-negative microorganisms. Oxygen is used by AAB as a terminal electron acceptor (Matsushita et al., 1992, 1994). This led to the belief that their aerobic growth requirements could be controlled easily by means of sound winemaking practices, such as maintaining anaerobic conditions with the sufficient use of sulphur dioxide (Amerine and Kunkee, 1968). To an extent, these criteria are still applicable to some AAB, although research has indicated that strains of *Gluconobacter* and *Acetobacter* are quite capable of surviving and growing

throughout oxygen-depleted conditions, such as during alcoholic fermentation (Drysdale and Fleet, 1988; Du Toit, 2000). The latter phenomenon is confirmed by studies that revealed the ability of AAB to use other compounds, such as quinones and reducible dyes, as a final electron acceptor in the place of oxygen (Aldercreutz and Mattiasson, 1984). The indication that *G. oxydans* could oxidise glycerol four-fold higher with *p*-benzoquinone rather than oxygen as electron acceptor and then re-utilise the byproduct (hydroquinone), clearly suggests their possible survival in wine conditions, especially as these phenolic compounds can occur in wine.

These microaerophilic organisms are well known for increasing their cell numbers following air contact. Joyeux et al. (1984a) found a 30- to 40-fold increase in the cell numbers of AAB in wine after about 7.5 mg/l oxygen had dissolved after air exposure. Various such studies have been reported and, in some cases, the biomass increase correlated with an increase in VA (Ribéreau-Gayon, 1985; Drysdale and Fleet, 1989b).

Special care must be taken during storage in wooden barrels, as the ethanol and water evaporation occurring over time may increase the surface area for AAB growth. However, oxygen is necessary for the sensory enhancement and stability of red wine (Ribéreau-Gayon, 2000a). The role of oxygen, which seemingly can be overcome by some AAB, is a critical factor that should be kept in mind during winemaking.

2.3.3.2 Temperature

AAB have a reasonably small temperature range for their optimal growth. The optimum growth temperature for *Acetobacter* and *Gluconobacter*, according to Holt et al. (1994), is 25-30°C, although a maximum growth temperature of 35°C has been noted for *A. aceti* (De Ory et al., 1998). Other thermotolerant AAB, such as *Gluconobacter frateurii*, that are able to grow at 37-40°C have been isolated (Saeki et al., 1997a). These thermotolerant AAB oxidise ethanol faster and more efficiently than the normal strains at 30°C and it is believed that their increased ethanol and acetic acid tolerance corresponds with their ability to grow at these raised temperatures (Ohmori et al., 1980). Lower temperatures, as often used in winemaking practices, seem to greatly affect AAB (Joyeux et al., 1984a). Weak growth of the species *A. aceti* has been observed at 10°C (Joyeux et al., 1984a), and this has been confirmed by De Ory et al. (1998), who found the same species unable to grow below 8°C. This sets the minimum temperature for the survival of AAB at around 10-12°C. Care should be taken, however, as it has been reported that an *A. aceti* increased its cell numbers by up to 40% after being stored in wine at 18°C over a period of one week (Joyeux et al., 1984a).

The elimination of *A. aceti* and *A. pasteurianus* from infected barrel wood by hot water (85°C) treatment for 20 minutes has been suggested (Wilker and Dharmadhikari, 1997).

2.3.3.3 pH

With regards to acidity, AAB can survive easily at the low pH conditions, ranging from 3.0 to 4.0, found in wine. Isolates of AAB from different Australian wines at pH values ranging from 3.02 to 3.85 support the previous statement (Drysdale and Fleet, 1985). The optimum pH for the normal growth of AAB is 5.5-6.3 (Holt et al., 1994). If this is considered, then the survival of these bacteria at pH values as low as 2.0-2.3 in acetate-containing media is concerning. This is why Kittelman et al. (1989) postulated that AAB from vinegar fermentations should be divided into three groups: acetophilic strains which grow at a pH of about 3.5; acetophobic strains, which can grow only above a pH of 6.5; and acetotolerant strains, which can grow between these ranges. The great adaptation ability of *Acetobacter* was also observed by Kösebalan and Özilgen (1992), who suggested the gradual conversion from acetophobic to acetophilic strains. This can also explain the prolonged survival of acetophilic strains in wine. The environmental acidity of AAB plays an important role with regards to their ethanol tolerance. It was found that, at a pH of 3.0, an *A. pasteurianus* strain displayed an ethanol tolerance of up to 8.2%. However, at a higher pH of 3.4, the same strain survived at ethanol levels of 12.5%. Contrary to the inhibition of most LAB by low pH values, AAB clearly are of greater concern from the point of view of quality control by the winemaker. Maintaining a low pH in wine is crucial for various reasons. Low pH values are not only necessary for better colour extraction, particularly in red wines, but they possibly also contribute to a greater ageing potential (Zoecklein et al., 1995). Another important reason associated with low wine pH is the antimicrobial effectiveness of SO_2, as more of the molecular form is present (Ribéreau-Gayon et al., 2000a).

2.3.3.4 Ethanol

AAB have been classified taxonomically on the basis of their ability to utilise ethanol as carbon source and oxidise it to acetic acid. This ability enables AAB to grow in wine, fermented cider, sake and kombucha tea, thus resulting in the production of various kinds of beverages and vinegar. Different studies have indicated that fluctuating ethanol concentrations determine their growth capability. However, limited information is available on the ethanol tolerance of *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* at concentrations above 8% (Drysdale and Fleet, 1988). A study performed by De Ley et al. (1984) revealed that only 58% of *Acetobacter* strains that were tested grew in media containing 5% ethanol. However, the growth number was reduced to 13% when the ethanol concentration was increased to 10%. According to De Ley and Swings (1984), none of the strains of *A. aceti* and 20% of the strains of *A. pasteurianus* grew in media containing 10% ethanol. They also reported that only 42% of *G. oxydans* are able to grow in media with an ethanol concentration of up to 5%. It has become quite evident that AAB can easily survive high ethanol concentrations in the range of 10 to 15%, as supported by isolations from various

wines (Joyeux et al., 1984a; Drysdale and Fleet, 1985; Du Toit and Lambrechts, 2002).

Although ethanol sensitivity is known to be strain dependent and associated with pH and temperature, the great adaptability of these significant microorganisms is once again questioned. A small ethanol increase of 1% has been reported to cause an initial lag phase before actual oxidation, which suggests that these bacteria have acquired ethanol tolerance (Saeki et al., 1997a). The main factor overruling all known evidence and suggesting a greater need of interest, however, is the fact that isolates have been obtained from strong alcoholic beverages such as *saké* and tequila (Swings and De Ley, 1981).

2.3.3.5 Sulphur dioxide

The use of sulphur dioxide during normal winemaking is believed to not fully and sufficiently control AAB. However, SO_2 definitely contributes to the inhibition of AAB in wine and other food and beverage products (Du Toit, 2000). As mentioned earlier, SO_2 consists of a bonded and a free form in wine, of which the latter, molecular form is the most active. At normal wine pH, Ribéreau-Gayon et al. (2000a) reported that only 5% of the free SO_2 occurs in the molecular form. Other factors that also determine SO_2 sensitivity are the temperature and the strains involved. A study has revealed that different molecular SO_2 concentrations were necessary to eliminate *A. pasteurianus* (0.6 mg/l), *A. aceti* (0.2 mg/l), *Ga. liquefasciens* (0.1 mg/l) and *G. oxydans* (0.05 mg/l) (Du Toit, 2000). It was evident that *Ga. hansenii* was the most resistant to SO_2, being eliminated only by more than 0.7 mg/l. Watanabe and Iino (1984) found that the inhibition of an *Acetobacter* species in grape must was obtained only with about 100 mg/l total SO_2. Even a pH value of 3.46 with a total SO_2 concentration of 81.6 mg/l was not sufficient enough to prevent *A. pasteurianus* from reaching cell counts of 10^5 in Japanese red wine (Drysdale and Fleet, 1985). This suggests that further research is required to investigate SO_2 sensitivity and its correlation to ethanol, acidity and the temperature used during winemaking. Additionally, the production by AAB of compounds such as 2-keto-D-gluconic, 5-keto-D-gluconic and 2,5-diketo-D-gluconic acids from glucose (Attwood et al, 1991; Qazi et al., 1991) and dihydroxyacetone from glycerol (De Ley and Swings, 1984) has the potential to diminish the antimicrobial function of SO_2. These compounds are known for their ability to bind SO_2, thus lowering the free active amount that is required (Eschenbruch and Dittrich, 1986).

The use of free SO_2 (250 mg/l) for the elimination of AAB from wooden staves and during storage (Wilker and Dharmadhikari, 1997) will probably soon come under scrutiny. Increasing consumer awareness of the health risks and safety of foods and beverages has motivated investigations into the use of "biopreservation" in these industries. Together with other preservatives, such as benzoic acid, fumaric acid and sorbic acid, which still need to be researched in wine with regard to AAB, alternative methods of controlling spoilage bacteria need to be applied. Current investigations

that may assist the winemaker with regard to biopreservation in the near future include the use of bacteriocins for the inhibition of LAB (Du Toit and Pretorius, 2000).

2.3.4 Interaction with other wine-related organisms

The interest regarding the intrinsic compilation through which several microorganisms contribute to wine quality has become an exploration process with various great expectations. The interaction of other wine-related microbial populations, especially with respect to AAB, has resulted in numerous suggestions about their survival and spoilage capabilities during the winemaking process.

2.3.4.1 *Botrytis cinerea*

The development of fungi (*Botrytis cinerea*) on grapes is advantageous for the development of AAB. *Botrytis* determines the exposure of the grape's intracellular contents, thereby making the sugar accessible to AAB and their growth and the consequent formation of acetic acid therefore will commence earlier. *Botrytis* hereby indirectly contributes to the negative influence that AAB exert on the yeasts via acetic acid. The occurrence of *Botrytis* is a direct consequence of the health status of the grapes. Species of *G. oxydans* are known for their dominance on grapes due to their high sugar preference (Drysdale and Fleet, 1988). However, with grape damage and consequential *Botrytis* development, species of *A. aceti* and *A. pasteurianus* have been reported to occur at higher cell numbers (up to 10^6 cells per ml) than *G. oxydans*, which normally is the dominant AAB flora on grapes (Drysdale and Fleet, 1988; Fugelsang, 1997). This is of great significance, as *Botrytis* produces glycerol, which serves as carbon source in the place of glucose, especially for *Acetobacter* species, and results in the formation of DHA (Drysdale and Fleet, 1989a; Barbe et al., 2001). Alternatively, the elevated glucose levels, also as a result of *Botrytis*, are preferred by *Gluconobacter* species and are converted into gluconic and ketogluconic acids (Ruiz-Argüeso and Rodriguez-Navarro, 1973; Barbe et al., 2001). It has been shown that up to 80% of *Gluconobacter*'s oxidation products, especially DHA and gluconic acid, can bind SO_2 (Barbe et al., 2001). The increased populations of AAB producing the yeast-inhibitory metabolite acetic acid definitely are determined by the occurrence of *Botrytis*.

2.3.4.2 *Saccharomyces* and non-*Saccharomyces* yeasts

Stuck or sluggish fermentations have been in the spotlight in recent years and, by using the possible causes indicated by previous investigations as basis, various attempts to find solutions have been exploited. These causes include ethanol toxicity, nutrient deficiency, yeast strain variability, bacterial strain dependency and inhibitory compounds such as acetic and other fatty acids Rasmussen et al., 1995; Henschke, 1997; Bisson, 1999, Du Toit, 2000). All of these are influenced directly by the presence of spoilage organisms such as AAB.

The ability of the yeast to ferment must to dryness was investigated in a study in which both the yeast and the AAB were inoculated simultaneously (Drysdale and Fleet, 1989a). It was found that both the yeast's ability to ferment to dryness and its growth (to a lesser extent) were affected. This corresponds to the finding that AAB can inhibit wine-associated yeasts (Gilliland and Lacey, 1964; Grossmann and Becker, 1984; Joyeux et al., 1984b). The more glucophilic nature of *Acetobacter* is also believed to cause a glucose/fructose imbalance. As most strains of *S. cerevisiae* are also glucophilic, the former imbalance may well result in sluggish or even stuck fermentations. Additionally, the presence of wild or non-*Saccharomyces* yeasts, such as *Candida*, *Kloeckera*, *Hanseniaspora* and *Metschnikowia*, which initiate natural fermentations with a corresponding ethanol build-up, can lead to the earlier formation of acetic acid by AAB. Some other yeasts, including *Dekkera* and *Brettanomyces*, have also been known to produce acetic acid in co-culture with *S. cerevisiae* (Fugelsang et al., 1993). It is known that the inhibitory effect of acetic and other fatty acids on the yeast is increased at higher temperatures (Du Toit, 2000). Therefore, the existence of thermotolerant AAB (Saeki et al., 1997a) should also be taken into consideration, as the increased production of these inhibitory acids by AAB at elevated temperatures could further enhance this inhibitory action. If inefficient temperature control is applied during winemaking, unnecessarily high temperatures may increase the concentrations of unwanted metabolites produced by some thermotolerant *Acetobacter* species. This, together with the decreased ethanol and acetic acid resistance of *S. cerevisiae* following raised temperatures (De Ory et al., 1998), clearly is indicative of another possible spoilage mechanism of AAB. The fact that high levels of VA from different wineries have been associated with the occurrence of sluggish or stuck fermentations has triggered numerous investigations (Eglinton and Henschke, 1999). Of the AAB, some strains of *Ga. hansenii* and *A. pasteurianus* have been found to inhibit *S. cerevisiae* the most (Arneborg et al., 1995). Although this correlated with their production of acetic acid, other investigations should be done, as the possibility of alternative inhibitory mechanisms cannot be excluded (Du Toit, 2000).

2.3.4.3 Lactic acid bacteria

LAB are also well known for their role in winemaking, specifically in malolactic fermentation (MLF). Joyeux et al. (1984b) mentioned that the growth of either *G. oxydans* or *A. aceti* prior to the inoculation of *Oenococcus oeni* (Dicks et al., 1995) stimulated MLF. This raises the possibility that AAB might produce secondary metabolites that are favourable for LAB. Some species of LAB (*Pediococcus*, *Lactobacillus*) are prominent spoilers that contribute through their production of metabolites serving as substrates. Recent evidence indicates that heterofermentative lactobacilli produce mannitol through the reduction of fructose to fructose-6-phosphate (Du Toit and Pretorius, 2000). Mannitol is recognised as a carbon source for AAB and is used for their isolation. This contribution, along with others, may very

well contribute to the occurrence of AAB in wine. The interaction between AAB and LAB during fermentation should still be investigated, however, as a possible synergistic effect will be of great significance.

2.3.4.4 Bacteriophages

The presence of bacteriophages in wines is believed to influence the survival and growth of AAB (Drysdale and Fleet, 1988). Bacteriophages have been reported that are active specifically against unidentified *Acetobacter* strains (Bradley, 1965). Bacteriophages that are active against *G. oxydans* have also been isolated (anonymous author). The occurrence of these bacteriophages in decaying apples suggests the possibility of their existence on grapes, and thus also in grape must. Although some phages also have been isolated from *Acetobacter*, strains of *Gluconobacter* are known to be the more common hosts (Swings, 1992). The use of bacteriophages could well be applied for the control of AAB and detailed research in this regard is recommended.

2.4 KNOWN INHIBITORY AND ANTIMICROBIAL COMPOUNDS

Microbial spoilage in the winemaking process can occur at any stage. In this regard, the contribution of AAB currently is limited to the inhibitory action it exercises via acetic acid. However, many microorganisms, especially yeasts, are sensitive to organic acids, such as formic acid, benzoic acid and, more specifically, acetic acid and other medium-chain fatty acids (Rasmussen et al., 1995; Casal et al., 1996; Du Toit, 2000). Acetic acid affects the chemical composition of grape must and wine and, at high concentrations (> 0.8 g/l), it results in the wine having a poor quality. However, the major problem of high VA or acetic acid is its influence on the wine yeast, *S. cerevisiae*. Acetic acid affects the yeast by releasing a proton into the cytoplasm after diffusion, thus causing intracellular acidification (Rasmussen et al., 1995). This reduction in the intracellular pH directly affects the yeast's metabolism and decreases its vitality during fermentation (Antoce et al., 1997; also refer to Du Toit (2000) for the influence of other medium-chain fatty acids in this regard). The suppression of yeast vitality then contributes to the occurrence of sluggish or stuck fermentations. The significance of AAB as a spoilage organism is evident from this.

The use of chemical preservatives (SO_2) has come under threat because of increasing consumer bias regarding its health and safety. Therefore, numerous investigations considering biological approaches currently are being undertaken (Du Toit and Pretorius, 2000). The use of biological compounds for preservation (biopreservation) focuses on the use of antimicrobial peptides to control bacterial cell numbers. These so-called bacteriocins, commonly associated with the Gram-positive LAB, are being used in the food industry and researched in the beverage industry (Jack et al., 1995). This approach could very well serve as an alternative in the attempts to control AAB, but, thus far, no known antimicrobial activity of

proteinaceous origin or bacteriocin, from or active against AAB, has been shown. Stadelmann (1961) has mentioned that additional antimicrobials, other than acetate, seem to be present in traditional tea fermented by AAB. In support of this, Sreeramulu et al. (2000) reported the antimicrobial activity of Kombucha tea after fermentation with yeasts and AAB. The antimicrobial activity also was exhibited after heating and at a pH of 7 and inhibited *Escherichia coli*, *Salmonella enteritidis*, *Salmonella typhimurium* and *Shigella sonnei* (Sreeramulu et al., 2000). The unfermented tea had no antimicrobial activity against these microorganisms, even though it is known to contain high levels of polyphenols, such as catechins, which have bacteriostatic properties against other microorganisms (Greenwalt et al., 2000). This implies the existence of antimicrobial compounds that could be proteinaceous in nature. Further research is needed to verify this.

It is also known that rice wine or saké has been reported to exhibit antimicrobial activity, but this was ascribed largely to the degradation of phenolic compounds (Yoshizawa et al., 1970). Nevertheless, the possibility of proteinaceous substances from AAB with bactericidal abilities cannot be ignored. Novel bacteriocin-like proteins will contribute greatly to the wine industry, as well as to the inhibitory abilities of Gram-negative bacteria.

2.5 CONCLUDING REMARKS

From this review it is evident that acetic acid bacteria are of great significance, not only during winemaking, but also in other processes of industrial importance. These bacteria are an example of evolutionary development and their good adaptation abilities were mentioned numerous times. Although AAB are renowned for their negative contributions to food and beverage products, other, more positive, uses are increasingly being announced. Technological advances resulting directly from this extension of knowledge are being used to explore AAB for use as starter cultures in some beverage preparations.

AAB, with all their implications for sluggish or stuck fermentations and sensorial spoilage, are currently considered to be the winemaker's foe. In the next decade, the significance of AAB in the winemaking process will be exploited and the knowledge gained will enable the winemaker to control this microorganism.

"A foe can always become a friend given the right circumstances"

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CHAPTER 3

Research results

The isolation and preliminary characterisation of the antimicrobial peptides produced by acetic acid bacteria isolated from South African wines

3. RESEARCH RESULTS

The isolation and preliminary characterisation of the antimicrobial peptides produced by acetic acid bacteria isolated from South African wines

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Abstract

Acetic acid bacteria (AAB) isolated from wine were screened for the production of antimicrobial peptides. At a pH of 6.5, the supernatant of two isolates exhibited bioactivity against other strains of AAB with the agar diffusion method. By means of biochemical tests and PCR-RFLP analysis of the 16S rDNA, the two producer strains were identified as *Acetobacter aceti* and *Gluconobacter frateurii*. The identification of the producer isolate AO286 as *Gluconobacter frateurii* revealed the occurrence of an AAB species that has not yet been found in the winemaking environment. Antimicrobial activities were found against *Gluconacetobacter liquefasciens* AOII23 and an unknown AAB AOI10 isolate from wine. Other Gram-negative microorganisms that also were affected were *Escherichia coli* DH5α and *Agrobacterium tumefasciens* GV 3101. The antibacterial compound was determined to be a proteinaceous substance, since it lost its activity after proteolytic enzyme treatment. The molecular weight could only be established between 3 and 10 kDa. Preliminary characterisation indicated that the antimicrobial substance was stable in a pH range from 3.0 to 8.0 and that it was temperature sensitive, with the activity diminishing slightly with the temperature increase from 4°C to 65°C and thereafter, all activity was lost. The mechanism of activity of the antimicrobial substance was mostly bacteriostatic. Of the various media tested, YPM supplemented with 0.5% Tween 80 yielded the best antimicrobial production. The production of antimicrobial substances from Gram-negative AAB is considered novel, as no evidence of such behaviour has been reported.

Keywords: Acetic acid bacteria, Antimicrobial, Gram-negative, Wine

3.1 INTRODUCTION

The occurrence of bacterial spoilage in wineries all around the world has triggered numerous investigations over the past decade, in an attempt to identify, evaluate and solve any problems that pose a threat to the winemaking process. A specific group of bacteria has been highlighted that is considered to be one of the most common microbial spoilers affecting wine quality worldwide. These microorganisms are known as acetic acid bacteria (AAB).

AAB are Gram-negative, catalase-positive rods that belong to the family *Acetobacteriaceae* (De Ley et al., 1984; De Ley and Swings, 1984; Holt et al., 1994). Their taxonomical classification has changed substantially over recent years due to the complexity of their identification (Swings, 1992; Yamada et al., 1997; Trček and Teuber, 2002). Currently, AAB are divided into five genera: *Acetobacter (A.), Gluconobacter (G.), Gluconacetobacter (Ga.), Acidomonas (Ac.)* and *Asaia (As.)*. Of these, *Acetobacter aceti, Acetobacter pasteurianus, Gluconacetobacter hansenii* (formerly *Acetobacter hansenii*), *Gluconacetobacter liquefasciens* (formerly *Acetobacter liquefasciens*) and *Gluconobacter oxydans* thus far have been associated with the vinification process (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002). Although other species, such as *Gluconacetobacter frateurii, Gluconacetobacter asaii* and *Gluconacetobacter xylinus,* also have been isolated from sugar-rich environments, their existence on grapes cannot be excluded.

AAB have gained attention due to the increased occurrence of sluggish/stuck fermentations. Some of the causes responsible for sluggish/stuck fermentations include nutrient deficiency, ethanol toxicity and inhibitory compounds, such as acetic and other fatty acids that cause yeast distress (Bisson, 1999; Du Toit, 2000). AAB have been implicated in sluggish/stuck fermentations, as acetic acid constitutes more than 90% of the volatile acidity of wine and high levels of acetic acid or volatile acidity (VA) have often been associated with wines that have undergone sluggish/stuck fermentations. Additionally, the interest in AAB was fueled further when these organisms, which were previously believed to be strict aerobes, were isolated from fermentation tanks in which an anaerobic environment had been maintained. This indicated their ability to survive and grow during the winemaking process and possibility to have a negative effect on wine quality (Drysdale and Fleet, 1985, Poblet et al., 2000; Du Toit and Lambrechts, 2002).

Today, the significance of AAB stretches from their popular use in the production of acetic acid from ethanol, resulting in wine acetification, to other, more visible, traits, such as the production of surface films, turbidity and colour changes (Fugelsang, 1997). Thus, these bacteria not only affect the chemical composition of the must and wine, but also directly affect the yeast's metabolism and vitality (Bisson, 1999). All of these contribute directly to a decrease in the sensory quality of the final product. It therefore is important to prevent the growth of these spoilage organisms throughout the winemaking process.

In order to control the growth of unwanted spoilage microorganisms during winemaking, chemical preservatives, such as sulphur dioxide (SO_2), are applied. SO_2 has long been used in winemaking for its antioxidative and antimicrobial properties. However, it is known that AAB exert varying degrees of resistance to this antimicrobial agent (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002) and, in addition, high amounts of SO_2 also can detract from the organoleptic quality of wines. This, together with an ever-increasing consumer resistance against the excessive use of chemical preservatives (Fugelsang, 1997) that pose possible health risks, has led to the exploration of alternative methods.

The use of biological preservatives derived from plants, animals or microorganisms to combat the natural occurrence of spoilage has been applied successfully in the food industry. It specifically is the use of antimicrobial compounds or bacteriocins from mostly Gram-positive lactic acid bacteria that has initiated the move to biopreservation (Du Toit and Pretorius, 2000). However, further research on the possible application of such proteinaceous antimicrobial compounds, especially in the beverage industry, still needs to be carried out.

An enormous gap regarding the significance of Gram-negative bacteria from wine, specifically AAB, is evident from the literature. As mentioned earlier, the negative influence of AAB on wine quality has become increasingly evident in recent years. As per definition, bacteriocin-like or antimicrobial compounds are peptides produced by bacteria that inhibit other closely-related organisms of the same species (Klaenhammer, 1988). Therefore, a study to find inhibitory compounds of a proteinaceous nature produced by AAB isolated from wine seemed to be a good approach to the attempt to find compounds that could possibly inhibit their growth.

Preliminary results from a study conducted at the Institute for Wine Biotechnology (IWBT, Stellenbosch University, South Africa) involving the screening of 117 AAB isolated from wine for the production of antimicrobial peptides revealed the existence of a possible inhibitory compound of proteinaceous nature (unpublished data). Therefore, this study was conducted in an attempt to isolate and characterise the antimicrobial peptides produced by AAB.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and growth conditions

The strains used in this study are listed in **Table 3.1** and **Table 3.3**. All wine isolates of AAB were grown in YPM (2.5% D-mannitol, 0.5% yeast extract, 0.3% peptone and 2% agar) and GYC (5% D-glucose, 1% yeast extract, 3% calcium carbonate and 2% agar) media. The reference strains from DSM (Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) were grown on the media as specified by the supplier. *Escherichia coli* and *Agrobacterium tumefasciens* (laboratory strains) were grown in Luria-Bertani (LB) medium. Yeasts were grown aerobically in YPD broth (yeast extract,

peptone, dextrose). All cultures were grown aerobically at 30°C, except for *E. coli*, which was grown at 37°C. Lactic acid bacteria (LAB) were cultivated microaerophilically on MRS media (De Man, Rogosa, Sharpe) (De Man et al., 1960) at 30°C (all nutrient broths and agars used were obtained from Biolab, Merck, South Africa). The cultures used during the experimental work were sub-cultured once in their respective media after an initial inoculation from a single colony.

Table 3.1
List of microorganisms used during this study, with their respective media and growth temperatures

Microorganism	Strain	Media	Temperature
AAB Isolate (producer)	AO286	YPM	30°C
AAB Isolate (producer)	AOII13	YPM	30°C
AAB Isolate (sensitive)	AOII23	YPM	30°C
AAB Isolate (sensitive)	AOI10	YPM	30°C
Acetobacter aceti	[1]DSM 3508[T]	YPM	30°C
Gluconacetobacter liquefasciens	DSM 5603[T]	YPM	30°C
Gluconacetobacter hansenii	DSM 5602[T]	YPM	30°C
Acetobacter pasteurianus	DSM 3509[T]	YPM	30°C
Gluconobacter oxydans	DSM 7145[T]	YPM	30°C
Gluconobacter frateurii	DSM 7146[T]	YPM	30°C
Escherichia coli	DH5α	LB	37°C
Agrobacterium tumefasciens	GV 3101	LB	30°C
Leuconostoc mesenteroides	[2]NCDO 529	MRS	30°C
Lactobacillus plantarum	DSM 10754	MRS	30°C
Saccharomyces cerevisiae	WE372	YPD	30°C
Saccharomyces cerevisiae	VIN13	YPD	30°C

[1] DSM: Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany
[2] NCDO: National Collection of Dairy Organisms, National Institute for Research in Dairying, Shinfield, England

3.2.2 Identification of acetic acid bacteria

Isolates of AAB were identified by means of biochemical tests as described by Drysdale and Fleet (1988) (**Table 3.2**) and additionally validated by means of restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR)-amplified 16S rDNA (Ruiz et al., 2000). Standard procedures for the isolation of genomic DNA (gDNA) were used (Sambrook et al., 1989) when colonies failed as templates for PCRs. PCR amplification was carried out in 45 µl samples and single colonies were used to obtain the bacterial extract. The 45 µl amplification mixture contained 15 pmol of each primer, 200 µM of each of the dNTPs (PCR dNTP mix, Roche), 5 µl 10x *Taq* amplification buffer (Bioline, Germany), 5 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (Bioline). The primers that were used for the PCR amplification were the same as those prescribed by Ruiz et al. (2000). These primers were designed from the conserved

regions of the 5'-end (16Sd, 5'-GCTGGCGGCATGCTTAACACAT-3') and the 3'-end (16Sr, 5'-GGAGGTGATCCAGCCGCAGGT-3') of the 16S rDNA.

Colony-PCRs were carried out in a thermocycler machine (PCR express, Hybaid). Samples were incubated for 3 min at 94°C and then cycled 35 times at 94°C for 1 min, 58°C for 1 min and 72°C for 2 min. The samples were then incubated at 58°C for 5 min and at 72°C for 5 min for a final extension. The 1 450 bp amplified PCR-product was removed from the gel and purified using a High Pure PCR Purification Kit (Roche, Germany).

For the restriction of the amplified DNA, 5 µl of each PCR product was digested with the restriction endonucleases, *Taq*I and *Rsa*I, as specified by the manufacturer (Roche). Restriction fragments were analysed on a 3% (w/v) agarose gel in 1 x TAE buffer. The reference strains that were included for identification purposes are listed in **Table 3.3**.

3.2.3 Preliminary screening for antimicrobial activity by acetic acid bacteria

Various isolates of AAB were grown overnight in YPM and GYC media. A 100 µl suspension of the producer cells was spotted into wells made in YPM agar. YPM plates were chosen for the purpose of better visualisation. 50 µl of the sensitive organism was spread-plated by means of a hockey stick (over the wells) and incubated for 2 days at 30°C. The approach of cross-streaking the different AAB was also performed on YPM agar.

Table 3.2
Biochemical tests used for differentiating genera and species of acetic acid bacteria isolated from wine^{ø}

Characteristic	*A. aceti*	*A. pasteurianus*	*Ga. hansenii*	*Ga. liquefasciens*	*G. oxydans*
Growth on carbon sources:					
Ethanol	+	d	-	+	d
Sodium acetate	+	d	-	d	-
Dulcitol	-	-	d	-	d
Ketogenesis from:					
Glycerol	+	-	+	+	+
Sorbitol	+	-	+	+	nd
Mannitol	d	-	+	+	nd
Total oxidation of ethanol	+	+	+	+	-
Oxidation of D-, L-lactate	+	d	+	+	-
Formation of water-soluble brown pigments on GYC*	-	-	-	+	+
5-Ketogluconic acid from D-glucose	+	-	d	d	+

ø   From Drysdale and Fleet (1988)
d   Strains strongly positive
*   See section 3.2.1 for media composition
nd not determined

Table 3.3
Type strains used in this study for the identification of the AAB isolates

Species	Strain	Origin
Gluconobacter oxydans	DSM 7145^T	Beer
Gluconobacter frateurii	DSM 7146^T	*Fragaria ananassa*
Gluconobacter asaii	DSM 7148^T	*Rheum rhabarbarum*
Acetobacter aceti	DSM 3508^T	Beechwood shavings, vinegar plant
Acetobacter pasteurianus	DSM 3509^T	Beer
Gluconacetobacter hansenii	DSM 5602^T	Vinegar
Gluconacetobacter liquefasciens	DSM 5603^T	Dried fruit
Gluconacetobacter xylinus	DSM 5615^T	Mountain ash berries
Gluconacetobacter europaeus	DSM 6160^T	Unknown
Gluconacetobacter diazotrophicus	DSM 5601^T	Sugarcane roots
Frateuria aurantia	DSM 6220^T	*Lilium aurantum*

3.2.4 Inhibitory activity assay and partial purification

Producer cells of AAB were allowed to grow for 2 days at 30°C while being shaken continuously for aeration. Thereafter, the cells were separated by centrifugation (Sorvall, MC 5C, Du Pont) at 10 000 rpm for 15 min to obtain the supernatant. The supernatant was further subjected to syringe filtration through a 0.22 μm pore (Osmonics) to assure complete cell removal. The pH of the supernatant was adjusted to between 6.0 and 7.0 with 10N sodium hydroxide (NaOH). This solution was designated the Cell-Free Supernatant (CFS). To eliminate protease activity, the CFS was treated with 1 mM phenyl methyl sulphonyl fluoride (PMSF) (Sigma) as protease inhibitor.

The CFS was tested for inhibitory activity by means of the agar-diffusion method. Wells of 100 μl were made in the relevant media (YPM agar for AAB, LB agar for *E. coli* and *Agrobacterium*, MRS agar for LAB and YPD agar for yeasts). Plates were left to dry in a laminar flow at room temperature for 2 h. The wells were filled with the CFS, left to diffuse and then re-spotted (agar-diffusion assay). Ten minutes thereafter, 50 μl of the selected sensitive organism was spread-plated and then they were incubated overnight under their respective conditions (refer to section 3.2.1. for incubation details).

The crude protein extract was obtained from the supernatant by means of ammonium sulphate (Biolab) precipitation. A saturation of up to 80% (52g/100 ml supernatant) was performed in an ice water slurry (below 4°C) as described by Bollag and Eidelstein (1991). The precipitate that was formed as a floating pellicle was scooped up and dissolved in 50 mM phosphate buffer (pH 6.0) or sterile double purified water. The remaining precipitated CFS was centrifuged at 10 000 rpm for 10 min at 4°C and dissolved in the same buffer. The precipitates were dialysed overnight against sterile double distilled water at 4°C and this was considered as the crude antibacterial substance.

3.2.5 Effect of media on production of antimicrobial compounds

The following media were used to cultivate AAB to determine their effect on the production of the antimicrobial compound: standard YPM broth consisting of 2.5% D-mannitol, 0.5% yeast extract and 0.3% peptone; GY broth consisting of 5% D-glucose and 1% yeast extract; MRS broth containing the nutrient components as specified by the manufacturer (Biolab); standard YPM broth supplemented with 0.5% Tween 80, 0.015% magnesium sulphate, 0.5% sodium acetate, 0.2% potassium di-hydrogen phosphate, 0.2% di-ammonium citrate and 0.5% casamino acids (Difco, Detroit, USA); and All Purpose Tween (APT) broth consisting of 1.25% peptone, 1% glucose, 0.75% yeast extract, 0.5% sodium chloride, 0.2% Tween 80, 0.5% tri-sodium citrate, 0.5% di-potassium hydrogen phosphate, 0.08% magnesium sulphate, 0.014% magnesium chloride, 0.004% ferric sulphate and 0.0001% tri-ammonium chloride.

Producer strains were cultivated in each of these media for 6 days at 30°C while being shaken for aeration. The crude antibacterial substance produced in each of the media was obtained by precipitation as described in section 3.2.4. The total protein concentration of each of the active fractions was determined by means of the Bradford method (Protein assay, Biorad). This was designated the antibacterial protein spike. The sensitive AAB strains, AOEI10 and AOII23, were inoculated into 5 ml of YPM liquid broth (5 µl from an overnight pre-culture) and the antibacterial spike, produced from each of the respective media, was added at a final concentration of 25 µg total protein. The unspiked cultures served as positive controls. The cultures were incubated for 16h at 30°C on a turning wheel before they were evaluated.

3.2.6 Effect of enzymes, temperature and pH on the activity

For preliminary characterisation of the antimicrobial compound, the producer strains were cultivated in YPM broth (supplemented only with 0.5% Tween 80) for 6 days at 30°C. The crude precipitate (section 3.2.4) that was obtained was subjected to proteolytic enzymes, heat treatments and pH ranges.

Firstly, the effect of proteinase K, pronase and α-chemotrypsin (Sigma) on the antimicrobial activity was tested (Zamfir et al., 1999). All enzymes were dissolved in 3 mmol/l sodium phosphate buffer (pH 6.8) and added to the crude protein sample at a final concentration of 0.5 mg/ml. After incubation for 1 h at 37°C, all the enzymes, except for proteinase K, were heat-inactivated for 20 min at 65°C before the inhibitory activity was tested. Proteinase K was inactivated at 80°C for 20 min. To completely eliminate possible inhibitory activity by hydrogen peroxide, the same sample was also treated with catalase (5 mg/ml) pre-dissolved in 50 mmol/l potassium phosphate buffer (pH 7.0) at 25°C. In order to ensure that the antibacterial activity was not the result of proteolytic enzymes, specifically proteases, the crude supernatant was treated with a cocktail of protease inhibitors (Protease inhibitor tablets, Roche). The YPM agar used for the activity assays contained 50 mmol/l of the protease inhibitor cocktail. This buffered the activity spots against possible proteolytic activity from the sensitive organism.

Secondly, the heat sensitivity was tested by incubating the antimicrobial samples at 4°C, 30°C, 37°C, 42°C and 65°C for 30 min respectively. The sample was also subjected to autoclaving at 121°C for 15 min. The activity of the samples was tested directly after these treatments.

Thirdly, to test the influence of pH, the pH of the crude precipitated samples was adjusted from 3.0 to 9.0 (with increments of 1) with 1 M HCl and 1 M NaOH. After mixing, the samples were allowed to stabilise at room temperature. The pH was verified before the inhibitory activity was tested.

3.2.7 Mode of action

In order to determine if the exerted antibacterial activity was bactericidal or bacteriostatic, indicator organisms were pre-cultured in their respective media under their respective conditions up to an optical density ($OD_{600\ nm}$) of 0.5. Of these, 10 µl was inoculated into 10 ml of liquid media and these organisms were then spiked with a partially purified active sample (produced in various media, section 3.2.5) containing 5 µg/ml of total protein. The cultures that didn't receive antimicrobial spikes served as negative controls. After 16 h, the cultures were investigated and their ODs determined.

3.2.8 Molecular size determination and overlay

To determine the molecular mass of the antimicrobial peptide, the dialysed ammonium sulphate precipitate was first fractionated by means of centrifugal filter units (Centricon, Millipore, USA), with cut-offs of 3 000 Da and 10 000 Da respectively. The active fraction was then subjected to Tricine-SDS-PAGE on a 10% polyacrylamide gel with a Tris-HCl pH 8.3 discontinuous buffer system. The samples for the Tricine-SDS-PAGE were diluted with one volume of sample buffer (Sigma Aldrich, Germany) and boiled for 3 min. The molecular weight marker (ultra-low range Sigma Marker) was diluted 1:20 with 1x sample buffer and heated for 2 min at 65°C. The protein bands, together with the protein standard, were visualised by means of silver staining, as outlined by Swain and Ross (1995).

Native gel electrophoresis[#] was performed as described by Hames (1990). After fixation, the gel was washed for 4 h with sterile millipore water that was replaced every hour. Finally, the gel was transferred to YPM agar and overlaid with YPM soft-agar (0.8%) containing the indicator organism, *A. liquefasciens* DII23. The other indicator bacteria that were used included AAB strain AOI10. The lawns were prepared by cultivating the indicator bacteria to an $OD_{600\ nm}$ of 0.5 and adding 150 µl of cell suspension to 3.5 ml overlay agar. The plates were incubated for up to 24 h at 30°C.

3.2.9 Electrospray Mass Spectrometry (ES-MS)

Peptides and proteins were characterised by mass spectrometry, utilising electrospray ionisation as described by Loo and Loo (1997). For this purpose, the peptide (fractionated on a G10 Sephadex column) was dissolved in acetonitrile/water/formic acid: 50/50/0.1 (v/v/v). Five µl of this solution was directly injected into the electrospray

[#] http://www.uwcm.ac.uk/study/medicine/medical_microbiology/Postgrad/page.htm

ionisation source of a Quattro triple quadropole mass spectrometer (Micromass, UK), using acetonitrile/water: 50/50, at a flow rate of 20 µl/minute, as carrier solvent. Ionisation was in the positive mode using a capillary voltage of 3.5 kV with the cone voltage set at 60 V and the source temperature at 80°C. Data was acquired by scanning from m/z = 100-2 000 at 100 amu/second, using the continuum mode. A representative spectrum was made by combining the scans under the elution peak and subtracting the background.

3.2.10 Plasmid isolation

For plasmid isolation, the cultures were grown in YPM broth overnight at 30°C. Plasmids were isolated by means of the High Pure Plasmid isolation Kit (Roche). The products were analysed on a 0.8% agarose gel.

3.3 RESULTS

3.3.1 Identification of acetic acid bacteria

PCR-amplified products corresponding to the 16S rDNA gene were obtained from the four AAB isolates (**Table 3.1**) and from the type strains (**Table 3.3**). PCR products of the expected length of approximately 1 450 bp were obtained after purification (results not shown).

From **Fig. 3.1** it can be seen that the band patterns of one of the producer strains (AO286) corresponds to the type strain of *G. frateurii*. It was also very similar to the species *G. oxydans,* but the single band of 300 bp obtained with the *Rsa*I digestion in **Fig. 3.2** distinguished it as *G. frateurii*. Additionally, the phenotypic growth patterns of strain AO286 obtained from the biochemical tests performed in **Table 3.4** supported the identification as being the closest to *G. frateurii*. From **Fig. 3.3** it can be seen that the band patterns of the other producer strain, AOII3, corresponded to the type strain *A. aceti* DSM 3508. This identification was again verified by the biochemical tests (**Table 3.4**), although the ketogenesis from mannitol differed. However, strain variation (>10%) is evident for certain species during biochemical tests (Drysdale and Fleet, 1988). The sensitive strain, AOII23, had the typical growth patterns and characteristics of *Ga. liquefasciens*. However, a clear distinction of this strain could not be obtained with the RFLP-PCR analysis. No band patterns could be obtained for AAB sensitive strain AOI10. The identification was up to the biochemical tests performed and this left us with an unidentified culture. This isolate appeared to be an AAB as it tested catalase positive, Gram-negative and had typical AAB cell morphology. However, the possibility that it belongs to a completely different genera cannot be excluded.
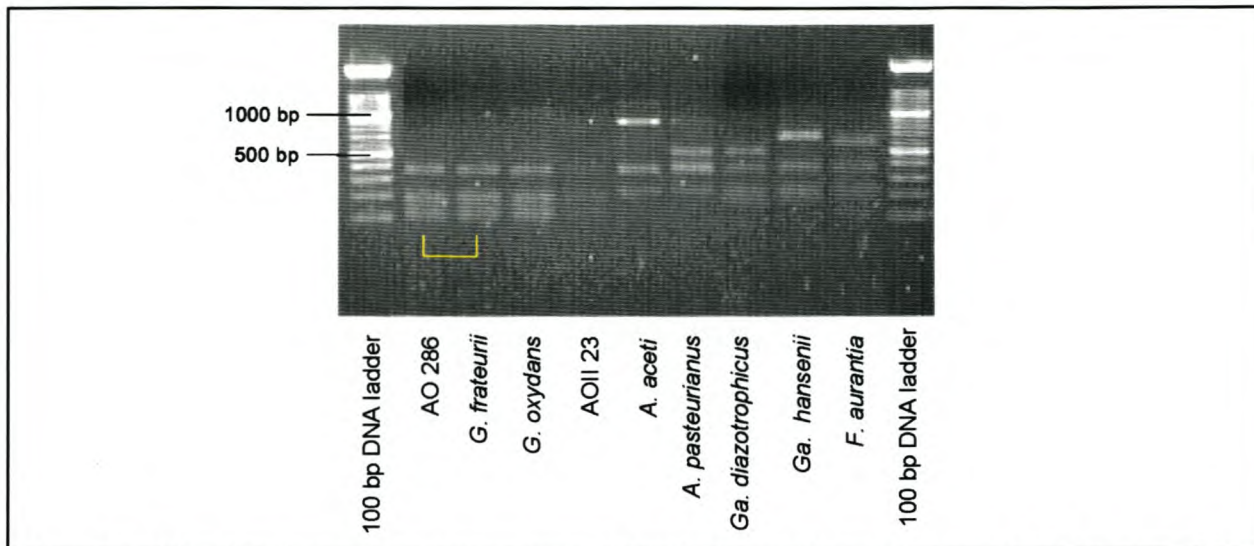
Fig. 3.1. Identification of producer strain AO286 from 16S rDNA PCR-RFLPs after *Taq*I digestion.
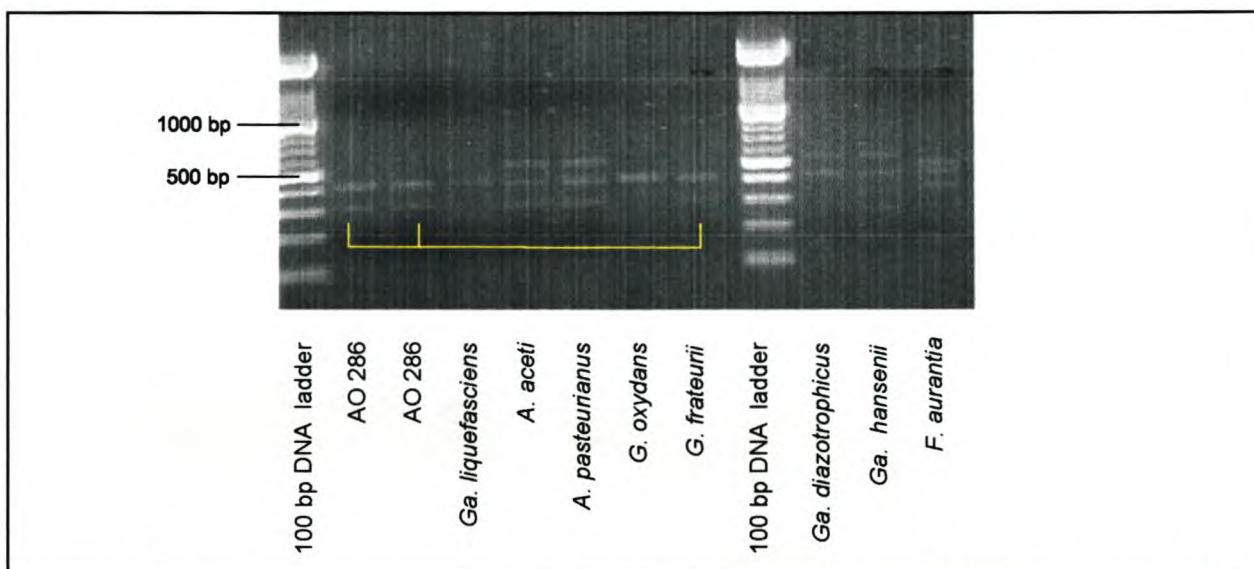

Fig. 3.2. Identification of producer strain AO286 from 16S rDNA PCR-RFLPs after *Rsa*I digestion.

3.3.2 Preliminary screening for antimicrobial activity by acetic acid bacteria

The inhibitory spectrum that was found was very narrow. The producer strains *A. aceti* AOII13 and *G. frateurii* AO286 exhibited inhibition towards two other strains of AAB out of the 87 strains that were tested, namely *Ga. liquefasciens* AOII23 and unknown isolate AOI10 (**Fig. 3.4**). The observed inhibitory action of the two sensitive AAB strains fluctuated in consistency and in zone sizes with the agar well-diffusion assay. **Fig. 3.5** shows the inhibitory pattern by *A. aceti* AOII13 against *Ga. liquefasciens* AOII23 after cross-streaking the AAB as an initial screening procedure. Inhibition against *Escherichia coli* DH5α and *Agrobacterium tumefasciens* GV 3101 was also observed, but these cultures overcame the inhibition within 12h of incubation after the initial inhibitory activity was observed. No inhibition was observed against the industrial *S. cerevisiae* yeast strains VIN13 and WE372.
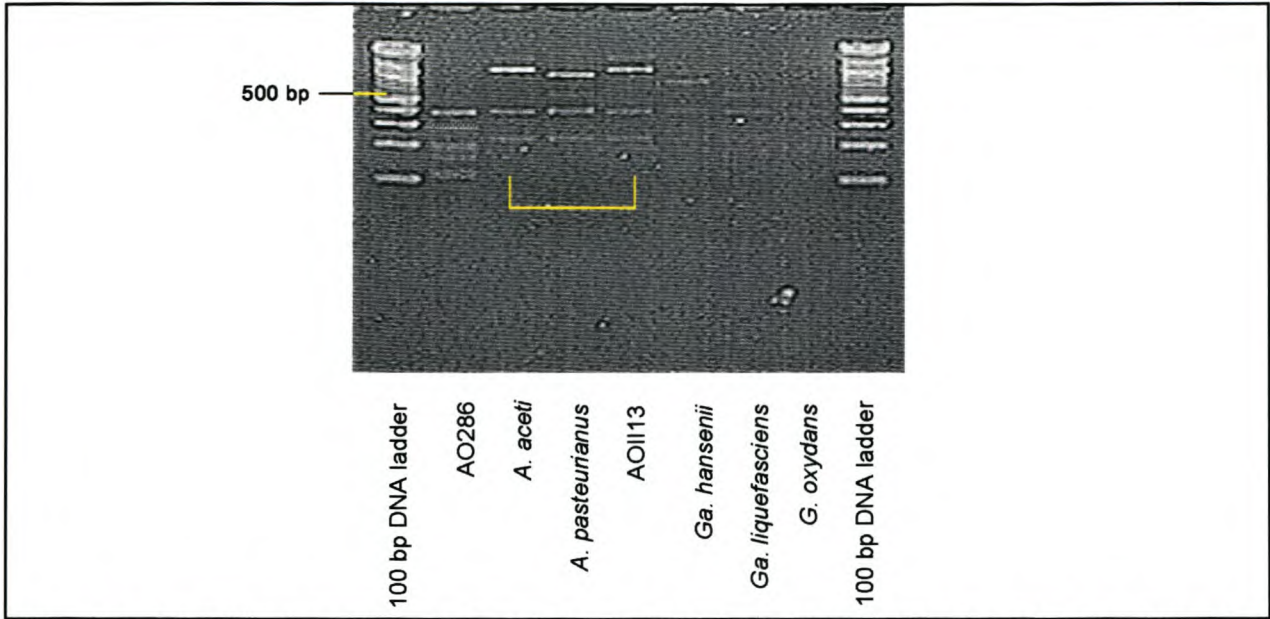
Fig. 3.3. Identification of producer strain AOII13 from 16S rDNA PCR-RFLPs after *Taq*I digestion.

Table 3.4
Biochemical tests used for differentiating genera and species of AAB isolated from wine

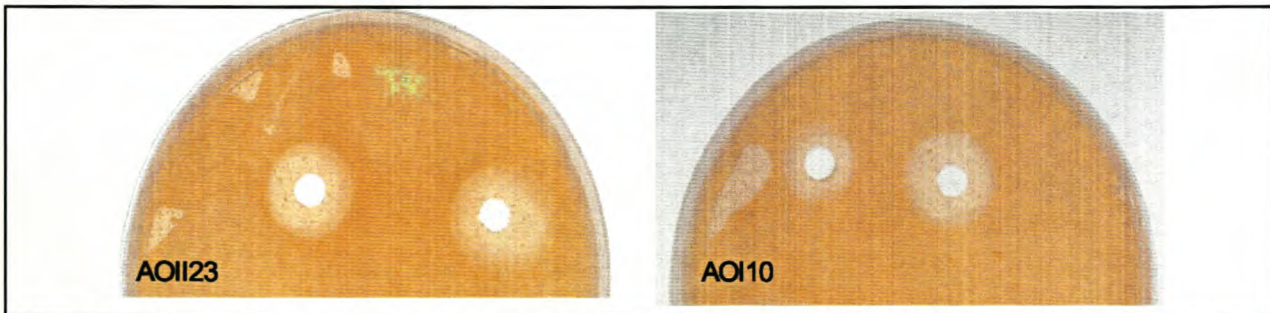| Characteristic	AO286	AOII23	AOII13	AOI10	*G. frateurii*	*A. aceti*
Growth on carbon sources:						
Ethanol	d	+	d	+	+	d
Sodium acetate	d	-	d	+	d	-
Dulcitol	d	-	d	+	d	d
Ketogenesis from:						
Mannitol	+	+	-	-	+	-
Acid production (zones) on GYC	+	+	-	-	+	-
Formation of water-soluble brown pigments on GYC*	-	+	-	-	-	-

[d] growth was evident, but very little

Fig. 3.4. The inhibition of strain *Ga. liquefasciens* AOII23 and unknown strain AOI10 by the supernatant of *G. frateurii* AO286 during the initial screening.
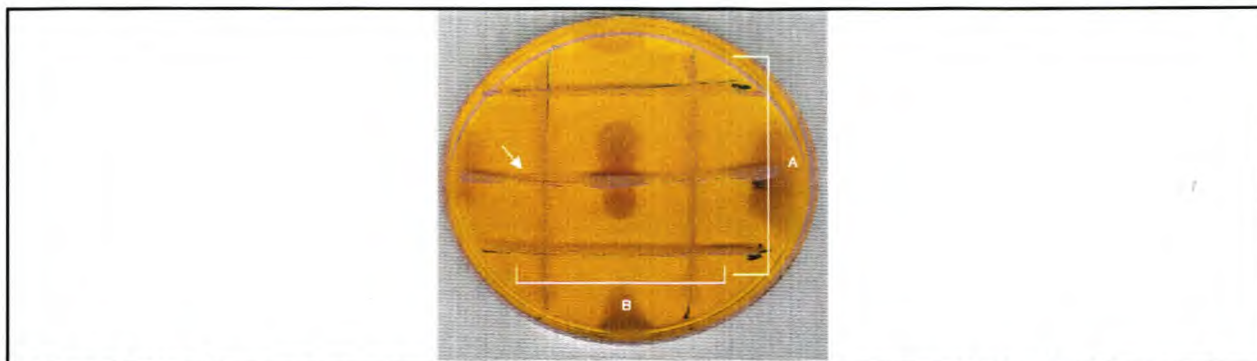
Fig. 3.5. Inhibitory action observed by cross-streaking the sensitive strain *Ga. liquefasciens* AOII23 (A) over the producer strain *A. aceti* AOII13 (B).

3.3.3 Mode of action

An interesting phenomenon occurred with the cultures that were spiked with the antimicrobial substance. Although no clear-cut effect was observed against the unknown sensitive strain AOI10, the presence of the partially purified fraction clearly influenced the sensitive *Ga. liquefasciens* AOII23. From **Fig. 3.6** it is quite evident that the cell numbers of *Ga. liquefasciens* AOII23 differed from that in the control. The antimicrobial spikes, as produced in the various media by both the producer organisms, *A. aceti* AOII13 and *G. frateurii* AO286, had varying effects of inhibition. As all of the cultures grew from their initial inoculums, it is believed that the mode of action more likely is bacteriostatic than bactericidal. The initially planned cell density measurements (OD readings) were complicated due to the fact that the cells aggregated together. The observations and conclusions that were made from the antimicrobial-spike test were included for interest sake and a more quantifying assay is suggested.

3.3.4 Preliminary characterisation of the antimicrobial substance

From **Fig. 3.7** it can be seen that the proteolytic enzymes (proteinase K, α-chemotrypsin and pronase) completely destroyed the antimicrobial activity after incubation for 1 h at 37°C. This suggested that the antimicrobial substance was of proteinaceous nature. Catalase treatment indicated that the possible inhibitory action was not the result of hydrogen peroxide (**Fig. 3.7**). The addition of the protease inhibitor (PI) cocktail to the samples revealed that there was proteolytic activity in the supernatant spots. **Fig. 3.8** depicts that the supernatant of *G. frateurii* AO286, which contained the PI cocktail, exerted a greater inhibition towards the indicator organism, *A. aceti* AOII23. The PI cocktail, which was dissolved in sterile distilled water, served as negative control, as it did not cause inhibition (**Fig. 3.8**).

The sensitivity of the inhibitory compound to heat treatment can clearly be seen in **Fig. 3.9**. An increase in temperature treatment clearly influenced the activity with the lowest temperature, 4°C, having the largest inhibition. The compound was still active up to 65°C, but the activity was lost after autoclaving (121°C).

The effect of the pH range (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) can be seen in **Fig. 3.10**. This indicated that the antimicrobial peptide was active in a wide pH range and showed that

the inhibition was not the result of organic acids such as acetic or lactic acid. The activity was lost at a pH above 9.0.
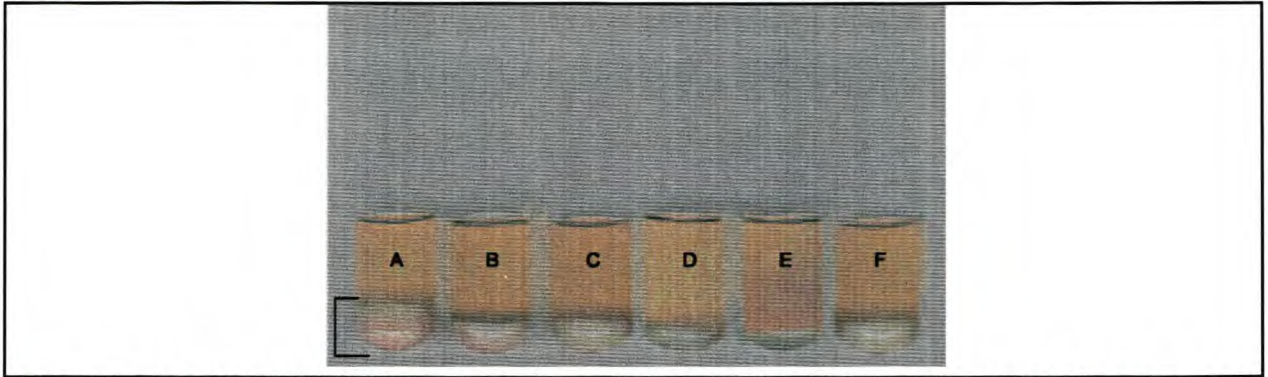

Fig. 3.6. The effect of the different media on the production of the antibacterial substance. The sensitive organism, *Ga. liquefasciens* AOII23, was cultivated in YPM media that had received a protein spike. The amount of cells was measured in millimetres as indicated. (A) Positive control that did not receive an antimicrobial protein spike – 8 mm. (B) spike AO286 produced in APT - 5 mm. (C) spike AO286 produced in MRS – 6 mm. (D) spike AOII13 produced in YPM with 0.5% Tween – 4 mm. (E) spike AO286 produced in YPM with 0.5% Tween – 3 mm. (F) spike AOII13 produced in APT – 6 mm.

3.3.5 Molecular size determination and overlay

The molecular size separation on the Tricine-SDS-PAGE gel yielded only two distinctive bands. The size of the active sample was estimated at between 3 and 10 kDa by means of centrifugal column separation (**Fig. 3.11**). No inhibition was observed on the gel overlays. Therefore, the exact size of the active fraction could not be determined.

3.3.6 Analysis of ES-MS

The result from the ES-MS of sample AO286 contained background and was very unclear. The selected area of the chromatogram in **Fig. 3.12** appeared to have a characteristic proteinaceous profile. The consistency of the background peaks suggests the presence of detergents or possibly salts. The ES-MS result of sample AOII13 was not optimal. No conclusion could be made and therefore it was not included.
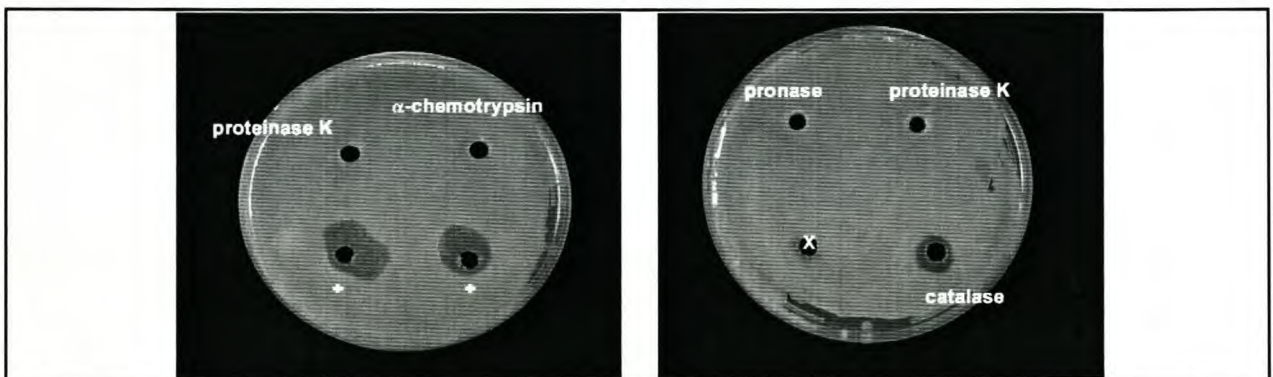

Fig. 3.7. The effects of the proteolytic enzymes and catalase treatment on the crude antimicrobial substance produced by *G. frateurii* AO286. The sensitive organism was isolate AOI10.

3.3.7 Plasmid isolation

No plasmids were found within *G. frateurii* AO286. This suggests that the possible antibacterial activity is encoded by the chromosomal DNA. It seemed as if *A. aceti* AOII13 contained plasmids (**Fig. 3.13**), but this result should be re-investigated before the activity from *A. aceti* AOII13 could be linked to it.
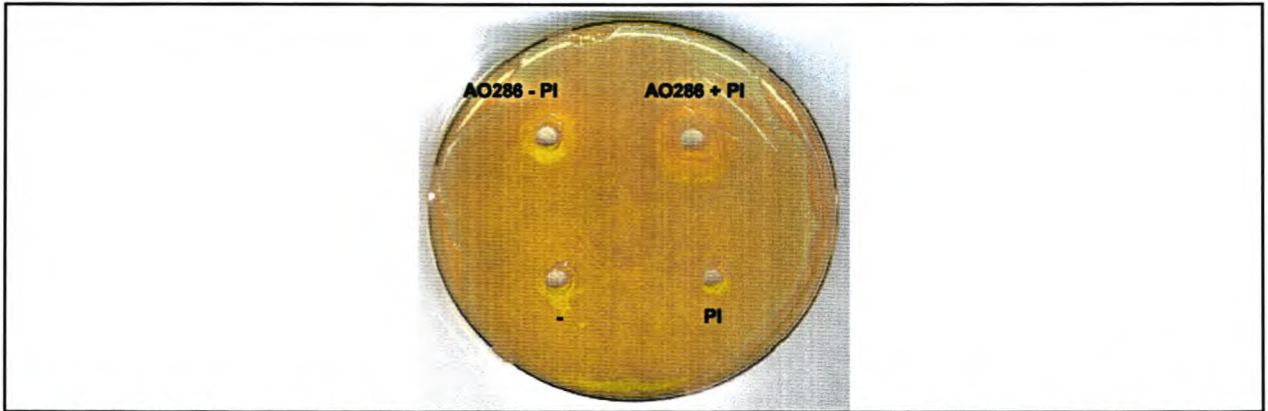
Fig. 3.8. The increased inhibitory effect of the crude supernatant of *G. frateurii* AO286 with the addition of protease inhibitor (PI) cocktail. The supernatant (AO286-PI) that did not receive the PI cocktail treatment had a smaller inhibition. The sensitive organism was *Ga. liquefasciens* AOII23.
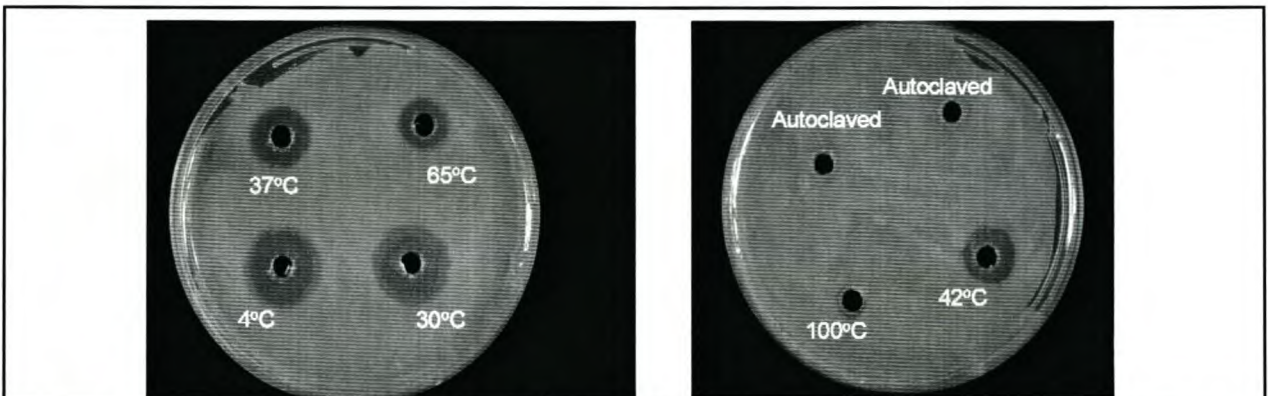
Fig. 3.9. The effect of temperature treatments on the activity of the crude antimicrobial substance of *G. frateurii* AO286. The sensitive organism was isolate AOI10.
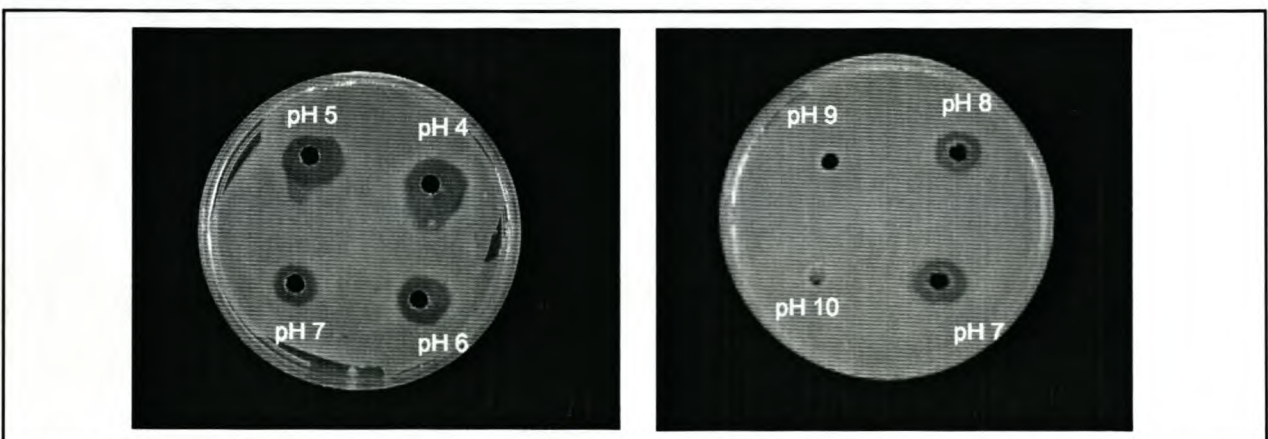
Fig. 3.10. The effect of pH adjustment on the activity of the crude antimicrobial substance of *G. frateurii* AO286. The sensitive organism was isolate AOI10.
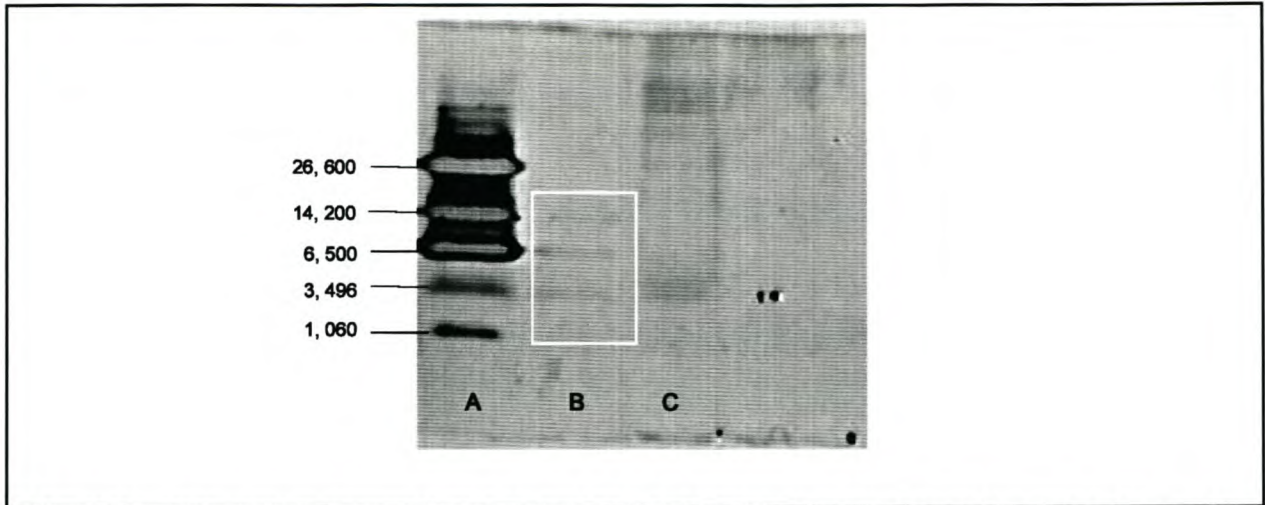
Fig. 3.11. Protein bands on the Tricine-SDS-PAGE. (A) Ultra-low weight molecular marker. (B) Two distinctive bands appeared from sample AO286 after it was separated on centrifugal columns between 3 kDa and 10 kDA. (C) The protein profile of the supernatant of AO286.
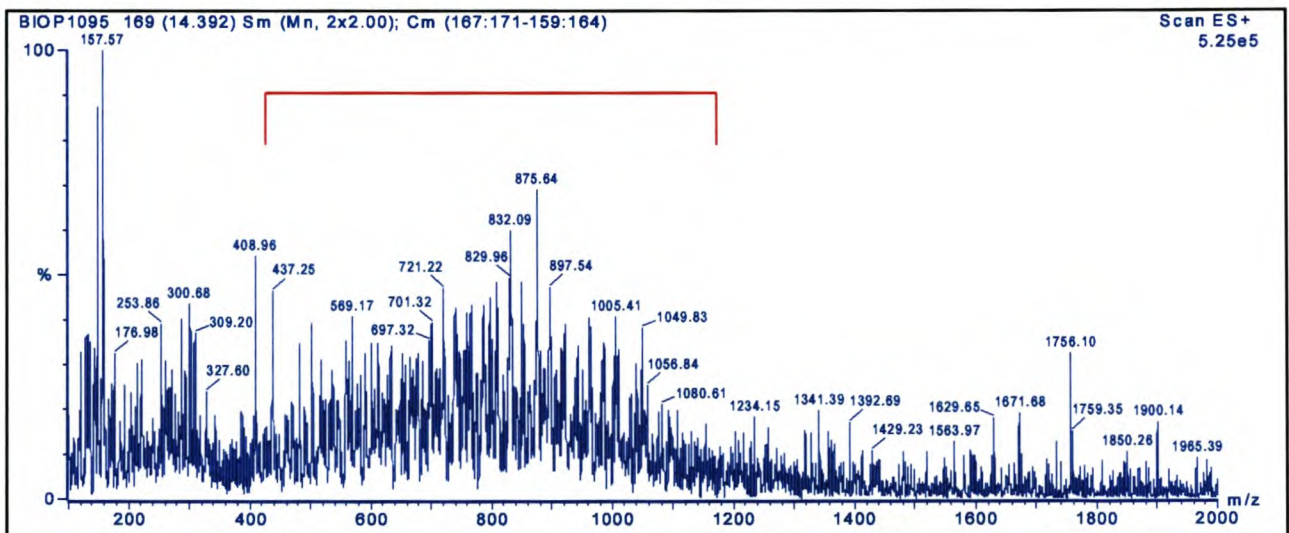

Fig. 3.12. The chromatogram from the ES-MS of sample AO286. The sample was first fractionated on a G10 Sephadex column.
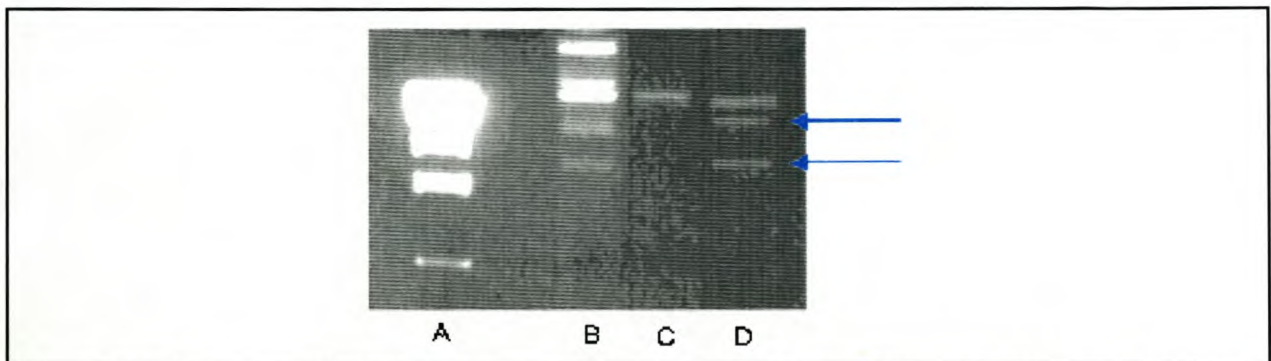
Fig. 3.13. The plasmid isolation performed on the producer strains. (A) λ *Bst*EII-digested marker. (B) *G. oxydans* DSM 7145[T]. (C) *G. frateurii* AO286, no plasmids visible. Visible band possibly result of genomic DNA contamination. (D) *A. aceti* AOII13, possible plasmids indicated by the arrows.

3.4 DISCUSSION

3.4.1 Identification of acetic acid bacteria

AAB are widely distributed in nature and therefore the occurrence of new species in the winemaking environment should always be evaluated. With the development of new molecular techniques for the differentiation of AAB to species level, this task has become significantly easier and more reliable than measuring the biochemical activities.

Preliminary results of the RFLP analysis of the amplified 16s rDNA gene revealed the occurrence of *G. frateurii* in the winemaking environment. Although this identification corresponded strongly to the biochemical tests, an additional method, such as DNA/ rDNA hybridisation, should be performed to verify the identification. The producer strain, *A. aceti* AOII13, and the sensitive strain, *Ga. liquefasciens* AOII23, were consistent in their behaviour and this was advantageous for their identification. Contrary to this, it was extremely difficult to perform a reliable identification of the other sensitive strain, AOI10. This specific AAB had a rapid growth rate and was prone to produce a slimy compound in the culture medium. It is more than likely that the sliminess is cellulose, as some AAB, especially *Ga. xylinus*, are well known for cellulose formation (Klemm et al., 2001). The isolate was compared with some cellulose producers, such as *A. pasteurianus* and *Ga. xylinus*, but it differed completely. The identification tests were performed several times and the phenotypic characteristics often yielded contradictory results. Future work will have to include the identification of this isolate, as this Gram-negative, catalase-positive rod might reveal an additional AAB species to the winemaking environment, or even a completely new species.

3.4.2 Preliminary screening of antimicrobial activity

From the onset of the preliminary screenings it was evident that the inhibitory activities were inconsistent. This was observed specifically during the cross-streaking screening procedure. The agar diffusion method was preferred, as it proved most reliable.

The activities often, but not always, correlated with different phenotypic behaviour. The two producer strains, AO286 and AOII13, together with many of the other AAB that were screened for sensitivity, often exhibited behaviour that was contradictory to their typical character. The same cultures grown on the same media under their optimal conditions occasionally appeared to form smaller colonies, sometimes producing a cellulose-like substance and even flocculating. Nevertheless, when supernatant containing the active fraction was obtained, the best inhibition was observed against other species of AAB. From the 87 wine isolates that were tested, only two strains AO286 and AOII13, showed inhibitory activity after the pH was adjusted to approximately 6.5. On the same account, the two AAB isolates, AOI10 and AOII23, seemed most promising for sensitive organisms. The addition of *E. coli* DH5α and *A. tumefasciens* GV 3101 to the indicator panel was performed only at a later stage of the study. Although they seemed to overcome the inhibition after more than 12 h, they

often were included for the purpose of increasing the indicator panel and to serve as backup in the cases where the AAB panel failed due to their alternate behaviour.

The fact that no inhibition was exerted on the industrial wine yeast strains, VIN13 and WE372, was very promising. This finding holds good prospects for genetically engineered wine yeasts that could combat AAB.

3.4.3 Influence of media on the production of the antimicrobial peptide

Producer cultures of AAB grew actively in all the media tested (YPM, YPM with 0.5% Tween, APT, MRS with 0.5% Tween) and no differences were observed. However, the media influence became evident in the precipitation of the proteins by means of ammonium sulphate. The proteins in the media containing the Tween additions all precipitated very quickly and a thick, toffee-like precipitate formed as a floating pellicle. This toffee-like substance had to be scooped up, heated at 42°C and dissolved in sterile double distilled water. The amount of protein precipitate obtained specifically from the YPM and MRS liquid media (both with 0.5% Tween 80), in the case of AO286 and AOII13 production, was significantly higher. The fact that the addition of Tween 80 increased the production of the antimicrobial substance correlated with previous findings (Ivanova et al., 2000; Xu-liang et al., 2000).

When the activities of the precipitates were tested (section 3.2.7), it was clear that the specific active fraction within the 25 µg protein spike varied in concentration. This suggests that the different media influenced the production of the antimicrobial compound. It was found that YPM with 0.5% Tween 80 yielded the best results.

3.4.4 Preliminary characterisation

By neutralising the supernatant and buffering the precipitates at a pH of between 6.0 and 7.0, the inhibitory action, as a result of organic acids, such as acetic acid, was eliminated. Although AAB are renowned for their acid resistance, specifically against acetic acid (Toda et al., 1997), the possibility of some acid-sensitive AAB should not be excluded.

The proteolytic enzymes that destroyed the activity suggested that the antibacterial substance was of a proteinaceous nature. Although the activity fluctuated constantly throughout the process, from the crude supernatant to the partially purified protein, it was always evident that it was eliminated by proteinase K treatment. Additionally, it can be seen from **Fig. 3.8** that the crude supernatant of AO286 without the PI cocktail had lower activity, which indicates one of two things. Firstly, it could indicate that the sensitive organism (AOII23) produced proteolytic activity that gradually affected the antibacterial substance. Secondly, the concentration of the PI cocktail in the agar might not have been sufficient enough to buffer the effect of the proteolytic activity from the sensitive organism and that could be why the AO286 sample containing the PI addition yielded a larger activity zone. This further verified the involvement of a proteinaceous substance in the activity.

The activity of the proteinaceous substance in a wide pH range (3.0-8.0) compares

to most small-sized bacteriocins of LAB (Jack et al., 1995; Du Toit, 2002). This can be used to determine the ionic status of the substance, as most bacteriocins are cationic at a pH of 7.0. This, together with the fact that similar bacteriocins also have high isoelectric points, enables them to interact with the anionic surface of the bacterial membranes in order to perform their lethal activity. However, both ion-exchange columns and isoelectric focussing were without success performed in an attempt to determine the ionic nature and isoelectric point (P_I) respectively (details not discussed).

The heat stability of the antibacterial substance is another major feature correlating with low-molecular weight peptides. It was evident that temperature played an important role in the stability of the substance and the observation that the lowered activity corresponded with increased temperature also supported the peptide-like nature of the substance.

It must be mentioned that most of the characterisation was performed on the active fractions of AO286. The production form this isolate was more consistent than that of the other producer, AOII13, and it was preferred in the attempt to optimise the characterisation and purification of the proteinaceous compound.

3.4.5 Mode of action

Due to the great variety in their molecular structures, proteinaceous antimicrobial peptides, such as bacteriocins, affect different essential functions of the living cell (transcription, translation, replication and cell wall synthesis). The different modes of action of various types of bacteriocin-like molecules have been reviewed by several authors (Jack et al., 1995; Daw and Falkiner, 1996). The most commonly known mode of action is the destruction of the cell's potential through the disruption of the proton motive force. Bacteriocins form voltage-dependent channels in the phospholipid bilayer membranes, resulting in the leakage of the intracellular ions. Nevertheless, the mode of action is responsible mainly for either completely killing the sensitive cells (bactericidal) or inhibiting the cellular processes, causing a delay in cell number increase (bacteriostatic).

From this study it was evident that the sensitive organisms, AAB isolates AOI10 and AOII23, were inhibited temporarily. These findings were inconsistent and the time of inhibition observed on the agar often varied. The inhibitory action on both the *E. coli* and *A. tumefasciens* strains clearly was temporary. Therefore, the preliminary results suggest a bacteriostatic character. It is too early to confirm this, however, and certainty will only be established once a more purified and stable proteinaceous compound is obtained. The assays should be performed in liquid media in which the ODs can be monitored.

In addition, the bacteriostatic nature of the antimicrobial substance correlated with the findings that zones were observed within the first 12 h and thereafter disappeared gradually.

3.4.6 Molecular size determination

The molecular size of the antimicrobial compound could not be determined. The protein profiles obtained from the Tricine-SDS-PAGE and native gels were inconsistent and too unreliable. Also, none of the overlays yielded significant results. Various additional approaches (size separation columns, ion-exchange columns, isoelectric focussing, HPLC) were also investigated (details not discussed), but without success. It was found that the dialysed partially purified antimicrobial substance exhibited activity after it was fractionated by means of the centrifugal filter units. This leaves the size estimation in the range of 3 000 to 10 000 Da.

The conclusions from the ES-MS chromatogram regarding molecular size were also insignificant. The profile that was obtained (**Fig. 3.12**) appeared to be similar to that of a characteristic protein profile, but this can only be speculated because of all the background. The consistency in the peaks indicates the presence of large quantities of salts and/or detergents. The presence of salts is very likely, as sodium hydroxide was used to neutralise the supernatant and sodium phosphate was used as buffer solution. The presence of Na^+ ions that could associate with the protein could explain the difficulty associated with the purification attempts (http://www.clontech.com/proteomics). Regarding the presence of detergents, Tween 80 was used in the cultivation medium of the specific sample as it has been reported to increase the production of antimicrobial peptides (Xu-liang et al., 2000). It is suggested that these solutions should be avoided and that alternative buffers should be investigated.

3.4.7 Plasmid isolation

Although it seemed as if the producer strain AOII13 contained plasmids, a more intrinsic investigation would be more viable (only a quick investigation was performed due to time limitations). Plasmids isolation was performed on a few selected strains and as *G. oxydans* DSM 7145 was selected as control because it contained plasmids. No plasmids were found in isolate AO286. From this investigation it is concluded that the possibility of plasmids as genetic determinants of the antimicrobial compounds should be investigated.

Antimicrobial peptides, such as bacteriocins, are known to be plasmid mediated, while others appear to be arranged in multi-gene operon-like structures (Jack et al., 1995). Some bacteriocins, like nisin, have also been reported to be transposon associated. This is of significance, as AAB have been reported to contain insertion elements that migrate through the genome, often causing spontaneous mutations at high frequencies (Beppu, 1993; Takemura et al., 1993). Phenomena such as these can drastically affect the phenotypic and genetic characterisation of AAB and might explain why problems were experienced during the identification procedures and the characterisation of the antimicrobial production.

3.5 CONCLUSION

No evidence of antimicrobial peptides or bacteriocin-like compounds has been reported for AAB in the literature. If the difficulty that was experienced throughout this study is taken into consideration, this can be well believed. Nevertheless, it is evident from this study that AAB are of great significance, especially during winemaking, and that research should probe the behaviour of these microorganisms in greater depth. The isolation and purification of antimicrobial compounds from AAB would contribute greatly to the latest developments in biopreservation, especially for application during winemaking.

3.6 ACKNOWLEDGEMENTS

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CHAPTER 4

General discussion
&
conclusions

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 GENERAL DISCUSSION AND PERSPECTIVES

It has been apparent for a long time that the process of making good wine requires vision, feeling and compassion, which are special traits resembling art. However, the fact that the intrinsic compilation of unique characteristics defines true quality makes it increasingly evident that the art of winemaking can be complemented by the field of science, which is notorious for giving specific attention to detail. Over the centuries, the appreciation of this vine-derived drink has increased and today wine plays a major role in society. It is important to remember, however, that this role would not have been possible without the scientific advances that have been made over the centuries.

The amount of research that has been done on wine has revealed several aspects that need to be probed. Some of the aspects that have received considerable interest in recent years include, the improvement of the sensorial quality of wine and the protection of wine against microbial spoilage. The winemaking environment contains a large population of microflora, which affect each other with regard to their metabolism, extracellularly produced products and consequent inhibition. These microorganisms include yeasts, fungi and lactic acid bacteria (LAB). Another microorganism that has been investigated is acetic acid bacteria (AAB), although a serious shortcoming, regarding its occurrence during winemaking, is evident.

The significance of AAB during winemaking has been indicated (Drysdale and Fleet, 1988; Du Toit and Pretorius, 2002) and it is specifically their implication in sluggish/stuck fermentations that have brought them under the spotlight (Henschke, 1997; Bisson, 1999). The ability of AAB to survive and consequently produce high amounts of acetic acid or volatile acidity (VA) during fermentation have been shown to affect the wine yeast's capability to ferment the must to dryness (Rasmussen et al., 1995). As these organisms, which previously were believed to be strict aerobes, were shown to exhibit various degrees of sulphur dioxide (SO_2) resistance (Du Toit and Pretorius, 2002), it was proposed that further attention should be given to finding alternative methods of controlling the cell numbers of AAB during winemaking, especially during fermentation. It also has been proposed that the use of SO_2 at high concentrations should be avoided, as it not only affects the organoleptic quality of the wine, but, because of the associated health risks clashes with consumer demands. This is why alternative methods to chemical preservatives have been investigated.

The use of biological compounds produced by microorganisms has initiated a trend of biopreservation. The use of bacteriocins produced specifically by LAB, together with other enzymes, has been investigated for application in the wine industry (Du Toit, 2002). However, bacteriocins are known to inhibit a wide range of Gram-positive bacteria, but only a small range of Gram-negative bacteria (Du Toit, 2002). Of these Gram-negative bacteria, AAB are excluded and that is why an investigation of

antimicrobial peptides that could inhibit the growth of AAB would be of significance. Per definition, bacteriocins are antimicrobial peptides produced by bacterial species that usually, but not always, inhibit other closely related species (Ennahar et al., 1999). Therefore, in an attempt to find antimicrobial peptides active against AAB, an investigation was done by screening various AAB isolated from wine. Several aspects of AAB became evident from this study.

The possibility that AAB species other than *G. oxydans*, *Ga. hansenii*, *Ga. liquefasciens*, *A. aceti* and *A. pasteurianus* occur in the winemaking environment has been indicated. The preliminary identification of *G. frateurii,* isolated from South African wine, supports the speculation by Du Toit (2000) that the existence of other species of AAB in wine cannot be excluded. *G. frateurii* was originally isolated from the flower, *Fragaria ananassa*, yet this species appeared to exist in the grape must. In support of this, the genus *Frateuria* (*F. aurantia*) was isolated from raspberries (Swings et al., 1980) and it was shown that it could withstand ethanol concentrations of up to 5% (Holt et al., 1994). Although this makes their occurrence in wine very unlikely, it is indications such as these that should inspire the research community to monitor the occurrence of AAB in the vineyard.

The numerous unsuccessful attempts to identify one of the sensitive AAB isolates (AOI10) indicated that a large gap still existed in the identification of these microorganisms. This specific isolate grew at a rapid rate and often produced large quantities of a cellulose-like substance, specifically from GYC media. AAB, especially *Ga. xylinus,* are renowned for the production of gluconic acid and for cellulose formation from glucose (Jonas and Farah, 1997). If the high levels of glucose in grape must are considered, the existence of other species and consequent spoilage by AAB should be investigated seriously.

One of the most concerning findings during this study was the fact that the AAB isolates often yielded contradictory results. These findings ranged from various phenotypic characteristics, such as flocculation, extended cultivation periods and fluctuations in sensitivity, to genetic alterations observed during identification. On occasion, an *A. aceti* revealed completely different band patterns after RFLP analysis of the PCRed 16S rDNA. All of these findings, however, question the influence of the insertion elements that have been identified in AAB. The presence of these insertion elements has been indicated to cause spontaneous mutations at high frequencies, often resulting in the inactivation of alcohol dehydrogenase (Beppu, 1993; Takemura et al., 1993) and decreasing the acetic acid resistance of AAB (Ohmori et al., 1980). This additionally could explain why difficulty was experienced in obtaining constant results during the cultivation and identification procedures.

Another aspect that has serious implications for antimicrobial peptide production is the composition of the growth medium, which greatly affects the production and consequent activity. Various approaches were tried with YPM media, as well as MRS and APT media, as these have been used for the production of bacteriocins by lactic

acid bacteria. Although YPM was the preferred medium for AAB, revealing the most promising results, the influence of MRS should be re-investigated. It is evident, however, that specific individual components should also be experimented with. Different concentrations of peptones (which form part of YPM media) have been reported to play a role in the production (Rammelsberg et al., 1990). The same is applicable to the addition of detergents, such as Tween. Although Tween 80 has been indicated on numerous occasions to increase the activity, it has also been reported to interfere with the purification process (Daba et al., 1993; Carolissen-Mackay et al., 1997). From this study it was evident that Tween 80 played a critical role in both the production and the purification of the antimicrobial substance. It was found that it was advantageous for the purposes of production, but its presence could explain the difficulty that was experienced during further purification steps (see ES-MS results). The influence of medium composition should therefore always be investigated in order to establish benchmark results.

From the antimicrobial peptide-like substances produced by *A. aceti* AOII13 and *G. frateurii* AO286, it is clear that the purification process still needs optimisation. It was found that ammonium sulphate precipitation yielded two phases of significance. Firstly, a clear, floating pellicle was formed, which was scooped up, and secondly, the remaining supernatant mixture was centrifuged to yield another precipitant. The strongest activity was observed from the scooped-up pellicle. This phenomenon has previously been reported and the activity varies between the phases (Carolissen-Mackay et al., 1997). As this is usually the first crucial step in obtaining the crude protein extract, it should be evaluated properly.

AAB are commonly associated with spoilage, especially during the winemaking process. Amongst all the wine AAB species, no real correlation has been established between specific species and specific types of spoilage. Evidence from wineries worldwide has indicated that *G. oxydans* dominated in the fresh must, whereas *Ga. liquefasciens* and *A. pasteurianus* dominated later during alcoholic fermentation (Drysdale and Fleet, 1988; Du Toit and Lambrechts, 2002). Nevertheless, winemakers should always be aware of the potential of these Gram-negative, catalase-positive cells and that conditions of oxygen limitation and controlled SO_2 usage may not always be sufficient to suppress their growth.

Contrary to their negative connotation, AAB have also been shown to be of great industrial importance. The application of bacterial cellulose produced by *Ga. xylinus* and *A. pasteurianus* in medicine (Klemm et al., 2001) is one example. This, together with their application in the production of self-tanning lotions and vitamin C via dihydroxyacetone and L-ascorbic acid respectively, further indicates their significance (Hancock and Viola, 2002).

A paradigm shift should evolve from the finding of this study that antimicrobial substances produced by AAB might exist. The long-standing finding that *Acetobacter*-fermented tea exhibits strong antimicrobial activity (Greenwalt et al., 2000) strongly

correlates with this and therefore the common association of AAB with spoilage organisms may soon be changed. Considering that the use of AAB as starter cultures for vinegar production is currently being investigated (Sokollek and Hammes, 1997), it is possible that a whole new dimension, involving the use of AAB in various applications, will be entered soon. Similarly, the unimaginable potential of gene technology could very well establish AAB on the applicatory shelf, rather than in the problem bin. The possibility exists that antimicrobial peptides produced by AAB, which are natural inhabitants of wine, could be utilised in the engineering of tailored wine yeasts. This would have great benefits, not only to expand the diversity of high quality wines, but also for the wine consumer (Pretorius and Bauer, 2002).

This study again indicated the large void, which still needs to be filled, regarding information on the occurrence and significance of AAB during winemaking. It is quite evident that AAB have unique characteristics that enable them to overcome the challenges imposed on them. This should inspire the research community to drastically increase the number of investigations of AAB to further the available knowledge.

To conclude, the earlier question is repeated: "Acetic acid bacteria, friend or foe?" The evidence in this thesis points clearly to the former. It of course is highly debatable to make connotations such as this. Nevertheless, this indicates that we should be cautious before we define our science, as changes can occur. As bacteria use extracellular molecules to communicate with each other to coordinate their activities, so we should define our science in order to educate the public and position ourselves on a highly competitive level.

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CHAPTER 5

Addendum
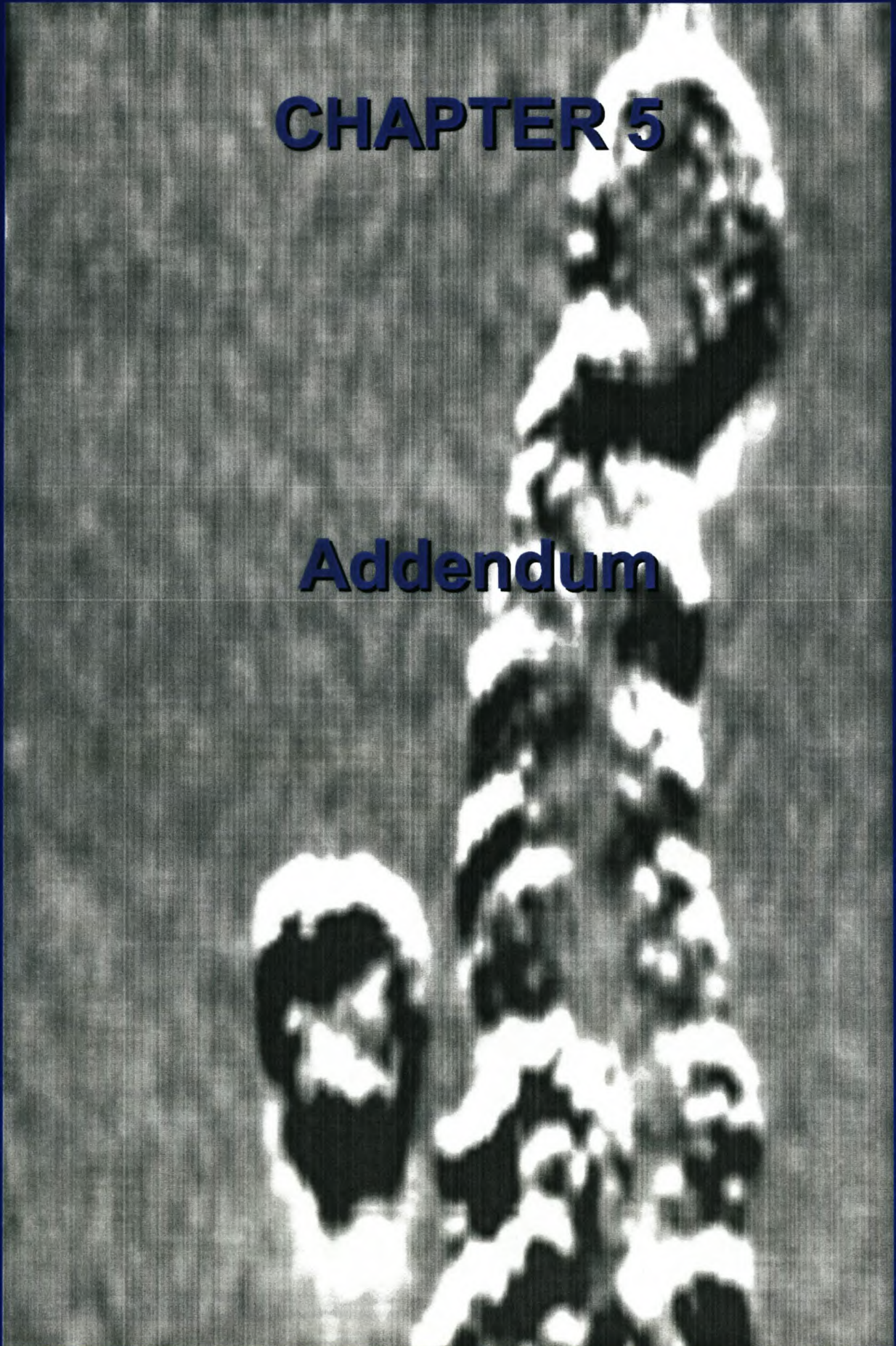
5. ADDENDUM

5.1 FURTHER CHARACTERISATION OF ACETIC ACID BACTERIA ISOLATED FROM SOUTH AFRICAN WINE

Acetic acid bacteria (AAB) possess the ability to affect grape must and wine quality negatively through the formation of acetic acid. Acetic acid constitutes more than 90% of wine's volatile acidity (VA) and elevated levels of acetic acid associated with sluggish/stuck fermentations have been known to cause yeast inhibition. At high concentrations, acetic acid decreases the intracellular pH of the yeast, which consequently affects their metabolism and vitality during fermentation. Additionally, elevated levels of acetic acid result in the wine having a vinegary off-flavour, which is why the legal limit for VA in South African wines is 1.2 g/l.

The spoilage by AAB is commonly associated with aerobic conditions. However, AAB have previously been isolated from fermentation tanks (Drysdale and Fleet, 1985) and it is known that they can use other substances, such as quinones and reducible dyes, as final electron acceptors in the place of oxygen to survive (Adlercreutz and Mattiasson, 1984). Therefore, their ability to endure anaerobic conditions, which prevail during winemaking, has proposed a short study on the characterisation of the VA caused by AAB under oxygen-limited conditions.

Various strains of AAB isolated from South African red wine fermentations were tested in Chenin blanc must for their ability to cause high levels of volatile acidity under various conditions. A comparative study between the species of *Gluconobacter oxydans*, *Acetobacter aceti*, *Acetobacter pasteurianus*, *Gluconacetobacter hansenii* and *Gluconacetobacter liquefasciens* was conducted after 5 days of growth in aerobic and microaerophilic conditions. AAB were also screened for their inhibitory action towards the industrial wine yeast, *Saccharomyces cerevisiae* VIN13.

5.1.1 MATERIALS AND METHODS

5.1.1.1 Bacterial strains

AAB were isolated from South African red wine fermentations during the 1998 and 1999 harvest seasons. Strains were selectively enumerated, identified and characterised according to the methods outlined by Du Toit and Lambrechts (2002). Sixty-two of the AAB strains (now part of the IWBT culture collection) were pre-cultured on GYC and YPM agar plates. These strains are listed in **Table 5.1**.

5.1.1.2 Media for VA tests

AAB cultures were cultivated and maintained on GYC and YPM agar plates. The GYC medium contained glucose (5% m/v), yeast extract (1% m/v), $CaCO_3$ (from 1 to 3% m/v) and bacteriological agar (2% m/v) and the YPM medium contained mannitol (2.5% m/v),

yeast extract (0.5% m/v), peptone (0.3% m/v) and bacteriological agar (2% m/v). The GYC and YPM liquid media had the same composition, just without the agar. All the medium components and the agar used are available from Merck (South Africa). The plates were incubated at 30°C for 2 to 5 days. Chenin blanc must with a sugar content of 26°B was used. After autoclaving, it was kept at 4°C until required.

5.1.1.3 VA production

Pre-inoculums were grown up in their respective liquid media to an optical density (OD) of 1.0 at 600 nm. At this OD, 1 ml of the culture was inoculated into 100 ml of sterile Chenin blanc must (26°B) in a 250 ml Erlenmeyer flask and allowed to grow for 5 days at 30°C. Two Erlenmeyers of each strain were inoculated; one was placed on a shaker (aerobic) and the other was kept stationary (microaerophilic). The VA tests were performed in duplicate. After 5 days, 10 ml of the must was removed and the VA was determined as outlined by Iland et al. (2000). Once 100 ml of distillate was obtained, a few drops of phenolphthaline (0.3%) were added as indicator and the distillate was titrated with 0.01 M NaOH. The VA was then calculated using the formula:

$$VA = \frac{[\text{Sample titre (ml)} - \text{Blanko titre (ml)}]}{\text{Volume of must analysed}} \times \text{Mol. NaOH} \times 60$$

5.1.1.4 Anaerobic tests

Twelve strains of various AAB species were chosen randomly from our culture collection and tested for anaerobic growth **(Table 5.2)**. Strains were cultivated in their respective liquid media (10 ml in test tubes) until an $OD_{600\ nm}$ of 1.0. The purpose of this test was two-fold. Firstly, the selected strains were tested for growth (indicated by turbidity). Secondly, they were tested for their ability to produce VA. The same inoculation method as mentioned previously for VA production was then used in 100 ml of must. Cells were incubated for 13 days in a plastic jar (sealed off with vacuum grease and parafilm) containing a gas-generating kit (Oxoid, Anaerobic System BR 038B) to establish an anaerobic environment. This was incubated at 25°C.

5.1.1.5 Inhibition tests

The inhibitory action of AAB on the industrial wine yeast *Saccharomyces cerevisiae* VIN13 was tested. The yeast cells were grown to an $OD_{600\ nm}$ of 0.6 in YPD medium. 50 µl of this culture was then inoculated into YPD (yeast extract, peptone, dextrose) containing 0.8% agar (Merck, South Africa) before the plates were poured. After the plates settled, they were placed in a laminar flow (sterile conditions) for 2h to dry. The inhibitory affect was tested by means of the agar-diffusion method. The crude extract of the AAB strains was obtained by cultivating the bacteria overnight in YPM broth at 30°C and then centrifuging them to retain the supernatant. 80 µl of this was spotted into the wells. The plates containing the yeasts were incubated at 30°C for 36 h before they were analysed for zones.

5.1.2 RESULTS AND DISCUSSION

5.1.2.1 Volatile acidity

Table 5.1 shows the amounts of VA (g/l) produced by the 62 different AAB strains. On average, the VAs produced varied only slightly between these tests. A few exceptions did occur, e.g. GI 9 (*Ga. liquefasciens)* produced one value of 0.91 g/l (VA 2, aerobic) that seemed quite unusual. All of the other VA values for GI 9 were below 0.3 g/l. This indicated the variability of these bacteria under the same growth conditions, with other bacteria showing similar variations. FII 13 (*Ga. liquefasciens*) delivered the highest values, with 3.8 g/l VA (aerobic) and 3.57 g/l VA (microaerophilic). Again, the second test (VA 2) had much higher values than the first test (VA 1). Samples of CI 18 (*A. aceti*) and HII 8 (*Ga. hansenii*) produced higher levels of VA after being cultivated under microaerophilic conditions. This indicates the ability of some of these microbes to produced higher amounts of volatile acids, such as acetic, propionic and butyric acid, under anaerobic conditions. *Ga. liquefasciens* generally produced the highest VA values (> 1.0 g/l), followed by *A. aceti*. The average VA value for all the AAB tested was 0.54 g/l. For more comprehensive deductions, the tests should have been performed in triplicate.

5.1.2.2 Anaerobic growth

Except for strain CI 17 (*A. pasteurianus*), the VA values of the selected cultures in **Table 5.2** appear considerably lower for the strict anaerobic test in comparison to the highest values obtained from the microaerophilic test in **Table 5.1**. The higher VA value of strain CI 17 once again proved the ability of some AAB to produce high amounts of VA under anaerobic conditions. Although the values for the other strains were lower, this test still indicated that these organisms are quite capable of contributing to microbial spoilage under anaerobic conditions, specifically by increasing the VA. Some of the strains, e.g. EI 21 (*Ga. hansenii*), appeared to be affected drastically by the anaerobic conditions. This correlated with the extended lag phase that was observed before the increase in cell density.

The ability of some strains to survive and grow during anaerobic conditions correlated with the previous findings of such behaviour during winemaking.

5.1.2.3 Inhibition tests

The supernatants of all the AAB listed in **Table 5.1** were tested against the wine yeast, *S. cerevisiae* VIN13. Of these, only the 12 bacterial strains listed in **Table 5.3** caused yeast inhibition. **Fig. 5.1** is an example of an inhibitory effect, which is indicated by the clear zone. Looking at the inhibitory strains Cab 256, HII 13, CI 32 and EI 38 in **Table 5.3** and comparing their VA values in **Table 5.1**, it can be seen that they are low producers of VA or acetic acid. Acetic acid is well known for its toxic affect on the yeast cell (Grossman and Becker, 1984; Rasmussen et al., 1995), but as low VA producers also caused inhibition, some of the inhibition was suggested to be the result of compounds other than acids. As the crude supernatant was used without pH adjustment (pH 4), the inhibitory

affect could have been caused by anything, ranging from organic acids to bacterial substances of a proteinaceous nature. Nevertheless, it was quite evident that the culture medium of AAB contained an inhibitory compound that influenced yeast growth negatively.

Table 5.1
VA production (in g/l) by acetic acid bacteria during aerobic and microaerophilic conditions

Sample	Spp	VA1		VA2		Sample	Spp	VA1		VA2	
		A[a]	M[a]	A	M			A	M	A	M
BI[b] 15	*A. pasteurianus*	0.30	0.36	0.67	0.71	BII[c] 8	*A. pasteurianus*	0.20	0.17	0.23	0.29
BI 20	*A. pasteurianus*	0.17	0.13	0.35	0.58	CII 17	*A. pasteurianus*	0.80	0.40	0.31	0.69
CI 7	*G. oxydans*	0.18	0.53	0.29	0.39	CII 19	*A. pasteurianus*	0.67	0.29	0.24	0.35
CI 11	*A. pasteurianus*	0.02	0.55	0.05	0.11	CII 23	*Ga. liquefasciens*	0.65	0.62	0.53	0.27
CI 17	*A. pasteurianus*	0.11	0.29	0.36	0.37	CII 26	*A. pasteurianus*	0.90	0.55	0.24	0.23
CI 18	*A. aceti*	0.11	0.65	0.19	0.82	DII 23	*Ga. liquefasciens*	1.04	0.44	0.15	0.22
CI 21	*A. pasteurianus*	0.33	0.53	0.30	0.64	EII13	*Ga. liquefasciens*	0.25	0.29	0.08	0.24
CI 32	*Ga. hansenii*	0.07	0.23	0.20	0.64	EII 19	*A. pasteurianus*	1.52	0.82	1.16	1.03
CI 38	*Ga. hansenii*	0.39	0.99	0.26	0.75	EII 21	*Ga. liquefasciens*	1.14	0.97	1.55	0.80
EI 10	*A. pasteurianus*	0.25	0.22	0.69	0.15	EII 25	*A. pasteurianus*	0.26	0.07	0.17	0.25
EI 17	*G. oxydans*	0.84	0.97	0.75	0.56	EII 28	*A. pasteurianus*	0.27	0.23	0.61	0.87
EI 21	*Ga. hansenii*	1.15	1.03	0.13	0.15	FII 2	*Ga. liquefasciens*	1.10	0.85	0.12	0.15
EI 22	*A. pasteurianus*	0.07	0.07	0.10	0.10	FII 6	*Ga. liquefasciens*	0.73	0.56	0.63	0.45
EI 38	*Ga. hansenii*	0.01	0.30	0.10	0.74	FII 13	*Ga. liquefasciens*	2.67	1.80	3.57	3.81
FI 4	*G. oxydans*	0.72	0.21	0.60	0.18	FII 16	*Ga. hansenii*	0.06	0.21	0.11	0.28
FI 8	*A. pasteurianus*	0.17	0.17	0.23	0.19	FII 20	*A. pasteurianus*	0.14	0.39	0.13	0.32
FI 10	*Ga. hansenii*	0.53	0.32	0.57	0.32	GII 4	*Ga. liquefasciens*	2.70	0.80	0.63	0.64
FI 13	*Ga. hansenii*	0.03	0.25	0.20	0.59	GII 9	*Ga. liquefasciens*	1.40	0.65	1.13	0.55
FI 25	*A. pasteurianus*	0.23	0.25	0.39	0.23	HII 1	*G. oxydans*	1.30	1.20	0.94	1.18
GI 4	*Ga. liquefasciens*	0.30	0.33	0.45	0.33	HII 8	*Ga. hansenii*	0.86	2.17	0.27	0.52
GI 9	*Ga. liquefasciens*	0.22	0.19	0.91	0.29	HII 10	*A. pasteurianus*	0.40	0.09	0.09	0.51
Cab 3	*G. oxydans*	0.32	0.25	0.17	0.19	HII 13	*A. pasteurianus*	0.10	0.13	0.12	0.22
Cab 6	*G. oxydans*	0.17	0.26	0.73	0.14	HII 16	*A. pasteurianus*	0.17	0.43	0.11	0.34
Cab 74	*G. oxydans*	0.15	0.15	0.21	0.16	Cab 177	*Ga. hansenii*	0.32	0.25	0.20	0.14
Cab 86	*G. oxydans*	1.20	1.36	1.41	0.64	Cab 187	*A. pasteurianus*	0.31	0.36	0.18	0.15
Cab 100	*A. pasteurianus*	0.24	0.19	0.15	0.14	Cab 190	*A. aceti*	1.33	0.56	1.63	0.76
Cab 110	*A. pasteurianus*	0.29	0.23	0.17	0.18	Cab 286	*A. aceti*	1.96	1.43	1.57	1.96
Cab 128	*A. pasteurianus*	0.08	0.25	0.16	0.29	F23	*A. aceti*	0.12	0.24	0.13	0.18
Cab 139	*Ga. liquefasciens*	1.91	1.66	1.55	1.20	F37	*A. aceti*	1.91	0.66	1.80	0.56
Cab 156	*Ga. liquefasciens*	1.76	0.89	1.32	0.67	F76	*A. pasteurianus*	0.17	0.19	0.19	0.15
Cab 256	*A. pasteurianus*	0.20	0.72	0.09	0.17	Cab 114	*A. pasteurianus*	0.30	0.28	0.18	0.16

[a] A = aerobic; M = microaerophilic
[b] I and Cab strains cultivated on YPM media
[c] II strains cultivated on GYC media

It can also be seen that all of the species of AAB (*G. oxydans, A. pasteurianus, A. aceti, Ga. hansenii* and *Ga. liquefasciens*) that have been isolated from winemaking environments thus far are represented in **Table 5.3**. This therefore suggests that none of these species can be distinguished as being of less significance, as they all are capable of contributing to spoilage.

A shift in focus occurred, however, after what was thought to be a contamination problem revealed a pattern of possible bacterial inhibition. One sample, EI 10, kept on growing out of the well covering the surface of the plates. However, the growth or lawn pattern of this strain was inhibited strongly when it came in close proximity to the wells of other AAB strains (**Fig 5.2**). Of all the strains tested, this was the only sample that exhibited this behaviour. When the inhibitory action was observed again, a closer investigation of the antimicrobial activity of AAB was performed. As no information could be obtained, this inhibitory observation led to the proposal for this Master's study.
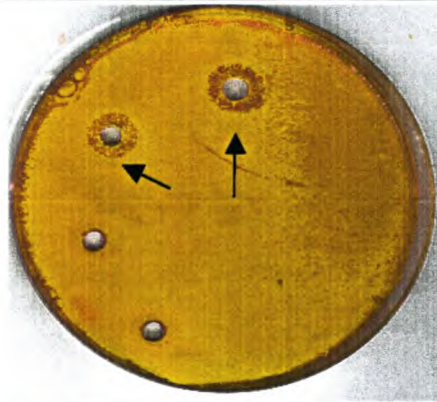

Fig. 5.1. The inhibition effect (clear zones) caused by Cab 139 (*Ga. liquefasciens)* on the growth of the yeast *S. cerevisiae* VIN13.
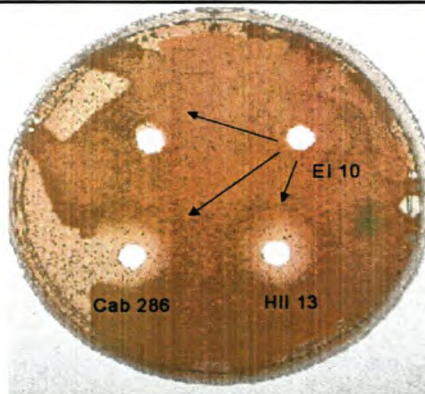

Fig. 5.2. The inhibitory effect caused by the crude supernatant of AAB strains HII 13 and Cab 286 on the growth pattern of AAB strain EI 10.

Table 5.2
Anaerobic VA production by acetic acid bacteria in Chenin blanc grape must

Sample	Species	VA	VA (H)[d]
C I 17	*A. pasteurianus*	0.86	0.37
C I 32	*Ga. hansenii*	0.58	0.64
E I 17	*G. oxydans*	0.50	0.97
E I 21	*Ga. hansenii*	0.20	1.15
E I 38	*Ga. hansenii*	0.23	0.74
Cab 86	*G. oxydans*	0.26	1.41
Cab 139	*Ga. liquefasciens*	0.68	1.91
Cab 156	*Ga. liquefasciens*	0.54	1.76
Cab 286	*A. aceti*	0.51	1.96
Cab 256	*A. pasteurianus*	0.23	0.72
F23	*A. aceti*	0.24	0.24
F37	*A. aceti*	0.54	1.91

[d] This is the highest VA value produced by the strains from the aerobic/microaerophilic test in
Table 5.1.

Table 5.3
Strains of acetic acid bacteria that caused inhibition of *S. cerevisiae* VIN13

Strains	Species
CI 32	*Ga. hansenii*
HII 1	*G. oxydans*
HII 8	*Ga. hansenii*
HII 13	*A. pasteurianus*
EI 38	*Ga. hansenii*
F23	*A. aceti*
Cab 86	*G. oxydans*
Cab 139	*Ga. liquefasciens*
Cab 156	*Ga. liquefasciens*
Cab 190	*A. aceti*
Cab 256	*A. pasteurianus*
Cab 286	*A. aceti*

5.2 SCREENING FOR EXTRACELLULAR ENZYME PRODUCTION

Enzymes are the major driving forces that catalyse various biotransformation reactions throughout the winemaking process. The occurrence of enzymes during winemaking originates from microorganisms associated with the vineyards and wine cellars. These microorganisms include the yeasts, fungi, bacteria and even the grapes themselves. These endogenous enzymes, together with the application of commercially prepared enzymes, play a critical role in improving the processing and clarification of wine, as well as increasing varietal aromas (Van Rensburg and Pretorius, 2000).

Although studies have been performed on yeasts and lactic acid bacteria regarding the production of extracellular enzymes of importance to the wine industry (Van Rensburg and Pretorius, 2000), very little, if any, focus has fallen on AAB. Therefore the screening of AAB for the production of enzymes significant to the winemaking process was performed.

5.2.1 MATERIALS AND METHODS

5.2.1.1 Bacterial strains and culture conditions

The strains of AAB used in this study were isolated from various South African red wine fermentations during the 1998 and 1999 harvest seasons. The strains were enumerated and identified by means of different biochemical and physiological tests by Du Toit and Lambrechts (2002). For details regarding the cultivation of the AAB strains, refer to sections 5.1.1.1 and 5.1.1.2.

5.2.1.2 Media involved in screening of extracellular enzymes

Screening methods were based on the work by Strauss et al. (2001), with slight modifications to best suit the growth of AAB. Single colonies of the selected strains were inoculated into 5 ml of GY (GYC medium without $CaCO_3$) or YPM liquid broth and left to grow for 48 h at 30°C on a turning wheel. 10 µl of each of these cultures was spotted onto agar plates containing the various substrates necessary for the enzymatic assays. Where staining was necessary, the various dyes were left on the plates for 1-2 hours.

Cellulase activity: Assessment of cellulase activity was made by spotting the strains onto GY or YPM containing 0.4% carboxymethylcellulose (CMC, Sigma). After incubation, the colonies were rinsed off the plates with distilled water, before the plates were stained with 0.03% Congo red. This was followed by destaining with 1 M NaCl. Positive cellulase activity was identified by a clear zone around the colony (Teather and Wood, 1982). In addition, strains were also tested for cellobiase activity on plates containing 2% cellobiose as the only carbon source. Growth on the plates indicated cellobiase activity.

β-Glucosidase activity: The production of β-glucosidase was determined by spotting the strains on YPM media containing 0.5% arbutin (Sigma, Germany) and 2% bacteriological agar. The pH of the media was adjusted to 5 before autoclaving. Two millilitres of a

filter-sterilised ferric ammonium citrate solution (1%) was added to the media before pouring the plates. After incubation, positive activity was identified by the discolouration of the media to a brown halo around the colonies.

Xylanase activity: AAB were tested for hemicellulase activity on YPM plates containing 0.2% Remazol Brilliant Blue Xylan (RBB-Xylan, Sigma). RBB-Xylan was first autoclaved dry before the addition of autoclaved distilled water. Finally, the RBB-Xylan was added to the selective medium before the plates were poured. Activity was identified by a pale clearing zone around the colonies.

Starch-degrading activity: A 2% starch solution (Merck, South Africa) underwent boiling before the addition of the growth medium (YPM agar). Cultures were spotted and the agar plates were incubated for 2 days at 30°C. After sufficient growth had occurred, the plates were placed at 4°C to precipitate for 2 to 4 days. A clear zone around a colony identified that particular strain as having starch-hydrolysing activity. Production of α-amylase was assessed using YPM agar containing 40 phadebas (Pharmacia & Upjohn, Sweden) pills per litre of medium. A clear zone around the colony identified positive activity.

Protease activity: The production of extracellular proteases was done by spotting strains on YPM plates containing 2% casein (BDH laboratories, England). A clear zone around the colony indicated protease activity.

Pectinase activity: For the determination of polygalacturonase production, a method was used that involved the use of a dinitrosalicyclic (DNS) assay: 1 ml of culture (grown up in GY or YPM) was centrifuged (2 min, 6 rpm). The supernatant (500 μl) was added to 900 μl of substrate (1.25% polygalacturonic acid in 0.68% potassium phosphate buffer with the pH adjusted to between 3.5 and 4) and left in a 30°C waterbath for 1 h. Thereafter, 1.5 ml of DNS was added to stop the reaction and the mixture was boiled at 100°C for 15 min. This assay was performed as described by Miller et al. (1960). 500 μl of uninoculated GY or YPM medium added to 900 μl of buffered substrate served as the negative control.

5.2.2 RESULTS AND DISCUSSION

5.2.2.1 Extracellular enzyme production

The results of the enzymatic activity assays can be seen in **Table 5.4**. All of the strains of AAB that have been isolated from the winemaking environment, namely *G. oxydans*, *A. aceti*, *A. pasteurianus*, *Ga. hansenii* and *Ga. liquefasciens*, possessed the ability to hydrolyse certain substrates that commonly are encountered in the grapes and must or wine.

The ability to degrade cellulose was the most common occurrence of hydrolysing activity among the AAB tested in this study. **Fig. 5.3** shows the clearing zones caused by two cellulose-degrading *G. oxydans* species, Cab 86 and Cl 17. Carboxymethylcellulose

(CMC) is broken down by the enzyme cellulase, which cleaves at the β-1,4 linkages. Cellulase activity can play a role in extracting more colour and flavour from the skins of the grapes. It is also possible that this activity can alter the composition of the tannins in the wine and shorten the fermentation time on the skins (Strauss et al., 2001).
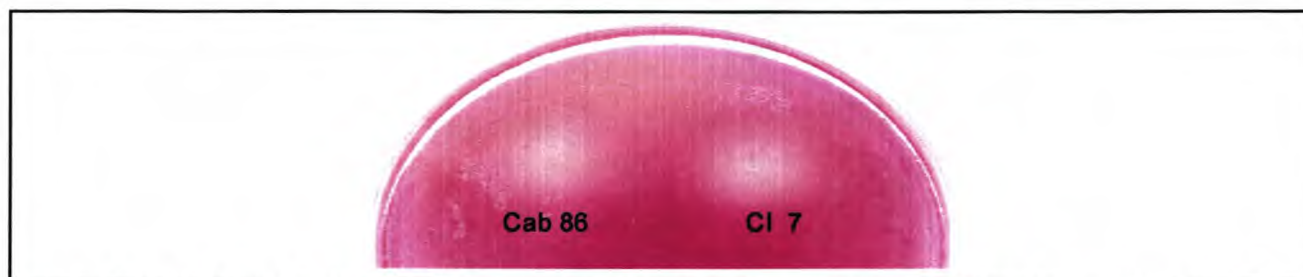

Fig. 5.3. The ability of strains Cab 86 and Cl 7 to degrade cellulose is shown by the formation of the clearing zones in the medium where the colonies grew.

Both cellulose and hemicellulose represent the primary structural polysaccharides of the plant cell wall and form the largest reservoir of fixed carbon in nature. Hemicellulose is a complex carbohydrate polymer containing xylan as its main component.

The degradation of hemicellulose is performed by a complex set of enzymes, including β-D-galactanases, β-D-mannases and β-D-xylanases (Thomson, 1993). In this study, only the latter enzyme was of interest. The results of the breakdown of RBB-xylan are shown in **Table 5.4.** Except for four of the strains, the strains tested negative for xylanase activity. It was mainly the strains of the specie *Ga. hansenii* (Cl 32, El 38 and HII 8) that revealed positive activity, in addition to a *Ga. liquefasciens* strain (Cab 156).

A possible explanation for these negative results is that endoxylanases are often prevented from cleaving the xylan backbone by the presence of substituents, which must first be removed before extensive degradation can occur. Not only do xylanases contribute to the filtration and clarification of wine by degrading the structural polysaccharides that interfere with these processes, but they also possibly could contribute to wine aroma by increasing the amount of monoterpenyldiglycosidase precursors in must (Van Rensburg and Pretorius, 2000).

Some AAB posses the ability to grow on cellobiose as the only carbon source (data not shown; work performed by colleague, D.R. Garner). What made this observation somewhat surprising was the fact that all sources of nitrogen (yeast extract and peptone) were removed from the media (along with the other carbon sources, glucose and mannitol) to avoid false positives, leaving only cellobiose and agar. Although colony growth was small, Cl 11 (*A. pasteurianus*) and Cl 38 (*Ga. hansenii*) were two organisms that managed to grow quite convincingly. The plates were incubated for no less than 5 days to give the colonies a chance to adjust to their less than optimal nutritional media.

The cellobiase enzyme is a member of the β-glucosidase group and is responsible for the hydrolysis of cellobiose to glucose. β-glucosidase activity can be measured using β-glucoside analogues, such as p-nitrophenyl-β-D-glucopyranoside (p-NPG). However, as 1,3-glucanase activity can also hydrolyse this substrate, p-NPG was unreliable and arbutin was the chosen substrate (Strauss et al., 2001).

Since β-glucosidase also acts on the β-glucosidic bonds of cellobiose to form glucose, one would expect positive results similar to that of cellobiase activity. However, very little β-glucosidase was observed. **Fig. 5.4** shows two strains that exibited β-glucosidase activity. A *Ga. liquefasciens* strain (EII 13) showed a small amount of activity, whereas an *A. aceti* strain (F 23) revealed strong activity.

Table 5.3
Screening of acetic acid bacteria for the production of extracellular enzymes

Strain	Species	Cellulase activity	Xylanase activity	Starch degradation	∝-amylase	Pectinase activity	Protease activity
BI 15	*A. pasteurianus*	-	-	-	+	-	+
CI 7	*G. oxydans*	+	-	-	-	+	+
CI 17	*A. pasteurianus*	+	-	-	-	-	+
CI 32	*Ga. hansenii*	-	+	-	-	-	-
CI 38	*Ga. hansenii*	+	-	-	+	-	-
EI 10	*A. pasteurianus*	-	-	-	-	+	+
EI 17	*G. oxydans*	+	-	-	-	+	-
EI 21	*Ga. hansenii*	+	-	-	-	-	-
EI 38	*Ga. hansenii*	+	+	-	+	-	-
FI 4	*G. oxydans*	-	-	-	-	-	-
FI 13	*Ga. hansenii*	+	-	-	-	-	-
GI 4	*Ga. liquefasciens*	+	-	-	-	-	-
CII 17	*A. pasteurianus*	-	-	-	-	+	-
CII 23	*Ga. liquefasciens*	-	-	-	-	+	+
CII 26	*A. pasteurianus*	-	-	-	-	+	+
EII 13	*Ga. liquefasciens*	-	-	-	-	+	+
EII 19	*A. pasteurianus*	+	-	-	-	-	-
EII 28	*A. pasteurianus*	+	-	-	-	+	+
FII 2	*Ga. liquefasciens*	+	-	-	+	-	+
FII 6	*Ga. liquefasciens*	-	-	-	-	-	-
FII20	*A. pasteurianus*	-	-	-	-	-	+
GII 4	*Ga. liquefasciens*	+	-	-	-	+	-
HII1	*G. oxydans*	-	-	-	-	-	-
HII 8	*Ga. hansenii*	+	+	-	-	-	+
HII 13	*A. pasteurianus*	-	-	-	-	+	-
HII16	*A. pasteurianus*	-	-	-	-	+	+
Cab 74	*G. oxydans*	-	-	-	-	-	-
Cab 86	*G. oxydans*	+	-	-	+	-	+
Cab 100	*A. pasteurianus*	+	-	+	+	-	+
Cab 128	*A. pasteurianus*	+	-	-	-	-	-
Cab 156	*Ga. liquefasciens*	+	+	-	-	+	-
Cab 187	*A. pasteurianus*	+	-	-	+	-	-
Cab 286	*A. aceti*	-	-	-	-	-	-
F 37	*A. aceti*	+	-	-	-	-	-
F 23	*A. aceti*	-	-	+	+	-	+

Fig. 5.4. The brown halo around the growth of strains Ell 13 and F 23 is indicative of their β-glycosidase activity.

Since it has been speculated that the presence of glucose could have an inhibitory effect on the production of β-glucosidases (Strauss et al., 2001), YPM medium was used instead of GY, regardless of the strain's preference as listed in **Table 5.1**. It is interesting to note that, on some of the plates, very faint browning of the media was observed and, in addition, faint clearing zones occurred around some of the colonies. This possibly indicates β-glucosidase activity or, alternatively, some other substance being broken down. Whatever the reason, this group of enzymes should not be disregarded prematurely and the media used should perhaps be reassessed and optimised for AAB.

Only two strains of AAB with the ability to degrade starch were found (**Table 5.3**). The clear zones as a result of starch-degrading activity can also be seen around the colonies of strains F 23 (*A. aceti*) and Cab 100 (*A. pasteurianus*) in **Fig. 5.5**. This correlated with the findings that the same two strains also exibited α-amylase activity on the phadebas plates (**Fig. 5.6**). This suggested that the amylolytic activity enabling AAB to degrade starch could be ascribed to α-amylase activity. However, it is interesting to note that a number of strains that tested positive for α-amylase activity, showed no degradation of starch (**Table 5.3**). This can be explained by the fact that α-amylase could have been too specific, suggesting that it only exerted activity towards the phadebas in the plates, rather than towards the starch media. Alternatively, the acids that are produced by AAB could also affect the phadebas plates resulting in zones. This phenomenon has also been found in the screening of non-*Saccharomyces* yeasts during α-amylase assays (unpublished data).

Pectolytic activity was observed in a number of AAB strains. Initially, the test was carried out on GY and YPM plates, but the dye, ruthenium red, had difficulty sinking into the media and precipitated on top of the plate instead. This possibly was the result of the acetic acid produced by the AAB causing a pH decrease in the medium. The adopted DNS assay yielded results that proved much more trustworthy. A number of strains showed positive pectinase activity, with strains of the species *A. pasteurianus* and *Ga. liquefasciens* prevailing (**Table 5.4**). Care was taken not to exceed the specified amount of glucose when making up the media, as glucose has been reported to completely inhibit pectolytic enzymes at high concentrations (Strauss et al., 2001).
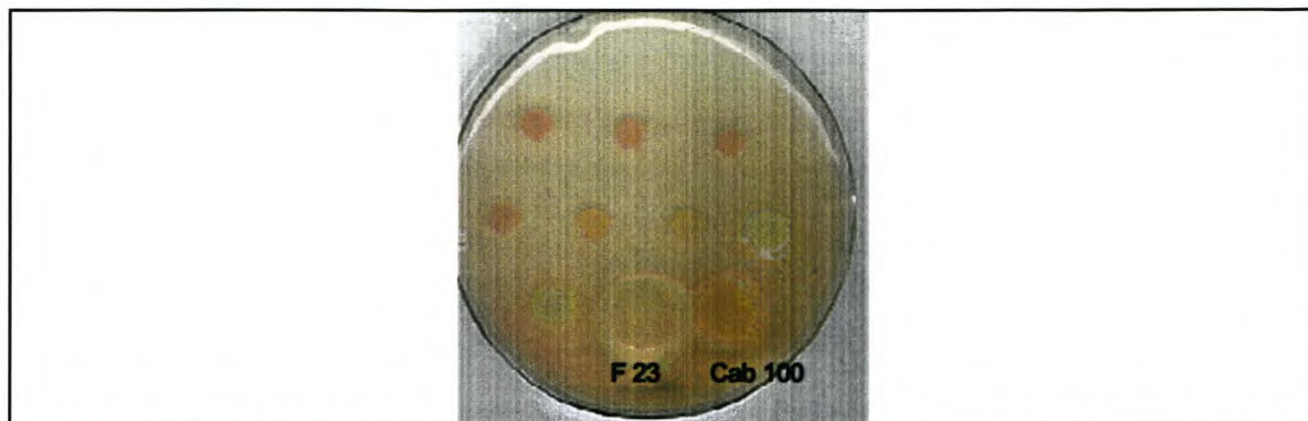
Fig. 5.5. Starch degradation is visible by the clearing zones surrounding the growth of strains Cab 100 and F 23.
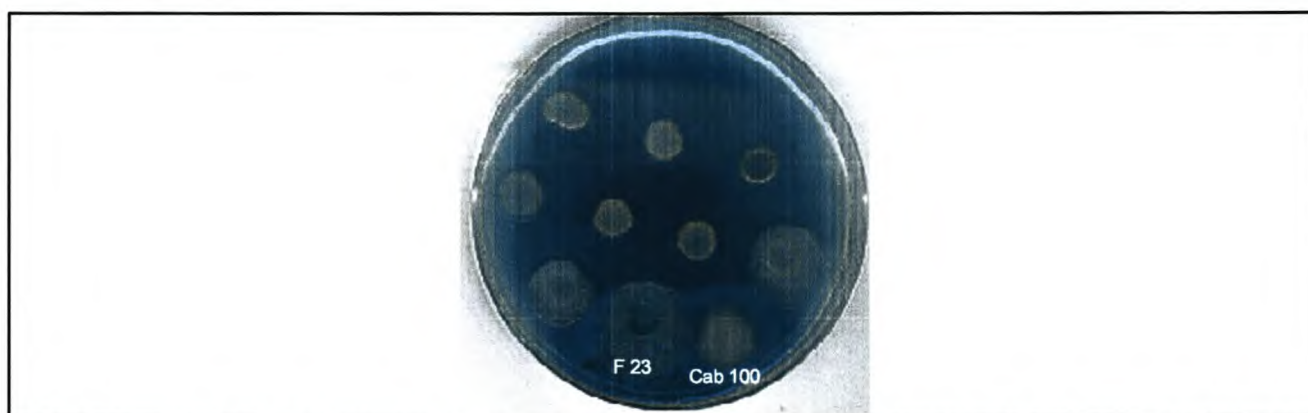

Fig. 5.6. α-Amylase activity can be seen by the clear zones around the growth of strains Cab 100 and F 23 on the phadebas plates.

From **Table 5.4**, it can be seen that proteolytic activity was observed in some of the AAB tested. Protease activity was observed mainly in strains of *A. pasteurianus,* although strains of each of the other representative wine species (*G. oxydans, A. aceti, Ga. hansenii* and *Ga. liquefasciens*) also showed positive activity. However, due to the discovery that the presence of a readily available nitrogen source can repress extracellular protease production (Charoenchai et al., 1997), these tests rather should be repeated and the growth conditions be optimised.

5.3 CONCLUSION

This study has covered various aspects of AAB, ranging from their anaerobic growth ability and their production of volatile acidity under various conditions to their potential to produce extracellular enzymes of significance to the winemaking process. It has again been shown that regulated levels of oxygen (and sulphur dioxide) should not be regarded as sufficient to control the numbers of these microorganisms. Some AAB can survive strenuous conditions, which implies that they could exert a negative effect on wine quality.

The significance of the existence of AAB throughout the winemaking process should not be underestimated. The ability of AAB to produce extracellular enzymes questions their role and consequent contribution to the chemical composition of the must. If it is considered that AAB could determine some of the enzymatic activities during winemaking, then they also could influence the sensory properties of the wine. This, together with their high potential to cause VA, shows that AAB can have a detrimental effect on wine aroma and these microbes therefore should always be monitored. A proper investigation of the production of extracellular enzymes by AAB has not been conducted previously and this should be a focus point for future research.

5.4 ACKNOWLEDGEMENTS

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