

**Fermentation optimization of pediocin PD-1  
production and a comparative study of the  
effect of pediocin PD-1, plantaricin 423 and  
nisin on biofilms of *Oenococcus oeni***



Thesis presented in partial fulfillment of the requirements for the degree of Master  
of Science at the University of Stellenbosch

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**December 2001**

## **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.

**Signature:**

**Date:**

## SUMMARY

Lactic acid bacteria are present in many foods and beverages and are used as starter cultures in the production of a variety of fermented products. Many of these bacteria produce ribosomally synthesized antimicrobial peptides (bacteriocins), which inhibit the growth of bacteria genetically closely related to the producer cell. Since many of these target bacteria include foodborne pathogens such as *Bacillus* spp., *Clostridium* spp., *Listeria* spp., and *Staphylococcus* spp., the practical importance of these peptides as food preservatives has been well documented and, in the case of nisin and pediocin PA-1, commercially explored.

The increased demand from health conscious consumers for foods with no chemical preservatives is putting renewed pressure on the producer to supply a “clean and green” product, but with the same or even an extended shelf life. Various research groups are screening lactic acid bacteria for production of novel broad-spectrum antimicrobial peptides or are exploring the possibilities of altering known bacteriocins to inhibit Gram-negative bacteria, yeasts and molds.

Pediocin PD-1, produced by *Pediococcus damnosus* NCFB 1832, belongs to the class IIa bacteriocins, i.e. heat-stable *Listeria*-active peptides, containing the YGNGV-consensus sequence in the N-terminal region. Little is known about the production and mode of activity of pediocin PD-1.

In this study, production of pediocin PD-1 was significantly increased by optimizing the growth medium, De Man Rogosa and Sharpe (MRS) broth. Addition of bacteriological peptone (1.7%, w/v), manganous sulphate (0.014%, w/v) and Tween 80 (3%, v/v), and lowering of the pH during fermentation stimulated pediocin PD-1 production and the level of organic acids produced. Maximum levels of bacteriocin activity were recorded at an initial pH of 6.7 in the latter medium. Under these conditions the specific bacteriocin activity increased by a factor of approximately six after 55 h of fermentation.

The effect of pediocin PD-1, plantaricin 423, produced by *Lactobacillus plantarum* 423, and commercial grade nisin (Aplin and Barrett Ltd., Trowbrige, Wilts, England) was tested against planktonic cells of *Oenococcus oeni* and a biofilm of the cells established on stainless steel surfaces identical to those used in wineries. After 5 h of treatment with 3000 AU (arbitrary units)/ml of each bacteriocin, all planktonic cells of *O. oeni* in a modified Chardonnay must medium were killed. All viable cells in the biofilm were killed after only 1 h in the presence of

3000 AU/ml of any one of the bacteriocins. In addition, pediocin PD-1, plantaricin 423 and nisin removed the biofilms from the surfaces and reduced the biomass either completely, as in the case of pediocin PD-1, or by 58% and 50% as in the case of plantaricin 423 and nisin, respectively. These same results were recorded after 5 h of treatment with 3000 AU/ml in a modified Chardonnay must medium.

To our knowledge this is the first report of controlling biofilm formation of malolactic bacteria on stainless steel surfaces with natural antimicrobial peptides. This implies that, apart from being very effective in controlling the cell numbers of free-living cells of *O. oeni*, the three bacteriocins, especially pediocin PD-1, could also be used as natural sanitizers. The fact that the production and activity levels of pediocin PD-1 could be increased without genetically modifying the producer strain is an added advantage.

## OPSOMMING

Melksuurbakterieë is teenwoordig in verskeie soorte voedsel- en drankprodukte en word as suurselkulture in die produksie van 'n verskeidenheid gefermenteerde produkte gebruik. Baie van hierdie bakterieë produseer ribosomaal-vervaardigde antimikrobiese peptiede (bakteriosiene) wat die groei van ander bakterieë, geneties naverwant aan die produserende organisme, inhibeer. Omdat baie van hierdie bakterieë voedselpatogene soos *Bacillus* spp., *Clostridium* spp., *Listeria* spp. en *Staphylococcus* spp. insluit, is die praktiese belang van hierdie peptiede reeds deeglik ondersoek en word, soos in die geval van nisien en pediosien PA-1, kommersieel gebruik.

Die toenemende behoefte van die verbruiker na voedselprodukte met geen chemiese preserveermiddels plaas nuwe druk op die vervaardiger om veilige voedselprodukte te produseer, maar met dieselfde of selfs langer rakleefyd. Verskeie navorsingsgroepe bestudeer melksuurbakterieë vir die produksie van unieke antimikrobiese peptiede met 'n wye spektrum van inhibisie en ondersoek ook die moontlikhede om hierdie bakteriosiene geneties te manipuleer ten einde Gram-negatiewe bakterieë, giste en swamme te inhibeer.

Pediosien PD-1, geproduseer deur *Pediococcus damnosus* NCFB 1832, word as 'n klass IIa bakteriosien geklassifiseer. Hierdie groep sluit in die hitte-stabiele *Listeria*-aktiewe peptiede, met 'n YGNGV-konsensus volgorde in die N-terminale deel van die peptied. Min is egter bekend oor die meganisme van werking van hierdie bakteriosiene.

In hierdie studie is die produksie van pediosien PD-1 betekenisvol verhoog met die optimalisering van die vloeibare groeimedium De Man Rogosa en Sharpe (MRS). Die toevoeging van bakteriologiese peptone (1.7%, m/v), mangaan sulfaat (0.014%, m/v) en Tween 80 (3.0%, v/v) en 'n afname in die pH gedurende groei het pediosien PD-1-produksie gestimuleer en sodoende ook die vlak van organiese sure wat geproduseer is. Maksimum vlakke van bakteriosien-aktiwiteit is in hierdie medium met 'n aanvangs-pH van 6.7 waargeneem. Onder hierdie omstandighede, en na 55 uur van fermentasie, het die spesifieke aktiwiteit van die bakteriosien met 'n faktor van ongeveer ses verhoog.

Die effek van pediosien PD-1, plantarisien 423, geproduseer deur *Lactobacillus plantarum* 423, en 'n kommersiële graad nisien (Aplin and Barrett Ltd., Trowbride, Wilts, Engeland) is teen die planktoniese selle van *Oenococcus oeni* en 'n biofilm van hierdie selle, gevestig op 'n vlekvrystaal oppervlak identies aan wat in wynkelders gebruik word, getoets. Na 5 ure van behandeling

met 3000 AE (arbitrêre eenhede)/ml van elke bakteriosien, is al die planktoniese selle van *O. oeni* in 'n gemodifiseerde Chardonnay mos-medium vernietig. Alle lewensvatbare selle in die biofilm is ook na slegs 1 uur in die teenwoordigheid van 3000 AE/ml van enige een van hierdie bakteriosiene vernietig. Verdermeer het pediosien PD-1, plantarisien 423 en nisien ook die biofilm op die vlekvrystaal-oppervlak verwyder. In die geval van pediosien PD-1 is 'n totale afname van die biomassa-oppervlak waargeneem, terwyl plantarisien 423 en nisien 58% en 50% van die totale biomassa verwyder het. Hierdie resultate is na 5 ure van behandeling (3000 AE/ml) in 'n gemodifiseerde Chardonnay mos-medium waargeneem.

Sover ons kennis strek is hierdie die eerste verslag rakende die gebruik van natuurlike antimikrobiële peptiede om biofilm-vorming deur appel-melksuurbakterieë op vlekvrystaal oppervlaktes te beheer. Dit impliseer dat bakteriosiene, spesifiek pediosien PD-1, benewens die beheer van planktoniese selle van appel-melksuurbakterieë, ook as natuurlike oppervlak-reinigingsgebruik kan word. Die feit dat die produksie en aktiwiteitsvlakke van pediosien PD-1 verhoog kon word sonder om die organisme geneties te modifiseer is 'n verdere voordeel.

## **BIOGRAPHICAL SKETCH**

Hannes Augustinus Nel was born in Moorreesburg on 6 August 1977. He matriculated at Dirkie Uys High School, Moorreesburg, in 1995. In 1998 he obtained a B.Sc. degree at the University of Stellenbosch, majoring in Microbiology and Genetics. He completed his B.Sc. (Hons.) degree the following year at the same institute.

## **ACKNOWLEDGEMENTS**

I sincerely wish to thank:

Our Creator and Almighty God, for blessing me with the ability, discipline and perseverance to complete this study.

My mother, brother and dear friends for their wonderful and continual support, interest, encouragement and prayers for the duration of this study.

My promoter, Prof. L.M.T. Dicks, Department of Microbiology, University of Stellenbosch, Stellenbosch, for his guidance, support, enthusiasm and ability to understand the students private needs.

My co-promoter, Prof. G.M. Wolfaardt, Department of Microbiology, University of Stellenbosch, for his encouragement and motivation.

Prof. E.J. Vandamme, the staff and students of the Laboratory of Industrial Microbiology and Biocatalysis, Department of Biochemical and Microbial Technology, Ghent University, Ghent, Belgium, for their hospitality, generosity, assistance, support and friendship during my time in their laboratory.

The co-workers and staff of the laboratory for their advice, encouragement, friendship and always making it a fun and productive environment to work in.

The National Research Foundation and Winetech for financial support.



## **DEDICATION**

I dedicate this thesis to the Lord, God Almighty, who has blessed me with the capability and opportunity to have a very tiny peek into His astonishing and enthralling creation.

## PREFACE

This thesis is presented as a compilation of manuscripts. Each chapter is introduced separately and is written according to the style of the respective journal. Two scientific papers stemmed from this research. An additional two papers were written for *Wineland* and are included in two appendixes.

- Chapter 6 Nel, H.A., Bauer, R., Vandamme, E.J. and Dicks, L.M.T. (2001) Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1. *Journal of Applied Microbiology* (in press). Accession number: JAM /2001/6.
- Chapter 7 Nel, H.A., Bauer, R., Wolfaardt, G.M. and Dicks, L.M.T. (2001) The effect of bacteriocins pediocin PD-1, plantaricin 423 and nisin on biofilms of *Oenococcus oeni* on a stainless steel surface. *American Journal of Enology and Viticulture* (submitted for publication).
- Appendix A Nel, H.A., Moes, C. and Dicks, L.M.T. (2001) Sluggish/stuck fermentation in Chardonnay: Possible causes. *Wineland*, July.
- Appendix B Nel, H.A., Bauer, R., Wolfaardt, G.M. and Dicks, L.M.T. (2001) Is this the new age of sanitizers and biocontrol? *Wineland* (in press).

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# **CHAPTER 1**

## **INTRODUCTION**



## CHAPTER 1

### INTRODUCTION

It is becoming increasingly important to replace chemical preservatives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate and nitrite, which may have an adverse effect on human health, with biological preservatives that are safe for human consumption and environmentally friendly (Dziezak 1986). In addition, it is also one of the most persistent challenges for food and beverage industries to replace sanitizers and detergents with biological substances. These issues, and the demand for minimally processed foods have resulted in large sums of money and time spent on research focussed on naturally occurring metabolites produced by lactic acid bacteria (LAB) (Harlander 1993; De Vuyst and Vandamme 1994; Holah *et al.* 1994; Zottola and Sasahara 1994; Kumar and Anand 1998).

LAB are inextricably linked to man and have been either isolated from various fermented food products, or used as starter cultures, especially in milk, meat, vegetables and wine. If controlled, these organisms alter the flavour, texture and appearance of raw food products in a desirable way. Since humans and animals have consumed these organisms for centuries without any adverse effects, it has been given GRAS (generally regarded as safe) status.

The antimicrobial substances produced by LAB include organic acids, H<sub>2</sub>O<sub>2</sub> and diacetyl. This coincides with the reduction of pH and removal of carbohydrates from the fermentation medium (Daeschel 1989; Davidson and Hoover 1993; De Vuyst and Vandamme 1994). Some of these bacteria also produce antimicrobial peptides known as bacteriocins, which are recognized as a potential source of food biopreservatives, particularly in minimally processed food (Davidson and Hoover 1993). Bacteriocins have an antagonistic effect against strains genetically closely related to the producer organism (De Vuyst and Vandamme 1994), are mostly heat resistant, stable in food, digestible, have GRAS status, and are active at low concentrations. These characteristics render bacteriocins a logical choice as natural food preservatives. The antimicrobial spectrum of most bacteriocins is restricted to Gram-positive bacteria, and often includes species such as *Bacillus cereus*, *Clostridium perfringens*, *Listeria monocytogenes* and *Staphylococcus aureus* (De Vuyst and Vandamme 1994). Furthermore, several factors in food,

such as proteolytic enzymes, lipid content and concentrations of salts, e.g. sodium chloride may influence the antimicrobial activity of bacteriocins (Ray 1994).

The first recorded observations of antagonistic interactions between bacteria were in 1676 and 1877 by Antonie van Leeuwenhoek and Louis Pasteur, respectively. Colicin V, produced by *Escherichia coli*, was the first bacteriocin described (Jack *et al.* 1995). Nisin, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis*, was discovered in 1927, but its molecular structure was only characterized in 1971. Ever since, numerous bacteriocins produced by LAB have been described, especially in the last 10 to 15 years. Research on the genetic engineering of bacteriocin-producing LAB has escalated during the past 10 years (Harlander 1993; Hoover and Steenson 1993; Jack *et al.* 1995; Parente and Ricciardi 1999; Johnsen *et al.* 2000).

Apart from being used as food preservatives, bacteriocins can possibly also be applied to health care products such as soaps, skin care products, toothpaste, household cleaning agents and even as an alternative therapeutic agent for the treatment of infections caused by antibiotic-resistant Gram-positive bacteria (Harlander 1993; Ross *et al.* 1999). Nisin is a good example of the latter applications. Although it is the only approved food-grade bacteriocin, it is also used to prevent mastitis in cows, control oral infections, dental caries and acne (Harlander 1993; Ross *et al.* 1999).

Since the control of bacterial biofilms represents one of the biggest challenges in the food, beverage and other industrial environments, various chemical sanitizers have been used. However, increased emphasis is falling on adopting different strategies, especially biological methods aimed at biofilm elimination. Bacteriocins may very well be considered as biocontrol agents to remove biofilms in the food and beverage industries (Holah *et al.* 1994; Zottola and Sasahara 1994; Kumar and Anand 1998). Little is known about the effect bacteriocins may have on biofilms. In the past most of the research in this field focussed on understanding the interaction of factors affecting bacterial attachment and biofilm formation with the ultimate goal of devising strategies to control the problem. In this regard the bacterium *L. monocytogenes*, which forms biofilms on stainless steel food processing surfaces, have been mostly studied.

Malolactic fermentation (MLF), mostly conducted by *Oenococcus oeni*, is considered important for the deacidification, flavor modification and microbial stability of wine, especially in wines produced from grapes grown in cool climates which often have a high acid (tartrate plus malate) content. MLF is, however, also desired in some white and red wines produced from

grapes grown in warmer climates, since it often introduces favourable organoleptic compounds (Henick-Kling 1994). Since MLF is a delicate process, it is important to control the presence of natural occurring malolactic bacteria and, if needed, effectively remove them from stainless steel surfaces. If these biofilms are not effectively destroyed, they may contaminate wine in which MLF is undesirable or even lead to the development of bacteriophages which may cause stuck or sluggish MLF in wines dependent on a secondary fermentation. MLF in wines, especially Chardonnay and Merlot, are often, for no obvious reason, not completed.

The first goal of this study was to optimize the production of pediocin PD-1, produced by *Pediococcus damnosus* NCFB 1832, by adding readily available nitrogen sources to the growth medium. The second goal was to determine the effect pediocin PD-1 has on *O. oeni* in an established biofilm on a stainless steel surface. Comparative studies were conducted with plantaricin 423, produced by *Lactobacillus plantarum* strain 423 and commercial grade nisin, produced by *Lactococcus lactis* subsp. *lactis*.

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# **CHAPTER 2**

## **LACTIC ACID BACTERIA**

## CHAPTER 2

### LACTIC ACID BACTERIA

#### 2.1 PRACTICAL IMPORTANCE OF LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) are Gram-positive, non-sporulating and microaerophilic with lactic acid as main fermentation product. These bacteria are ubiquitous in distribution, non-pathogenic and do not produce toxins. One of the most important roles LAB have is the preservation of food and beverage products, amounting to billions of dollars spent worldwide. Although fermented foods date back into antiquity, it was only in 1857 when Louis Pasteur, for the first time proved that lactic acid fermentation was due to the presence of a microorganism. Historically, food preservation by lactic acid fermentation was an empirical process in which raw foods were observed to undergo textural and organoleptic changes, resulting in different food products with enhanced keeping qualities (Daeschel 1989; De Vuyst and Vandamme 1994b). Today we know that the fermentation of sugars with a subsequent decrease in pH is the main reason for the natural preservation of foods (Mora *et al.* 2000).

Fermentation processes were gradually improved and a wide variety of industries exist that produce fermented products, including milk, meat, fish, fruits, cereals, vegetables, wine and ciders, by lactic acid fermentation (Daeschel 1989; Salovaara 1993; De Vuyst and Vandamme 1994b; Lücke 1996; Motarjemi and Nout 1996). Milk alone can be fermented to more than 1000 products, each different in flavour and texture. Where refrigeration or hot storage is not available, lactic acid fermentation is an affordable method to keep food safe until consumed, provided that rapid and adequate acidification is attained.

Lactic acid fermentation is also associated with the degradation of anti-nutritional factors, the increased bioavailability of minerals, the improvement of the digestibility of proteins, i.e. in tannin-rich cereals, and the degradation of indigestible oligosaccharides (Motarjemi and Nout 1996). Moreover, certain amino acids may be synthesized, the availability of B-group vitamins increased and the colour formation of the product accelerated (Motarjemi and Nout 1996). With the lowering of pH, lactic acid fermentation also provides optimal conditions for the enzymatic degradation of phytate complexes in proteins of polyvalent cations such as iron, zinc, calcium,

and magnesium (Motarjemi and Nout 1996). This process is important in cereals. Soaking of cereal grains in water for 12-14 hours reduces the phytate levels and increases the amount of soluble iron, zinc, and calcium several fold. In some cereals the tannin content is also reduced, leading to increased absorption of iron. Vegetables fermented with LAB showed an increase in the preservation of Vitamin C, which in turn also facilitates the absorption of iron in a phytate-rich diet (Motarjemi and Nout 1996). The presence of other iron-promoting factors in fermented vegetables due to lactic acid fermentation have also been reported (Motarjemi and Nout 1996).

Lactic acid produced by LAB is usually a mixture of the L-(+) and D-(-) isomers. Since humans cannot metabolize the latter, excessive intake can result in acidosis, which is a disturbance in the acid-alkali balance in the blood (Motarjemi and Nout 1996). Production of odors, off-flavours (especially in wine), biogenic amines and slime are other negative factors associated with the growth of LAB which can result in enormous financial losses (Fleming *et al.* 1986; Cerning 1990; Buckenhüskes 1993).

A number of the strains display probiotic properties by protecting the host against pathogenic enteric and urinary tract bacteria. Some strains keep cholesterol levels within acceptable limits by affecting cholesterol ingestion in the diet, hydrolyse bile salts and/or modulate the ratio of high-density to low-density lipoprotein/cholesterol (De Vuyst and Vandamme 1994b). LAB can also minimize colon cancer as they convert, degrade or absorb carcinogenic compounds, degrade antinutritional factors (trypsin inhibitor, glucosinolates, phytic acid, etc.) and reduce fecal bacterial enzymes, e.g.  $\beta$ -glucuronidase, azoreductase and nitroreductase (De Vuyst and Vandamme 1994b). These strains stimulate the immune system to produce macrophages, resulting in tumor suppression and may even play a positive role in the inactivation of the human immunodeficiency virus (HIV) (De Vuyst and Vandamme 1994b).

## 2.2 GENERAL CLASSIFICATION OF FOOD-RELATED LACTIC ACID BACTERIA

Gram-positive bacteria form two phylogenetic lines of descent, the *Clostridium* branch, with a G+C content of less than 50 mol% and the *Actinomyces* branch, with a G+C content higher than 50 mol% (Schleifer and Ludwig 1995). Phylogenetically, LAB are members of the *Clostridium-Bacillus* subdivision of Gram-positive eubacteria. The genus *Bifidobacterium* (G+C content of more than 50 mol%) groups with the lactobacilli, but is the most ancient group of the second

subdivision of the Gram-positive eubacteria, the *Actinomycetes* (De Vuyst and Vandamme 1994b, Schleifer and Ludwig 1995) and is not considered a member of LAB. Propionibacteria, microbacteria and brevibacteria also belong to this subdivision, but they too appear to be offshoots of non-lactic acid bacteria (Stackebrandt and Teuber 1988).

LAB are Gram-positive, catalase negative, non-sporeforming rods, cocci or coccobacilli with a G+C content of less than 53% (Stiles and Holzapfel 1997) and have complex nutritional requirements. On the basis of their hexose catabolism, LAB are divided into two groups: homofermentative, using the Embden-Meyerhof-Parnas pathway, and heterofermentative, using the 6-phosphogluconate pathway (Orla-Jensen 1919). Homofermentative LAB convert hexoses to lactic acid and do not ferment pentoses or gluconate. However, some heterofermentative LAB also ferment hexoses to acetic acid, ethanol and formic acid under glucose limitation (Pot *et al.* 1994). Obligately heterofermentative LAB convert glucose to lactic acid, carbon dioxide, ethanol and/or acetic acid and pentoses to lactic acid and acetic acid (Pot *et al.* 1994). Facultative heterofermentative LAB in turn ferment hexoses to lactic acid and pentoses to lactic acid and acetic acid.

LAB comprise both cocci (*Aerococcus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus* and *Vagococcus*) and rods (*Carnobacterium* and *Lactobacillus*). *Leuconostoc oenos* was reclassified as *Oenococcus oeni* by Dicks *et al.* (1995) and *Leuconostoc paramesenteroides* and related organisms included in the genus *Weissella* (Collins *et al.* 1993). The latter genus comprises of both cocci and rods.

The taxonomy of LAB is currently primarily based on phenotypic and genetic characteristics, rather than on morphological and physiological differences (Klein *et al.* 1998). Mode of action, configuration of the lactic acid produced, growth at different temperatures, growth at high salt concentrations, acid or alkaline tolerance, fatty acid composition and mobility of lactate dehydrogenase are some of the phenotypic characteristics used to group LAB into different genera (Axelsson 1993). Methods used for phenotypic and phylogenetic classification methods include comparative analysis of SDS-PAGE of whole cell protein patterns, 16S and 23S rRNA sequence analysis, DNA base composition, DNA homologies and RAPD-PCR (random amplified polymorphic DNA – polymerase chain reaction) banding patterns (Pot *et al.* 1994; Stiles and Holzapfel 1997).



Gram-positive, non-sporulating LAB not involved with food production or food spoilage include the genera *Aerococcus* (Collins *et al.* 1990; Stiles and Holzapfel 1997), *Alloiococcus* (Aguirre and Collins 1992) and *Melissococcus* (Bailey and Collins 1982). New species and strains with novel phenotypic characteristics are continually being described. The classification and characterization of LAB remains, therefore, a dynamic and intensive study-field (Pot *et al.* 1994).

### 2.3 ANTIMICROBIAL SUBSTANCES OF LACTIC ACID BACTERIA

In recent years it has become clear that the overall inhibitory action of LAB is due to more complex antagonistic systems than merely lactic acid. These compounds are produced in smaller quantities than lactic acid and acetic acid and include formic acid, free fatty acids, ammonia, ethanol, hydrogen peroxide, diacetyl, acetoin, 2,3-butanediol, acetaldehyde, benzoate, bacteriolytic enzymes, bacteriocins and antibiotics, as well as several less well-defined or unidentified inhibitory substances (Klaenhammer 1988; Daeschel 1989; Lindgren and Dobrogosz 1990; Piard and Desmazeaud 1991, 1992; Schillinger 1990; Vandenberg 1993; De Vuyst and Vandamme 1994a).

#### 2.3.1 Fermentation end-products

Production of fermentation end-products and accumulation thereof in the surrounding environment depends on the species, chemical composition of the environment and physical conditions (Kandler 1983; Gilliland 1986; Condon, 1987; Lindgren and Dobrogosz 1990).

##### 2.3.1.1 Organic acids and fatty acids

Lactic acid is the major end-product of homo- and heterofermentative LAB. It is produced from pyruvate by lactate dehydrogenase to regenerate pyridine nucleotides necessary in the metabolism of carbohydrates (De Vuyst and Vandamme 1994a). At least 50% of the end-product formed by LAB is lactic acid (Orla-Jensen 1919). Only a few non-lactic bacteria are able to survive and grow at levels below the threshold pH of between 3.8 and 4.4 (Piard and

Desmazeaud 1991; De Vuyst and Vandamme 1994a). In addition to the decrease in pH, the undissociated form of the acid molecule causes a collapse in the electrochemical proton gradient, leading to eventual death of susceptible organisms (Earnshaw 1992).

Other organic acids that are produced include acetic acid, formic acid, malic acid, succinic acid and citric acid. Acetic acid ( $pK_a = 4.75$ ) is a more effective growth inhibitor than lactic acid which has a  $pK_a$  of 3.08 (Davidson and Hoover 1993). Preservation is, however, usually affected by the simultaneous presence of various acids and/or other antimicrobial compounds (Davidson and Hoover 1993).

Under optimal conditions some LAB produce significant amounts of fatty acids (Earnshaw 1992). Short chain fatty acids, such as acetic-, propionic- and sorbic acids, are also known to act as food preservatives, while medium-chain fatty acids are used as surface active or emulsifying agents (Jay 1992). Gram-positive bacteria are primarily sensitive to fatty acids with a carbon chain length of 12 to 16 ( $C_{12}$ – $C_{16}$ ), while Gram-negative bacteria are not inhibited by fatty acids (Jay 1992). Monolaurin is the most active antimicrobial lipid (Kabara *et al.* 1977; Jay 1992).

#### 2.3.1.2 Hydrogen Peroxide

In the presence of oxygen, LAB produce  $H_2O_2$  through electron transport via flavin enzymes (Kandler 1983; Condon 1983, 1987). The dismutation of endogenous short-living superoxide anions ( $O_2^{\cdot -}$ ), formed through partial transfer of electrons from sugar metabolism, to the dioxygen molecule can also contribute to the accumulation of  $H_2O_2$ . This is due to the action of a superoxide dismutase present in most LAB (particularly lactococci), or by manganese ions, present in high concentrations in the cytoplasm of lactobacilli, leuconostocs and pediococci lacking superoxide dismutase (Archibald and Fridovich 1981; Daeschel 1989). Manganese-containing non-heme pseudocatalase can also convert this reaction, as in the case of *Lactobacillus plantarum* (Kono and Fridovich 1983). Furthermore, in the presence of  $H_2O_2$ , these superoxide anions result in the formation of inhibitory hydroxyl radicals ( $OH\cdot$ ).

Hydrogen peroxide can also react with other components to form inhibitory substances. In raw milk,  $H_2O_2$  generated by LAB reacts with endogenous thiocyanate, which is catalyzed by lactoperoxidase to form intermediary oxidation products that are inhibitory to microorganisms.

This process, the “lactoperoxidase antibacterial system,” extends the shelf-life of unrefrigerated raw milk and has been well reviewed (Björck *et al.* 1979; Reiter and Härnulf 1984).

#### 2.3.1.3 Carbon dioxide

The major end-product in fermentation of hexoses by heterofermentative LAB is CO<sub>2</sub>. A number of LAB, including homofermentative species, produce CO<sub>2</sub> from malate and citrate (Flemming *et al.* 1986; London 1990). Its role in creating an anaerobic environment by replacing existing molecular oxygen, its extra- and intracellular pH-decreasing effect and its destroying effect on the cell membrane (Eklund 1984) make CO<sub>2</sub> an inhibitory substance towards several microorganisms (Clark and Takacs 1980). This protective role of CO<sub>2</sub> is especially important in the fermentation of vegetables and silages to prevent growth of molds (Lindgren and Dobrogosz 1990).

#### 2.3.1.4 Diacetyl

Diacetyl (2,3-butanedione) is a metabolic end product of citrate-fermenting LAB, including *O. oeni* (Cogan 1986; Sandine 1986; Hugenholtz 1993; Lonvaud-Funel 1999). Diacetyl is better known for the buttery aroma associated with cultured dairy products, wine, brandy, roasted coffee and silage (Daeschel 1989; De Vuyst and Vandamme 1994a; Lonvaud-Funel 1999). Although diacetyl is generally recognized as safe, its application as a food preservative is limited because of the relatively high levels (400 µg/ml) needed for inhibition (De Vuyst and Vandamme 1994a). Diacetyl is inhibitory at a level of 200 µg/ml for yeasts, 300 µg/ml for Gram-negative and non-lactic Gram-positive bacteria, and more than 400 µg/ml for LAB (Jay 1982). The intense aroma of diacetyl would preclude its use to preserve non-fermented foods and may only be useful as an aseptic agent for surfaces and utensils (Daeschel 1989; Davidson and Hoover 1993; De Vuyst and Vandamme 1994a).

### 2.3.1.5 Ethanol and acetaldehyde

Acetaldehyde formed during carbohydrate metabolism of heterofermentative LAB is reduced to ethanol in the re-oxidation of pyridine nucleotides. In the absence or repression of alcohol dehydrogenase, LAB may secrete acetaldehyde. Although acetaldehyde appears to possess antagonistic properties (Piard and Desmazeaud 1991), its antagonistic effect has been relatively undocumented (De Vuyst and Vandamme 1994a). Acetaldehyde is responsible for the typical aroma of yogurt fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* (Piard and Desmazeaud 1991; De Vuyst and Vandamme 1994a).

Acetaldehyde, produced by LAB, can also inhibit some foodborne pathogens e.g. *E. coli*, *Salmonella typhimurium* and *S. aureus*) at concentrations of 10-1000 ppm (Kulshrestha and Marth 1974a,b,c). In yoghurt, only 25 ppm of acetaldehyde is produced during fermentation, which suggests that it plays a minor antagonistic role (De Vuyst and Vandamme 1994a).

### 2.3.1.6 Other compounds

Reuterin is a low-molecular-weight, non-proteinaceous, highly soluble and pH-neutral product produced by *Lactobacillus reuteri*. It is a broad-spectrum antimicrobial agent active against certain Gram-negative and Gram-positive bacteria, yeast, fungi and protozoa (Daeschel 1989).

Antimicrobial compounds such as methylhydantoin, mevalonolactone and benzoic acid are produced by *L. plantarum* VTT-E-78076 (Niku-Paavola *et al.* 1999). Various unidentified low-molecular mass non-proteinaceous antimicrobial substances produced by LAB have also been reported (De Vuyst and Vandamme 1994a). Other factors that play a role in the antimicrobial potential of LAB include the competition for nutrients and phage-induced antibacterial proteins (lysins).

### 2.3.2 Bacteriocins

Bacteriocins produced by LAB are defined as biologically active proteins or protein complexes (protein aggregates, lipocarbohydrate proteins, glycoproteins, etc.) with a bactericidal mode of action towards Gram-positive bacteria, in particular closely related species occupying the same

ecological niche (Hoover and Steenson 1993; De Vuyst and Vandamme 1994a). The narrow specificity of their action and their protein nature distinguishes them from other (classical) antibiotics (Daw and Falkner 1996).

The potential use and application of bacteriocins as biocontrol and biopreservative agents, produced by species from all the genera of LAB, resulted in the extensive exploitation of this research field. Bacteriocins are further discussed in detail in Chapter 3.

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# **CHAPTER 3**

**BACTERIOCINS OF LACTIC  
ACID BACTERIA, WITH  
SPECIAL EMPHASIS ON THE  
GENERA *PEDIOCOCCUS*,  
*LACTOBACILLUS* AND  
*LACTOCOCCUS***

## CHAPTER 3

### **BACTERIOCINS OF LACTIC ACID BACTERIA, WITH SPECIAL EMPHASIS ON THE GENERA *PEDIOCOCCUS*, *LACTOBACILLUS* AND *LACTOCOCCUS***

#### 3.1 INTRODUCTION

One of the strategies bacteria use to compete and survive in the same ecological niche is the production of antimicrobial peptides, termed bacteriocins. Bacteriocins are usually membrane-permeabilizing cationic peptides with less than 50 amino acid residues and with a bactericidal mode of action towards genetically closely related species that occupy the same ecological niche (Hoover and Steenson 1993; De Vuyst and Vandamme 1994a; Johnsen *et al.* 2000). Notable exceptions include the lanthionine-containing peptides such as nisin and subtilin, which exhibit bactericidal activity against Gram-positive bacteria in general. Bacteriocin-producing strains protect themselves by an immunity protein specific to its own bacteriocin (Quadri *et al.* 1995).

Because of their long-term and beneficial association with humans and their food supply, there has been an increased interest in examining lactic acid bacteria (LAB) for bacteriocins. The multitude of bacteriocins produced by these bacteria offer great promise as biological food preservatives and as disease control agents, especially because they have GRAS (generally regarded as safe) status. The removal of biofilms in food and beverage processing industries, with the subsequent decreased use of chemical additives such as sulfur dioxide, benzoic acid, sorbic acid, nitrate and nitrite is another promising application of bacteriocins. To date nisin and pediocin PA-1 are the only two bacteriocins approved as food preservatives (Montville and Chen 1998).

During the last century, numerous bacteriocins produced by LAB have been described with newly discovered peptides being added to the list continually. Despite this, relatively few of the bacteriocins have been fully characterized (Piard and Desmazeaud 1992; Nettles and Barefoot 1993; Carolissen-Mackay *et al.* 1997). Bacteriocins produced by members of the same species are often identical, as in the case of pediocin PA-1 and pediocin AcH, both produced by *Pediococcus acidilactici* (Gonzalez and Kunka 1987; Bhunia *et al.* 1988). On the other hand, identical bacteriocins have been isolated from different organisms, such as sakacin A, produced

by *Lactobacillus sakei* and curvacin A, produced by *Lactobacillus curvarus* (Holck *et al.* 1992; Tichaczek *et al.* 1993). Some strains produce more than one bacteriocin, e.g. several identical leucocins produced in the same habitat (Papathanasopoulos *et al.* 1997). Several of the bacteriocin-producing strains have initially been identified incorrectly, for instance the producer of pediocin 5 produced by *Ped. acidilactici* UL5 (Daba *et al.* 1991; Huang *et al.* 1996) and bavaricin MN produced by *Lactobacillus bavaricus* MN (Kaiser and Montville 1996).

### 3.1.1 Bacteriocins produced by *Pediococcus* spp.

Species of the genus *Pediococcus* are distinguished from other LAB by grouping of their spherical cells into two planes at right angles to form tetrads. Cells may also be arranged in pairs. Single cells are rarely found (Ray 1994). Pediococci are facultative anaerobes and homofermentative with an optimum growth temperature between 25°C and 40°C (Holt *et al.* 1994). Glucose is fermented to DL or L(+)-lactic acid by the Embden-Meyerhof-Parnas pathway. They do not produce CO<sub>2</sub> from the fermentation of glucose. Some strains produce diacetyl and acetoin from pyruvate. They are catalase negative, but some strains contain pseudocatalase (Holt *et al.* 1994). They are nonmotile, non-encapsulated and are chemoorganotrophs, requiring a rich medium containing nicotinic acid, pantothenic acid and biotin (Ray and Hoover 1993).

The genus contains eight species. *Ped. acidilactici*, *Ped. damnosus*, *Ped. parvulus* and *Ped. pentosaceus* are phylogenetically closely related based on DNA/DNA homology and some physiological and biochemical characteristics. *Ped. acidilactici* is closely related to *Ped. pentosaceus* and *Ped. damnosus* to *Ped. parvulus* (Collins *et al.* 1990). *Ped. cerevisiae*, previously included in the genus, has been replaced with *Ped. damnosus*. Some strains that were included in the species *Ped. acidilactici* have now been designated *Ped. damnosus*, *Ped. pentosaceus* and *Ped. acidilactici* (Kitahara 1974; Gherna and Pienta 1989; Holt *et al.* 1994). The other species in this genus are *Ped. inopinatus*, *Ped. dextrinicus*, *Ped. halophilus* and *Ped. urinaeequi* (Holt *et al.* 1994). *Ped. halophilus* has been reclassified as *Tetragenococcus halophilus* (Collins *et al.* 1990).

The bacteriocins produced by pediococci are usually classified as class IIa (discussed in 3.2.1.2.1), i.e. small heat-stable *Listeria*-active peptides (Klaenhammer 1993; Horn *et al.* 1998).

They are characterized by a YGNGV-motif and a disulfide bridge in a highly conserved N-terminal region. Pediocins are antilisterial and have a membrane-permeabilizing mode of action (Chikindas *et al.* 1993; Chen *et al.* 1997b; Nes *et al.* 2000). Some of the pediocin-like bacteriocins, such as pediocin PA-1, also contain a disulfide bridge in the C-terminal region, whereas others, such as sakacin P, do not (Fimland *et al.* 2000). The highly conserved N-terminal region is hydrophilic and cationic, and it has been proposed that this region mediate the initial binding of the peptide to target cells through electrostatic interactions (Chen *et al.* 1997a). Comparative studies of natural bacteriocins have led to the suggestion that the second disulfide bridge, located in the C-terminal region, is an important determinant of bacteriocin activity (Eijsink *et al.* 1998). The somewhat less conserved C-terminal half is hydrophobic and/or amphiphilic and is thought to penetrate into the hydrophobic part of the target cell membrane, thereby mediating membrane leakage (Fimland *et al.* 1996; Miller *et al.* 1998). Structural analysis indicated that a 15- to 20-residue stretch from the middle towards the C-terminal end forms an amphiphilic  $\alpha$ -helix upon interaction with membrane-like structures and that the remaining C-terminal residues are relatively unstructured (Fregeau *et al.* 1997; Wang *et al.* 1999).

A number of bacteriocins produced by *Pediococcus* spp. have been described, viz. pediocin A produced by *Ped. pentosaceus* FBB-61 (Etchells *et al.* 1964; Flemming *et al.* 1975), PA-1 produced by *Ped. acidilactici* PAC 1-0 (Gonzales and Kunka 1987), AcH produced by *Ped. acidilactici* H (Bhunja *et al.* 1987, 1988), JD produced by *Ped. acidilactici* JD-23 (Richter *et al.* 1989), SJ-1 produced by *Ped. acidilactici* SJ-1 (Schved *et al.* 1993), N5p produced by *Ped. pentosaceus* (Strasser de Saad *et al.* 1995), 5 produced by *Ped. acidilactici* UL5 (Huang *et al.* 1996) and pediocin L50 that has been renamed as enterocin L50 (Cintas *et al.* 1998). Pediocin PD-1, produced by *Ped. damnosus* NCFB 1832 is structurally different from other pediocins (R. Bauer, personal communication) and unique in the sense that it does not inhibit other *Pediococcus* spp. (Green *et al.* 1997).

Pediocins have a broad spectrum of activity. Pediocin A is active against species of the genera *Bacillus*, *Clostridium* (including *Cl. botulinum*), *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria*, *Micrococcus*, *Pediococcus* and *Staphylococcus* (Ray 1994). Pediocin AcH is active against strains from many genera, including *Bacillus*, *Brochothrix*, *Clostridium* (including *Cl. botulinum* E), *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria*, *Pediococcus*,

*Propionibacterium* and *Staphylococcus* (Ray 1994). Pediocin PA-1 is inhibitory to strains and species from the genera *Lactobacillus*, *Leuconostoc*, *Listeria* and *Pediococcus*. Pediocin PD-1 is inhibitory to species of *Bacillus* (including *B. cereus*), *Clostridium*, *Enterococcus* (including *Ent. faecalis*), *Lactobacillus*, *Leuconostoc*, *Oenococcus*, *Listeria*, *Micrococcus*, *Propionibacterium*, *Staphylococcus* and *Streptococcus* (Green *et al.* 1997). Not all strains and species are always sensitive to the same pediocin. This is probably also true for other bacteriocins of LAB.

Pediocin-like bacteriocins of LAB are normally not antibacterial to Gram-negative bacteria. However, limited studies have shown that when Gram-negative bacteria are sublethally injured by freezing, or when treated with chelating agents such as EDTA, they become sensitive (Ray 1992).

### 3.1.2 Bacteriocins of *Lactobacillus* spp.

Among the members of LAB, the lactobacilli are composed of a diverse group of homofermentative and heterofermentative species (Klaenhammer 1993). The cells of the genus *Lactobacillus* are rod-shaped and usually regular. However, when under stress, for instance towards the end of growth, some species covert to a more coccoid morphology. They are nonsporing, facultative anaerobic, often described as being microaerophilic, with optimum growth temperatures between 30 and 40°C. They are chemoorganotrophs requiring rich, complex media. They have a fermentative and saccharoclastic metabolism and at least half of their end-product carbon is lactate. Nitrate is not reduced, gelatin is not hydrolysed, and all strains are catalase and cytochrome negative (Holt *et al.* 1994).

The first antimicrobial activity associated with *Lactobacillus* spp. were reported by Grossowics (1947). This heat-stable antimicrobial substance was produced by an unidentified homofermentative *Lactobacillus* strain, active against a number of Gram-positive and Gram-negative bacteria, but not against other lactobacilli. Ever since, numerous bacteriocins (more than 50) produced by the same species were reported, although they did not always meet the criteria for bacteriocins and the antimicrobial activity were in many cases due to the presence of hydrogen peroxide. Most of these bacteriocins have not been fully characterized or are produced by unidentified *Lactobacillus* spp., and the extent of the diversity of these bacteriocins cannot be determined. Those bacteriocins that have been characterized usually belong to class II (as

described in section 3.2.1.2), while helveticin J and caseicin 80 belong to class III (as described in section 3.2.1.3). Recent studies indicated that plantaricin, produced by *Lactobacillus plantarum* 423, may fall into class IIa bacteriocins (Van Reenen *et al.* 2001).

Numerous plantaricins, produced by strains of *L. plantarum*, have been described and include plantaricin B (West and Warner 1988), A (Daeschel *et al.* 1990), K (Olukoya *et al.* 1993), C19 (Atrih *et al.* 1993), S (Jiménez-Díaz *et al.* 1993), C (González *et al.* 1994), 154 (Kanatani and Oshimura 1994), 149 (Kato *et al.* 1994), LC74 (Rekhif *et al.* 1994), F (Fricourt *et al.* 1994; Paynter *et al.* 1997), SA6 (Rekhif *et al.* 1995), KW30 (Kelly *et al.* 1996), UG1 (Enan *et al.* 1996), D (Franz *et al.* 1998), NA (Olasupo 1998), 1.25 $\alpha$  and 1.25 $\beta$  (Remiger *et al.* 1999), ST31 (Todorov *et al.* 1999), and 423 (Van Reenen *et al.* 1998).

The organisms inhibited by one or many of these bacteriocins include *Bacillus cereus* and *Bacillus stearothermophilus*, *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium tyrobutyricum*, *Enterobacter* spp., *Enterococcus faecalis*, *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Lactobacillus helveticus*, *L. plantarum*, *Leuconostoc lactis*, *Leuconostoc mesenteroides*, *Leuconostoc paramesenteroides*, *Listeria monocytogenes*, *Listeria grayi*, *Micrococcus* spp., *Oenococcus oeni*, *Ped. acidilactici*, *Ped. damnosus*, *Ped. cerevisiae*, *Ped. pentosaceus*, *Propionibacterium acidipropionici*, *Propionibacterium jensenii*, *Propionibacterium theonii*, *Pseudomonas aeruginosa*, *Streptococcus* spp., *Staphylococcus aureus* and *Staphylococcus thermophilus* (De Vuyst 1994a; Klaenhammer 1993).

### 3.1.3 Bacteriocins from *Lactococcus* spp.

A variety of bacteriocins are produced by *Lactococcus* spp., which includes the lantibiotic nisin (Rogers and Whittier 1928; De Vuyst van Vandamme 1994b; De Vuyst 1994b), diplococcin (Davey and Pearce 1982), lactostrepcins (Kozak *et al.* 1978), lactococcins (Van Belkum *et al.* 1992; Nissen-Meyer *et al.* 1992) and lacticins (Piard *et al.* 1990; Ryan *et al.* 1996).

Nisin is one of the best-studied bacteriocins and is extensively reviewed by numerous authors (Hansen 1991; De Vuyst 1994b; De Vuyst and Vandamme 1994b; Teuber 1995). It is the first bacteriocin to be granted GRAS status and is used commercially worldwide in various food products (especially dairy products) as preservative (Hansen 1991; De Vuyst 1994b; De Vuyst

and Vandamme 1994b; Teuber 1995). Nisin is produced by many strains of *L. lactis* subsp. *lactis*. The host range of nisin includes most Gram-positive bacteria such as corynebacteria, enterococci, lactobacilli, leuconostocs, listeria, oenococci, pediococci, *Mycobacterium tuberculosis*, and germinating spores of bacilli and clostridia (Teuber 1995).

Lactococcins A, B and M are produced by *L. lactis* subsp. *cremoris* 9B4 and *L. lactis* subsp. *diacetylatis* DPC938 and are inhibitory only to other lactococci (Teuber 1995; Van Balkum 1992). *L. lactis* subsp. *cremoris* LMG 2130 (Holo *et al.* 1991) and *L. lactis* subsp. *diacetylatis* WM4 (Stoddard *et al.* 1992) also produce lactococcin A. *L. lactis* CNRZ 481 produce lacticin 481 (Piard *et al.* 1990), which is inhibitory to most *Lactococcus* spp. and some *Clostridium*, *Lactobacillus* and *Leuconostoc* spp. *L. lactis* subsp. *lactis* ADRIA 85LO30 produce the lantibiotic lactococcin DR, which is identical to lacticin 481 (Rince *et al.* 1994). Lactococcin G, produced by *L. lactis* LMG 2081 inhibits various LAB and different clostridia (Nissen-Meyer *et al.* 1992). Other bacteriocins produced by *Lactococcus* sp. include lacticin 3147 by *L. lactis* DPC3147 (Ryan *et al.* 1996), diplococcin by *L. lactis* subsp. *cremoris* strain 347 (Davey and Richardson 1981) and lactostrepsin by *L. lactis* subsp. *cremoris* strain 202 (Zajdel *et al.* 1985).

#### 3.1.4 Other bacteriocins produced by lactic acid bacteria

Bacteriocins produced by *Enterococcus* spp. include enterococcin Sf25 (Reichelt *et al.* 1984); enterocin 81 (Ennahar *et al.* 1998); enterocin 01 (Olasupo *et al.* 1994); enterocin I (Floriano *et al.* 1998); enterocin L50A and B (Cintas *et al.* 1998); enterocin EJ97 (Gálvez *et al.* 1998); enterocin AS-48 (Gálvez *et al.* 1989); enterocins 1071A and B (Balla *et al.* 2000) and enterocin 012 (Jennes *et al.* 2000). These bacteriocins either do not belong in any of the currently described groups (see section 3.2.1), or have not been characterized to the extent they can be classified (see section 3.2.1.2.4).

Bacteriocins produced by *Carnobacterium* spp. have been fully characterized and belong to the class II group of bacteriocins as described in section 3.2.1.2. The following bacteriocins have been reported: Carnocin U149 (Stoffels *et al.* 1992), divergicin 750 (Ahn and Stiles 1990; Lewus *et al.* 1991), carnobacteriocins BM1 and B2 (Ahn and Stiles 1990; Quadri *et al.* 1994), carnocin CP51 and CP52 (Herbin *et al.* 1997), piscicocin V1a and V1b (Bhugaloo-Vial *et al.* 1996), piscicolin 126 (Jack *et al.* 1996), piscicolin 61 (Holck *et al.* 1994), divergicin 750 (Holck *et al.*



1996), divergicin A (Worobo *et al.* 1995) and divercin V41 (Métivier *et al.* 1998; Pilet *et al.* 1995).

Bacteriocins produced by *Leuconostoc* and *Weissella* spp. mostly belong to the class IIa group as described in section 3.2.1.2.1. Examples are carnocin LA54A (Keppler *et al.* 1994), dextranin 24 (Revol-Junelles and Lefebvre 1996), leucocin A-UAL 187 (Hastings and Stiles 1991), leuconocin S (Lewus *et al.* 1992), leucocin A-TA33a, which is identical to leucocin A-UAL 187 (Papathanasopoulos *et al.* 1997; 1998), leuconcin C-TA33c (Papathanasopoulos *et al.* 1997), leuconocin J (Choi *et al.* 1999), leucocin H (Blom *et al.* 1999), leucocin F10 (Parente *et al.* 1996), leucocin B-TA33b (Papathanasopoulos *et al.* 1997), leucocin C-LA7a (Hastings *et al.* 1996), mesentericin Y105 (Hécharad *et al.* 1992a, b), mesentericin 52A, which is identical to mesentericin Y105 (Revol-Junelles *et al.* 1996) and mesentericin 52B (Revol-Junelles *et al.* 1996). Mesentericin 52B and leucocin B-TA33b cannot be classified in the currently described groups (see section 3.2.1).

Tagg (1992) reported the production of numerous bacteriocin-like inhibitory substances by streptococci, which at the time were still identified using the Lancefield groupings. *Streptococcus* spp. produce the bacteriocins thermophilin 13 (Marciset *et al.* 1997), thermophilin 347 (Villani *et al.* 1995) and thermophilin T (Aktypis *et al.* 1998). Thermophilin 13 (Marciset *et al.* 1997) belongs to group IIb.

## 3.2 CHARACTERISTICS OF BACTERIOCINS

Bacteriocins differ widely in their spectrum of bacterial inhibition, amino acid composition and amino acid sequence, although differences in antimicrobial activity cannot be attributed to particular amino acids or sequence of amino acids. They also differ in three-dimensional structures, molecular weight, pI, and the formation and release of biologically active bacteriocins (Jack *et al.* 1995). Most of the low molecular-weight bacteriocins are cationic at pH 7, and many have greater antimicrobial activity at low pH. The mode of action (adsorption to Gram-positive cell surfaces, penetration, etc.) differs among bacteriocins, with maximum adsorption at or above pH 6 (Jack *et al.* 1995).

### 3.2.1 Classification of bacteriocins

Klaenhammer (1993) defined four distinct classes to group the bacteriocins of LAB: Class I (lantibiotics), class II (small, < 10 kDa, heat-stable membrane-active peptides), class III (large, > 30 kDa, heat-labile proteins) and class IV (complex bacteriocins). The class II bacteriocins were later divided into *Listeria*-active peptides with N-terminal consensus sequences (class IIa), poration complexes requiring two different peptides for activity (class IIb) and thiol-activated peptides that require reduced cysteine residues for activity (class IIc).

Recently, Moll *et al.* (1999) reclassified the bacteriocins. He defined class I as lantibiotics and further divided it into types A and B. Type B lantibiotics are compact, with globular structures, are enzyme inhibitors and immunologically active (De Vuyst and Vandamme 1994b). The class II non-lanthionine peptides is further divided into four groups: Class IIa, which consists of *Listeria*-active peptides with an N-terminal consensus sequence; Class IIb, consisting of two-peptide complexes; Class IIc, consisting of sec-dependent bacteriocins and Class IId, the small heat-stable non-lanthionine bacteriocins that do not belong to any of the three groups within class II. Class III consists of large heat labile bacteriocins. Most of the bacteriocins belong to class I or class II, since they are the most abundant and have the best potential for industrial application (Nes *et al.* 1996). Complex bacteriocins are grouped into Class VI (Nes *et al.* 1996).

Cintas *et al.* (1998) proposed a new class of bacteriocins that are not related to class II. A good example of the latter is enterocin L50A and L50B, produced by *E. faecium* L50. These peptides are secreted without an N-terminal leader sequence or signal peptide and are more related to a group of cytolytic peptides secreted by staphylococci.

#### 3.2.1.1 Class I: Lantibiotics

Lantibiotics (*lan*thionine-containing *anti*biotic peptides) are small (less than 5 kDa, with 19 to 38 amino acids), membrane active peptides that contain unusual, posttranslationally modified amino acids such as lanthionine (Lan),  $\beta$ -methyl lanthionine (MeLan) and dehydrated residues (Sahl and Bierbaum 1998). Lantibiotics are only produced by Gram-positive bacteria (Nes and Tagg 1996). Usually the gene cluster encoding lantibiotic peptides also contain a gene or genes that encode specific enzymes able to facilitate the dehydration of certain residues in the propeptide region,

followed by the addition of cysteine residues to form characteristic Lan and MeLan sulfur ring structures (Nes and Tagg 1996). Considerable differences in the leader peptides and amino acid sequence of type A lantibiotics have been observed. The mode of action of nisin, which falls into this class, is the most thoroughly studied of all bacteriocins (Giffard *et al.* 1997; Chen *et al.* 1997a).

### 3.2.1.2 Class II: Small heat-stable bacteriocins

These bacteriocins do not contain any unusual amino acids and can be defined as small (less than 10 kDa). They contain between 30 and 60 residues and are usually positively charged at a neutral pH (Eijsink *et al.* 1998). They are membrane active and heat resistant at temperatures up to 100°C. Klaenhammer (1993) classified these bacteriocins by their so-called double-glycine (G-G) processing site in the bacteriocin precursor. Bacteriocins in this class have a high content of small amino acids such as glycine and are strongly cationic with pI's between 8 and 11, and possess hydrophobic and amphiphilic domains (Abee *et al.* 1995).

#### 3.2.1.2.1 Class IIa: *Listeria*-active bacteriocins

Bacteriocins in this group are also referred to as pediocin-like bacteriocins and are produced by a number of LAB. The first pediocin-like bacteriocins that were identified and thoroughly characterized were pediocin PA-1 (Gonzalez and Kunka 1987), leucocin A-UAL 187 (Papathanasopoulos *et al.* 1998), mesentericin Y105 (Revol-Junelles *et al.* 1996), sakacin P (Tichaczek *et al.* 1994) and curvacin A (Fimland *et al.* 2000). Today, at least nine of the 14 pediocin-like bacteriocins in this group have been isolated and characterized (Johansen *et al.* 2000). Despite similarities in their primary structures, the pediocin-like bacteriocins have different target cell specificities (Fimland *et al.* 2000). Although the antimicrobial spectrum of these bacteriocins is different, they are all active against *Listeria* spp. and share the same conserved amino acid sequence, YGNGV, in their structure. The function of this consensus motif is not clear (Chen *et al.* 1997a). Recent studies indicated that plantaricin 423, produced by *L. plantarum* 423, show homology with pediocin AcH and may also fall into this class (Van Reenen *et al.* 2001).

#### 3.2.1.2.2 Class IIb: Two-peptide complexes

The activity of these bacteriocins depends on the complementary activity of two peptides. Some two-peptide bacteriocins such as lactococcin G and lactococcin M (Nissen-Meyer *et al.* 1992) need both peptides for activity, while one or both peptides of plantaricin S (Jiménez-Díaz *et al.* 1993), lactacin F (Fremaux *et al.* 1993) and thermophilin 13 (Marciset *et al.* 1997) are active. The combined effect of the two peptides of these bacteriocins is much greater than the total activity calculated from the individual effect of these peptides (Cintas *et al.* 1998).

#### 3.2.1.2.3 Class IIc: Sec-dependent bacteriocins

Bacteriocins that belong to this class do not possess a double-glycine leader peptide, but are synthesized with a Sec-type N-terminal leader sequence, leading to secretion and processing via the Sec pathway (Nes *et al.* 1996). Divergicin A (Worobo *et al.* 1995) and enterocin P (Cintas *et al.* 1997) are examples.

#### 3.2.1.2.4 Class IId: Unclassified small heat-stable non-lanthionine membrane-active bacteriocins

Bacteriocins that do not meet any of the criteria of the previous sections within the other class II bacteriocins are included in this class. Moll *et al.* (1999) included acidocin 8912 (Kanatani *et al.* 1995), enterocin B (Casaus *et al.* 1997), enterocin I (Floriano *et al.* 1998), carnobacterium A (Worobo *et al.* 1994), curvaticin FS47 (Garver and Muriana 1994), lactococcin A (Holo *et al.* 1991) and lactococcin B (Van Belkum *et al.* 1992) in this group.

#### 3.2.1.3 Class III: Large heat-labile bacteriocins

Bacteriocins in this group are more than 30 kDa in size. The bacteriocin helveticin J is grouped into this class (Fremaux and Klaenhammer 1994).

#### 3.2.1.4 Class IV: Complex bacteriocins

Bacteriocins in this class contain a protein and one or more chemical moieties, such as lipids or carbohydrates. The existence of this class was supported by the inactivation of the bacteriocins by glycolytic and lipolytic enzymes. Several bacteriocins produced by *Leuconostoc* spp., such as leuconocin S and carnocin LA54A, are inactivated by  $\alpha$ -amylase (Lewus *et al.* 1992; Keppler *et al.* 1994). Subsequent studies suggested that these complexes may be artifacts caused by interaction between cell constituents or growth medium and regular peptide bacteriocins (Nes *et al.* 1996).

### 3.3 PRODUCTION OF BACTERIOCINS BY LACTIC ACID BACTERIA

#### 3.3.1 Kinetics of bacteriocin production

Bacteriocin production in LAB is correlated with bacterial growth, implying that the volumetric bacteriocin production is dependent on the total biomass formation (De Vuyst and Vandamme 1992; Parente *et al.* 1994; Parente and Ricciardi 1994; Mørtvedt-Abildgaard *et al.* 1995; Kim *et al.* 1997). Production occurs throughout the growth phase and ceases at the end of the exponential phase or sometimes before the end of growth, often followed by a decrease in bacteriocin titre (Parente *et al.* 1997; Lejeune *et al.* 1998).

Optimal bacteriocin production in batch fermentation usually requires complex media and well-controlled physical conditions, such as temperature and pH (Parente and Ricciardi 1994; Leroy *et al.* 1999; Callewaert and De Vuyst 2000). Bacteriocin production is strongly dependent upon pH and temperature. Most studies indicated that the highest bacteriocin levels are obtained at temperatures and pH values lower than the optima for growth. This was the case for enterocin 1146 (Parente and Ricciardi 1994), lactococcin A (Parente *et al.* 1994), lactocin S (Mørtvedt-Abildgaard *et al.* 1995), amylovorin 1471 (De Vuyst *et al.* 1996), nisin Z (Matsusaki *et al.* 1996) and mesenterocin (Krier *et al.* 1998).

Since adsorption of bacteriocins to cells is maximal at pH 5.5 - 6.5 (Yang *et al.* 1992) and decreases at low pH, it is not surprising that reduction of bacteriocin titre is often observed in fermentations without pH control (De Vuyst and Vandamme 1992; Yang and Ray 1994).

However, after reaching a maximal bacteriocin activity in the fermentation medium during the active growth phase, a drastic decrease in soluble bacteriocin activity often occurs. This disappearance of activity is ascribed to proteolytic inactivation (Joerger and Klaenhammer 1986; De Vuyst and Vandamme 1992), protein aggregation (De Vuyst *et al.* 1996), and adsorption of the bacteriocins to the cell surface of the producer cells (Yang *et al.* 1992; De Vuyst *et al.* 1996; Parente *et al.* 1994, 1997). The addition of ethanol to the fermentation medium can, however, prevent aggregation (Mørtvedt-Abildgaard *et al.* 1995). The use of calcium in the fermentation medium prevented the adsorption of nisin Z to the producer cells (Matsusaki *et al.* 1996).

Only a few studies have been conducted on the optimization of growth medium with respect to bacteriocin production (Aasen *et al.* 2000). However, LAB are fastidious with respect to nutrient requirements and rich growth mediums with yeast extract and protein hydrolysates are required for growth and bacteriocin production (Parente and Hill 1992; Dada *et al.* 1993; De Vuyst *et al.* 1996). Fed-batch fermentation technology allows for the obtaining of high cell densities through the continuous supply of fresh medium. The growth rate can be controlled by the application of growth-limiting feeding strategies. Bacteriocin production in fed-batch fermentation has been described for epidermin, gallidermin (Ungermann *et al.* 1991) and nisin (De Vuyst and Vandamme 1991). Bacteriocin production was, however, modeled in only a few cases (Parente and Ricciardi 1994; Parente *et al.* 1997; Lejeune *et al.* 1998; Leroy and De Vuyst 1999). Strong non-linear relationships of peptide production have also been observed for nisin in batch (Kim *et al.* 1997) and continuous cultures (Meghrouh *et al.* 1992) and for plantaricin C in continuous culture (Bárcena *et al.* 1998). The non-linear relationship between nisin production and growth is probably a result of the complexity of nisin biosynthesis and regulation compared to class II bacteriocins. Maximization of growth does not necessarily result in optimal bacteriocin production (Parente and Ricciardi 1999). An approximate linear relationship between bacteriocin production and growth rate for some bacteriocins was, however, obtained in batch and continuous fermentations (Kaiser and Montville 1993; Parente *et al.* 1994; De Vuyst *et al.* 1996; Bhugaloo-Vial *et al.* 1997).

Despite the apparent primary metabolic kinetics, the maximum bacteriocin levels or production rates do not always correlate with or growth rate (Kim *et al.* 1997; Bogovic-Matijasic and Rogelj 1998). The enhanced production at non-optimum pH and temperature implied that a low growth rate or unfavourable growth conditions (“stress”) in general, might stimulate

bacteriocin production (De Vuyst *et al.* 1996; Aasen *et al.* 2000). Bacteriocin production per unit biomass is therefore affected by several factors, such as the producer strain, media (carbohydrate and nitrogen sources, cations, etc.) and fermentation conditions, i.e. pH, temperature, agitation, aeration and dilution rate in continuous fermentations (Parente and Ricciardi 1999).

### 3.3.2 Factors affecting bacteriocin production in lactic acid bacteria

#### 3.3.2.1 Microbial strain

A specific bacteriocin can be produced by several strains of the same species (Jack *et al.* 1995; Rodriguez *et al.* 1995). However, the level of bacteriocin production varies considerably between different species within the same genus. Nisin and leuconocin Lcm1 production varies among different strains, while pediocin AcH shows less variation (Yang and Ray 1994). De Vuyst (1994b) screened 21 nisin-producing and 6 non-producing strains of *L. lactis* for nisin production and immunity. Nisin titres varied from 1 to 1886 IU ml<sup>-1</sup> and did not correlate with the number of copies of the *nis* genes nor with their transcription or translation levels. Differences among strains were attributed to expression level and activity of maturing enzymes and, to a lesser extent, nisin immunity.

On the other hand, Quiao *et al.* (1997) showed that nisin resistance may dramatically affect nisin production. Nisin production of strain LAC48, a spontaneous mutant of *L. lactis* N8, was ten times higher than the parental strain, even with the expression of the *nis* genes being similar in both strains. Increase in nisin resistance by introducing plasmids containing nisin immunity determinants has recently been shown to result in increased nisin titres and faster growth compared to the parent strains (Kim *et al.* 1998).

A “ceiling” for bacteriocin production has also been observed for nisin (Kim *et al.* 1997) and amylovoryn L471 (De Vuyst *et al.* 1996; Lejeune *et al.* 1998). It has been demonstrated that both nutrient availability and nisin inhibition (Kim *et al.* 1997) affect the “ceiling” for nisin production.

### 3.3.2.2 Medium composition

Bacteriocin production is affected by the type and level of the carbon, nitrogen and phosphate sources, cations, surfactants and various inhibitors. Bacteriocins can be produced from media containing different carbohydrate sources.

Nisin Z can be produced from glucose, sucrose and xylose by *L. lactis* IO-1 (Matsusaki *et al.* 1996; Chinachoti *et al.* 1997), but better results were obtained with glucose (4000 IU ml<sup>-1</sup>) compared to xylose (3000 IU ml<sup>-1</sup>). Glucose, followed by sucrose, xylose and galactose, were the best carbon sources for the production of pediocin AcH in an unbuffered medium (Biswas *et al.* 1991). Sucrose was found to be a better carbon source than glucose for enterocin 1146 production; fructose or lactose again results in comparable levels of biomass but low levels of bacteriocin (Parente and Ricciardi 1994). Catabolite repression has been used to explain the production of plantaricin C at higher dilution rates (0.1 - 0.12 h<sup>-1</sup>) with sucrose and fructose compared to glucose (0.055 h<sup>-1</sup>) in continuous cultures (Bárcena *et al.* 1998).

Since LAB are nutritionally fastidious microorganisms, growth and bacteriocin production are often limited by organic nitrogen sources rather than by carbon substrates. Nisin levels increased when the levels of organic nitrogen in the medium were increased (Kim *et al.* 1997). However, at any given growth rate, the specific nisin production rate decreased with an increase in nitrogen (Kim *et al.* 1997). The type of nitrogen source also affects bacteriocin production. Comparing the effect of nitrogen sources on nisin production in a complex medium, De Vuyst and Vandamme (1993) found that the best results (2500 IU ml<sup>-1</sup>) were obtained with cotton-seed meal but high nisin yields (> 2000 IU ml<sup>-1</sup>) were also obtained with yeast extract and fish meal. *L. lactis* subsp. *lactis* ATCC11454 produced 1.5 times more nisin in a filtered stillage-based medium (a raw nitrogen source) compared to LTB broth (Van't Hul and Gibbons 1996). The differential effect of nitrogen sources was confirmed by factorial experiments for enterocin 1146 (Parente and Hill 1992) and lactocin D (Parente and Hill 1992).

Phosphate anions and cations (Mg<sup>2+</sup> and Ca<sup>2+</sup>) affect bacteriocin production, but their effect may be strain specific. Phosphate improves nisin production (Matsusaki *et al.* 1996), while Mg<sup>2+</sup> increases pediocin AcH production (Biswas *et al.* 1991). The addition of CaCl<sub>2</sub> (0.1 mol l<sup>-1</sup>) in batch fermentations resulted in increased maximum nisin concentrations and a specific production rate, but did not affect growth or lactate production from xylose and glucose at



controlled pH (Matsusaki *et al.* 1996; Chinachoti *et al.* 1997). This was explained by the increased immunity of the leader peptidase (NisP) (by protecting the integrity of the cytoplasmic membrane) or by nisin Z displacement from the cell surface due to the presence  $\text{Ca}^{2+}$  (Parente and Ricciardi 1999).

Tween 80 stimulated the production of mesenteroides 5 (Daba *et al.* 1993), nisin Z (Matsusaki *et al.* 1996) and plantaricin 423 (Verellen *et al.* 1998). A possible explanation for the increase in bacteriocin activity is the fact that Tween 80 could be acting as a surfactant, thereby preventing peptide adsorption on polypropylene and glass surfaces (Joosten and Nuñez 1995). The addition of ethanol (1%, v/v) to these fermentations further improved the production of lactocin S (Mørtvedt-Abildgaard *et al.* 1995) and amylovorin L471 (De Vuyst *et al.* 1996). It has also been reported that the use of calcium in the fermentation medium prevented the adsorption of nisin Z to producer cells (Matsusaki *et al.* 1996).

### 3.3.2.3 Effect of fermentation conditions

Few studies seem to have been conducted on optimization of the growth medium with respect to bacteriocin production. However, as lactic acid bacteria are fastidious with respect to nutrient requirements, a rich medium with yeast extract and protein hydrolysates is required for good growth and production (Parente and Hill 1992; Daba *et al.* 1993; De Vuyst and Vandamme 1993; De Vuyst *et al.* 1996).

Bacteriocin production strongly depends on pH and temperature. Most studies indicated that the highest bacteriocin levels are obtained at pH values and temperatures lower than the optima for growth [Parente *et al.* 1994 (lactococcin A); Parente and Ricciardi 1994 (enterocin 1146); Mørtvedt-Abildgaard *et al.* 1995 (lactocin S); De Vuyst *et al.* 1996 (amylovorin 1471); Matsusaki *et al.* 1996 (nisin Z); Krier *et al.* 1998 (mesenterocin)]. Since pH control improves the growth of LAB, it also results in improved bacteriocin production. However, the optimal pH for bacteriocin production is usually 5.5 - 6.0 (Meghrous *et al.* 1992; Kaiser and Montville 1993; Parente *et al.* 1994; Matsusaki *et al.* 1996; Chinachoti *et al.* 1997), which is often lower than the optimal pH for growth. A few bacteriocins are produced only at the low pH of 5.0 (Biswas *et al.* 1991; Yang and Ray 1994; Mørtvedt-Abildgaard *et al.* 1995; Bárcena *et al.* 1998). For pediocin AcH this was attributed to the pH needed for posttranslational processing of the bacteriocin.

However, this effect may be strain or species-dependent, since pediocin AcH is produced at pH 6.0 by *Lactobacillus plantarum* WHE2 (Ennaher *et al.* 1996). Optimal pH may also be affected by the culture medium. Nisin Z production with strain IO-1 is optimal at pH 6.0 in a xylose-containing medium (Chinachoti *et al.* 1997) and at pH 5.5 in a glucose-containing medium (Matsusaki *et al.* 1996).

Bacteriocin production is growth-associated, implying that the volumetric bacteriocin production is dependent on the total biomass formation (De Vuyst and Vandamme 1992; De Vuyst *et al.* 1996). This usually requires complex media and well-controlled physical conditions, such as temperature and pH (Parente *et al.* 1994; Parente and Ricciardi 1994; De Vuyst *et al.* 1996; Kim *et al.* 1997). Growth at optimal temperature therefore usually results in optimal bacteriocin production (Meghrous *et al.* 1992; Daba *et al.* 1993; Matsusaki *et al.* 1996; Chinachoti *et al.* 1997; Lejeune *et al.* 1998). Despite the apparent primary metabolite kinetics, the maximum bacteriocin levels or production rates do not always correlate directly with cell mass or growth rate (Kim *et al.* 1997; Bogovic-Matijasic and Rogelj 1998). Because of the enhanced production at non-optimum pH and temperature, it has been suggested that a low growth rate, or unfavourable growth conditions (“stress”) in general, may stimulate bacteriocin production (De Vuyst *et al.* 1996; Lejeune *et al.* 1998). A decrease in bacteriocin concentration after the cessation of growth is a common observation that has been explained by proteolytic activity, aggregation and/of absorption to the cells (Parente and Ricciardi 1994; Parente *et al.* 1994; De vuyst *et al.* 1996; Lejeune *et al.* 1998).

Some LAB produce more than one bacteriocin. Interestingly, the optimal pH and temperature for production of the two bacteriocins of *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 (mesenterocin 52A and 52B; Krier *et al.* 1998) were different, thus allowing manipulation of the ratio of the two bacteriocins by changing growth conditions.

Agitation and aeration also play a role in bacteriocin production. Small differences in production occurred with different agitation speeds for nisin Z, while aeration significantly reduced its production, perhaps because of chemical degradation (Chinachoti *et al.* 1997). As observed for other stress factors, fermentation in the presence of oxygen resulted in an increased bacteriocin production in the case of amylovorin L471 (De Vuyst *et al.* 1996), despite lower final bacteriocin titres.

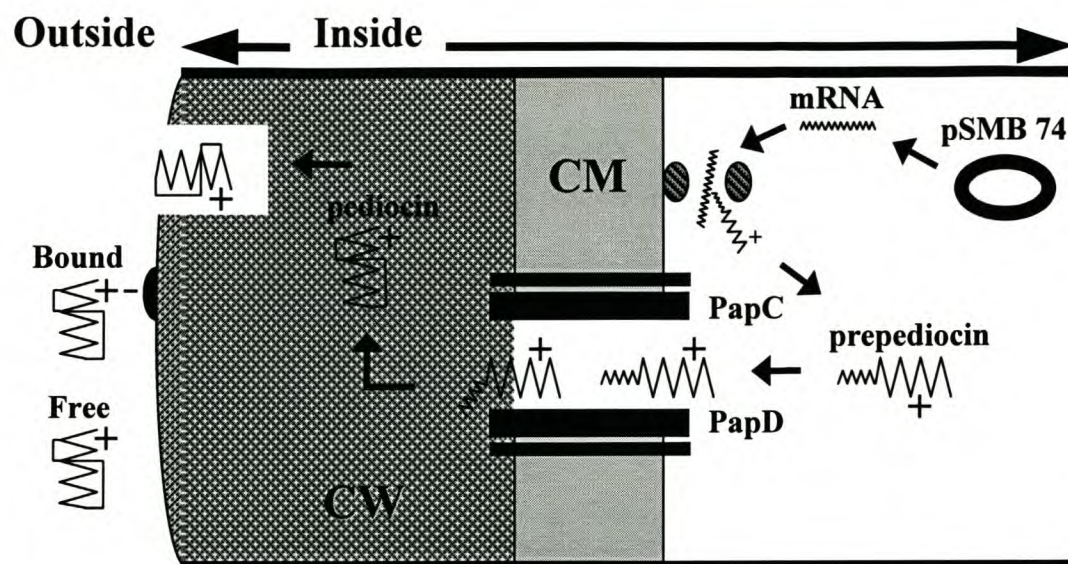
### 3.4 MODE OF ACTION OF PEDIOCIN-LIKE (CLASS IIa) BACTERIOCINS

Bacteriocins produced by Gram-positive bacteria are ribosomally synthesized polypeptides, are usually membrane-permeabilizing and cationic with less than 50 amino acid residues. Based on models for nisin/membrane interactions, bacteriocins form poration complexes in the cell membrane through a multi-step process of binding and insertion. Although the mode of action of lantibiotics (class I), particularly nisin, has been studied in detail (Jack *et al.* 1995; Klaenhammer 1993; Montville and Chen 1998), much less is known about the interaction between class II bacteriocins and the membrane interactions with their target organisms.

Details of the posttranslational events which occurs during the maturation of the non-lanthionine-containing bacteriocins, including propeptide modifications, cell envelope translocation, and cleavage of the leader peptide, are not yet fully understood. One model, which is based on observations with pediocin AcH, predicts that, since active pediocin molecules are likely to destabilize the cytoplasmic membrane, translocation should occur in the prepediocin form, i.e. prior to cleavage of the leader peptide (Fig. 1). This hypothetical model, proposed by Jack *et al.* (1995), represents the transcription of mRNA from the *pap* gene cluster in pSMB 74, translation of prepediocin, translocation through the cytoplasmic membrane, processing to remove the leader peptide, formation of disulfide bonds in prepediocin, and secretion of active pediocin through the cell wall. The PapD protein has both translation and processing functions and PapC may, in association with PapD, assist in efficient translocation of prepediocin across the cell membrane. Active pediocin AcH molecules are formed away from the cytoplasmic membrane, secreted through the wall and, depending on the pH of the environment, either remain bound to the cell wall or exist in a free form (Fig. 1).

Pediocin producer cells may also have an intracellular peptidase, which inactivates pediocin AcH (Ray *et al.* 1993). Although the modification of prepediocin by the formation of two disulfide bonds is probably nonenzymatic and could thus theoretically occur either before or after translocation of the molecule, significant spatial constraints on membrane passage would probably be imposed from the folding of the molecules by the disulfide bonds, especially those between Cys-24 and Cys-44 (Jack *et al.* 1995). A model developed for the lantibiotic subtilin suggested that folding, due to thioether linkages, occurs after translocation of unfolded molecules through

the membrane. Folding prior to secretion may provide a barrier, i.e. being too big for the traversal of the membrane by the known mechanisms (Hansen *et al.* 1991).



**Fig. 1** Hypothetical model of the translocation of the prepediocin form, prior to cleavage of the leader peptide. Reproduced from Jack *et al.* 1995.

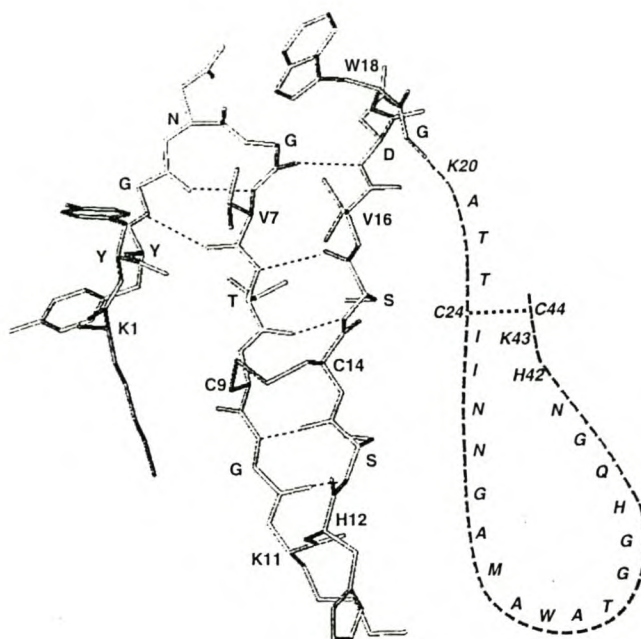
Aromatic residues in class IIa bacteriocins appear to have a critical role in antimicrobial activity and the entire sequence is essential for the activity of the peptide (Montville and Chen 1998). However, conservative amino acid substitutions in pediocin-like bacteriocins appear to have little effect on antimicrobial activity. Class IIa bacteriocins further share a high degree of homology in the N-terminus of the peptide, and a low to moderate degree of sequence homology in the C-terminus (Montville and Chen 1998).

The mode of action of the class IIa bacteriocins indicate that antimicrobial activity does not require a specific receptor and is enhanced by, but not fully dependent on, membrane potential (Eijsink *et al.* 1998). Based on their primary structures, pediocin-like bacteriocins may roughly be divided into two regions: a hydrophilic, cationic, and highly conserved N-terminal half and a less-conserved hydrophobic and/or amphiphilic C-terminal half (Fimland *et al.* 1996). The N-terminal half mediates the initial binding of these bacteriocins to target cells through electrostatic interactions (Chen *et al.* 1997a). The C-terminal half is responsible for the penetration into the

hydrophobic part of the target cell membrane, thereby mediating membrane leakage (Miller *et al.* 1998). Structural analysis indicated that a 15- 20-residue form the middle towards the C-terminal end forms an amphiphilic  $\alpha$ -helix upon interaction with membrane-like structures. The remaining C-terminal residues are relatively unstructured (Fregeau *et al.* 1997; Wang *et al.* 1999). The propensity of the bacteriocin to form this amphiphilic  $\alpha$ -helical conformation is a determining factor for poration (Montville and Chen 1998). An amphiphilic structure would facilitate formation of a barrel-stave pore. Spatial arrangement of amino acid residues to form amphiphilic structures can, however, also be achieved by  $\beta$ -sheet conformations (Montville and Chen 1998). The hydrophobic or amphiphilic C-terminal half also appears (in part) to mediate target cell specificity, since hybrid bacteriocins containing N- and C-terminal regions from different pediocin-like bacteriocins have an antimicrobial spectra similar to that of the bacteriocin from which the C-terminal region is derived (Fimland *et al.* 1996).

In addition to the conserved disulfide bridge in the N-terminal half, a few pediocin-like bacteriocins contain a second disulfide bridge, located in the C-terminal half. Comparative studies of natural bacteriocins have confirmed that this second disulfide bridge is essential for bacteriocin activity (Fimland *et al.* 1996, 2000).

The 44 amino acid pediocin PA-1, which is identical to pediocin AcH, is an example of a bacteriocin with a second disulfide bridge (Motlagh *et al.* 1992). Sequence alignments and secondary structure predictions for the N-terminus of pediocin PA-1 and other class IIa bacteriocins predict that it contains three  $\beta$ -sheets, maintained in a hairpin conformation, which is stabilized by the first disulfide bond (Fig. 2). The only defined structure that could be predicted for pediocin PA-1 is the second disulfide bond between Cys24 and Cys44, because of the high degree of conformational freedom. This second disulfide bond is essential for its activity (Chen *et al.* 1997b). The predicted  $\beta$ -sheet conformation at the N-terminus gives an amphiphilic characteristic. Two positively charged residues (Lys-11 and His-12), located at the tip of the  $\beta$ -hairpin between the second and the third strand, may form a positive patch together with Lys-1 (Montville and Chen 1998). A hydrophobic patch consists of the residues Val at position 7, Cys at position 9, Cys at position 14, Val at position 16, and Trp at position 18. The positive patch has been reported to mediate binding of the molecule to target membranes (Chen *et al.* 1997a) and the hydrophobic patch in the N-terminus may be important for the insertion of pediocin PA-1 into the membrane.



**Fig. 2** Predicted  $\beta$ -sheet conformation of the N-terminal domain of pediocin PA-1 (amino acid residues 1-19). The sequence of residues 20-44 is indicated without a structure, except for the Cys22-Cys44 disulfide bond. From Chen *et al.* (1997b).

In this context, it is interesting to note that the antimicrobial range of class II bacteriocins seems to be related to their disulfide bond (Jack *et al.* 1995). Pediocin PA-1 (containing two disulfide bonds) has a wider range than leucocin A and mesentericin Y 105, which contains one disulfide bond. However, they in turn have a wider range than lactococcin B (containing no Cys residue). Pediocin PA-1 is the only known class II bacteriocin with two disulfide bonds and has the broadest range of activity, since it is lethal to many species in the genera *Clostridium*, *Enterococcus*, *Lactobacillus*, *Leuconostoc*, *Listeria* and *Pediococcus* (Henderson *et al.* 1992; Mazzotta *et al.* 1997). In contrast, lactococcins A and B only inhibit closely related strains of *Lactococcus* spp. (Van Belkum *et al.* 1991; Venema *et al.* 1993). It may be that defined structural elements, especially the second disulfide bond which forms after pediocin PA-1 and binds to membrane lipids, play an important role.

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# **CHAPTER 4**

## **THE ROLE OF LACTIC ACID BACTERIA IN WINE-MAKING**



## CHAPTER 4

### THE ROLE OF LACTIC ACID BACTERIA IN WINE-MAKING

#### 4.1 INTRODUCTION

Enologists first identified lactic acid bacteria (LAB) in wine about 45 years ago (Peynaud 1956). Today, the wine-making process includes two main steps: alcoholic fermentation by yeasts, followed by malolactic fermentation (MLF) conducted by LAB. The secondary fermentation entails deacidification of the wine by the conversion of L-malic acid to L(+)-lactic acid and carbon dioxide (Wibowo *et al.* 1985).

At harvest, grape berries, stems, leaves and soil contains LAB, yeasts, acetic acid bacteria and molds. Yeasts and LAB are present in and on cellar equipment (Wibowo *et al.* 1985; Henick-Kling *et al.* 1994). Acetic acid bacteria survive in low cell numbers and molds are repressed, both because of the low redox potential of the must.

In grape must, up to 10 species of LAB can be identified. They represent the genera *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Oenococcus*. The facultative heterofermentative species include *Lactobacillus plantarum* and *Lactobacillus casei*, and the obligately heterofermentative species include *Lactobacillus hilgardii*, *Lactobacillus brevis* and *Lactobacillus fructivorans*. The homofermentative cocci are mainly *Pediococcus damnosus* and *Pediococcus pentosaceus*. *Pediococcus parvulus* has also been isolated from Washington State wines (Edwards *et al.* 1990) and Australian wines (Davis *et al.* 1988). The heterofermentative cocci are classified in the genus *Leuconostoc* that, until 1995, consisted of the species *L. mesenteroides* and *L. oenos*. The latter species is the only representative of the acidophilic branch of the genus *Leuconostoc* and is phylogenetically, based on RNA/DNA hybridization studies, not closely related to other *Leuconostoc* spp. (Garvie 1981). The molecular approach to taxonomy, based on 16S rRNA and 23S rRNA sequencing, led to the creation of a new genus, namely *Oenococcus*, with the sole species *O. oeni* (Dicks *et al.* 1995). This species is only found in wine.

Yeasts are better adapted to grow in grape must, which is normally high in sugar content (>210 g l<sup>-1</sup>) and acid (pH 3.0 – 3.3) (Lonvaud-Funel 1999). The alcoholic fermentation,

therefore, starts quickly. When all reducing sugars are fermented to ethanol, yeast levels decline, the cell numbers ( $10^6$  CFU/ml and higher) of LAB increases and MLF commences. However, throughout alcoholic fermentation and adaptation to this harsh environment, a natural selection leads to the dominance of *O. oeni*. This is due to interactions between yeasts and bacteria, among bacteria and the ability of *O. oeni* to adapt to harsh environments (Lonvaud-Funel 1999).

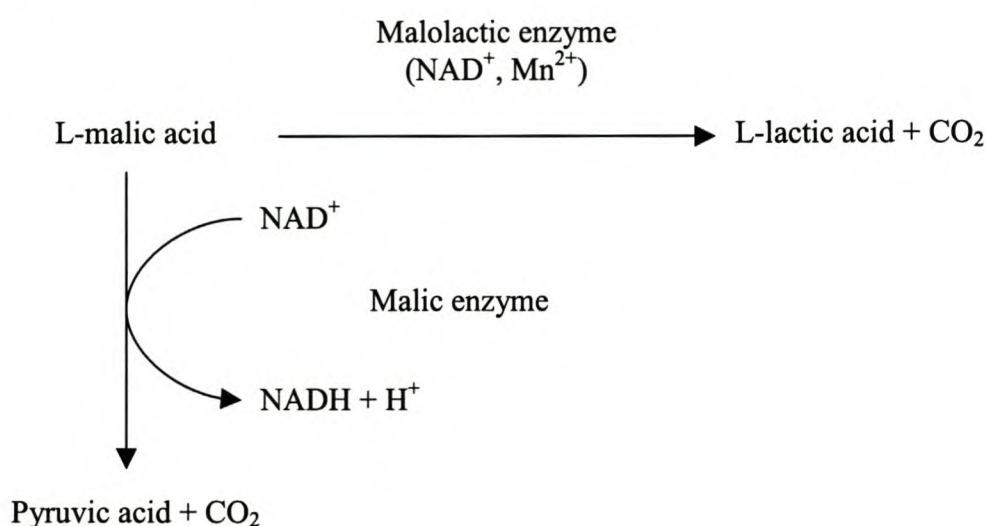
Apart from malic acid conversion, many other substrates, such as citric acid and certain amino acids are also metabolized, thus providing the cells with carbon sources and energy (Henick-Kling *et al.* 1994). Diacetyl produced from citric acid is one of the most important organoleptic compounds formed during MLF (Lonvaud-Funel 1999). On the contrary, an excess of other fermentation end-products, such as acetic acid, exopolysaccharides, glucane, biogenic amines and precursors of ethylcarbamate, are undesirable in wine (Lonvaud-Funel 1999). All these end-products of MLF greatly influence the sensory and hygienic quality of wine. The most striking trait of LAB in wine is, however, their capacity to adapt to a hostile environment (Henick-Kling *et al.* 1994). This helps wine-makers and oenologists to select for the most desirable malolactic strains to improve the organoleptic quality for each different wine.

Traditionally, MLF has been allowed to develop spontaneously from the LAB naturally present in the wine and cellar. However, most wine-makers are now aware of contamination by spoilage bacteria. The natural organisms occurring in the must and cellar equipment is eliminated by adding  $\text{SO}_2$  (50-80 ppm for red wines and 20-40 ppm for white wines) during crushing and separation of the must (Lonvaud-Funel *et al.* 1998; Henick-Kling and Park 1994). Sulfur dioxide is at this stage of wine-making the only permitted and efficient agent for the microbial stabilization of wine. Yeast and LAB starter cultures are then added to the must at required times, depending on the must quality and the final characteristics that are preferred in the wine.

The control of yeast and bacterial growth is also becoming more of a necessity in wine-making, even after starter cultures is added. During maturation and aging they have to be eliminated from the wine (Fugelsang 1997). Another concern in the wine industry is the effective cleaning and removal of yeast and LAB biofilms from the surface areas of fermentation tanks and cellar equipment (discussed in Chapter 5).

## 4.2 MALOLACTIC FERMENTATION IN WINE

LAB, mainly *O. oeni* deacidifies the wine by decarboxylating L(-)-malic acid to L(+)-lactic acid (Wibowo *et al.* 1985; Henick-Kling 1994; Guerzoni *et al.* 1995; Vivas *et al.* 1997; Edwards *et al.* 1998; Poblet-Icart *et al.* 1998). The reaction is catalyzed by malate carboxylase, the so-called malolactic enzyme, and requires the coenzyme  $\text{NAD}^+$  and  $\text{Mn}^{2+}$  (Fig. 3). Unlike the formation of lactic acid from the fermentation of glucose, only the L-isomer is produced during MLF (Fugelsang 1997; Lonvaud-Funel 1999).



**Fig. 3** Transformation of L-malic acid by the malolactic and malic enzymes.

This secondary fermentation may occur during or after alcoholic fermentation and is one of the most difficult steps to control (Lonvaud-Funel 1999). Normally, MLF occurs after completion of the alcoholic fermentation and represents a very important process in all quality red wines and some white wines, particularly in wines aged in oak barrels or matured in bottles.

*L. plantarum* is often used for conducting a pre-alcoholic MLF when partial malic acid degradation is desired. However, the species converts malic acid to lactic acid without the production of all the various favourable fermentation end-products and does not have the same impact on the flavours in the wine as observed when fermented with *O. oeni* (Nygaard 2001).

#### 4.2.1 Influence of malolactic fermentation on wine flavour

The wine industry is becoming increasingly aware that MLF not only affects the taste of wine through deacidification, but that it also contributes to other flavour characteristics. These malolactic flavours have been described as ‘malolactic’, ‘buttery’, ‘lactic’, ‘nutty’, ‘yeasty’, ‘oaky’ and ‘sweaty’. Furthermore, it is believed by some that MLF can enhance the fruity character of a wine (Henick-Kling 1994). MLF also contributes to wine flavour by giving it a smoother mouth feel when the strong green taste of malic acid is replaced by the less aggressive taste of lactic acid. It also increases the microbial stability of the wine due to the presence of lactic acid and possibly bacteriocins (Henick-Kling 1994) and increases the pH by 0.1 – 0.3 units (Lonvaud-Funel 1999). Deacidification is very important in wines produced from grapes grown in cold climates and with a high acid (tartrate and malate) content and low pH (Henick-Kling 1994). Decreased acidity of wines, produced from grapes grown in warm climates is generally not desirable. These wines are characterized by having too little acidity and a too high pH (Henick-Kling *et al.* 1994; Lonvaud-Funel 1999). Nevertheless, MLF is desired in some red and white wines produced from grapes grown in warm climates because of flavour changes. The descriptive analysis of the aroma of wines fermented by different bacteria and bacterial strains also show that significant differences can even exist between LAB. This indicates the uniqueness between the different LAB strains used to increase the organoleptic quality of the wine (McDaniel *et al.* 1987; Hugenholtz 1993).

MLF is also important in the production of sparkling wines by the “*méthode champenoise*” where it is used to advance the maturity of the wines used in the base blends. It also gives the wine some of its characteristic ‘nutty’ and ‘yeasty’ aromas, and stabilizes the wine against MLF during the second fermentation in the bottle (Pool and Henick-Kling 1991).

##### 4.2.1.1 Citric acid metabolism

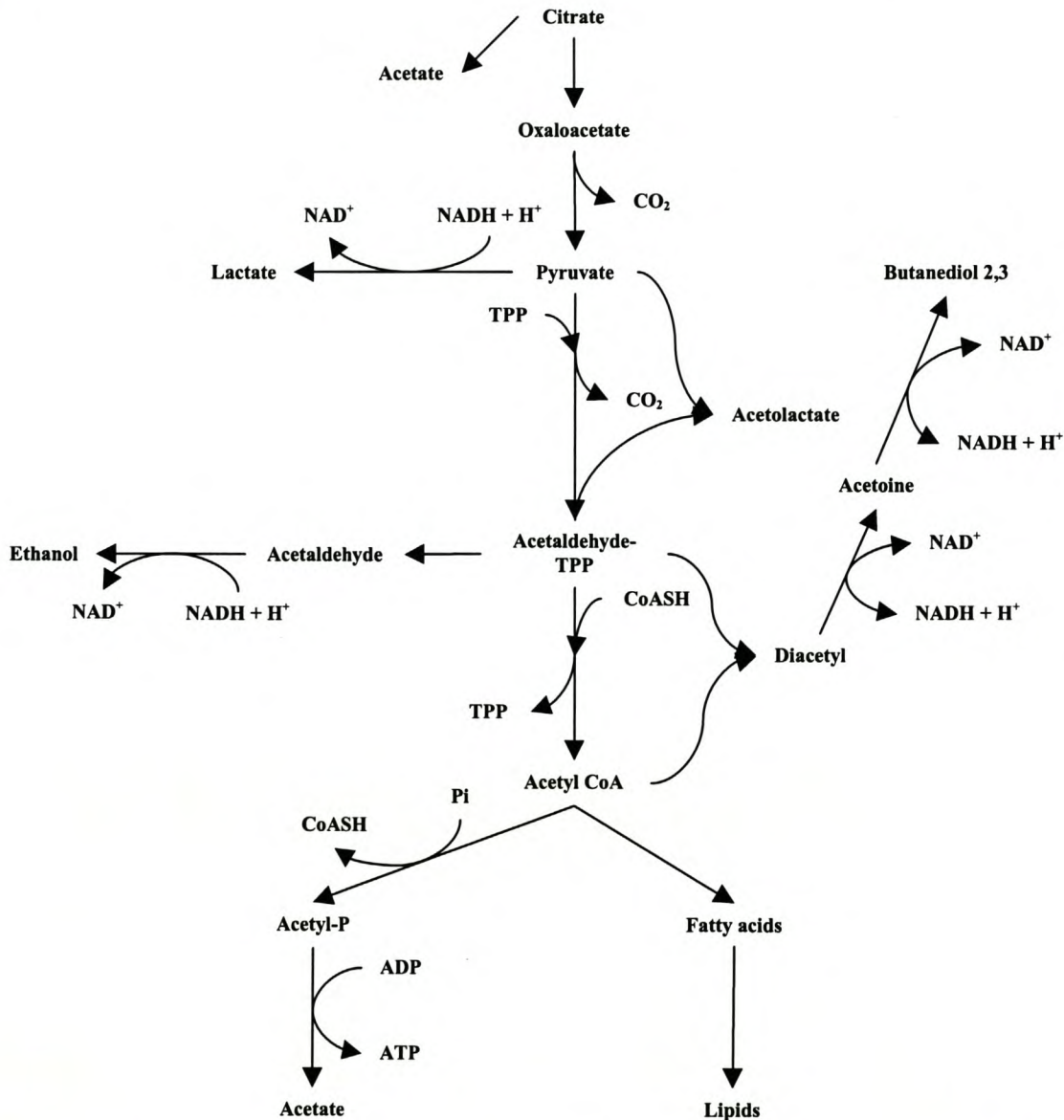
Wine flavour is not only modified by the degradation of malic acid, but also the degradation of citric acid by LAB (Fig. 4). All heterofermentative cocci (*Leuconostoc* and *Oenococcus*) and facultative heterofermentative lactobacilli e.g. *L. plantarum* and *L. casei*) in wines degrade citric acid. *O. oeni* metabolizes citric acid slower than malic acid. This metabolism continues until all

the citric acid is converted (Henick-Kling 1994; Garmyn *et al.* 1996). One of the intermediary compounds in the citric acid metabolism is diacetyl, which is considered to be one of the most important flavours produced during MLF (Rankine *et al.* 1969; Bertrand *et al.* 1984; Davis *et al.* 1985). Diacetyl gives wine the characteristic buttery or nutty taste when present at a concentration above the sensory threshold (Nielsen 2001). According to Martineau *et al.* (1995) this threshold values vary in different wines from approximately 0.2 mg/l in Chardonnay to 0.9 mg/l in Pinot Noir and 2.8 mg/l in Cabernet Sauvignon. Concentrations higher than these thresholds are not appreciated by all people and should be kept below these levels.

Diacetyl is further reduced by *O. oeni* to acetoin and 2,3-butanediol, which in normal concentrations have no influence on wine aroma (Nielsen 2001). The major products of citric acid degradation are acetic acid and acetoinic compounds (C4 compounds), which significantly affects the taste of wine (Henick-Kling 1994; Fugelsang 1997). Acetic acid, catalyzed by citrate lyase, is produced in the first reaction. Acetoinic compounds are produced from pyruvate resulting from the activity of citrate lyase and oxaloacetate decarboxylase (Fugelsang 1997; Lonvaud-Funel 1999). Pyruvate is usually reduced to lactate and reoxidizes NADH (Fig. 4). However, when additional pyruvate is produced from citric acid in the absence of sugar, it is shifted to the production of acetoin and butanediol (Henick-Kling 1994; Fugelsang 1997). The pathway for the production of C4 compounds entails that the C5  $\alpha$ -acetolactate is produced from two pyruvate molecules (Fig. 4). The enzyme  $\alpha$ -acetolactate decarboxylase catalyzes the reaction to acetoin, which accumulates in the medium or is reduced to butanediol (Fig. 4). Diacetyl could be produced by the chemical decarboxylation of  $\alpha$ -acetolactate (Hugenholz 1993; Henick-Kling 1994; Fugelsang 1997). Finally, the diacetyl reductase activity leads to acetoin fermentation. The key enzymes in this reaction are citrate lyase,  $\alpha$ -acetolactate synthase and  $\alpha$ -acetolactate (Garmyn *et al.* 1996).

Accumulation of diacetyl, acetoin and acetic acid in wine can vary according to the rate of MLF. When MLF is rapid, acetic acid production from a given amount of citric acid is relatively high and the resultant diacetyl and acetoin concentration low. On the contrary, when bacteria multiply slowly, less acetic acid and more diacetyl and acetoin are excreted (Henick-Kling 1994; Fugelsang 1997). Pyruvate in this pathway can also be shifted to the production of acetyl CoA and finally to fatty acids (Harvey and Collins 1963). It may also be assumed that in fast growing conditions (high pH and temperature) the need for the molecule to synthesize lipids and fatty

acids is much higher. Under conditions like these pyruvate is then converted towards acetyl CoA and less to acetoic compounds. When growth is limited (acidic pH and low temperatures) an excess of pyruvate leads to C4 compounds. Thus, in addition to the initial amount of citric acid in wine, the diacetyl level varies with the conditions during MLF (Henick-Kling 1994; Fugelsang 1997).



**Fig. 4** Citric acid metabolism by lactic acid bacteria. Redrawn from Lonvaud-Funel (1999).

The final concentration of diacetyl in wine is further affected by the diacetyl reductase activity of both yeast and bacterial lees. The prolonged contact of wine with lees reduces the diacetyl

concentration, while early racking or clarification enhances its concentration (Nielsen and Prah 1996; Nielsen 2001). In addition, diacetyl also combines irreversibly with sulfur dioxide like many other ketonic compounds of wine. The influence of citric acid metabolism on the sensory quality of wine, due to the complexity caused by diacetyl is, therefore, determined by several biochemical factors (Fugelsang 1997).

#### 4.2.1.2 Methylglyoxa

LAB might also be involved in the production of methylglyoxa. The concentration of this C3 compound (analogous to diacetyl) increases during MLF, but the aroma impact is probably much lower than that of diacetyl, since C3 compounds are lighter in aroma intensity than C4 compounds (De Revel and Bertrand 1993).

#### 4.2.1.3 Yeast-like aroma compounds

Some organoleptic compounds, revealed by yeast during alcoholic fermentation, disappear or change after MLF. This explains why MLF is sometimes favourable for red and white wines where the fruitiness attributed to the grape variety is not that essential. These wines are more complex in taste, with an improved mouthfeel that is often subjected to aging for a long time in barrels, and need bottle-aging to reach their plenitude. On the contrary, light red wines and some white wines, are characterized by the organoleptic quality of the specific grape variety and by having a “full body”, which can both fade when the wine undergoes MLF.

#### 4.2.1.4 Other aroma changes

Not all aroma changes that occur during MLF are attributed to citrate metabolism. The fruitiness of wine is enhanced while vegetative aroma compounds are reduced during MLF, for both white (Sauvignon and Semillon) and red wines (Cabernet Sauvignon) (Henick-Kling 1994). To date very few substances have been identified to explain such variations.

At completion of MLF, red wine colour and body are also affected as a result of modifications in phenolic compounds. MLF can also significantly decrease the reaction between tannins and

anthocyanins. During MLF, some of the phenolic compounds also precipitate or undergo structural changes. MLF in barrels again confers specific modifications which all combine to improve colour stabilization (Vivas *et al.* 1995).

#### 4.2.2 Lactic acid bacteria as wine spoilage organisms

##### 4.2.2.1 Increased wine acidity (volatile acidity)

LAB can spoil wine during wine-making or during maturation and bottle aging. During wine-making the heterofermentative bacteria (mainly *O. oeni*) may start to grow too early when they multiply at the end of alcoholic fermentation and not thereafter, as they should (Henick-Kling 1994). As a consequence, they ferment carbohydrates, particularly hexoses, which have not been totally fermented by the yeast. The volatile acidity of the wine increases due to the over-production of acetic acid and, when in limited amounts, causes the wine to depreciate. The quantity of acetic acid produced depends on the amount of hexoses and pentoses fermented and on the total bacterial population. If the wine exceeds the limit of 1 g/l volatile acidity (expressed as acetic acid) the wine is unmarketable. This (“piqûre lactique”) happens when the end of alcoholic fermentation is too slow or when it stops, due to high sugar concentrations, low pH, nitrogen deficiency and the excretion of toxic yeast metabolites (Lonvaud-Funel 1999).

Normally the interactions between yeast and bacteria, together with the effect of SO<sub>2</sub> added to the grape must, prevents premature bacterial growth. Difficulties can, however, be predicted when grapes are over-ripe, particularly when the climate is hot and dry when the grapes are harvested. Under these conditions, the high sugar and low acidity of must are both factors which are favourable for sluggish or stuck alcoholic fermentation or MLF (Henick-Kling 1994; Fugelsang 1997; Lonvaud-Funel 1999).

Fortified wines are also affected by volatile acidity or “piqûre lactique”. These wines are made with the addition of brandies or wine spirits (Cognac or Armagnac) to grape must which are more or less at the end of alcoholic fermentation. They contain a variable, but rather high concentration of hexoses and have a 16 to 20% ethanol level. Despite the high level of ethanol, heterofermentative lactobacilli often multiply in these wines. These bacteria are not only tolerant to ethanol, but may also depend on it for the repression of other bacteria, hence their own survival



(Couto and Hogg 1994). Most of the time *L. hilgardii* and *L. fructivorans* are the bacteria of concern. Since their generation time in the latter environment is long, multiplication is most often only evident during barrel aging or after bottling.

#### 4.2.2.2 Tartaric acid

The extent to which bacterially mediated tartaric acid degradation occurs in wines is unknown, but when observed, is part of a general spoilage scenario (Sponholtz 1993). Although not discounting the potential for bacterial conversion, the precipitation of tartrate, by itself, may account for the change (Kunkee, 1967; Rice and Mattick 1970). *L. brevis* and *L. plantarum* utilizes L(+)-tartaric acid (Krumperman and Vaughn 1966), whilst *L. brevis* first converts tartaric to oxaloacetic acid (Radler and Yannissis 1972). Subsequently, a portion of the intermediate is decarboxylated, yielding acetic acid and CO<sub>2</sub>. The rest of the intermediate is reduced and dehydrated to succinic acid. By comparison, *L. plantarum* decarboxylates oxaloacetic acid to pyruvic acid, which is subsequently reduced to lactic acid and decarboxylated to acetic acid (Radler and Yannissie 1972).

#### 4.2.2.3 Ropiness of wine

Pasteur described this “wine disease” in 1860 as ‘graisse’ and it is a real concern in many wines. It is characterized by an abnormal increase in viscosity, to such an extent that the wine turns thicker than oil. These ropy wines contain polysaccharides produced from residual sugars (less than 1 g/l) by strains of *P. damnosus*. This species is normally present in the grape must, but disappears almost completely during the wine-making process. Most of the *P. damnosus* strains are not spoilage agents and may even play an important part in MLF. Not all *Pediococcus* strains, however, synthesize exocellular polysaccharides (EPS) from glucose (Llauberes *et al.* 1990). This ability appears to be related to the presence of a 4 kb plasmid. The ropy strains are also much more tolerant to ethanol (Lonvaud-Funel 1999). They are also very tolerant to hostile conditions and even to SO<sub>2</sub> (perhaps because of the EPS layer around the cell). In most cases, the ropiness develops very slowly and only becomes evident several weeks or months after bottling.

#### 4.2.2.4 Production of off-flavours

During and after wine-making, off flavours may develop. Although these flavours have not been fully characterized, the yeast *Brettanomyces* (Chatonnet et al. 1995; Lonvaud-Funel 1999) ascribes for some of the volatile phenols in red wines in that it decarboxylates and reduces p-coumaric and ferulic acid.

However, LAB, especially *L. plantarum*, *L. brevis* and *Pediococcus*, could metabolize the phenol carboxylic acids, ferulic and p-coumaric acids (Carvin et al. 1993). *L. plantarum* may, however, not be involved in the deterioration due to its low frequency in wine during the wine-making process. The possible existence of *Pediococcus* strains capable of such activity should be more worrying.

In a complex interaction between the carbohydrate and amino acid metabolism in the presence of ethanol, heterofermentative species of lactobacilli and *O. oeni* produce 'mousy' off-flavours in wine when the heterocyclic volatile bases 2-acetyltetrahydropyridine, 2-ethyltetrahydropyridine and 2-acetyl-1-pyrroline are metabolized (Lonvaud-Funel 1999).

#### 4.2.3 Influence of lactic acid bacteria on the hygienic quality of wines

Biogenic amines and ethylcarbamate are two compounds that are released in wine by LAB during MLF (Lonvaud-Funel 1999). These compounds are, however, undesirable for the hygienic quality of wine.

In wine most of the aliphatic, aromatic and heterocyclic amines have been identified and include diaminobutane (putrescine), diaminopentane (cadaverine), ethylamine, isoamylamine, phenylethylamine, tyramine and histamine. They are produced by the decarboxylation of the corresponding amino acids isoamylamine, methylamine and putrescine, which are present in low concentrations in grape musts (Lonvaud-Funel and Joyeux 1994). Some strains of *O. oeni* are responsible for the production of histamine from histidine, although *L. hilgardii* also produces histamine (Corton et al. 1998a; Poolman 1993; Le Jeune et al. 1995). The time of production is, however, not restricted to the duration of MLF and can increase during wine storage, as reported by Conton et al. (1998b).

Another concern in wine is ethylcarbamate, known as a possible carcinogen. Precursors in the wine are urea, produced by yeast, citrulline, ornithine, carbamyl phosphate and ammonia produced by LAB via the arginine pathway (Hogg *et al.* 1996). *L. hilgardii* and strains of *O. oeni* are capable of performing the latter reactions (Liu *et al.* 1994, 1995, 1996).

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# **CHAPTER 5**

## **BIOFILMS IN THE FOOD AND BEVERAGE INDUSTRIES**



## CHAPTER 5

### BIOFILMS IN THE FOOD AND BEVERAGE INDUSTRIES

#### 5.1 INTRODUCTION

In any aqueous environment, suspended particles of organic and inorganic origin have the tendency to react with solid surfaces, thereby creating a conditioned film on the surface with higher nutrient levels which attracts biologically active particles, including planktonic (free-floating) organisms such as bacteria, bacteriophages, bacterial spores, yeast and molds (Zottola and Sasahara 1994). Following the initial attachment, the cells become irreversibly attached, grow and actively multiply to form micro-colonies (Kumar and Anand 1998). With time these colonies become large enough to entrap organic and inorganic debris, nutrients and other microorganisms leading to the formation of a complex biofilm community.

These biofilms may be of benefit or detrimental to the environment where they form (Zottola and Sasahara 1994). The ability of bacterial cells to attach to a surface, form a biofilm and act as a source of contamination is a major concern in the food, beverage, packaging, bottling and other industries. Processing facilities typically have an abundance of exposed surfaces in contact with liquids on which microorganisms can attach and form biofilms (Holah *et al.* 1994; Willcock *et al.* 1997; Eginton *et al.* 1998). There are numerous reports on biofilm formation by food borne pathogens (especially *Listeria monocytogenes*) and bacteria that contaminate related food products after adhesion to stainless steel surfaces (Zottola and Sasahara 1994; Kumar and Anand 1998). However, the effective removal and prevention of biofilm formation on these surfaces is still an unsolved matter that needs much more research.

#### 5.2 ATTACHMENT OF MICROORGANISMS TO SURFACE AREAS

Attachment of microorganisms to a negatively charged, conditioned surface and the development of a biofilm involve complex passive and active mechanisms that include bacterial motility or transport of planktonic cells by gravity, diffusion or fluid dynamic forces from the surrounding fluid phase (Kumar and Anand 1998). The adhesion of microorganisms to surfaces is also

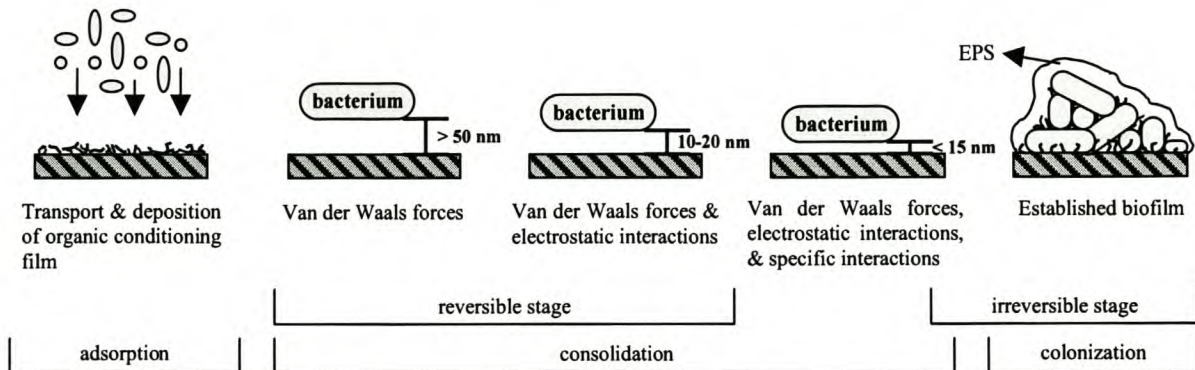
dependent on the species, growth stage of the cells, nutrient availability in the surrounding medium and other environmental factors.

### 5.2.1 Reversible and irreversible adhesion

Marshall *et al.* (1971) divided the adhesion process into two distinct phases, viz. reversible and irreversible adhesion, related to the force required for removal of the cells from the surface (Fig. 5). During reversible adhesion, bacteria along with other organic and inorganic molecules like proteins first deposit on the substratum forming a conditioned film (Characklis and Cooksey 1983). Subsequently, microbial adhesion to the conditioned surface occurs and is affected by surface free energy, electrostatic charges, cations and exopolymeric substances (EPS). These initial weak interactions between the bacterial cells and the substratum are referred to as reversible adhesion. During this stage, bacteria still show Brownian motion and can easily be removed by the shear forces of the fluid, e.g. merely by rinsing (Marshall *et al.* 1971; Busscher and Weerkamp 1987). Various long-range interaction forces which may influence the reversible adhesion process are the van der Waals attraction forces, electrostatic forces and hydrophobic interactions (Fig. 5) (Oliveira 1992; Zottola and Sasahara 1994; Flint *et al.* 1997; Kumar and Anand 1998). The nutrient transfer is also more rapid in a biofilm than for bacterial cells in aqueous phase, which in turn favours biofilm formation, depending on the type of competitive culture associated with the biofilm (Wolfaardt and Cloete 1992; Jeong and Frank 1994). The physiochemical properties of the bacterial cell surface are also important in the attachment process, especially in determining the adhesion of cells during the initial attachment phase (Van Loosdrecht *et al.* 1990).

Irreversible attachment of bacterial cells is the next crucial step in biofilm development. Repulsive forces prevent the bacterial cells to make direct contact with the surface at first. The contact then occurs due to the production of surface appendages by the bacteria such as flagella, fimbriae, pili and the exopolysaccharide fibrils on their surface area (Allison and Sutherland 1987; Costerton *et al.* 1987; Hancock 1991). Various short-range forces involved in irreversible adhesion include dipole-dipole interactions, hydrogen, ionic and covalent bonding, and hydrophobic interactions. If allowed to remain associated with the substratum, the cells eventually synthesize EPS (composed of carbohydrates and proteins) that exude from the cell

surface and directly binds the cell to the substratum. This bridge which is formed between the cell surface and solid substratum adhere the cell irreversibly to the surface to form an established biofilm (Marshall *et al.* 1971). The biofilm is then a functional consortium of microorganisms attached to the surface and is embedded in the EPS produced by the microorganisms (Costerton *et al.* 1987).



**Fig. 5** A summary of the stages involved in the formation of biofilms as reported by various authors. Modified from Oliveira 1992; Zottola and Sasahara 1994; Flint *et al.* 1997 and Kumar and Anand 1998.

Recently, electron micrographs of the ultrastructure of *L. monocytogenes* cells did not show the presence of EPS when studied with ruthenium red (Fletcher 1992). This suggested the possible presence of another EPS-producing microorganism that entraps cells of other microorganisms, i.e. *L. monocytogenes*, within their own EPS matrix (Sasahara and Zottola 1993). In the food industry, the high level of organic material remaining on food contact surfaces could result in an conditioned film onto which organisms can attach more easily, even those microorganisms that do not adhere tenaciously, i.e. *L. monocytogenes* (Sasahara and Zottola 1993).

Biofilm formation reaches a steady state when the cells at the edge of the biofilm, which protrude into the bulk liquid phase, are replenished when old biofilm cells are sloughed off (Costerton and Lappin-Scott 1989; Marshall 1992). In flowing systems, a continuous supply of nutrients ensures that cells are metabolically active at the outermost layer during steady state. In

static systems, this may not occur and biofilms become inactive until nutrients are again available for further growth (Costerton and Lappin-Scott 1989; Marshall 1992).

### 5.2.2 Surface areas

The topography of the substratum on which the biofilm establishes itself also plays an important role in the rate and efficiency of adhesion (Flint *et al.* 1997; Korber *et al.* 1997; Arnold and Bailey 2000). Bacteria can attach more easily to rough or uneven surfaces. The pH, temperature and charge of the contact surface also have an influence on the degree of adhesion of microorganisms (Herald and Zottola 1988a; Herald and Zottola 1988b; Shea *et al.* 1991). Removal of the cells at this stage requires much stronger forces, such as scrubbing or scraping (Marshall *et al.* 1971).

Stainless steel is an important surface concerning biofilm formation, as it is the most frequently used material for construction of vessels, piping, valves, and various types of equipment used in the food and beverage processing and storage industries (Kumar and Anand 1998; Arnold and Bailey 2000; Leriche and Carpentier 2000; Flint *et al.* 2001). It is a chemically and physiologically stable surface at a variety of processing temperatures (inert), easy to clean, and has a high resistance to corrosion (Stone and Zottola 1985; Zottola and Sasahara 1994). Many inert surfaces are negatively charged (Van Loosdrecht *et al.* 1989). The cell surface of bacteria at neutral pH also has a negative charge and varies depending on the microorganism and growth environment (Fletcher 1977). Due to their cell envelope, both Gram-positive and Gram-negative microorganisms may be regarded as negatively charged particles (Ward and Berkeley 1980). Although the net charge surrounding the cells do not have uniform density, it is the charge similarity that causes repulsion between cells and the substratum.

The other common surface areas involved in biofilm accumulation are the floors, waste water pipes, bends in pipes, rubber, nylon, polyvinyl, seals and conveyor belts. Buna-N and Teflon seals have also been implicated as important sites for biofilm formation (Blackman and Frank 1996). Aluminosilicate, Teflon and glass are also surfaces of concern (Kirtley and McGuire 1989; Sasahara and Zottola 1993). Since the substratum charge has a role in bacterial adherence, maximum cell adhesion occurs on highly charged substrates, such as glass and less on lower charged substrates such as polystyrene (Dexter *et al.* 1975).

Many studies have also shown the attachment of different microorganisms to poultry (Lillard 1988) and meat surfaces (Butler *et al.* 1979; Schwach and Zottola 1982). These organisms are not only associated with slaughtering processes, but are also responsible for cross contamination of uncontaminated carcasses (Anand *et al.* 1989).

### 5.2.3 Detachment and spreading of biofilms

For bacteria to survive and colonize new niches in their environment, the attached bacteria in an aging biofilm must be able to disperse from the biofilm. Bacteria from the biofilm, mainly daughter cells, get detached individually or are sloughed off. Sloughing is a discrete process whereby periodic detachment of relatively large particles or lumps of biomass from the biofilm takes place (Kumar and Anand 1998). This process in a biofilm can be due to various factors such as the fluid dynamics and the shear effects of the bulk fluid (Rittmann 1989; Applegate and Bryers 1991), presence of certain chemicals in the fluid environment or altered surface properties of the bacteria or substratum. The presence of air-borne microflora in the dairy industry is also considered to be major sources of contamination (Schröder 1984). The released bacteria may be transported to new locations in the environment where it can attach and again restart biofilm formation (Marshall 1992). The persistence of accumulated microorganisms in the formation of a biofilm may eventually cause post-processing contamination, leading to lowered shelf life of the product (Zottola 1994).

## 5.3 RESISTANCE OF BACTERIAL BIOFILMS

Various authors reported that, in addition to the important role in initial adhesion and firm anchorage of bacteria to a solid surface, EPS may also protect the bacteria from dehydration, antimicrobial agents (biocides, disinfectants and sanitizers), phagocytic cells and cleansing chemicals, which may facilitate in the trapping and retaining of nutrients from the environment (Ophir and Gutnick 1994; Somers *et al.* 1994; Oh and Marshall 1996; Fugelsang 1997; Eginton *et al.* 1998; Kumar and Anand 1998).

Other possible explanations for the increased resistance of a biofilm community, in addition to EPS, could be the (i) variation of physical and chemical environments associated with individual

bacterial cells or regions within the biofilm (e.g. variation in pH, osmotic strength or nutrients), (ii) variation in metabolic activity of cells within the biofilm, (iii) interaction of antimicrobial agents with the biofilm matrix (cells and polymer), and (iv) limitation of the free diffusion of antimicrobial agents through the biofilm matrix (Brown *et al.* 1988; Anwar *et al.* 1990; Gilbert 1990; Korber *et al.* 1993; Korber *et al.* 1997).

Bacteria attached to surfaces are physiologically different from planktonic cells (reviewed by Fletcher 1991). The biofilm can, therefore, survive for extended periods under severe stress conditions and only slowly becomes desiccated. It has also been demonstrated that bacterial biofilms develop increased resistance to antimicrobial treatments (Mustapha and Liewen 1989; Frank and Koffi 1990; Krysinski *et al.* 1992; Norwood and Gilmour 2000). Biofilms are known to be 10-1000 times more resistant to biocides (Cheung and Beech 1996). This resistance is attributed to the combined mechanisms and varied properties associated with the biofilm, including reduced diffusion, physiological changes due to reduced growth rates, the production of enzymes which degrade antimicrobial substances and the presence of an EPS matrix embedded with the component cells. EPS may act as a diffusion barrier, molecular sieve and adsorbent (Boyd and Chakrabarty 1995), therefore, the resistance to antimicrobials may decrease as soon as the 3-dimensional structure of the biofilm is disrupted (Faber *et al.* 1990; Hoyle *et al.* 1990; Hoyle *et al.* 1992; Stewart 1996). In cases of extensive biofouling, thick biofilms are formed which may include many metabolically dormant cells, with altered growth rates and physiology, also resulting in an increased resistance to antimicrobial agents (Gilbert *et al.* 1990; Evans *et al.* 1991; McFeters *et al.* 1995).

Biofilms can again rapidly regenerate in most environments after removal with clean-in-place processes, using cleansing chemicals and disinfectants (Jones 1994). They are also more resistant to detergents and sanitizers following treatment and can, therefore, provide a source of re-contamination for the next line or batch of production (Wirtanen and Mattila-Sandholm 1992a,b).

#### 5.4 BIOFILMS IN THE FOOD AND BEVERAGE INDUSTRIES

The formation of biofilms is a constant concern in the food and beverage industries, although their effect in the beverage industry is poorly studied. There are numerous reports on biofilm formation by food-borne pathogens and bacteria that contaminate related food products after

adhesion to processing surfaces and equipment, as discussed in section 5.2.2. The attachment of contaminant and spoilage bacteria to these products, packaging material and processing contact surfaces leads to serious food spoilage and hygienic problems (Carpenter and Cerf 1993; Kumar and Anand 1998). Foodborne pathogens and food spoilage bacteria on food contact surfaces include the organisms *L. monocytogenes* (Leriche and Carpentier 2000), *Yersinia enterocolitica* (Kumar and Singh 1994), *Campylobacter jejuni* (Stern and Kazmi 1989), *Escherichia coli* spp. (Dewanti and Wong 1995), *Pseudomonas* spp. (Boyd and Chakrabarty 1995), *Bacillus* spp. (Flint *et al.* 2001), *Staphylococcus* spp. (Leriche and Carpentier 2000), *Streptococcus* spp. (Zottola and Sasahara 1994), *Salmonella* spp. (Lillard 1986, 1988), *Shewanella* spp. (Bagge *et al.* 2001) and *Lactobacillus* spp. (Wong 1998). Previously, most of the research in this field focused on the adhesion (Arnold and Bailey 2000; Leriche and Carpentier 2000; Bagge *et al.* 2001), growth and development (Wolfaardt *et al.* 1994; Wong 1998; Flint *et al.* 2001), chemical removal (Eginton *et al.* 1995; Korber *et al.* 1997; Eginton *et al.* 1998) and control of the bacteria associated with stainless steel surfaces; mostly in the dairy, poultry and related processing industries.

The term 'biofouling' describes instances where biologically active biofilms are considered deleterious. A prevailing concept is that cells at the center of the biofilm are protected from chemical and physical forces such as antibiotics and sloughing. Although biofilm formation of bacterial starter cultures can, therefore, be considered as an advantage, they may act as a potential 'breeding ground' for bacteriophages also leading to stuck or incomplete fermentations, especially in the dairy and wine industries (Henick-Kling 1994; Wong 1998). Biofouling in heat exchangers and cooling towers has also been a major problem for many years. The bacterial attachment greatly reduces the heat transfer and operating efficiency of the processing equipment (Bott 1992; Lehmann *et al.* 1992; Mattila-Sandholm and Wirtanen 1992).

Biofouling is also common in the food industry as foulants in filtration membranes that impede proper and efficient filtration of milk and other liquid products. In recent years, membrane technologies like ultrafiltration (UF) and reverse osmosis (RO) have been widely used in the dairy, food and beverage industries and in waste water treatment processes (Glover 1985; Cheryan 1986). In the RO process for wastewater treatment, the development of a microbial biofilm contributes to a significant reduction in water flux and the deterioration of overall membrane performance (Ridgeway *et al.* 1983; 1984). In the case of the dairy and food processing industries, the UF/RO membrane systems find major application in the fractionation

and concentration of liquid foods like skim milk and whey, and the clarification of beverages, fruit juices and wine (Fugelsang 1997). An inherent feature of these processes is that the active membrane surface will come in contact with the feedstock. Even a small degree of adsorption causes pore blockage and as a result the filters get clogged, a phenomenon called fouling leading to a reduction in permeate flux rate and loss in production yields (Cheryan 1986; Flemming *et al.* 1992; Fugelsang 1997). This fouling of the membranes may further also favour the formation of biofilms (Kumar and Anand 1998). Filters with surface charges or hydrophobicities that can resist early colonization of microorganisms have been suggested as a partial solution for this problem (Zottola and Sasahara 1994).

#### 5.4.1 Control and removal of biofilms

The control of biofilms represents one of the most persistent challenges within the food, beverage and industrial environments. Increased emphasis is falling on adopting different strategies such as physical, chemical and biological methods to control and eliminate biofilms. In addition, biological means has been the latest field of interest for the biocontrol of bacterial biofilms.

The formation of biofilms in drinking water distribution systems leads to decreased water velocity and carrying capacity, clogging of pipes, increase in energy consumption and a decreased efficiency of operations (Ridgeway and Olson 1981; LeChevalier *et al.* 1987). Not even the presence of high levels of residual chlorine can prevent biofilm formation (Marshall 1992).

In the food processing industry routine clean-up programs utilize detergents or cleaning agents, sanitizers or disinfectants, or a combination, in their routine clean-up processes. The detergents or cleansing agents are composed of various chemicals that wet and penetrate the attachment area. This make it easier to remove alkalis that saponify fats and oils, agents to solubilize proteins, chelating compounds that bind and remove minerals, acids to remove deposited minerals, rinsing, deflocculating agents, dispersing agents to prevent redeposition of the bacterial soil and surfactants that act as wetting agents (Troller 1983; De Goederen *et al.* 1989; Zottola and Sasahara 1994). Detergents are essential to remove bacterial soils that contain or cover attached bacteria (Jackson 1985). Bacterial soil in a food processing system is



composed of both inorganic and organic matter, the latter in the form of proteins, fats and carbohydrates (Zottola and Sasahara 1994).

Sanitizers or disinfectants are used after the application of detergents to kill undesirable and pathogenic microorganisms associated with objects or materials, so that they pose no threat or disease (Black 1993). The chemical sanitizers or disinfectants that are used include the halogens chlorine and iodine, quaternary ammonium compounds, and various acids (Troller 1983). High temperatures can also be used as a sanitizing treatment (Graham-Rack and Binsted 1973). Enzymes have also proved effective in removing the EPS of an established biofilm. This would be another aid in the removal of biofilms (Kumar 1997). The choice of sanitizer to be used depends, however, on several factors that can vary with different applications.

Similar to other systems, biofilms on food-contact surfaces are showing increased resistance to conventional sanitizers like acid anionic biocides and quaternary ammonium compounds (Petrocci 1983; Mustapha and Liewen 1989; Frank and Koffi 1990; Kumar and Anand 1998). The reduced efficacy of reactive agents against biofilms is the result of incomplete penetration (Huang *et al.* 1995) and the wide variation in environmental conditions existing on food-contact surfaces (Kumar and Anand 1998). Biofilms can resist complete removal from wash-out flow processes or by cleansing chemicals and disinfectants (Jones 1994). This will lead to their rapid regeneration after treatment, providing a source of contamination for the next line or batch of production (Gilbert *et al.* 1997).

Comparative cleaning studies conducted on materials like stainless steel, glass, nylon and polyvinyl compounds showed no significant changes in the cleanability when the surfaces were still new (LeClercq-Perlat and Lalande 1994). However, with time, stainless steel exhibit better hygienic properties by resisting damage caused by the cleaning process. The use of sanitizers after cleaning processes, to eliminate residual bacteria, cause some degree of corrosion to these surfaces (Dunsmore 1981; Fugelsang 1997). The adherence of *Shewanella putrefaciens* (Bagge *et al.* 2001) and *Desulfovibrio vulgaris* (Jayaraman *et al.* 1999) to stainless steel and copper alloy surface coupled with their ability to produce sulfides and reduce iron, can also induce the corrosion of these surfaces. It has been estimated that corrosion damage due to these bacteria in the U.S. costs \$4-6 billion/year (Beloglazov *et al.* 1991).

The combination of chlorinated alkaline and alkaline detergents with sanitizers effectively reduce viable cell populations with more than 98% during cleaning and sanitizing procedures

(Czechowski and Banner 1992). Increased sanitizer contact times further reduced viable cell populations. Frequent cleaning and thoroughly drying of the process equipment after cleanup, provide another hurdle for biofilm-forming microorganisms to establishment a favourable niche (Czechowski and Banner 1992). Proper chemical composition and concentrations, the length of contact time, the temperature and pH of the substratum are also important factors for the efficient removal a biofilms regardless of the type and finish of the contact surfaces (Milledge and Jowitt 1980). The same requisites would apply for possible biological methods i.e. the use of bacteriocins.

Another important aspect essential in controlling biofilm and/or minimizing the biotransfer potential in food-processing equipment like tanks, pipelines, joints and other accessories are good design practices (Kumar and Anand 1998). These mainly include the proper choice of equipment, materials and accessories, type of finish of materials (refer to section 5.2.2), correct construction to avoid pits and cracks, process layout and process automation (Holah 1992; Mattila-Sandholm and Wirtanen 1992). Of the many surfaces in a food and beverage processing facility that could harbor potential biofilms, floors, floor drains and related non-food contact surfaces and processing equipment are especially difficult to clean and sanitize (Fugelsang 1997). Special care needs to be taken to clean these areas to avoid cross-contamination in the processing plant (Zottola and Sasahara 1994).

Although a few reports mentioned the effect of antimicrobial peptides and antibacterial agents on biofilms they focussed primarily on the effect these antibiotics have on biofilms of medical importance (Anwar *et al.* 1990; Widmer *et al.* 1990; Anwar *et al.* 1992; Khardori *et al.* 1995). The antimicrobial activity of surface-adsorbed nisin to food contact surfaces is another potential method to control pathogenic organisms during food and beverage processing (Bower *et al.* 1995). Food packaging material containing antimicrobial compounds has also gained practical importance in recent years. These antimicrobial compounds, incorporated in the packaging material, reduce and eliminate microbial contamination on the surfaces of packed foods (Ming *et al.* 1997; Kumar and Anand 1998). Similarly, the application of lactic cultures and their cell-free extracts have also been reported to selectively inhibit different spoilage and pathogenic microflora on the surfaces of dressed poultry (Anand *et al.* 1995). The application, therefore, of antimicrobial peptides/bacteriocins would not only have significant implications in the food

industry, but would also have applications in the beverage (e.g. wine, ciders, soft drinks, etc.), and the storage and packaging industries.

#### 5.4.2 Beneficial aspects of biofilms in food and related areas

Not all biofilms cause problems and can therefore also be positively used, i.e. in the manufacturing of vinegar (Frazier and Westhoff 1988). The dense growth of vinegar-producing organisms, such as species of *Acetobacter* and *Gluconobacter*, on free-floating wood chips increases surface area and efficiently converts substrate to product. Biofilms also found applications in the production of industrial products like ethanol and polysaccharides and are used for metal ore leaching (Bryers 1990; Macaskie *et al.* 1995). Even the gastrointestinal track are colonized by lactic acid bacteria and *Bifidobacterium* spp., which constitute a major part of the natural microflora (Fuller 1989), in turn also serves as a protective layer against the colonization of pathogenic bacteria. When these natural organisms are present in sufficient numbers it creates a healthy equilibrium between the beneficial and potentially harmful microflora in the gut (Collins and Hardt 1980; Vanbelle *et al.* 1989; Kumar and Anand 1998). These organisms also promote a probiotic effect when consumed through various fermented foods or even as a health supplement in freeze-dried form (Johannsen *et al.* 1993; Kumar and Anand 1998). The co-aggregation of bacteria can also enhance their attaching ability. In a review on co-aggregation among oral bacteria, Kolenbrander (1989) noted that human oral bacteria participate in intergeneric co-aggregation. The term “cooperative effects” among oral bacteria is used to describe the microbial process by which adhering cells modify their surrounding environment to designate a more favourable one for further attachment (Sjollema *et al.* 1990).

Established biofilms in trickling bed filters can be used for the microbial control and treatment of waste water effluent, sewage management and in waste gas treatment of the food and beverage industries (Pedersen *et al.* 1997; Raunkjaer *et al.* 1997). These established microbial biofilms are attached to the filter packing material where they remove organic and inorganic compounds from waste water so that the water can be reused or discharged into nearby lakes or streams (Zottola and Sasahara 1994). Bacteria present in these biofilms biodegrade many of the toxic compounds by minimizing the buildup of pollutants in the environment, thus also acting as pollutant monitors (Fuchs *et al.* 1996). The organic nutrient-trapping capabilities of biofilms are again responsible

for reducing the organic content of wastewaters before they are released into the water streams or used for irrigation. In recent years, considerable attention was also given to the use of biofilms for the bioremediation of various industrial effluents (Nigam *et al.* 1996), environmental disasters and in the nitrification process for the treatment of high strength nitrogen fertilizer wastewater (Cecen and Orak 1996).

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# CHAPTER 6

GROWTH OPTIMIZATION OF  
*PEDIOCOCCUS DAMNOSUS*  
NCFB 1832 AND THE INFLUENCE  
OF pH AND NUTRIENTS ON THE  
PRODUCTION OF PEDIOCIN PD-1

Accepted for publication in Journal of Applied  
Microbiology. Accession number: JAM /2001/6.

## Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1

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**Aims:** Optimization of the growth of *Pediococcus damnosus* NCFB 1832 and the production of pediocin PD-1 by traditional fermentation methods.

**Methods and Results:** Fermentation studies were conducted in De Man Rogosa and Sharpe (MRS) broth (Oxoid), pre-adjusted to specific pH values, and in MRS broth supplemented with various nitrogen sources, MnSO<sub>4</sub>, MgSO<sub>4</sub> and Tween 80. The production of pediocin PD-1 closely followed the growth curve of *Ped. damnosus* NCFB 1832. Maximum levels of bacteriocin activity (3249 AU ml<sup>-1</sup>/O.D.<sub>max</sub>) were recorded in MRS broth with an initial pH of 6.7. In media with an initial pH of 4.5 bacteriocin activity as low as 222 AU ml<sup>-1</sup>/O.D.<sub>max</sub> was recorded. The highest bacteriocin activity was recorded in growth conditions allowing the greatest pH variation (highest ΔpH). The addition of bacteriological peptone at 1.7% (w/v), MnSO<sub>4</sub> (0.014%, w/v) and Tween 80 (3%, v/v) to MRS and adjusting of the medium pH to 6.7, resulted in a further increase in activity (from 3249 to 5078 AU ml<sup>-1</sup>/O.D.<sub>max</sub>). The same medium, but an initial pH of 6.2 resulted in a 82.5% decrease in bacteriocin activity.

**Conclusions:** Pediocin PD-1 production is not only stimulated by the presence of specific growth factors (e.g. bacteriological peptone, MnSO<sub>4</sub> or Tween 80), but may also be stimulated by the lowering in pH during growth (highest ΔpH), and thus also the amount of organic acids produced.

**Significance and Impact of Study:** The production of pediocin PD-1 of the wild-type producer strain was significantly improved by using a defined medium and traditional fermentation methods.



## INTRODUCTION

Bacteriocins are defined as ribosomally synthesized antimicrobial peptides or proteins produced by bacteria, with antagonistic activity against bacteria genetically closely related to the producer strain (De Vuyst and Vandamme 1994b). The pediocin-like bacteriocins fall into class IIa and are defined as small heat-stable *Listeria*-active peptides (Klaenhammer 1993; Horn *et al.* 1998).

A number of bacteriocins produced by *Pediococcus* spp. have been described, viz. pediocin A produced by *Ped. pentosaceus* FBB-61 (Etchells *et al.* 1964; Flemming *et al.* 1975), PA-1 produced by *Ped. acidilactici* PAC 1·0 (Gonzales and Kunka 1987), AcH produced by *Ped. acidilactici* H (Bhunja *et al.* 1987, 1988), JD produced by *Ped. acidilactici* JD-23 (Richter *et al.* 1989), SJ-1 produced by *Ped. acidilactici* SJ-1 (Schved *et al.* 1993), N5p produced by *Ped. pentosaceus* (Strasser de Saad *et al.* 1995), 5 produced by *Ped. acidilactici* UL5 (Huang *et al.* 1996) and pediocin L50 that has been renamed as enterocin L50 (Cintas *et al.* 1998).

Little research has been done on the bacteriocins of *Pediococcus* spp., despite the fact that they cause spoilage in beer, wine and other high-pH food products. The spectra of antimicrobial activity of the pediocins produced by *Ped. pentosaceus* and *Ped. acidilactici* are very similar and the genes coding for their production share many conserved regions (De Vuyst and Vandamme 1994a). This is not surprising, since the latter two species are phylogenetically closely related (Collins *et al.* 1991). *Ped. damnosus* is, however, phylogenetically distant from *Ped. acidilactici* and *Ped. pentosaceus* (Collins *et al.* 1991). Furthermore, the spectrum of antimicrobial activity of pediocin PD-1, produced by *Ped. damnosus* NCFB 1832, is different from that recorded for other pediocins and unique in the sense that it does not inhibit *Pediococcus* spp. (Green *et al.* 1997).

Most of the research aimed at the increase of bacteriocin production is focussed on genetic manipulation of the producer strain. Little research has been done on fermentation optimization of bacteriocin-producing strains. For optimal bacteriocin production the producer strain usually requires complex nutrients, ions, Tween 80 and well-controlled growth conditions, such as temperature and pH (Biswas *et al.* 1991; De Vuyst and Vandamme 1992; Daba *et al.* 1993; Kaizer and Montville 1993; Parente and Ricciardi 1994; Parente *et al.* 1994; Yang and Ray 1994; Mørtvedt-Abildgaard *et al.* 1995; Leroy and De Vuyst 1999; Callewaert and De Vuyst 2000).

Bacteriocin production is usually growth associated, i.e. the level at which the peptide is produced is linked to the biomass formed (De Vuyst and Vandamme 1992; Mørtvedt-

Abildgaard *et al.* 1995; De Vuyst *et al.* 1996; Kim *et al.* 1997; Aasen *et al.* 2000; Callewaert and De Vuyst 2000). However, production rates do not always correlate with growth rate or biomass production (Kim *et al.* 1997; Bogovic-Matijasic and Rogelj 1998; Aasen *et al.* 2000). Enhanced production at non-optimum growth pH and/or temperature suggests that low growth rate, unfavorable growth conditions, or other less optimal environmental conditions may also stimulate bacteriocin production (De Vuyst *et al.* 1996; Aasen *et al.* 2000; Callewaert and De Vuyst 2000).

In this paper we report on the influence of fermentation conditions such as pH, various nitrogen sources, manganese, magnesium and Tween 80 on the growth of *Ped. damnosus* NCFB 1832 and the production of its bacteriocin, pediocin PD-1.

## **MATERIALS AND METHODS**

### **Bacterial strains and growth conditions**

The pediocin PD-1 producer strain, *Ped. damnosus* NCFB 1832, was grown in De Man Rogosa and Sharpe (MRS) broth (Oxoid, Basingstoke, Hampshire, UK) at 30 °C. The indicator strain, *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks *et al.* 1995) was grown in acidic grape broth (Dicks *et al.* 1990) at 30 °C.

### **Production of pediocin PD-1**

Production of pediocin PD-1 was followed during fermentation in 2 l MRS broth. The medium was inoculated with 1% (v/v) of a three-day-old culture of *Ped. damnosus* NCFB 1832 (O.D.<sub>600</sub> approx. 1.4). Fermentations were conducted at 30 °C for 115 h. Samples were withdrawn aseptically from the fermentation medium at regular time intervals and analyzed for cell growth (O.D.<sub>600</sub>) and bacteriocin production (AU ml<sup>-1</sup>), as described by Green *et al.* (1997).

### **Fermentation optimization of pediocin PD-1 production**

The inoculum size used in all fermentation experiments was 1% (v/v) of a three-day-old (O.D.<sub>600</sub> = 1.4) culture grown in MRS broth. All fermentations were conducted in duplicate, with three repeats of each fermentation run. The average of six pH measurements, optical density readings, and bacteriocin activity tests was thus determined.

**Growth pH.** The effect of pH on the growth of strain NCFB 1832 and the production of pediocin PD-1 were studied in 250 ml Erlenmeyer flasks. The pH of MRS broth was adjusted to 7.0, 6.7, 6.4 and 6.2 by adding sterile 5 N NaOH. To obtain initial pH values of 6.0, 5.8, 5.4, 5.0 and 4.5, the medium was adjusted with sterile 2 N HCl. All pH adjustments were done after autoclaving. Samples were taken at the end of the fermentation to determine the pH, maximum optical density (600 nm), bacteriocin activity (AU ml<sup>-1</sup>) and specific bacteriocin activity as described by Verellen *et al.* (1998).

**Effect of nitrogen sources.** The influence of different nitrogen sources were tested in MRS broth, supplemented with the following: 1.9% (w/v) meat extract (Oxoid), 1.9% (w/v) tryptone (Oxoid), 2.5% (w/v) yeast extract (Oxoid), 1.7% (w/v) bacteriological peptone (Oxoid), and 3.4% (w/v) casamino acids (Oxoid), respectively, as used by Verellen *et al.* (1998). Fermentations were conducted in sterile 2 l (Biostat<sup>®</sup> M, B. Braun Biotech International GmbH, Melsungen, Germany) fermentors with a maintained agitation speed of 50 rpm, without aeration and at a constant pH of 6.7 by automatic addition of sterile 5 N NaOH. The pH, optical density (O.D.<sub>600</sub>) of the culture and antimicrobial activity (AU ml<sup>-1</sup>) of pediocin PD-1 were determined directly after inoculation, after 5 and 10 h, and then every 7 h of fermentation.

**Effect of manganese, magnesium and Tween 80.** Fermentations were conducted in 200 ml MRS broth, supplemented with 1.7% (w/v) bacteriological peptone. The medium pH was adjusted to 6.7 by the addition of sterile 2 N NaOH. The concentrations of MnSO<sub>4</sub>·H<sub>2</sub>O used were between 0.004 and 0.064% (w/v). Magnesium was added in the form of MgSO<sub>4</sub>·7H<sub>2</sub>O (0.005%, w/v). Medium without MgSO<sub>4</sub>·7H<sub>2</sub>O served as control. Tween 80 was added at levels of 0.05%, 0.1%, 0.2%, 0.35%, 1.0%, 2.0% and 3.0% (v/v). Medium without Tween 80 served as control. Samples were taken after 27, 32, 48, 55, 72 and 96 h of fermentation. The pH, optical density of the culture and bacteriocin activity (AU ml<sup>-1</sup>) were recorded and the specific bacteriocin activity (AU ml<sup>-1</sup>/ O.D.<sub>600</sub>) calculated as described before.

**Optimization of growth medium and pediocin PD-1 production.** An optimal medium was compiled for the production of pediocin PD-1 based on results obtained from individual experiments. MRS broth was supplemented with 1.7% (w/v) bacteriological peptone, 0.014%

(w/v)  $\text{MnSO}_4$  and 3.0% (v/v) Tween 80 and adjusted to an initial pH of 6.7 with 5N NaOH before autoclaving. MRS broth not supplemented and inoculated at an initial pH of 6.2 served as control. The culture pH, optical cell density and activity ( $\text{AU ml}^{-1}$ ) of pediocin PD-1 were recorded after 27, 32, 38, 55, 72 and 96 h of fermentation.

## RESULTS

The pH measurements, optical density readings, and bacteriocin activity tests of the six fermentation runs did not vary by more than 5%. The data represents the average of six fermentation runs.

### **Pediocin PD-1 production during growth in MRS broth**

Growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1 were monitored in MRS broth without any additives (Fig. 1). The cells reached stationary phase after 84 h of fermentation. During this time the optical density of the culture increased to 2.13 (measured at 600 nm) and remained at that level for the remainder of the fermentation. Production of pediocin PD-1 more-or-less followed the growth curve and increased to  $1600 \text{ AU ml}^{-1}$  during the logarithmic phase of growth and remained at this level throughout the stationary phase. The pH decreased from 6.2 to 3.9 during the first 84 h of fermentation, but decreased further to pH 3.6 during stationary growth.

### **The effect of initial pH on growth and production of pediocin PD-1**

The effect of initial growth pH is shown in Table 1. The highest specific activity was obtained with an initial pH of 6.7, followed by growth at pH 7.0, 6.4, 6.2, 6.0, 5.8, 5.4, 5.0 and 4.5. Changes in pH that were recorded during growth ( $\Delta\text{pH}$ ) coincided with the  $\text{O.D.}_{\text{max}}$  values recorded for each of the fermentations, *i.e.* the culture with the highest cell density produced more acid, which in turn resulted in a lower end pH. The highest  $\Delta\text{pH}$  ( $\Delta\text{pH } 2.56$ ) was obtained for cells that started at an initial growth pH of 6.7.

### **The effect of different nitrogen sources on the growth and production of pediocin PD-1**

*Ped. damnosus* NCFB 1832 reached stationary growth phase within 38 h when the medium was supplemented with bacteriological peptone (Fig. 2A). Meat extract provided the second

best growth, followed by tryptone, yeast extract and casamino acids. Medium supplemented with bacteriological peptone and meat extract produced the highest level of pediocin PD-1 activity, followed by tryptone and yeast extract (Fig. 2B). Bacteriocin production occurred throughout logarithmic growth, but ceased at the onset of the stationary phase. For the remainder of the fermentation no reduction of bacteriocin activity was observed. All substrates resulted in similar maximum cell density ( $O.D._{max}$  between 2.0 and 2.3), except for casamino acids, which did not yield significant growth.

### **The effect of manganese, magnesium and Tween 80 on the specific activity of pediocin PD-1**

To study the effect of different concentrations of  $MnSO_4$ ,  $MgSO_4$  and Tween 80 on the growth of *Ped. damnosus* NCFB 1832 and pediocin PD-1 production, readings were taken at six specific time intervals and the results reported as  $AU\ ml^{-1}/O.D._{max}$ .

The maximum concentration of  $MnSO_4$  that was soluble in the experimental medium was 0.064% (w/v). The highest specific activity recorded in the presence of manganese was at an initial concentration of 0.014% (w/v)  $MnSO_4$  (Fig. 3). At this concentration the specific activity of pediocin PD-1 reached a peak of 2560 AU after 32 h of fermentation. Concentrations of 0.004% (w/v) and 0.014% (w/v)  $MnSO_4$  yielded specific activity levels of 1172 and 1800  $AU\ ml^{-1}/O.D._{600}$ , respectively, during the same growth period. The cultures reached similar optical density readings after 32 h of fermentation. After 55 h of fermentation in the presence of 0.014% (w/v)  $MnSO_4$  the specific activity decreased to approx. 2000  $AU\ ml^{-1}/O.D._{max}$ , which is similar to the maximum specific activity recorded when cultured in the presence of 0.024, 0.044 and 0.064  $g\ l^{-1}\ MnSO_4$ .

The addition of  $MgSO_4$  (0.05%, w/v) resulted in a very slight increase of growth, which was similar to that recorded with  $MnSO_4$ , but did not result in an increase in the specific production of pediocin PD-1 (data not shown).

Increased concentrations of Tween 80 stimulated the production of pediocin PD-1 (Fig. 4). The maximum concentration of Tween 80 that was soluble in the experimental medium was 3% (v/v). At this concentration the specific activity increased to 4000  $AU\ ml^{-1}/O.D._{max}$  after 55 hours of fermentation. Results recorded when cells were grown in the presence of 0.5, 1.0 and 2.0% (v/v) Tween 80 were similar. Lower concentrations of Tween 80 resulted in no drastic increase of pediocin PD-1 activity.

### Optimization of growth medium and pediocin PD-1 production

The effect on the production of pediocin PD-1 in 1 l MRS media supplemented with 1.7% (w/v) bacteriological peptone, 0.014 (w/v) MnSO<sub>4</sub> and 3% (v/v) Tween 80 is shown in Fig. 5. No significant difference with regard to maximum cell growth (O.D.<sub>max</sub>) and growth rate was observed between the optimized and control media (data not shown). However, specific bacteriocin activity increased by a factor of approx. 6 after 55 h of fermentation (Fig. 5 and Table 2).

### DISCUSSION

Pediocin PD-1 production occurred throughout logarithmic growth but stopped as the cells entered the stationary growth phase. A decrease in bacteriocin production after the cessation of growth is frequently observed for other bacteriocins (Parente and Ricciardi 1999). However, in our fermentation under controlled and uncontrolled pH no reduction in activity levels of pediocin PD-1 occurred during stationary growth phase. Thus, even though production of pediocin PD-1 is growth associated, conditions leading to higher maximum cell growth (O.D.<sub>max</sub>) did not necessarily result in higher levels of pediocin PD-1 production.

Many studies indicated that temperatures and pH levels lower than the optimal for growth resulted in higher levels of bacteriocin production. This was observed for lactococcin A (Parente *et al.* 1994), enterocin 1146 (Parente and Ricciardi 1994), lactocin S (Mørtvedt-Abildgaard *et al.* 1995), amylovorin 1471 (De Vuyst *et al.* 1996), nisin Z (Matsusaki *et al.* 1996) and mesenterocin (Kim *et al.* 1997). A few bacteriocins, including pediocin AcH and plantaricin C, are produced only at a pH below 5.0 (Biswas *et al.* 1991; Yang and Ray 1994; Bárcena *et al.* 1998). Pediocin AcH production at a low pH was attributed to posttranslational processing of the bacteriocin (Biswas *et al.* 1991). However, this effect is strain or species-dependent, since pediocin AcH is produced at pH 6.0 by *Lb. plantarum* WHE2 (Ennahar *et al.* 1996). In our case, a controlled pH of 6.7 resulted in a significantly lower pediocin PD-1 yield than at an uncontrolled initial pH of 6.7. In the latter case, the pH dropped dramatically to reach levels of approximately 3.5 in the stationary phase and allowed for the highest level of pediocin PD-1 production recorded. These results suggest that the production of pediocin PD-1 may increase when the pH level drops during the growth phase of the organism. A possible explanation for this observation may be found in less stringent binding of the peptide to the cell wall of *Ped. damnosus* NCFB 1832 as fermentation continues and the pH

decreases. A similar phenomenon has been described for nisin, produced by *Lactococcus lactis* subsp. *lactis* (Hurst and Dring 1968). More than 80% of nisin remained adsorbed to the producer cell at a pH of 6.8. However, at a pH below 6.0, more than 80% of the lantibiotic was present in the culture supernatant (Hurst and Dring 1968).

Bacteriocin production is strongly dependent on the composition and concentration of complex nutrients. Bacteriological peptone supported the fastest growth rate and resulted in the highest specific activity of pediocin PD-1. Despite the lower growth rate observed on meat extract, the level of pediocin PD-1 produced was similar to the level recorded in medium supplemented with bacteriological peptone. Otherwise, a more-or-less linear correlation was obtained between the rate of bacteriocin production and growth rate. All the nitrogen sources tested, except casamino acids, supported growth.

Both anions and cations affect bacteriocin production, but their influence may be strain specific (Parente and Ricciardi 1999).  $Mg^{2+}$  has been shown to increase pediocin ACh production (Biswas *et al.* 1991). The highest specific activity of pediocin PD-1 was achieved at an initial concentration of 0.014% (w/v)  $MnSO_4$ , approximately two times higher than recorded at a concentration of 0.004% (w/v). A further increase in  $MnSO_4$  concentration did not result in an increase in specific activity, but a slight decrease despite similar growth kinetics.

Increase of Tween 80 concentration resulted in the increase of specific pediocin PD-1 production up to the highest concentration tested (3%, v/v), despite a slight decrease in growth where a concentration of more than 1% (v/v) was added to the medium. Tween 80 appears to stimulate the production of some bacteriocins (Parente and Hill 1992; Daba *et al.* 1993; Matsusaki *et al.* 1996; Verellen *et al.* 1998). However, it may simply have the effect of preventing bacteriocin adsorption on polypropylene and glass surfaces (Joosten and Nuñez 1995), thus increasing apparent bacteriocin titres.

Conclusions from this study are that pediocin PD-1 production is not only stimulated by the presence of specific growth factors (e.g. bacteriological peptone,  $MnSO_4$  or Tween 80), but may also be stimulated by the lowering in pH during growth (highest  $\Delta pH$ ), and thus also the amount of organic acids produced.

## ACKNOWLEDGEMENTS

The authors would like to extend their gratitude to the post-graduate students and staff in the Laboratory of Industrial Microbiology and Biocatalysis, Ghent University, Ghent, Belgium for very valuable assistance and insights. This research was funded by Winetech.

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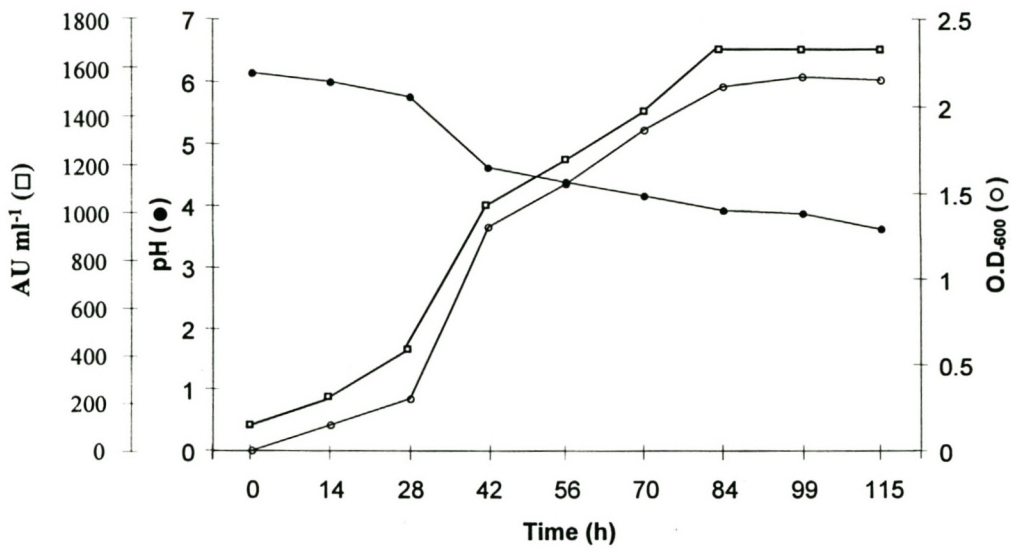
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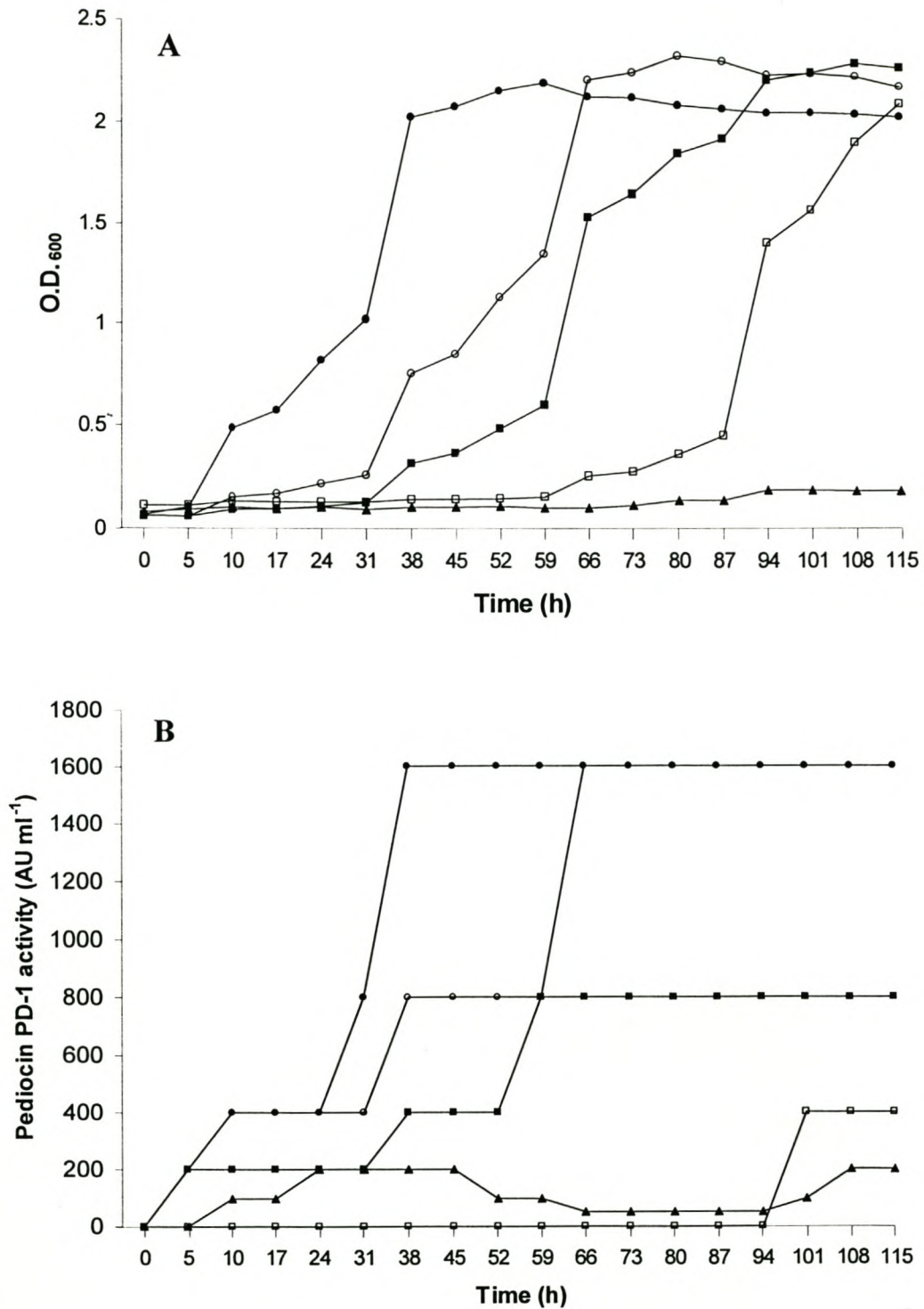
**Fig. 1** Production of pediocin PD-1 during growth of *Ped. damnosus* NCFB 1832.

●, Changes in pH; ○, optical density at 600 nm; □, pediocin PD-1 activity in AU ml<sup>-1</sup>.

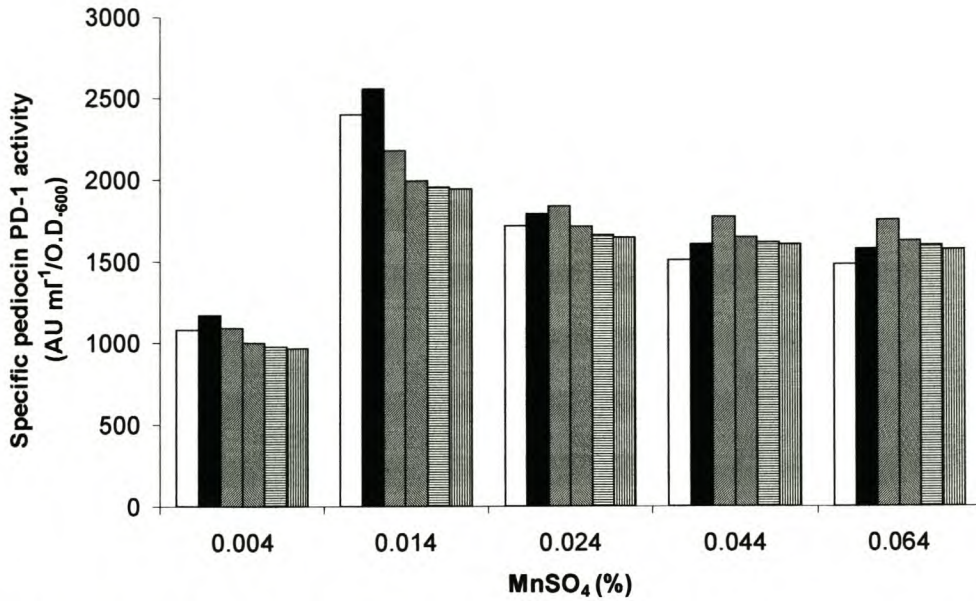
**Table 1** Effect of initial pH on the growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1\*

Initial pH	7.00	6.70	6.40	6.20	6.00	5.80	5.40	5.00	4.50
Final pH	5.04	4.14	4.40	4.37	4.39	4.34	4.29	4.25	4.43
$\Delta$ pH	1.96	2.56	2.00	1.83	1.61	1.46	1.11	0.75	0.07
O.D. <sub>max</sub>	1.039	1.970	1.890	1.864	1.721	1.663	1.484	1.008	0.451
Maximum bacteriocin production (AU ml <sup>-1</sup> )	2600	6400	3200	1600	1400	1200	800	400	100
Specific bacteriocin production (AU ml <sup>-1</sup> /O.D. <sub>max</sub> )	2502	3249	1693	858	814	722	539	397	222

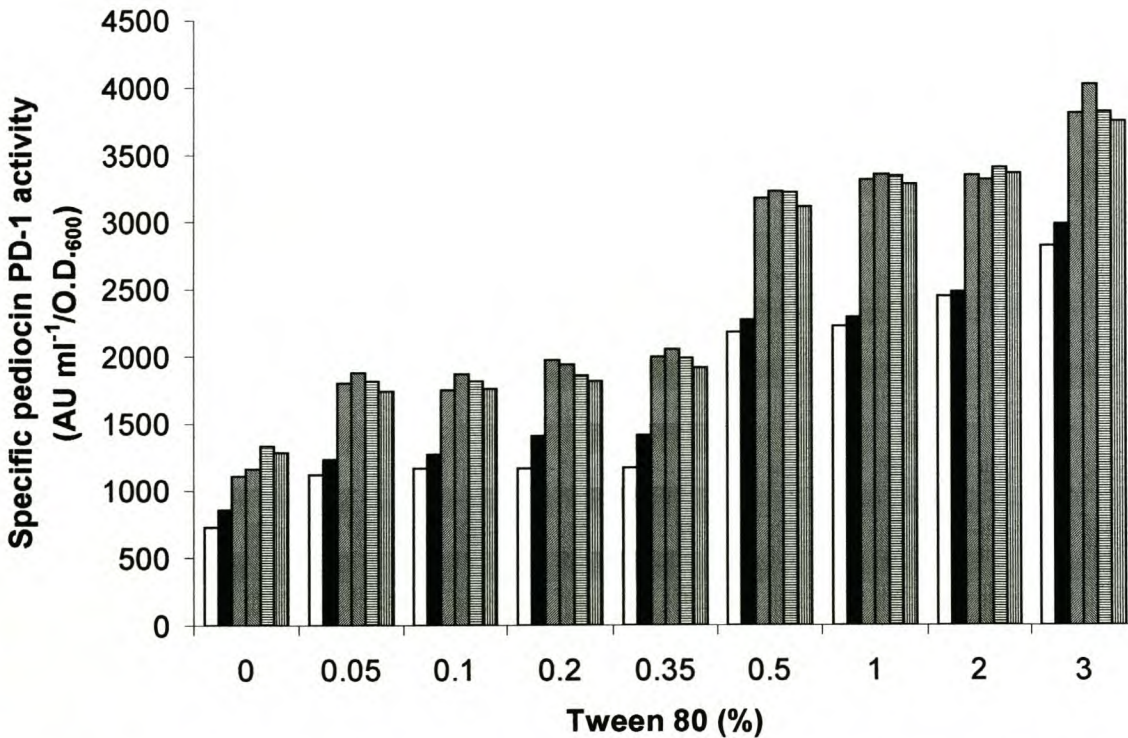
\*The values represent the average of two experiments.



**Fig. 2** (a) The effect of bacteriological peptone, meat extract, tryptone, yeast extract and casamino acids on the growth of *Ped. damnosus* NCFB 1832. (b) The effect of bacteriological peptone, meat extract, tryptone, yeast extract and casamino acids on the production of pediocin PD-1. ●, Bacteriological peptone; ○, meat extract; ■, tryptone; □, yeast extract; ▲, casamino acids.



**Fig. 3** The effect of MnSO<sub>4</sub> on the production of pediocin PD-1 (AU ml<sup>-1</sup>/O.D.<sub>600</sub>). Activity at: □, 27 h; ■, 32 h; ▨, 48 h; ▩, 55 h; ▤, 78 h; ▥, 96 h.



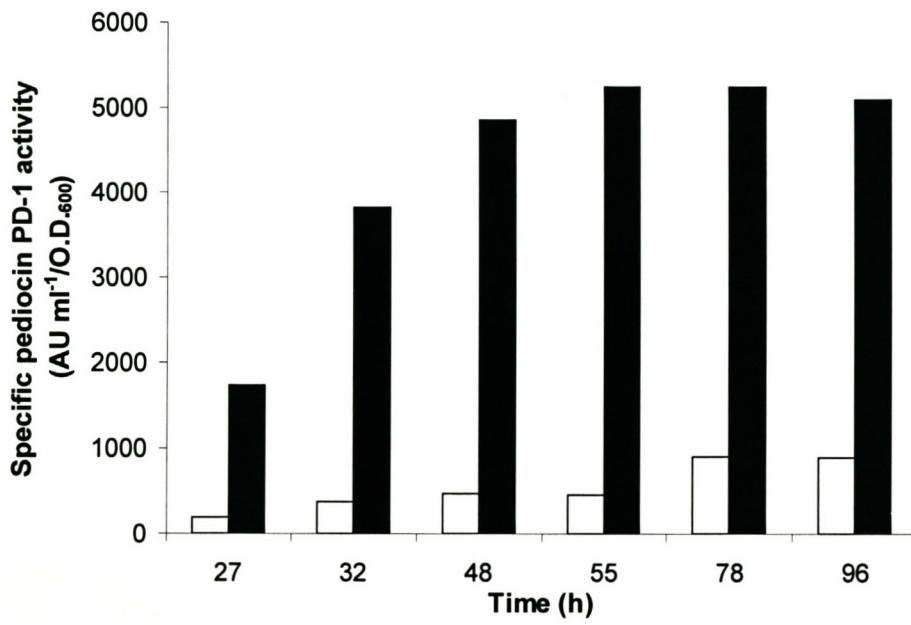
**Fig. 4** The effect of Tween 80 on the production of pediocin PD-1 (AU ml<sup>-1</sup>/O.D.<sub>600</sub>). Activity at: □, 27 h; ■, 32 h; ▨, 48 h; ▩, 55 h; ▤, 78 h; ▥, 96 h.

**Table 2** Effect of initial pH on the growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1 under optimized and control conditions \*

	Control	Optimized medium
Initial pH	6.2	6.7
Final pH	3.55	3.58
$\Delta$ pH	2.65	3.12
O.D. <sub>max</sub>	1.803	1.851
Maximum bacteriocin production (AU ml <sup>-1</sup> )	1600	9400
Specific bacteriocin production (AU ml <sup>-1</sup> /O.D. <sub>max</sub> )	887	5078

\*The values represent the average of two experiments.





**Fig. 5** The effect of the optimized (■) and control (□) media on the production of pediocin PD-1 (AU ml<sup>-1</sup>/O.D.<sub>600</sub>).

# CHAPTER 7

## THE EFFECT OF BACTERIOCINS PEDIOCIN PD-1, PLANTARICIN 423 AND NISIN ON BIOFILMS OF *OENOCOCCUS OENI* ON A STAINLESS STEEL SURFACE

Submitted for publication in American Journal of  
Enology and Viticulture.

## The Effect of Bacteriocins Pediocin PD-1, Plantaricin 423 and Nisin on Biofilms of *Oenococcus oeni* on a Stainless Steel Surface

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The effect of pediocin PD-1, plantaricin 423 and nisin was tested against established biofilms formed by a commercial starter culture of *Oenococcus oeni* on stainless steel disks and planktonic (free-living) cells. The percentage viable and non-viable cells were determined by using the BacLight™ viability probe and an epifluorescent microscope with image analysis. Pediocin PD-1 (3000 AU/ml) removed viable and non-viable cells of a biofilm of *O. oeni* in acidic grape medium after 5 h. Plantaricin 423 (3000 AU/ml) and nisin (3000 AU/ml) killed all viable cells in the biofilm. However, approximately 42% and 49% of non-viable cells remained on the stainless surfaces when treated with the latter two peptides, respectively. In a modified Chardonnay must, viable and non-viable cells of *O. oeni* in the biofilm were removed after 1 h when treated with pediocin PD-1 (3000 AU/ml). In similar experiments, treatment with plantaricin 423 and nisin killed all viable cells in the biofilm. Approximately 36% and 43% of the non-viable cells remained on the stainless steel surfaces after 5 h of treatment with the latter two peptides, respectively. After 5 h of treatment with the respective antimicrobial peptides (3000 AU/ml), the planktonic cell numbers of *O. oeni* decreased from  $1.37 \times 10^{10}$  cfu/ml to less than  $4.6 \times 10^{-5}$  cfu/ml in acidic grape medium. The same experiment performed in modified Chardonnay must yielded no detectable cells of *O. oeni*. To our knowledge, this is the first report on the application of bacteriocins in the destruction of malolactic biofilms on a stainless steel surface.

Malolactic bacteria occur throughout the winemaking process, but normally only commences after primary (alcoholic) fermentation (27). Malolactic fermentation (MLF) is considered important for the deacidification, flavor modification and microbial stability of wine, especially in wines produced from grapes grown in cool climates which often have a high acid (tartrate plus malate) content. MLF is, however, also desired in some white and red wines produced from grapes grown in warmer climates, since it often introduces favourable organoleptic compounds (15). Since MLF is a delicate process, it is important to control the presence of natural occurring malolactic bacteria.

Many wines, including Chardonnay, are fermented in stainless steel tanks prior to maturation in oak vats. Apart from this, the must and wine is pumped through stainless steel pipelines and valves. Adherence of malolactic bacteria to stainless steel surfaces may have a pronounced affect on the ability of malolactic bacteria to survive during the primary (alcoholic) fermentation and conduct MLF. Furthermore, if biofilms of malolactic bacteria are not effectively destroyed, they may contaminate wine in which MLF is undesirable or even lead to the development of bacteriophages which may cause stuck or sluggish MLF in wines dependent on a secondary fermentation.

Numerous papers have been published regarding biofilm formation by food-borne pathogens and bacteria that contaminate related food products after adhesion to stainless steel surfaces [reviewed by Kumar and Anand (25) and Zottola and Sasahara (36)]. Previously, most of the research in this field focused on the adhesion (2, 3, 26), growth and development (11, 35) and the chemical removal (9, 23) of bacteria from stainless steel surfaces. Although a few reports mentioned the effect of antimicrobial peptides and antibacterial agents on biofilms (1, 22), they focussed primarily on the effect these compounds have on biofilms of medical importance. Jayaraman *et al.* (20), however, reported on the effect of *in situ* production of gramicidin S by an established biofilm of *Bacillus brevis* prior to the colonization of the sulfate-reducing bacterium *Desulfovibrio vulgaris*, as a method to inhibit the growth of the latter bacterium on stainless steel surfaces. Bower *et al.* (4) reported on the antimicrobial activity of surface-adsorbed nisin to food contact surfaces to control pathogenic organisms (19).

Little is known regarding the effect bacteriocins have on stainless steel-adhered malolactic biofilm communities, hence nothing has been reported regarding the use of bacteriocins to control these biofilms in the wine industry. This study was aimed at determining if a commercial

starter culture of *O. oeni* is able to form biofilms on stainless steel and if so, if bacteriocins could be used to prevent biofilm formation.

## Materials and Methods

**Bacterial strains and culture conditions:** *Pediococcus damnosus* NCFB 1832 was grown in De Man Rogosa and Sharpe (MRS) broth (Oxoid) for 55 h at 30°C. The production of pediocin PD-1 was optimized by supplementing MRS broth (Oxoid) with bacteriological peptone (1.7%, w/v), MnSO<sub>4</sub> (0.014%, w/v) and Tween 80 (3%, v/v) and adjusting of the pH to 6.7 with 2N NaOH.

*Lactobacillus plantarum* 423 was cultured in MRS broth (Oxoid) for 15 h at 30°C. Optimal plantaricin 423 production was obtained in the latter medium, supplemented with tryptone, MnSO<sub>4</sub> and Tween 80 and adjusted to pH 5.8, as described by Verellen *et al.* (33). A strain of *Oenococcus oeni*, isolated from a commercial starter pack (Lallemand, Saint-Simon, France) was cultured in acidic grape broth (8) at 26°C.

**Preparation of bacteriocins:** Cell-free supernatants of pediocin PD-1 and plantaricin 423 were obtained by centrifuging 2 L of each culture at 9800 × g for 10 min at 8°C. These cell-free supernatants and a 1.3% (w/v) suspension of Nisin (1 × 10<sup>6</sup> I.U./g, Aplin & Barrett Ltd., Trowbrige, Wilts, England) were tested for antibacterial activity against metabolically active cells of *O. oeni* embedded in acidic grape soft agar (0.75% w/v agar), according to the spot-on-lawn technique (28), and expressed as AU (activity units)/ml. Based on the activity levels obtained, the supernatants of the three bacteriocins were diluted with sterile distilled water to represent equal activity levels (AU/ml). These supernatants were then individually freeze-dried and the lyophilisate of each re-dissolved in 10 ml sterile distilled water.

**The effect of pediocin PD-1, plantaricin 423 and nisin on biofilms formed by *O. oeni*:** Stainless steel (type 304, grade 2B finish), was cut into 75 × 25 × 2 mm disks. The disks were wiped with 96% (v/v) ethanol, rinsed five consecutive times in distilled water, submerged in distilled water and autoclaved. The disks were then aseptically transferred to a 250 ml screw-cap bottle containing 240 ml acidic grape medium and inoculated with *O. oeni* (1%, v/v, OD<sub>600</sub> = 0.6). After nine days of static incubation at 26°C the disks were aseptically removed from the bottle, rinsed for five seconds with sterile distilled water to remove non-adherent bacteria, stained

with 300  $\mu$ l of the Live/Dead *BacLight* viability probe (Molecular Probes Inc., Eugene, Oregon, USA) and left for 15 min in the dark at room temperature. Care was taken not to disturb the disks.

In a separate, but similar experiment, pediocin PD-1, plantaricin 423 and nisin suspensions were added to a 9-day-old culture of *O. oeni* to obtain final activity levels of 100, 500, 1000, 2000 and 3000 AU/ml, respectively. After periods of 1 h, 3 h and 5 h of incubation at 26°C, the disks were aseptically removed and studied under the microscope. Disks incubated in the absence of bacteriocins served as control.

Images of the biofilms on the disks were captured using a high performance CCD camera (Cohu) mounted on a Nikon Eclipse E400 epi-fluorescence microscope, equipped with an  $\times 60/1.4$  Dic H oil objective and filters. A minimum of 20 images was selected at random on each disk and the percentage viable and non-viable cells per optical field calculated. Corrections were made for spectral overlap and background fluorescence and the images analyzed with Scion Image software (U.S. National Institutes of Health; <http://rsb.info.nih.gov/nih-image>).

**The effect of pediocin PD-1, plantaricin 423 and nisin on planktonic cells of *O. oeni*:** Cells of *O. oeni* were grown for 9 days at 26°C to an optical density of approx. 1.1 (at 600 nm) in acidic grape broth (240 ml in a 250 ml screw-cap bottle), containing sterile stainless steel disks, as described before. The three bacteriocins were then added to the respective cultures to represent final concentrations of 100, 500, 1000, 2000 and 3000 AU/ml, respectively. Cultures to which no bacteriocins were added served as control. Stirring was kept to the minimum to maximize contact of the bacteriocin to the planktonic cells. By doing this, care was taken to avoid disturbance of biofilm formation on the disks. After 10 min, 30 min, 1 h, 3 h and 5 h, respectively, 100  $\mu$ l was withdrawn from each culture, diluted in sterile saline, plated out onto acid grape agar, incubated at 26°C and the number of viable cells determined after 14 days at 26°C.

**The effect of the three bacteriocins on biofilm formation and planktonic cells of *O. oeni* in a modified Chardonnay grape must medium:** Chardonnay must (2 L) was centrifuged at  $9800 \times g$  for 10 min at 8°C and the supernatant supplemented with 5% (w/v) yeast extract (Biolab) to stimulate bacterial growth. The must was then divided into 240 ml volumes and dispensed into 250 ml screw-capped bottles. The pH was adjusted to 4.5 with 2N NaOH and heated to boiling point. Sterile stainless steel disks were added to each of the three volumes and

inoculated with *O. oeni* and incubated at 26°C for nine days. The 9-day-old cultures then each received 3000 AU/ml of a specific bacteriocin. The effect of the bacteriocins on biofilm formation and the planktonic cells was recorded as described before.

**Replication of experiments:** All experiments were done in duplicate with a minimum of three repeats for each fermentation.

## Results and Discussion

Viable and non-viable cell counts obtained in the biofilms and planktonic cultures for all three repeats of each fermentation did not vary by more than 10%.

**Growth and attachment of *O. oeni* to stainless steel disks:** The planktonic cell density of *O. oeni* increased from 0.6 (O.D.<sub>600</sub>) to approximately 1.25 after nine days at 26°C. During this time the cells gradually adhered to the stainless steel surfaces to form mature and evenly dispersed biofilms. Approximately 75% and 86% of the surface areas of the disks submerged in acidic grape medium and in Chardonnay must, respectively, were covered with cells. Cells which emitted a green fluorescence had intact cell membranes and were viable, whereas cells which fluoresced red were in a bactericidal or bacteriostatic state. Based on these observations, only a small percentage of the cells in the biofilms (5 to 7%), whether generated in acidic grape broth or Chardonnay must, were non-viable.

**Effect of the bacteriocins on mature biofilms:** The addition of pediocin PD-1 to a 9-h-old biofilm of *O. oeni* resulted in a drastic decrease in viable cell numbers, as depicted in the differences recorded between the viable cell numbers in the control biofilm (no pediocin PD-1 added) versus the viable cell numbers recorded on the disks in the presence of the bacteriocin (Fig. 1A). In general the number of viable cells decreased as incubation in the presence of pediocin PD-1 increased from 1 to 5 h. After 1 h in the presence of 100 AU ml<sup>-1</sup> pediocin PD-1, less than 3% viable cells were detected in the biofilm and after a further 5 h of incubation, no viable cells were detected. The effect of pediocin PD-1 on the viable cells in the biofilm was more pronounced as the concentration increased to 500 AU/ml and above.

The non-viable cells in the biofilm increased from approximately 5% (control biofilm with no pediocin PD-1) to approximately 52% after 1 h in the presence of 100 AU/ml pediocin PD-1. The number of non-viable cells remained more-or-less the same after 5 h in the presence of

pediocin PD-1. However, at activity levels of 500 AU/ml and above, pediocin PD-1 resulted in a more efficient removal of the non-viable cells in the biofilm (from approx. 52% at 100 AU/ml to zero after 5 h at 3000 AU/ml).

In the presence of plantaricin 423 the number of viable cells in the biofilm decreased from 70% (control) to approximately 9% after 1 h and approximately 4% after 5 h at 100 AU/ml (Fig. 1B). As expected, very few viable cells were recorded after 1 h in the presence of 2000 AU/ml plantaricin 423 and no viable cells detected after longer incubation at this concentration or at higher levels of the bacteriocin. No drastic changes were recorded in the number of non-viable cells after 5 h in the presence of 100 AU/ml plantaricin 423 (Fig. 1B). However, a slight but consistent decrease in the number of non-viable cells were observed at higher levels of plantaricin 423 or when the biofilm remained for longer in contact with the same concentration of the bacteriocin. After 5 h of treatment with plantaricin 423 (3000 AU/ml) 42% of non-viable cells remained in the biofilm.

The biofilm treated with nisin (Fig. 1C) produced more-or-less the same pattern of viable cells than recorded for pediocin PD-1 (Fig. 1A) and plantaricin 423 (Fig. 1B). However, the number of non-viable cells in the biofilm increased at 100 and 500 AU/ml nisin as the contact time increased from 1 to 5 h, respectively (Fig. 1C). A decrease in non-viable cells were observed only at higher activity levels of nisin (1000 AU/ml and above). After 5 h of treatment with nisin (3000 AU/ml) 49% of non-viable cells remained in the biofilm.

**Effect of bacteriocins on planktonic cells:** After 9 days of incubation at 26°C the planktonic cells of *O. oeni* in the control medium, i.e. in the absence of bacteriocins, reached  $1.4 \times 10^{10}$  cfu/ml (not shown). An increase in the activity levels and contact time of each bacteriocin resulted in a more-or-less linear reduction in planktonic cell counts (Fig. 2A-C). Pediocin PD-1 (1000 AU/ml) reduced the planktonic cell numbers from  $1.4 \times 10^{10}$  cfu/ml to 630 cfu/ml after 3 h of contact (Fig. 2A). This correlates to a reduction of more than 99.99% in viable cell numbers. Similar results were recorded after 3 h with 2000 AU/ml plantaricin 423 (Fig. 2B) and 3 h with 500 AU/ml Nisin (Fig. 2C).

Based on results obtained in this study, Nisin proved to be the most bactericidal against planktonic cells (1 h at 3000 AU/ml), followed by plantaricin 423 (3 h at 3000 AU/ml) and pediocin PD-1 after (5 h at 3000 AU/ml) (Fig. 4).



**Effect of pediocin PD-1, plantaricin 423 and nisin on biofilm formation and planktonic cells of *O. oeni* in Chardonnay must:** The number of viable cells decreased from 80% (control) to almost zero after 1 h of incubation in the presence of 3000 AU/ml pediocin PD-1, plantaricin 423 and nisin, respectively (Fig. 3). In the presence of pediocin PD-1 the number of non-viable cells decreased to zero within 1 h (Fig. 3). However, in the presence of plantaricin 423 and nisin, the numbers of non-viable cells detected were 36% and 43%, respectively, after 1h of incubation, followed by a slight reduction after 3 and 5 h (Fig. 3).

The planktonic cells in the control medium, i.e. in the absence of bacteriocins, reached  $1.5 \times 10^{10}$  cfu/ml (not shown). Pediocin PD-1 reduced the planktonic cell numbers by 100% after 5 h of contact, whilst plantaricin 423 and nisin yielded the same result after 3 h and 1 h, respectively (Fig. 4).

It is well known that biofilms on food-contact surfaces show increased resistance when treated with conventional sanitizers such as acid anionic biocides and quaternary ammonium compounds (12, 16, 25). The reduced efficacy of antimicrobial agents is likely due to the ineffective penetration of biofilms (18) or variation in environmental conditions on the contact surface (25). In some studies where disinfectants proved to be effective, a rapid re-formation of biofilms were reported (21).

Concluded from the results obtained in the present study, the three bacteriocins not only removed viable cells of *O. oeni* from a mature biofilm, but also non-viable cells. Pediocin PD-1 proved to be the most effective, whether tested in acidic grape broth or Chardonnay must. This is a remarkable result, since microbial cells in biofilms are known to be as much as 1000 times more resistant to biocides (6, 24, 30, 34). In cases of extensive biofouling, thick biofilms are formed which may include many metabolically dormant cells with altered growth rates and physiology, resulting in increased resistance to antimicrobial agents (10, 13, 29).

Resistance of biofilms to antimicrobial agents is attributed to the combined mechanisms and varied properties associated with the biofilm, including reduced diffusion, physiological changes due to reduced growth rates, the production of enzymes degrading antimicrobial substances and often exopolysaccharide (EPS) matrixes (9, 23, 25). Although EPS may act as a diffusion barrier, molecular sieve and adsorbent (5), the resistance to antimicrobial compounds is lost as soon as the three-dimensional structure of the biofilm is disrupted (17, 31). Since many strains of

*O. oeni* are known to produce EPS (32), this is an important hurdle to take into consideration when selecting an antimicrobial agent to prevent biofilm formation.

The three bacteriocins included in this study are also active against other lactic acid- and malolactic bacteria, including certain food-spoilage bacteria (7, 14, 33). Similar studies have to be conducted to determine the effect of pediocin PD-1, plantaricin 423 and nisin in other food- and beverage environments.

The use of antimicrobial peptides to control the formation of biofilms offers a promising alternative to conventional treatment strategies, especially in the wine industry where the implementation of chemical disinfectants, including SO<sub>2</sub>, is becoming more restricted.

## Conclusions

The three bacteriocins included in this study, pediocin PD-1, plantaricin 423 and nisin, successfully killed all viable cells in an established biofilm of *O. oeni* which formed in acidic grape medium. Apart from being the most effective against viable cells of *O. oeni* in the biofilm, pediocin PD-1 also proved to be the most effective in the removal of non-viable cells from the stainless steel surfaces. Similar results were obtained in Chardonnay must. Based on results obtained in this study, it is safe to assume that all cells of *O. oeni* (viable and non-viable) will be removed from these stainless steel surfaces after 5 h at a concentration of 3000 AU/ml pediocin PD-1. These results also suggest that, of the three bacteriocins evaluated, pediocin PD-1 would be the best choice to prevent the potential re-formation of malolactic biofilms on stainless steel surfaces. The application of bacteriocins in the control of bacterial biofilm formation could be one of the answers to a safer and environmentally friendlier method of sanitation.

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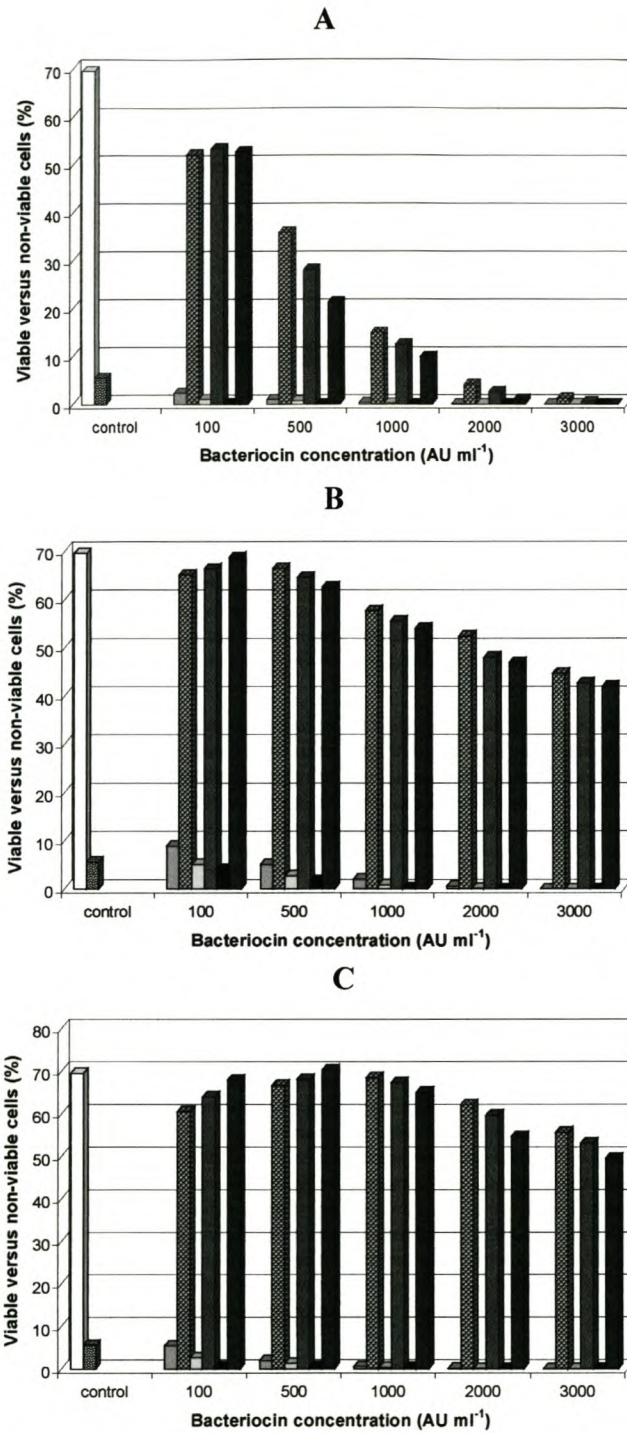


FIG. 1. The effect of different concentrations of pediocin PD-1 (A), plantaricin 423 (B) and nisin (C) on an established biofilm of *O. oeni* in acidic grape medium at specific time intervals.

Symbols: □ , control viable (i.e. no bacteriocin added); ▣ , control non-viable (i.e. no bacteriocin added); ■ , 1 h viable; ▤ , 1 h non-viable; □ , 3 h viable; ▤ , 3 h non-viable; ■ , 5 h viable; ▤ , 5 h non-viable.

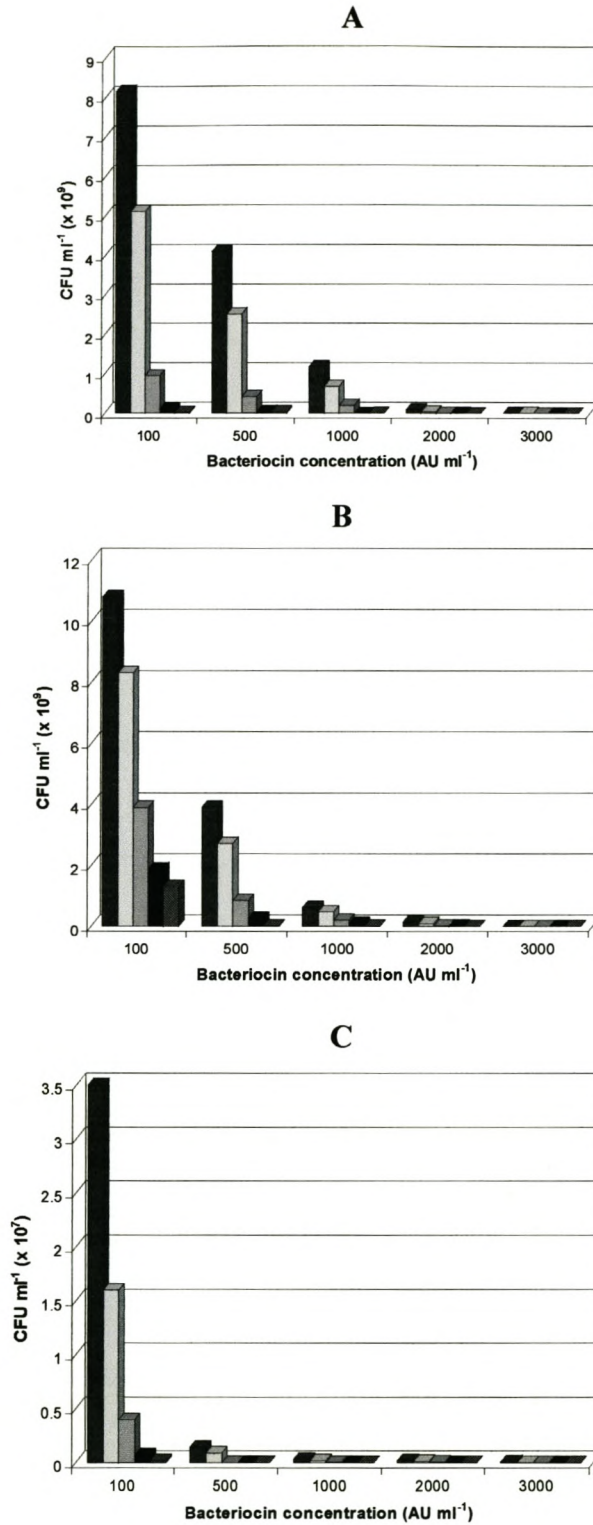


FIG. 2. The effect of different concentrations of pediocin PD-1 (A), plantaricin 423 (B) and nisin (C) on planktonic cells of *O. oeni* at specific time intervals.

Symbols: ■, 10 min; □, 30 min; ▒, 1 h; ■, 3 h; ▒, 5 h.

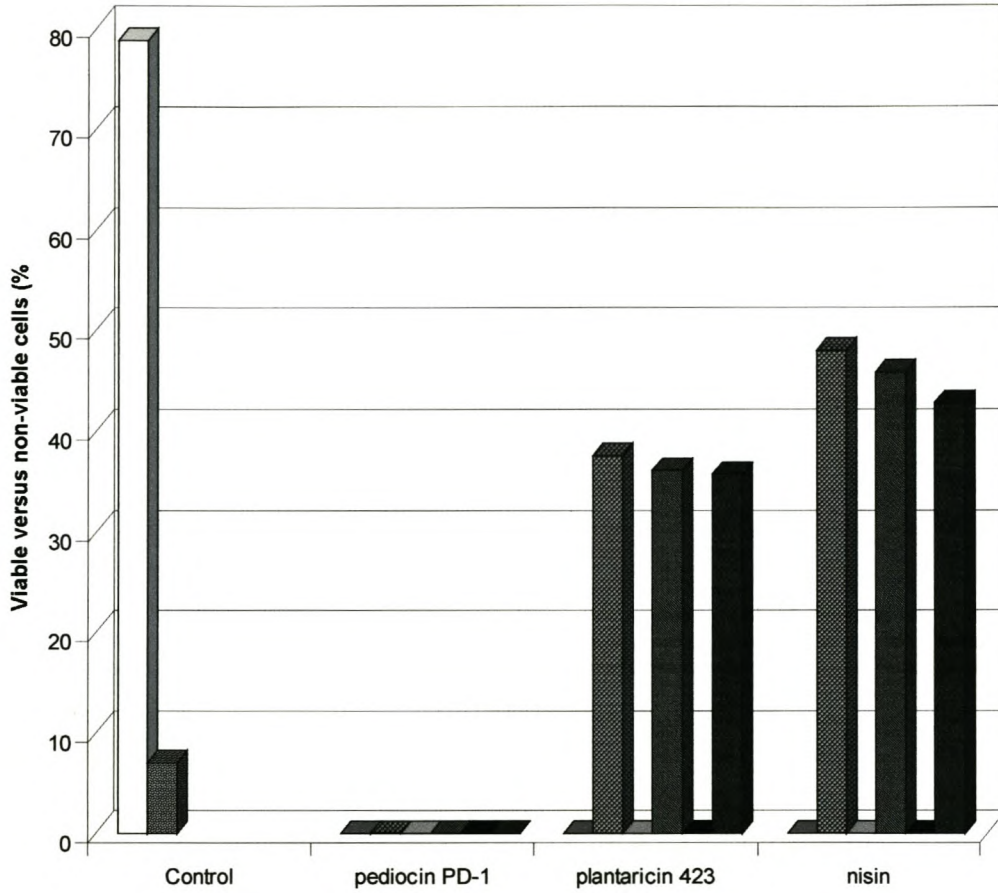


FIG. 3. The effect of 3000 AU ml<sup>-1</sup> of pediocin PD-1, plantaricin 423 and nisin on an established biofilm of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

Symbols: □, control viable (i.e. no bacteriocin added); ▣, control non-viable (i.e. no bacteriocin added); ■, 1 h viable; ▤, 1 h non-viable; □, 3 h viable; ▤, 3 h non-viable; ■, 5 h viable; ▤, 5 h non-viable.



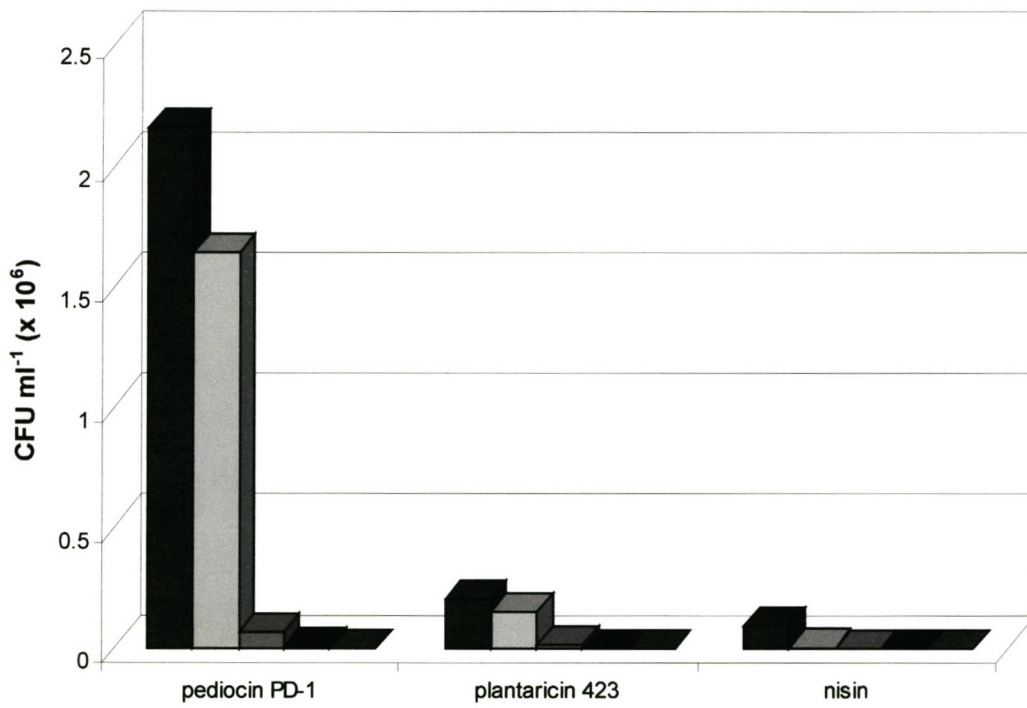


FIG. 4. The effect of pediocin PD-1, plantaricin 423 and nisin (3000 AU ml<sup>-1</sup>) on planktonic cells of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

Symbols: ■, 10 min; □, 30 min; ▒, 1 h; ■, 3 h; ▒, 5 h.

# **CHAPTER 8**

## **GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH PROSPECTS**

## CHAPTER 8

### GENERAL DISCUSSION, CONCLUSIONS AND FUTURE RESEARCH PROSPECTS

#### 8.1 INTRODUCTION

Lactic acid bacteria (LAB) are widespread in fermentable foods and have probably, unknowingly, been used by our ancestors ever since the Late Stone Age, throughout the infancy of the West and Middle Ages, until rediscovered in the nineteenth century. Today, the world depends on these organisms that are used for i.e. fermented milk, meat, vegetables and wine products. If controlled, they alter the flavour, texture and appearance of raw food and beverages in a desirable way. LAB have GRAS (generally regarded as safe) status and can, therefore, be commercially used in several food products. Since the demand for safe and healthy food and beverage products is on the increase, it is becoming increasingly important to use methods that are environmentally safe, e.g. free of chemicals and without genetically modified organisms.

In this study we optimized the production of pediocin PD-1, produced by the wild-type strain *Pediococcus damnosus* NCFB 1832, by adding readily available nitrogen sources to the fermentation broth. The affect of pediocin PD-1 on cells of *Oenococcus oeni* in an established stainless steel biofilm was determined as a possible means of control of malolactic fermentation. Comparative studies were conducted by including plantaricin 423 (previously optimized in our laboratory) and nisin in these studies.

#### 8.2 FERMENTATION OPTIMIZATION OF PEDIOCIN PD-1

The first aspect of this study was to determine the influence of fermentation conditions such as pH, various nitrogen sources, manganese, magnesium and Tween 80 on the growth of *Ped. damnosus* NCFB 1832 and the production of its bacteriocin, pediocin PD-1, in De Man Rogosa and Sharpe (MRS) broth (Oxoid). Pediocin PD-1 production occurred throughout logarithmic growth, but stopped as the cells entered stationary growth. A decrease in bacteriocin production after the cessation of growth is frequently observed for other bacteriocins (Parente and Ricciardi 1999). However, in this fermentation, under controlled and uncontrolled pH, no reduction in

activity levels of pediocin PD-1 occurred during stationary growth. Thus, even though production of pediocin PD-1 is growth associated, conditions leading to higher maximum cell growth ( $O.D._{max}$ ) did not necessarily result in higher levels of pediocin PD-1 production.

Many studies indicated that temperatures and pH levels lower than the optimal for growth resulted in higher levels of bacteriocin production. This was observed for lactococcin A (Parente *et al.* 1994), enterocin 1146 (Parente and Ricciardi 1994), lactocin S (Mørtvedt-Abildgaard *et al.* 1995), amylovorin 1471 (De Vuyst *et al.* 1996), nisin Z (Matsusaki *et al.* 1996) and mesenterocin (Kim *et al.* 1997). A few bacteriocins, including pediocin AcH and plantaricin C, are produced only at a pH below 5.0 (Biswas *et al.* 1991; Yang and Ray 1994; Bárcena *et al.* 1998). Pediocin AcH production at low pH levels was attributed to posttranslational processing of the bacteriocin (Biswas *et al.* 1991). However, this effect is strain- or species-dependent, since pediocin AcH is produced at pH 6.0 by *Lactobacillus plantarum* WHE2 (Ennahar *et al.* 1996). In our case, a controlled fermentation pH of 6.7 resulted in a significantly lower pediocin PD-1 yield compared to an uncontrolled initial medium pH of 6.7. In the latter case, the pH dropped dramatically to reach levels of approximately 3.5 in the stationary phase and allowed for the highest level of pediocin PD-1 production recorded. These results suggested that the production of pediocin PD-1 might increase when the pH levels decrease during growth. A possible explanation for this observation may be found in the less stringent binding of the peptide to the cell wall of *Ped. damnosus* NCFB 1832 as fermentation continues and the pH decreases. A similar phenomenon has been described for nisin, produced by *Lactococcus lactis* subsp. *lactis* (Hurst and Dring 1968). More than 80% of nisin remained adsorbed to the producer cell at a pH of 6.8. However, at a pH below 6.0, more than 80% of the lantibiotic was present in the culture supernatant (Hurst and Dring 1968).

Bacteriocin production is strongly dependent on the composition and concentration of complex nutrients. Bacteriological peptone supported the best growth rate and resulted in the highest specific activity of pediocin PD-1. Despite the lower growth rate observed in the presence of meat extract, the level of pediocin PD-1 produced was similar to the level recorded in medium supplemented with bacteriological peptone. Otherwise, a more-or-less linear correlation was obtained between the rate of bacteriocin production and growth rate. All the nitrogen sources tested, except casamino acids, supported growth.

Both anions and cations affect bacteriocin production, but their influence may be strain specific (Parente and Ricciardi 1999). Magnesium increased pediocin AcH production (Biswas *et al.* 1991). It did not, however, have any effect on the growth of *Ped. damnosus* NCFB 1832 and the production of pediocin PD-1. Growth and production increased, however, with a slight increase of  $\text{MnSO}_4$  concentration.

An increase in Tween 80 resulted in the increase of specific pediocin PD-1 production. The specific bacteriocin activity increased from 3249 AU/ml/O.D.<sub>max</sub> to 4000 AU/ml/O.D.<sub>max</sub> at a concentration of 3% (v/v) Tween 80. Tween 80 also appears to stimulate the production of some bacteriocins (Parente and Hill 1992; Daba *et al.* 1993; Matsusaki *et al.* 1996; Verellen *et al.* 1998). However, it may simply have the effect of preventing bacteriocin adsorption on polypropylene and glass surfaces (Joosten and Nuñez 1995), thus increasing apparent bacteriocin titres.

Pediocin PD-1 production was not only stimulated by the presence of specific growth factors, e.g. bacteriological peptone (1.7%, w/v),  $\text{MnSO}_4$  (0.014%, w/v) or Tween 80 (3%, v/v), but also by the lowering in pH during growth (highest  $\Delta\text{pH}$ ), and thus also the level of organic acids produced. A combination of all these factors resulted in the increase of specific bacteriocin activity of pediocin PD-1 with a factor of approximately 6, after 55 h of fermentation.

### 8.3 EFFECT OF BACTERIOCINS ON ESTABLISHED BIOFILMS OF *O. OENI*

Numerous papers have been published on biofilm formation, especially on stainless steel, by food-borne pathogens and bacteria that contaminate related food products (Zottola and Sasahara 1994; Kumar and Anand 1998). The control of biofilms represents one of the most persistent challenges within the food, beverage and industrial environments where microbial communities are problematic. Increased emphasis is falling, therefore, on adopting different strategies like physical, chemical and biological methods for biofilm elimination.

Previously, most of the research in this field was focused on the adhesion (Arnold and Bailey 2000; Leriche and Carpentier 2000; Bagge *et al.* 2001), growth and development (Wolfaardt *et al.* 1994; Wong 1998; Flint *et al.* 2001) and the chemical removal (Eginton *et al.* 1995; Korber *et al.* 1997; Eginton *et al.* 1998) of these bacteria from stainless steel surfaces. Very little has been reported on the effect bacteriocins have on a stainless steel-adhered biofilm community,

especially in the beverage industries. It is well established that bacterial biofilms exhibit an increased resistance to antimicrobial treatments (Frank and Koffi 1990; Kryszinski *et al.* 1992; Norwood and Gilmour 2000), since biofilms are known to be 10-1000 times more resistant to biocides (Cheung and Beech 1996). This resistance is attributed to the combined mechanisms and varied properties associated with the biofilm i.e. reduced diffusion, physiological changes due to reduced growth rates, the production of enzymes degrading antimicrobial substances and the presence of an exopolysaccharide (EPS) matrix embedded with the component cells as discussed in Chapter 5.

We have tested the effect of the three bacteriocins, pediocin PD-1, plantaricin 423 and nisin against an established biofilm of *O. oeni* on stainless steel. These bacteriocins were able to overcome the biofilm barriers, penetrated the EPS and successfully killed all living cells. Pediocin PD-1 furthermore totally removed the remaining non-viable cells from the stainless steel surface at a concentration of 3000 AU/ml. Treatment with plantaricin 423 and nisin also resulted in a decrease in non-viable cell numbers, with only 42% and 45% of non-viable cells remaining on the stainless steel surface, at a maximum concentration of 3000 AU/ml and 5 h contact time. The regenerative potential of the biofilms after treatment with the respective bacteriocins is therefore significantly reduced, whereas in the case of pediocin PD-1, the surface is totally cleansed of all cells. These bacteriocins were initially able to reduce the viable cell numbers in the biofilm much faster than in the case of planktonic cells.

We also showed that these peptides were more efficient under wine-making conditions, although a slightly higher initial cell yield was observed. These peptides, therefore, show potential to effectively control the numbers of *O. oeni* in the wine-making process.

This method of biofilm removal is a very exciting prospect as the detachment of bacteria from a surface, which act as a source for contamination, is more significant than killing the planktonic cells in the environment. This is because it has been demonstrated that for every free-floating bacterial cell in a natural aqueous environment an estimate of more than 500 cells can be attached to the surface areas of that environment (G.M. Wolfaardt, personal communication). These biofilms are known to be as much as 1000 times more resistant to biocides, chemical cleaning agents and sanitizers (Kumar and Anand 1998). Since all three these bacteriocins are active against *Listeria* spp., other food-borne pathogens and spoilage bacteria, this would not only have

significant effects and possibilities in the wine industry, but would also have significant implications in other beverage and food processing, storage and packaging industries.

#### 8.4 FUTURE RESEARCH PROSPECTS

Although many bacteriocins produced by LAB are effective against pathogenic and spoilage microorganisms *in vitro* and *in vivo*, only nisin and pediocin PA-1 is currently licensed to be used in a partially purified form in foods. Although some class II bacteriocins may indeed be more effective than nisin against some food-borne pathogens and other spoilage bacteria (Cintas *et al.* 1998; Eijsink *et al.* 1998; Parente and Ricciardi 1999), evidence regarding their effectiveness and stability in foods is still scarce (Stiles 1996). In some cases it may even be simpler and cheaper to use bacteriocin-containing food ingredients (Zottola and Sasahara 1994) or to produce the bacteriocin *in situ*. However, in several food and beverage products the growth of LAB may be undesirable and direct addition of purified bacteriocins may be a more viable alternative. This would, however, still require approval by regulatory agencies (Fields 1996), even though LAB are GRAS organisms and bacteriocins are likely to be present in all fermented foods (Stiles 1996). Bacteriocin-containing products or bacteriocinogenic cultures are already being marketed. The high costs for the approval of new bacteriocins as food additives often does not justify the application of bacteriocins. Their use as additives requires that they are sufficiently stable and consequently devoid of residues that are prone to potentially damaging chemical modifications.

Low yields and high costs may be further bottlenecks limiting the commercial production of bacteriocins. Low cost substrates such as carbon (cotton seed meal, filtered stillage) and nitrogen (bacteriological peptone, yeast extract, meat extract, tryptone) sources can, however, be successfully used to optimize production, without the need for any genetic manipulation or production in heterologous hosts to improve the production of existing bacteriocins. Since it is becoming increasingly evident that strains can produce more than one bacteriocin (Bhugaloo-Vial *et al.* 1996; Casaus *et al.* 1997; Eijsink *et al.* 1998), the use of purified bacteriocins for comparative analysis is, however, essential.

The continued cost of complex media for bacteriocin optimization may, however, still be more than the initial cost of genetic manipulation and protein engineering. Genetic manipulations and molecular technology can lend itself to numerous possibilities in the production and optimization

of bacteriocins. The consumer's acceptance of GMO's is, however, still a hurdle scientists need to overcome. Medium optimization is a promising alternative.

In the past, extensive studies have been performed on various aspects of biofilms. However, very little practical information has been gained. Similar to other systems, biofilms on food-contact surfaces are showing increased resistance to conventional sanitizers such as acid anionic biocides and quaternary ammonium compounds (Mustapha and Liewen 1989; Frank and Koffi 1990; Kumar and Anand 1998). The reduced efficacy of reactive agents against biofilms is likely to be the result of incomplete penetration (Huang *et al.* 1995) and the wide variation in the environmental conditions existing on food-contact surfaces (Kumar and Anand 1998). Combinations of chlorinated alkaline and alkaline detergents with sanitizers were effective in reducing the viable cell populations by more than 98% (Zottola and Sasahara 1994). With an increased sanitizer contact time, further reductions in viable cell populations were achieved. It is also suggested that frequent cleaning and thoroughly drying process equipment after cleanup would provide another hurdle for biofilm-forming microorganisms to prevent formation of a favourable niche (Zottola and Sasahara 1994; Kumar and Anand 1998). The proper chemical composition and concentrations, length of contact time, temperature and pH of the substrate are, however, essential for the efficient removal of biofilms, regardless of the type and finish of the contact surfaces (Kumar and Anand 1998). The same requisites would apply for bacteriocins.

Experiments related to the attachment of the microorganisms and the influence bacteriocins have on them in food processing environments must be conducted under the conditions existing in those environments. Such studies will help to fully understand the interactions between the biotic and abiotic entities in the food processing operations and to assess the risks posed by spoilage organisms and food borne pathogens (Kumar and Anand 1998). They are also needed for the effective analysis of the impact of cleaning and sanitation from a microbiological viewpoint. In effect it will also increase our understanding and knowledge of the organisms commercially used as starter cultures.

In view of the increased resistance of bacterial biofilms to cleaning treatments, such as sanitizers and detergents and their limited effectiveness, new strategies should be implemented for the control of biofilms. The application of bacteriocins are gaining increased importance in this field. The use of bacteriocins in combination with enzymes and other chemical cleansing



and disinfectant agents should also be considered. Each biofilm problem should however, be studied and analysed in detail.

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# **APPENDIX A**

## **SLUGGISH/STUCK FERMENTATION IN CHARDONNAY: POSSIBLE CAUSES**

Published in Wineland July 2001.

## SLUGGISH/STUCK MALOLACTIC FERMENTATION IN CHARDONNAY: POSSIBLE CAUSES

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### *Introduction*

Malolactic fermentation (MLF) entails the bacterial conversion of L-malic acid to L(+)-lactic acid and carbon dioxide (Wibowo *et al.*, 1985). This secondary fermentation, which may occur during or after alcoholic fermentation, is usually conducted by *Oenococcus oeni* (previously *Leuconostoc oenos*, Dicks *et al.*, 1995), but also by other lactic acid bacteria (LAB). *O. oeni* is the preferred starter culture due to its tolerance to low pH and high alcohol levels (Wibowo *et al.*, 1985). Depending on the strain(s) of LAB involved, several byproducts are produced that may impact on the sensory properties of wine. Chemically, the most significant changes observed during the course of MLF are increases in pH and corresponding decreases in titratable acidity. MLF is important for three reasons: (i) deacidification of the wine, (ii) flavour modification and (iii) microbiological stability (Henick-Kling, 1994; Zoecklein *et al.*, 1995).

Although MLF may occur spontaneously, it is considered necessary to inoculate wines with a specific commercial culture to conduct MLF efficiently. The success of induced MLF is, however, not always guaranteed. From practical experience and studies done at ARC Infruitec-Nietvoorbij (Loubser, 1999a, b) and Distillers (internal report), it is clear that slow or incomplete MLF usually occurs when the primary fermentation is performed with yeast strain N96. Wines that suffer from slow or sluggish MLF require more time to convert L-malic acid to L(+)-lactic acid, whereas wines with “stuck” MLF have an abundant amount of unconverted L-malic acid.

Slow or incomplete MLF is especially prominent in Chardonnay wines. However, the problem seems to vary from year to year. This led researchers to believe that stuck or sluggish MLF is influenced by a combination of factors, such as nutritional deficiencies in the must, less optimal pH, high SO<sub>2</sub> or alcohol levels, or unfavourable fermentation

temperatures (Van der Westhuizen and Loos, 1981). The exact reason(s) for slow or incomplete MLF are not known and may differ from one fermentation to the other. Other possible reasons could be bacteriophage contamination or even antimicrobial compounds (e.g. fatty acids) produced by yeast. Little is known about the interaction between yeast and lactic acid bacteria. Several papers have been published on the antimicrobial proteins or peptides (bacteriocins) produced by lactic acid bacteria and their effect on malolactic bacteria (Green *et al.*, 1997; Van Reenen *et al.*, 1998).

We have conducted a fermentation experiment with two commercially available yeast cultures to determine if they produce any antibacterial compounds that could lead to stuck or sluggish MLF. We have also tested the interaction among malolactic strains isolated from a commercial starter culture and recorded their influence on MLF.

### *Experimental Procedure*

Sterile Chardonnay grape must (derived from grapes of the Stellenbosch region during the 2000 season) was divided in 25-liter glass containers and fermented at 16°C with yeast strains N96 (*Saccharomyces bayanus* strain) and VIN 13 (*Saccharomyces cerevisiae* strain), respectively (Anchor Yeast, SA). The inoculum size was 0.02% (w/v). The presence of lauric acid (C<sub>12</sub>), myristic acid (C<sub>14</sub>), pentadecanoic acid (C<sub>15</sub>), palmitic acid (C<sub>16</sub>) and stearic acid (C<sub>18</sub>) were recorded at the end of the alcoholic fermentation. The gas chromatography method described by Marais and Houtman (1979) and M. Blom (personal communication, 1997) was used. Bacterial strains isolated from a commercial malolactic starter culture, Viacell™ (Lallemand), were grown to  $1.8 \times 10^{11}$  cfu/ml in acidic grape broth (Dicks *et al.*, 1990) and used to inoculate grape must at 5% (v/v). Autoclaved cells of the malolactic bacteria were used as control. The secondary fermentation was performed in duplicate at 20°C. Viable cell numbers and the conversion of L-malic acid to L(+)-lactic acid were monitored weekly. Standard microbiological methods were used (Sharpe, 1979). The organic acids were determined according to the method described by Schneider and co-workers (1987). In a separate experiment, the malolactic strains isolated from the commercial starter culture were tested for possible antimicrobial activity against each other and against yeast strains VIN13 and N96. The two yeast strains were also tested for

antimicrobial activity against the commercial malolactic strains. Antimicrobial activity tests were performed as described by Van Reenen and co-workers (1998).

### *Results and Discussion*

Chardonnay must fermented with the yeast starter cultures yielded very low levels of fatty acids and no significant differences were recorded between fermentations conducted by VIN13 and N96. MLF was completed after 15 weeks. Symptoms of sluggish MLF were observed for N96 and VIN 13 between days 24 to 77 (Fig 1). However, no antimicrobial compounds active against the commercial malolactic starter culture were detected in the wine sampled during this period. This implied that neither the malolactic bacteria, nor the yeast produced any antimicrobial substances that inhibited the growth of the malolactic bacteria. We could also not detect any antibacterial activity in sterile Chardonnay must. Further tests are needed to determine if other LAB, which are usually present in grape must, may produce antibacterial compounds against malolactic starter cultures. The possibility of antibacterial compounds produced by wild yeast is also not ruled out. Numerous other factors can attribute to sluggish MLF. Some possible causes for sluggish or stuck MLF and possible ways to combat this phenomenon are discussed below.

### *Causes and Recommendations*

#### **Bacteriophages**

Bacteriophages (bacterial viruses) are probably one of the most neglected reasons for sluggish or stuck MLF. This problem can be overcome if it is effectively assessed and the presence of bacteriophages prevented or restricted to a minimum (Henick-Kling, 1994).

As much as five litres of wine can penetrate the first few millimetres of a standard 300-liter barrel (Berthelot, 2000). The penetration of bacteria and their phages into the wood and their survival in empty barrels is therefore likely to happen. Contamination of fresh wine by these bacteria when aged in re-used barrels is thus very possible. The following precautions may be taken to prevent phage contamination and ensure an active malolactic starter culture:

- Prevent the possible build-up of bacteriophages in fermentation tanks from year to year by applying adequate cleaning methods.



- Use aseptic inoculation techniques and grow the starter culture in the presence of less than 20 mg/L free SO<sub>2</sub> to prevent possible inhibition (Henick-Kling, 1994).
- Use a stationary phase culture as inoculum. Cells of *O. oeni* harvested 18-24h after the culture had entered stationary phase, proved to be the most viable and yielded the highest malolactic activity (Krieger and Hammes, 1988). When harvested earlier in the growth phase only 18% of the cells survived after 5 days of MLF. These cells could also not induce MLF.
- Stimulate the growth of the starter culture by using an optimal growth medium instead of grape must. The cells should be grown to numbers exceeding 10<sup>8</sup> cfu/ml before inoculated into must.
- Rotate the starter culture. Use different strains of *O. oeni*, or even strains of *Lactobacillus* spp., which have been preselected and evaluated as suitable malolactic bacteria.
- Optimise the growth conditions in the must. Ideal growth conditions for *O. oeni* are 15°C-20°C, pH 3.4-4.2 and in the absence of SO<sub>2</sub>. Bacteriophages are most active at low temperatures and at the early growth phase of the host (Henick-Kling, 1994).
- Use malolactic bacteria harbouring temperate bacteriophages (i.e. lysogenic cells) as possible starter cultures. These bacteria are more resistant to phage infection. An estimated 50% of *O. oeni* strains isolated from wine contain temperate phages (Patel, 1990).

### **Managing malolactic fermentation**

It is more difficult to accomplish MLF in white and rosé wines than in red wines (Pilatte and Nielsen, 2000). The reasons for this are:

- White wines normally have a higher acidity and wider pH range (pH 3.0-3.5 after alcoholic fermentation).
- The low nutrient content in white wines, compared to red wines, is often inadequate to support high cell numbers of malolactic bacteria.

- White wines are produced at a lower temperature (18-22°C) compared to red wines (20-24°C).
- White wines have less phenolic compounds to which SO<sub>2</sub> can bind. Malolactic starter cultures in these wines are thus under more SO<sub>2</sub> stress than starter cultures in red wines.

### **Sulphur dioxide**

SO<sub>2</sub> is often produced by yeast during alcoholic fermentation. This may inhibit the growth of malolactic bacteria (Eksteen, 2000b; Henick-Kling and Park, 1994; Lonvaud-Funel *et al.*, 1988). The levels of free SO<sub>2</sub> produced are dependent on the yeast strain, the availability of nutrients (especially nitrogen) and the presence of compounds in the must to which SO<sub>2</sub> binds (Nygaard and Prael, 1996).

It is of utmost importance to keep the SO<sub>2</sub> levels at 50-80 ppm for red wines and 20-40 ppm for white wines during crushing and separation of the grape must to control the numbers of spoilage bacteria (Eksteen, 2000b). Lactizyme, a product of lysosyme, may be used in combination with SO<sub>2</sub>, but should only be considered in high-pH wines where the growth of pediococci are favoured. *Pediococcus* spp. can cause volatile acidity or even produce bacteriocins that may inhibit the growth of *O. oeni* (Eksteen, 2000a; Green *et al.*, 1997). It is not recommended to add SO<sub>2</sub> to must after alcoholic fermentation (Henick-Kling, 1994).

### **Parameters influencing the nutrient composition of the must**

The use of complex nutrients, such as amino acids and nitrogen sources by yeast during early alcoholic fermentation may retard or even prevent bacterial growth, especially in white wines (Nygaard and Prael, 1996). This is not surprising, since malolactic bacteria are considered fastidious organisms with limited means of synthesising growth requiring compounds (Fugelsang, 1996; Fourcassier *et al.*, 1992). They survive on low concentrations of hexoses and certain pentoses, organic acids (e.g. malic- and citric acids) and nitrogen in organic form (amino acids, peptides). Other inorganic elements (Mg<sup>++</sup>, Mn<sup>+</sup> and K<sup>+</sup>) and vitamins are also essential cofactors in enzymatic reactions. Towards the end of alcoholic fermentation, yeast cell lysis results in the release of nutrients that will favour the growth of malolactic bacteria (Nygaard and Prael, 1996). A slow alcoholic fermentation will inevitably lead to sluggish or stuck MLF. It is therefore essential that all the nutrients needed by the malolactic bacteria

are present in the grapes before crushing. Nutrient supplements for malolactic bacteria are commercially available and are usually used with direct inoculated starter cultures.

The nutrient content of the must and its turbidity is also affected during the clarification methods of white and rosé wines (static or dynamic, use of fining agents, temperature conditions, and duration of clarification). When MLF is desired in white wine, the intensity of the clarification should be adjusted in order to prevent any nutrient deficiency, which is likely to interfere with fermentability. Red wines usually have higher nutrient concentrations because of the prolonged maceration on the skins.

### **Environmental and chemical factors**

High levels of herbicides and pesticides left on the grapes, acidic acid accumulation, temperature fluxes and competition between bacteria, are also possible reasons for sluggish or stuck MLF. Must should be free of pesticides, since malolactic bacteria are more sensitive to these residues than yeast. Wild yeast strains and bacteria (e.g. *Lactobacillus brevis*) present on grapes or in the cellar often form high levels of acid, which inhibits MLF. Low temperatures and a pH value below 3.4 favours the growth of unwanted yeast strains (Gafner *et al.*, 2000).

The characteristic of the harvest (rainfall, maturity, the condition of the grapes) and wine growing practices (soil type, rootstock type, weeds and nitrogen fertilisation) have a major influence on the levels of acids and nutrients and thus also on the fermentability of the must. The presence of weeds may even cause the levels of malic acid and amino acids in the must to decrease significantly, particularly under dry conditions (Maigre *et al.*, 1995).

### *Conclusions*

No antimicrobial compounds could be detected in sterile Chardonnay must or in must fermented with yeast strains VIN13 and N96, respectively. We could also not detect any antibacterial compounds produced by the strains used as the malolactic starter culture. Other yeast and LAB are currently being screened for the production of antibacterial compounds. When all possible factors are taken into consideration, it is clear that the prevention of sluggish or stuck MLF starts in the vineyard long before the grapes find their way to the

cellar. The old cliché is still very relevant, viz. “no good wine can be made from bad grapes, but bad wine can be made from good grapes”.

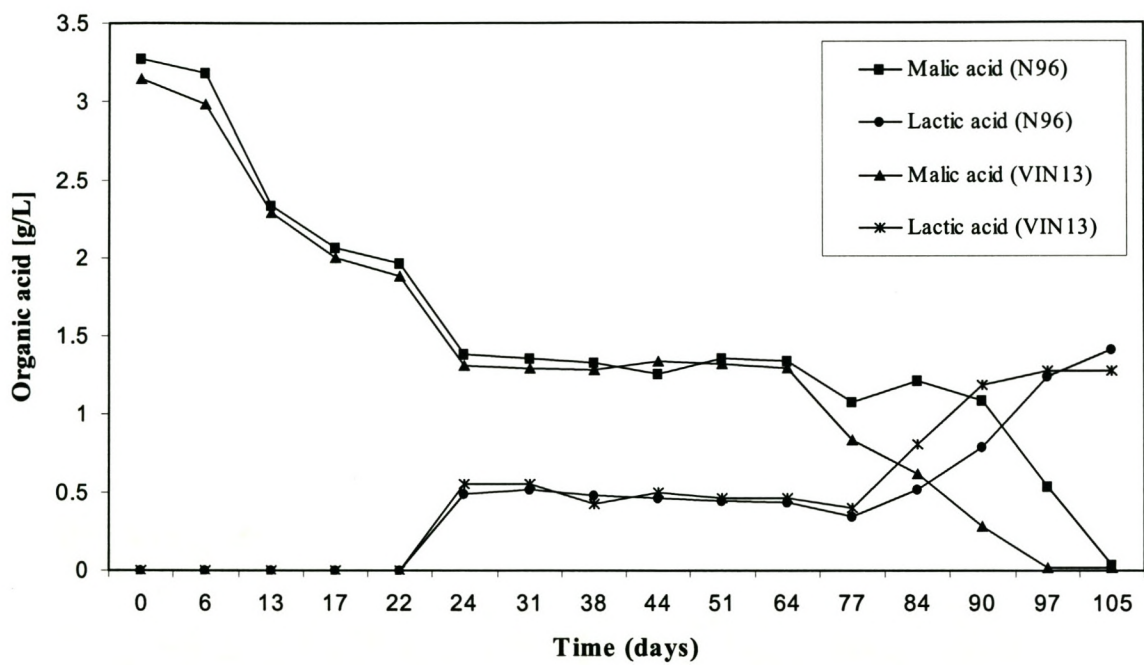
**Acknowledgements:** The authors wish to thank Marais Blom and Ludick Arnolds from Distillers for valuable assistance.

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**Figure 1: Malolactic fermentation in Chardonnay wine fermented with N96 and VIN13, respectively.**

# **APPENDIX B**

## **IS THIS THE NEW AGE OF SANITIZERS AND BIOCONTROL?**

Accepted for publication in *Wineland*.



## IS THIS THE NEW AGE OF SANITIZERS AND BIOCONTROL?

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### *Bacteria in the wine industry*

Enologists first identified lactic acid bacteria in wine 45 years ago (Peynaud 1956). Today the wine-making process includes two main steps: alcoholic fermentation by yeasts, followed by malolactic fermentation (MLF) conducted by lactic acid bacteria. The secondary fermentation entails the conversion of L-malic acid to L(+)-lactic acid and carbon dioxide (Wibowo *et al.* 1985).

At harvest, grape berries, stems, leaves and soil contains molds, yeasts, acetic acid bacteria and lactic acid bacteria. Yeasts and lactic acid bacteria are also present in and on cellar equipment (Henick-Kling 1994). Acetic acid bacteria normally only survive in low concentrations and molds are mostly eliminated as soon as the must are poured into fermentation tanks, due to the low redox potential of the must.

In grape must, up to ten different species of lactic acid bacteria have been identified. They represent the genera *Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*. Depending on the species and even on the strains, as well as the timing when they increase in numbers, lactic acid bacteria may be beneficial or detrimental to the quality of wine (Lonvaud-Funel 1999).

Although malolactic bacteria are present throughout the wine-making process, their activity normally becomes significant only after primary (alcoholic) fermentation. MLF is considered important for the deacidification, flavor modification and microbial stability of wine, especially in wines produced from grapes grown in cool climates which often have a high acid (tartrate plus malate) content. MLF is, however, also desired in some white (e.g. Chardonnay) and red (e.g. Merlot) wines produced from grapes grown in warmer climates, since it often introduces favourable organoleptic compounds (Henick-Kling 1994).

Traditionally, MLF has been allowed to develop spontaneously by the growth of lactic acid bacteria that is naturally present in the wine and cellar. However, most wine-makers are now aware of the many risks, for instance contamination by spoilage bacteria, associated with

spontaneous MLF, and use commercially available starter cultures to induce secondary fermentation instead.

Since MLF is a delicate process, it has therefore become a necessity to control the presence of natural occurring malolactic bacteria during the wine-making and maturation processes (Fugelsang 1997). The natural organisms occurring in the must and cellar equipment are therefore eliminated by adding sulfur dioxide (SO<sub>2</sub>) (50-80 ppm for red wines and 20-40 ppm for white wines) during crushing and separation of the must (Henick-Kling and Park 1994). Thus far SO<sub>2</sub> is the only authorized agent for the microbial stabilization and control in must and wine during the wine-making process. Side effects of consuming too much SO<sub>2</sub> can, however, range from nausea, vomiting, gastric irritation, allergies, primary asthmatics and headaches, depending on body size, age, drinking habits and gender.

It is, however, as important to effectively remove malolactic bacteria (that were added to induce MLF) from stainless steel and wood surfaces when the wine-making and maturation processes are complete. This is essential to assure hygienic and controlled wine-making practices and to prevent bacterial build-up resulting in bacteriophage contamination and possible sluggish or stuck fermentation in the next line of production (Nel *et al.* 2001c).

### ***Bacteriocins in the wine industry***

One of the natural methods for bacteria to compete and survive in the same ecological niche (e.g. must and wine) is the production of antimicrobial peptides, referred to as bacteriocins. These biologically active protein complexes display a bactericidal mode of action towards genetically closely related species and/or bacteria that occupy the same ecological niche (De Vuyst and Vandamme 1994a).

Because of their long-term association with humans and their food and beverage supply, lactic acid bacteria have been given GRAS (generally regarded as safe) status by the world health organization (WHO). Increased demands by the consumer to lower or omit chemical preservatives from wine, and their insistence for natural, higher quality and healthier products led to the exploitation of natural antimicrobial compounds like bacteriocins produced by lactic acid bacteria.

The multitude of bacteriocins produced by various species of malolactic bacteria and their ability to kill bacterial cells in biofilms further highlights the industrial importance of these

molecules as bio-preservatives and bio-sanitizers. The correct use of bacteriocins could result in the cut down of chemical additives such as sulfur dioxide and detergents and sanitizers. Nisin and pediocin PA-1 are examples of bacteriocins that have already found practical applications as food preservatives (Montville and Chen 1998).

In a purified form bacteriocins are colorless, tasteless and odorless and will not influence the organoleptic quality or bouquet of the wine. Extensive tests on a number of bacteriocins have proven that they are non-toxic and safe for human consumption. Since most bacteriocins have a narrow spectrum of antimicrobial activity, they have little affect on the normal population of intestinal bio-flora (Verellen *et al.* 1998; De Vuyst and Vandamme 1994b). Furthermore, the proteolytic enzymes in our intestinal tract degrade many of these proteins.

### ***Biofilms in the wine industry***

In any aqueous environment, suspended particles of organic and inorganic origin react with solid surfaces, creating a conditioned film on the surface with higher nutrient levels which attracts biologically active particles, including planktonic (free-floating) organisms that may include bacteria, bacteriophages, bacterial spores, yeasts and molds (Zottola and Sasahara 1994). With time these colonies become irreversibly attached, grow and actively multiply to form micro-colonies large enough to entrap organic and inorganic debris, nutrients and other microorganisms leading to the formation of a complex and established biofilm community (Kumar and Anand 1998).

The ability of bacterial cells to attach to a surface, form a biofilm and act as a source of contamination is a major concern in the food and beverage industries (Zottola and Sasahara 1994). Stainless steel and wood surfaces are no exception to the rule and may have a pronounced affect on the ability of malolactic bacteria to survive in grape must and during the primary (alcoholic) and secondary (malolactic) fermentation of wine. Most wines are fermented in stainless steel tanks prior to maturation in oak vats. Apart from this, the must and wine is pumped through stainless steel pipelines and valves.

It has been demonstrated that for every free-floating bacterial cell in a natural aqueous environment an estimate of more than 500 cells can be attached to the surface areas of that environment. These biofilms are known to be as much as 1000 times more resistant to biocides, chemical cleaning agents and sanitizers (Kumar and Anand 1998). Biofilms on must-contact

surfaces are also showing increased resistance to conventional sanitizers such as acid anionic biocides and quaternary ammonium compounds. The reduced efficiency of antimicrobial agents is likely due to the ineffective penetration of the biofilms or variation in environmental conditions on the contact surface (Kumar and Anand 1998). It can also be attributed to the combined mechanisms and varied properties associated with the biofilm, including reduced diffusion, physiological changes due to reduced growth rates, the production of enzymes degrading antimicrobial substances and often exopolysaccharide (EPS) matrixes. These EPS matrixes (composed of carbohydrates and proteins) are exuded from the cell surface and directly bind the cell to the substratum. Although EPS may act as a diffusion barrier, molecular sieve and adsorbent, the resistance to antimicrobial compounds is lost as soon as the three-dimensional structure of the biofilm is disrupted. In cases of extensive biofouling, thick biofilms are formed which may include many metabolically dormant cells with altered growth rates and physiology, also resulting in increased resistance to antimicrobial agents (Kumar and Anand 1998; Zottola and Sasahara 1994). In some studies where disinfectants proved to be effective, a rapid re-formation of biofilms were reported (Jones 1994).

If biofilms of malolactic bacteria are not effectively destroyed, they may contaminate the must and wine in which MLF is undesirable, or even be a breeding ground for bacteriophages which may cause sluggish or stuck MLF in the next line of production that depends on secondary fermentation (Nel *et al.* 2001c). MLF in wines, especially Chardonnay and Merlot, often experience this problem.

The use of bacteriocins to control the formation of biofilms offers a promising alternative to conventional treatment strategies, especially in the wine industry where the implementation of chemical disinfectants, including SO<sub>2</sub>, is becoming increasingly restricted.

### ***Fermentation optimization of pediocin PD-1***

The bacterium *Pediococcus damnosus* NCFB 1832 produces the bacteriocin pediocin PD-1 (Green *et al.* 1997), which penetrates the cytoplasmic membrane of the malolactic bacterium *Oenococcus oeni* and a few other lactic acid bacteria of the genera *Lactobacillus* and *Leuconostoc*, which are considered to be spoilage organisms.

We studied the growth of *P. damnosus* and optimized the production of pediocin PD-1 in modified De Man Rogosa and Sharpe (MRS) broth. MRS consists of glucose, nitrogen and ions

essential for the growth of *P. damnosus* NCFB 1832. When the bacterium was grown for 55 h at 30°C in the medium supplemented with bacteriological peptone (1.7%, wt/vol), MnSO<sub>4</sub> (0.014%, wt/vol) and Tween 80 (3%, vol/vol) and the pH adjusted to 6.7, an approximate 6-fold increase of pediocin PD-1 production was obtained (Nel *et al.* 2001a). These nitrogen sources are readily available in various forms and usually obtainable at affordable prices, counting in favour of the possible future industrial upscaling of pediocin PD-1 production.

Other bacteriocins that have similar inhibitory effects towards lactic acid bacteria and malolactic bacteria found in wine, especially *O. oeni*, are plantaricin 423 (produced by *Lactobacillus plantarum* 423, Verellen *et al.* 1998) and nisin (produced by *Lactococcus lactis* subsp. *lactis*, De Vuyst and Vandamme 1994b).

The affect of pediocin PD-1, plantaricin 423 and nisin was tested on an established biofilm of a commercial starter culture of *O. oeni* (Lallemand, Saint-Simon, France). These tests were conducted in a modified Chardonnay must medium (Nel *et al.* 2001b).

#### ***Application of bacteriocins to control biofilms***

Chardonnay must, supplemented with 5% (wt/vol) yeast extract and set at pH 4.5 was pasteurized (Nel *et al.* 2001b) and inoculated with 1% (vol/vol) of a commercially available *O. oeni* starter culture. To study the ability of *O. oeni* to form a mature and established biofilm, sterile stainless steel disks (type 304, grape 2B finish) were aseptically added to the modified Chardonnay must. After 9 days of incubation at 26°C the bacteriocins pediocin PD-1, plantaricin 423 and nisin were added to the mediums to achieve a final concentration of 3000 arbitrary units (AU)/ml, respectively. These concentration units portray the ability of the bacteriocin to kill *O. oeni* cells (Nel *et al.* 2001b). A contact time of 1 h, 3 h and 5 h with each bacteriocin were then allowed. The disks were removed at the specific time intervals, rinsed to remove all non-adherent cells, and stained with the Live/Dead *BacLight* viability probe. The probe stain cells green when viable and red when non-viable. The average percentage of viable and non-viable cells on the disks were calculated by taking a minimum of 20 images at random with an epi-flourescence microscope (equipped with a ×60/1.4 objective) after each contact time. Images were taken using a high performance CCD camera mounted on the microscope and the calculations were done using Scion image software. Inoculated disks without the addition of bacteriocins served as control (Fig. 1).

In the same experiment we determined the planktonic (free floating) cells (measured as colony forming units (CFU)/ml) in the medium after 10 min, 30 min, 1 h, 3 h and 5 h, respectively. Inoculated medium without the addition of bacteriocins served as control (Fig. 2).

Cells of *O. oeni* formed biofilms on the stainless steel disks. In the control experiment approximately 85% of the surface area was covered with cells of which 78% were found to be viable and 8% non-viable. When the respective bacteriocins were added, they not only killed all viable cells in the biofilm (after 1 h), but also were responsible for the reduction of non-viable cells on the surface area. In this regard pediocin PD-1 were responsible for the total removal of all cells after only 1 h of contact time. Approximately 36% and 43% of the non-viable cells remained on the stainless steel surfaces after 3 h of treatment with plantaricin 423 and nisin, respectively (Fig. 1).

Planktonic cells in the medium, i.e. in the absence of bacteriocins, reached  $1.5 \times 10^{10}$  CFU/ml (not shown). From the results it was evident that pediocin PD-1 destroyed all viable cells in the aqueous medium after 5 h, whilst plantaricin 423 and nisin achieved this after 3 h and 30 min, respectively (Fig. 2).

### **General discussion and conclusions**

To our knowledge this is the first report of biofilm formation by *O. oeni* under simulated wine conditions and the destruction thereof with bacteriocins. As far as we could determine, this is also the first report of bacteriocins, in particular pediocin PD-1, killing all viable cells in a biofilm, followed by destruction of all non-viable cells on the surface area. This result suggests that, of the three bacteriocins evaluated, pediocin PD-1 would be the best choice to prevent the potential re-formation of malolactic biofilms on stainless steel surfaces.

The use of these bacteriocins to control lactic acid bacteria and the formation of biofilms (especially *O. oeni*), therefore, offers a promising alternative to conventional treatment strategies in the wine industry. This is becoming increasingly important as the implementation of chemical additives, including SO<sub>2</sub> and the use of chemical detergents and sanitizers (e.g. alkalies, acids, iodine, chlorine-based sanitizers and quaternary ammonium compounds) in winery sanitation, is becoming more restricted. Bacteriocins may also be used as sanitizers and disinfectants for the cleaning of fermentation tanks, barrels and cellar equipment. The use of pediocin PD-1 to remove biofilms from surface areas and control bacterial build-up in tanks and barrels may thus

also reduce the occurrence of bacteriophages that may cause sluggish or stuck fermentations (Nel *et al.* 2001c).

SO<sub>2</sub> is, however, also necessary in wine to prevent oxidation during and after the wine-making process, something bacteriocins cannot achieve. Additional studies in our laboratory indicated that SO<sub>2</sub> and ethanol have no effect on the stability or functioning of the bacteriocins tested. Although these bacteriocins can function in a wide pH range, it is the main factor effecting the functioning of bacteriocins in a must and wine environment. Time, protein fining and different wine treatments can, however, also influence their effectiveness. Aspects such as the upscaling of the bacteriocin production process, the synergistic effect of SO<sub>2</sub> and bacteriocins, determination and comparison of the cost effectiveness compared to the use of SO<sub>2</sub>, normal sanitizers, cleaning agents and detergents, need to be studied.

Cells of *O. oeni* were able to attach and form biofilms on both French and American oak disks. It was, however, not possible to get any reliable results (as for the stainless steel disks) from the oak disks, due to the natural roughness of the wood surface area. Furthermore, cells of *Saccharomyces cerevisiae* (strain N96) were also able to attach to the surface areas of both stainless steel and oak disks. Similar results were also obtained when the yeast was inoculated together with *O. oeni*.

To conclude, it is evident from these results that bacteriocins, especially pediocin PD-1, offers a very promising alternative to current sanitizers, detergents and other cleaning agents for biocontrol in the wine-making industry. It is certainly a giant leap forward in the new age of sanitizers and biocontrol.

Will bacteriocins, and in particular pediocin PD-1, be the future buzzword in sanitizers and biocontrol in the wine industry?

### ***Acknowledgements***

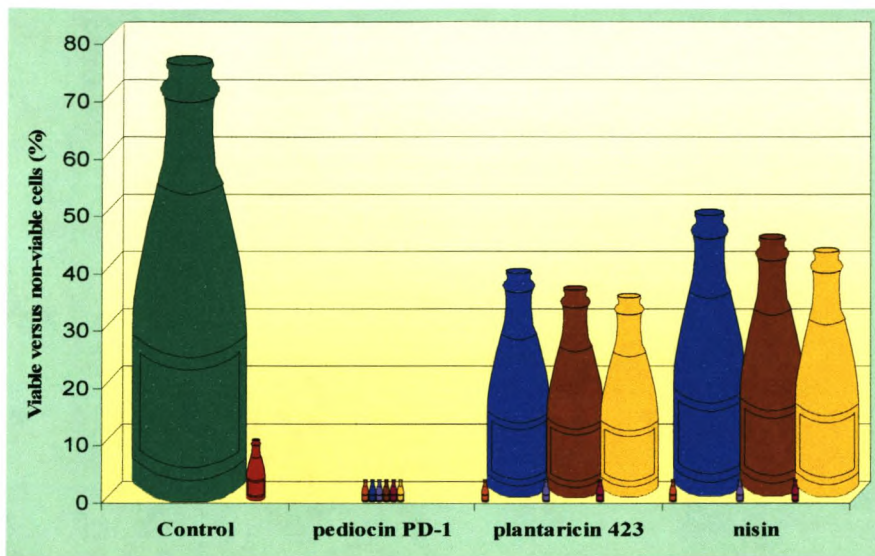
The authors would like to thank Distell Corporation for supplying the Chardonnay must, Wineland Engineers CC for supplying the stainless steel disks and Tonnellerie Radoux SA (Pty) Ltd for supplying the oak disks. The authors also extend their gratitude towards Winetech, THRIP, the National Research Foundation and the University of Stellenbosch for funding the research.

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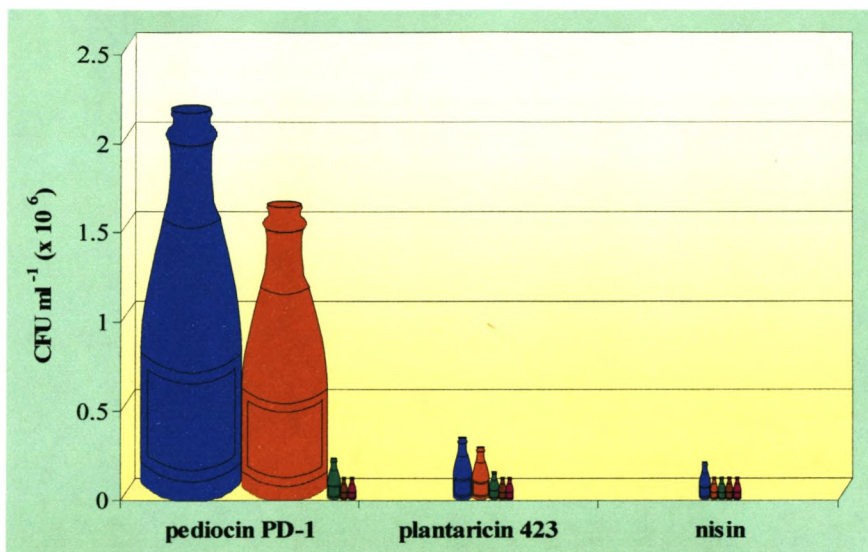


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**Figure 1: The effect of 3000 AU ml<sup>-1</sup> of pediocin PD-1, plantaricin 423 and nisin on an established biofilm of *O. oeni* in a modified Chardonnay must medium at specific time intervals.**

**Symbols:** 🍷, control viable (i.e. no bacteriocin added); 🍷, control non-viable (i.e. no bacteriocin added); 🍷, 1 h viable; 🍷, 1 h non-viable; 🍷, 3 h viable; 🍷, 3 h non-viable; 🍷, 5 h viable; 🍷, 5 h non-viable.



**Figure 2:** The effect of pediocin PD-1, plantaricin 423 and nisin (3000 AU ml<sup>-1</sup>) on planktonic cells of *O. oeni* in a modified Chardonnay must medium at specific time intervals.

Symbols: , 10 min; , 30 min; , 1 h; , 3 h; , 5 h.