Isolation and characterisation of an antimicrobial peptide from *Enterococcus* B9510

A thesis in partial fulfilment of the requirements for the degree of

Master of Technology in Biotechnology

at Massey University, Palmerston North, New Zealand.

Craig T Bennison

2005

ABSTRACT

This work deals with the antimicrobial activity of bacteriocins produced by *Enterococcus* B9510. The greatest importance demonstrated by this work was the range of activity exhibited by this bacterium, across both Gram positive and negative pathogens. This is not typical for bacteriocins, which tend to inhibit only closely related bacteria in similar environments. While attempts to identify the peptide sequence were unsuccessful the overall results of this investigation are enough to suggest that the bacteriocin from *Enterococcus* B9510 was novel and warrants further investigation.

Bacteriocins are small peptides that demonstrate antimicrobial properties and are produced by a number of lactic acid bacteria, including *Enterococcus sp.*. Bacteriocins are important because they are a possible alternative for food preservation. As a natural product they could be used to improve customer perceptions, due to the reduced need for chemical preservatives in products. From a range of lactic acid bacteria which were tested for antimicrobial activity across a selection of pathogens the strain *Enterococcus* B9510, which was a local isolate from bovine rumen, was selected due to its high level of activity. Using 16S ribosomal DNA (rDNA) analysis and sequence alignment with the BLAST network service, B9510 showed the highest homology (97%) to *Enterococcus faecalis* in the genome database.

Fermentation trials were carried out in order to maximise the concentrations of bacteriocins produced. In a controlled pH environment, cellular growth and activity was highest at pH 5.5; however, the effect of low pH showed a higher relative activity at pH 4, possibly due to pH interactions with the bacteriocin. Multi-factor trials were carried out to find the effect of glucose concentration, salt concentration and air saturation on the bacteriocin production. High salt concentrations showed a reduced production of bacteriocins and low cellular growth whereas aeration had little effect on growth but some affect on bacteriocin activity, possibly due to a variation in stress on the bacteria. Glucose concentration effected cellular growth rates, which may be a significant factor in the production of the antimicrobial activity.

Purification of the bacteriocins was carried out using two methods. The pH binding method of attaching the bacteriocins to the cell wall by neutralising the pH of the

fermentation broth was unsuccessful over a range of pH values, pH 6-9. However, the resin binding method was successful in binding the bacteriocins from a cell free broth.

An ion-exchange resin (Macro-Prep CM) was used to purify the active fraction and remove a large pigment component associated with the fermentation media. High Performance Liquid Chromatography provided an effective means to isolate samples for molecular weight determination and N-terminal sequencing.

Antimicrobial activity of the isolated fraction was tested under a range of environmental conditions including temperature, salt concentration and pH. Bacteriocin activity was still present after being held at 100°C for an hour. Optimal activity was observed at pH 3, but activity was shown by the peptide over the range of pH 1.7 - 11. Low levels of salt (<200 mM) increased the activity of the peptides; however, high concentrations (≥500 mM) reduced the effectiveness of the bacteriocin. Enzymes which acted upon peptide bonds were effective at inactivating the bacteriocin, while non-peptidase enzymes were ineffective. Testing against thirteen different bacteria, including *E. coli*, *Listeria* and *Bacillus*, showed that the peptide was active against both Gram positive and Gram negative bacteria.

Mass determination showed that the active peptide was between 1.27 and 1.35 kDa. Attempts to fully sequence the purified peptide were unsuccessful, however, an N-terminal sequence of LMPPYGVIMFF was predicted, which had a molecular weight of 1.314 kDa. This sequence showed no significant homology to any known sequences in the protein databases using the BLAST network service.

ACKNOWLEDGMENTS

So I guess the writing of this section means that I have finally finished, and it seems that this time it is actually for real. I want to thank all the people who have helped me around the lab and office and those that have let me vent or just kept an encouraging word handy, to all of you I say thank you.

To my supervisor Dr Pak-Lam Yu, thank you for you guidance, support and patience, for the interest you took in this thesis and ensuring the completeness achieved, even if we never did quite figure out the peptide sequence.

To the technicians, Mike Sahayam, Ann-Marie Jackson, Judy, Jean and John, it was always good having a laugh at others with you, and occasionally at me, thanks for all the help.

A special thanks to Rachel, you were my sounding board for ideas, good and bad, and enhanced the whole experience. I should also thank that husband of yours for letting me crash at your house after I'd moved away, Cheers Pete.

To Sapph, Steph and Abby, my good friends, your support and proof reading was greatly appreciated, even if half the time you had no idea what I was saying, or if it made any sense at all, but at least my grammars improving, I think, if not my spelling.

All the people who have been in and out and around the post-grad room Adi, Steve, Anna, Petja, Mossop, Kenny, Jenny, and the hoard of foreign students who I've meet and worked with, you enlivened the place with you energy.

To my blessing and my curse, Ultimate Frisbee, and all the people who managed to drag me away from work, yes that's right dragged, especially the guy's from the Princess Bride's, SPY and Fish, this was my vice.

And finally to Mum and Dad, it took longer than expected but your support was always there, this would not have been possible without you, I thank you most of all.

TABLE OF CONTENTS

TABLE OF FIGURES

TABLE OF TABLES

FREQUENTLY USED ABBREVIATIONS

ADP	Adenosine Di-phosphate
AMP	Adenosine Mono-phosphate
ATP	Adenosine Tri-phosphate
au	Arbitrary units
HIC	Hydrophobic Interaction Chromotography
HPLC	High Performance Liquid Chromatography
Da	Daltons
LAB	Lactic Acid Bacteria
LC-MS-MS	Liquid Chromatography Tandem Mass Spectrometry
MIC	Minimum Inhibitory Concentration
M	Molar
PCR	Polymerase Chain Reaction
sp.	Species
rpm	Revolutions per Minute

1 INTRODUCTION

1.1 REASON FOR RESEARCH

This research was carried out to investigate the bacteriocins from *Enterococcus* B9510, and to test the bacteriocin under a range of conditions. Bacteriocins are small peptides produced by bacteria as a form of physiological advantage in their natural environments, in order to kill other bacteria competing for the same resources. Interest in bacteriocins has grown around the desire for a natural preservative in food products and as an alternate to traditional antibiotics in medicine. Investigation of *Enterococcus* B9510 was undertaken after testing showed the antimicrobial activity of this bacterium was the greatest of a range of lactic acid bacteria tested, strength of the bacterial inhibition, against both Gram positive and negative bacteria ere measured. The stability of the activity demonstrated by *Enterococcus* B9510 when exposed to acidic conditions and high temperatures was also higher that the other samples tested.

1.2 PROJECT OBJECTIVES

This project had the following objectives:

- Optimise fermentation conditions for the production of bacteriocins in *Enterococcus faecalis* B9510.
- Compare methods of extraction and purification of the bacteriocins.
- Optimise one method to maximise the yield of bacteriocins.
- Test the spectrum of bactericidal activity of the semi-purified and pure bacteriocins.
- Test the effect of various environmental conditions on the bacteriocin activities.
- Determine molecular mass and amino acid sequence of purified bacteriocins.

2 LITERATURE REVIEW

2.1 SUMMARY

Enterococcus faecalis is a member of the lactic acid bacteria, and has been used in fermented foods, including dairy and meat products. These bacteria are added to enhance flavours and textures in various products. However, research interest in these bacteria is now in their bacteriocins, and the ability of these peptides to inhibit the growth of pathogenic bacteria. Enterococci produce a range of bacteriocins, most of these falling within the category of Class II bacteriocins. These are described as small, heat stable and non-lanthionine containing peptides, of importance to the food industry because they have a relatively wide spectrum of activity and are generally stable under the conditions processed foods are subjected to (including temperature, pH and a range of enzymes). It is believed that bacteriocins act by creating pores in the surface of susceptible bacteria, forming an ionic imbalance between the bacteria and the environment. The leakage of ATP and other ions causes the de-energisation of the cell which results in cell death.

There are a number of ways in which the peptides can be isolated from a fermentation broth. The most common method is using ammonium sulphate to precipitate the peptides from solution. Non-ionic resins and pH solubility methods can also be used for the purification of these peptides.

2.2 INTRODUCTION

The following literature review describes the *Enterococcus* family and the properties of bacteriocins produced by these bacteria. The review is divided into several sections which address, among others things, lactic acid bacteria, the function of bacteriocins, and the organisms that produce the bacteriocins, with the major focus of the review being on the genus *Enterococcus*. Common properties and functions of bacteriocins produced by lactic acid bacteria will be discussed; especially those from *Enterococcus faecalis* and *Enterococcus faecium*, and the discussion will include how the different classes of bacteriocins can be identified, along with how these classes differ from one another in form and function. The theorised mode-of action of bacteriocins will also be discussed, together with the possible interactions that occur between peptides and target cells. Finally, peptide purification methods, environmental factors that can affect bacteriocin production and activity in possible products will be examined.

2.3 LACTIC ACID BACTERIA

Lactic acid bacteria (LAB) belong to a group of bacteria that are Gram-positive, facultative anaerobes that produce lactic acid during fermentation. These bacteria are used extensively in food manufacture, especially in products such as fermented milk, vegetables and meats, and can also be found in the processing of products such as wine. LAB are widely valued for their various physiological traits, which may lead to new applications for these bacteria. While this review will mainly concentrate on the bacteriocins excreted from LAB, specifically from *Enterococcus*, there are a variety of other functions that these bacteria may be able to perform (48). One of these functions is as a probiotic, in which bacteria that are found in the flora of the gastrointestinal tract are used to influence the balance within the gut's microflora. These are most commonly added to fermented products or in pharmaceutical applications (32).

2.4 *ENTEROCOCCUS* SPECIES

The genus *Enterococcus* was first proposed in 1903 for a group of Gram positive diplococci of intestinal origin. This was further developed in 1937 when the *Streptococcus* genus was divided into four divisions: viridans, lactic, pyrogenic and enterococci. The taxonomy of *Enterococcus* remains uncertain and not well defined, with no phenotypic characteristics that separate them from other Gram-positive, catalase-negative, coccal-shaped bacteria.

Due to their regular inhabitancy of the intestinal tract, *Enterococcus* are regularly used as indicators of faecal contamination, and are therefore of great importance in public health and food microbiology (46) (84). Some of *E. faecium* and *E. faecalis* strains are leading causes of nosocomial infections, also known as "in hospital" infections, and strains resistant to most clinically used antibiotics have been isolated (25) (64). Other enterococci are used in food fermentations, especially fermented cheeses and other milk products (22) (51) (75). They are also associated with natural food fermentation in African products (63) and olives (28).

Enterococci make up a large proportion of the native bacteria associated with the mammalian gastrointestinal tract, specifically the species *E. faecium* and *E. faecalis*. Other common examples of enterococci from faecal samples include *E. durans*, *E. hirae* and *E. cecorum*. Enterococci also occur in soil samples, in surface waters and on plants and vegetables, *E. faecium* is also the most predominant micro-organism in unprocessed milk, making it significant to the dairy industry.

Enterococcus is significant to the food industry, firstly in the production of meat products, and secondly in cheese manufacture. Enterococci are commonly present in the gastrointestinal tract of animals; they are the chief cause of contamination of meat at the time of slaughter. Of the non-spore forming bacteria enterococci are among the most heat resistant, and due to this thermo-tolerance they can create a spoilage problem in cooked and processed meats. After heat processes, such as pasteurisation, where the core temperature of the product is raised to 60-70°C, the bacteria can still multiply and cause the spoilage of products such as luncheon meats and canned hams. This problem is sometimes compounded by the practice of reworking products. This occurs when processed product is placed with raw product, due to packaging damage during heat

treatment. The presence of any heat resistant bacteria would be compounded in the fresh product, as they can multiply during the reworking process and are likely to survive the heat treatment a second time. For this reason enterococci are frequently used as indicators for sanitary conditions in processing plants.

However, the undesirable nature of enterococci in meat products is not applicable to all food groups, as there are numerous uses of enterococci in fermented cheeses. This is most common in cheeses originating from southern European countries, including those manufactured from goat, sheep, and bovine milk sources. In these processes the bacteria are used in the development of aroma and ripening of the cheese. However, enterococci can also be a contaminant, where its presence is due to unhygienic practices, leading to detrimental flavours, colouration and smell. The presence of enterococci in many of the cheese production processes can be related to the varied conditions that the bacteria are able to survive under, including a wide temperature growth range, resistance to high temperatures, as well as salt and acid concentrations. The British Advisory Committee on Novel Foods and Processes has also approved a strain of *Enterococcus, E. faecium* K77D, for use as a starter culture in cheeses. Acceptance from this committee is required for any new process, and in this case it was applied by a food company for the production of fermented dairy products (27).

2.5 BACTERIOCINS

The term bacteriocin was originally used to describe colicin-type protein antibiotics, molecules predominantly characterised by lethal biosynthesis in the form of intraspecies killing activity (38). Present day applications of the term bacteriocin describes a range of chemical substances that are released extracellularly by predominately Gram positive bacteria. This can also be applied to other terms such as lantibiotics (for lanthionine-containing proteins) and BLIS (for bacteriocin-like inhibitory substances). Generally, these chemicals inhibit the growth of other closely related bacteria that may be competing for the same ecological niche.

The use of bacteriocins varies, and recent investigations revolve around the production of substances to be used against infectious diseases. In some cases bacteriocins have been shown to be just as effective as some currently used therapeutic medicines in the

treatment of staphylococcal infections and acne (37). The reason for the importance of bacteriocins in the medicinal field is in large part due to the general over-prescription of antibiotics to patients. This has caused an increase in the development of antibiotic-resistant strains of bacteria. Researchers are now looking into alternatives, with bacteriocins being one of the possible replacements for standard antibiotics. The antibacterial action of the bacteriocins means that no subsequent resistant strains of the targeted bacteria can form, as the cells are completely destroyed.

Another possible use of bacteriocins is in food preservation, an increasingly studied area. Bacteriocins from lactic acid bacteria are of particular interest as they can also produce or improve other beneficial food properties such as flavour and texture. The growth in studies in this area is due to growth in consumer demand for natural and minimally processed foods. Nisin, a Class I bacteriocin, is the most common of these agents and has been used in commercial food preparation in over 40 countries for some time, and is most prevalent in dairy products such as cheeses. Bacteriocins, such as pediocin and other similar bacteriocins have been shown to be more effective than nisin at killing a range of pathogens in meat products (19).

The isolation of new bacteriocins from starter cultures needs to include bactericidal activity that can operate at a wider range of temperatures and pH levels than those normally used in food processing. Also, they will also need to be tested for long periods of storage time and in food environments, where they may be subjected to the proteolytic enzymes sometimes found in raw foods. The continuous search for microbiological, biochemical and genetic characterisation of novel bacteriocins (with improved or expanded antimicrobial activities) will form the basis for future strategies to develop natural and engineered biological compounds to be used as food additives, and to develop unique starter cultures for *in situ* bacteriocin production for food preservation purposes.

2.6 CLASSIFICATION OF BACTERIOCINS

The wide range of bacteriocins has lead to the grouping of similar characteristics into five separate classes. These are Class I, Class II, Class III and Class IV bacteriocins, along with Class V bacteriocins, a proposed class of peptides.

2.6.1 Class I bacteriocins

Class I bacteriocins are the lantibiotic peptides, identified by the presence of dehydroamino acids and thioether amino acids, or lanthionine rings that are introduced by post-transitional modification (59). A suggested division of Class I bacteriocins into two types is based on the structural differences present in these peptides (61).

2.6.1.1 Class I, Type A bacteriocins

Class I, Type A bacteriocins are elongated shaped molecules, and include the bacteriocins nisin and epidermin. These peptides are also identified as flexible, cationic molecules, which act on the bacterial membranes (31).

2.6.1.2 Class I, Type B bacteriocins

Class I, Type B bacteriocins are globular peptides that can be further divided into two categories. The first category contains peptides with a characteristic head-to-tail cross linkage. The second category does not share this cross linkage but shares similar leader peptides. They both share other characteristics, including being negatively charged or neutral, and affecting bacterial enzyme functions. Examples of cross linked peptides include cinnamycin and ancovenin, while peptides which do not have the cross linkage include mersacidin and actagardine (31).

The primary structures of nisin, epidermin, cinnamycin and mersacidin are shown in Figure 2.1. There are structural differences between the Type A and B varieties, with the Type A being much more elongated and Type B being much more globular.

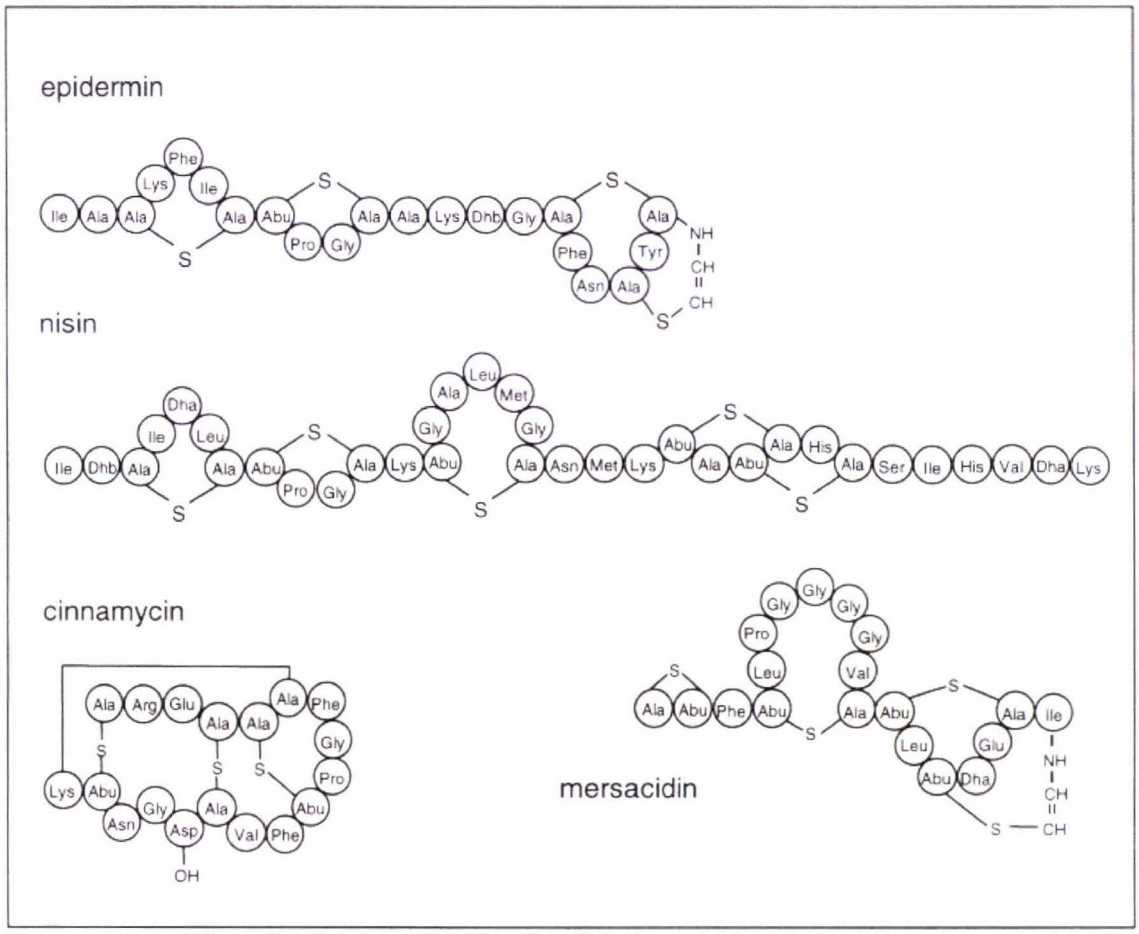


Figure 2.1 Primary structure of the lantibiotics epidermin, nisin, cinnamycin and mersacidin. Dha: 2,3-didehydroalanine, Dhb: 2,3-didehydrobutyrine, Abu: α-aminobutyric acid, Ala-S-Ala: lanthionine, Abu-S-Ala: 3-methyllanthionine (31).

Table 2.1 Comparison of the amino-acid sequences for a range of Class Ia bacteriocins.

	1 10	11 20	21 30	31 34
Nisin A	ITSISLCTPG	C.KTGALMGCN	MKTATCHCSI	HVSK
Nisin Q	ITSISLCTPG	C.KTGVLMGCN	LKTATCNCSV	HVSK
Nisin Z	ITSISLCTPG	C.KTGALMGCN	MKTATCNCSI	HVSK
Subilin	WKSESLCTPG	C.VTGALQTCL	FQTLTCNCKI	..SK
Epidermin	IASKFICTPG	CAKTGSFNSY	C.C	
Cannamycin	CRQSCSFGPF	TFVCDGNTK		
Mersacidin	CTFTLPGGGG	VCTLTSECIC		

Note: Cannamysin and Mersacidin (31) are Class Ib and were not compared using this system. Key: Residues coloured red are conserved > 90% of the sequences. Residues coloured blue are conserved > 50% of the sequences. The alignment was prepared with the Multalin program (version 5.4.1) using the Dayhoff-8-0 settings (15), (14). Epidermin, Nisin A (31), Nisin Z (68), Subilin (37) and Nisin Q (87).

2.6.2 Class II bacteriocins

Class II bacteriocins are generally recognised as small (less than 10 kDa), heat stable, non-lanthionine containing peptides. Class II can be spilt into three further subdivisions. Class IIa are separated from other bacteriocins on the basis of their similar amino acid sequences, particularly that of the N-terminal, and their strong antilisterial activity. These bacteriocins are also referred to as pediocin-like bacteriocins (24). Class IIb are the two-peptide bacteriocins and Class IIc bacteriocins operate synergistically with Class IIa bacteriocins. Examples of the amino acid sequences for Class II bacteriocins are shown in Section 2.7.

2.6.2.1 Class IIa bacteriocins

Class IIa bacteriocins are some of the best prospects for the production of useful materials. They are the most extensively researched bacteriocins because they generally show a higher biological activity and wider spectrum of activity when compared to other bacteriocins. Class IIa are a subgroup of the Class II bacteriocins characterised by their similar amino acid sequences, particularly that of the N-terminal, and their strong antilisterial activity. The potential application of these bacteria against pathogens and spoilage bacteria appears to be extensive. Activity of the bacteriocins is very effective against problem organisms such as *L. monocytogenes*, *Clostridium* sp. and *Staphylococcus* sp., creating the opportunity for these peptides to become bio-preservatives in a large range of foods. This group of peptides also includes the sec-dependent bacteriocins; these peptides were formally part of the Class IIc bacteriocins but have been reclassified, as the secretion mechanism is no longer used as a tool for the classification of bacteriocins. Examples of these bacteriocins include enterocin P and listeriocin 743A (10) (21).

A large body of work is being carried out into the investigation of the Class IIa bacteriocins. At least 14 members of this sub-class have been characterised (61), including both Enterocin A and Enterocin P, both of which have been purified from *Enterococcus* species. The purification of these peptides is becoming easier with the use of a standardised process for the isolation of these hydrophobic cationic peptides.

2.6.2.2 Class IIb bacteriocins

Class IIb bacteriocins cause inhibition through the use of two complementary peptides. The peptides are clearly different in sequence and comprise two independent bacteriocins. The key to Class IIb peptides are that they act synergistically, where the combination of the two peptides are more effective at inhibiting bacteria than either peptide individually (61). Examples of this type of peptide are enterocins 1071A and 1071B, isolated from *E. faecalis* BFE 1071. The genes encoding these peptides are located on the same plasmid, which is the other characteristic of Class IIb bacteriocins (4).

2.6.2.3 Class IIc bacteriocins

Class IIc bacteriocins are a small group of peptides that are similar to the Class IIb peptides in that they work with a partner bacteriocin to produce a synergism effect, but the partners of these peptides are characterised as Class IIa peptides with the N-terminus sequence. An example of this is found in *E. faecium* with Enterocin A being the Class IIa peptide and Enterocin B the Class IIc peptide (12).

2.6.2.4 Class III and Class IV bacteriocins

Bacteriocins with high molecular mass, greater than 30 kDa, and which are heat-labile represent Class III, and include many extracellular enzymes. An example of a Class III bacteriocin is helveticin J (68). Class IV bacteriocins require a lipid or carbohydrate moiety for activity, however, this class has not had a member characterised as yet, their existence is subject to some conjecture (44).

2.6.2.5 Class V bacteriocins

Class V bacteriocins were proposed by Kemperman *et al* 2003, to include those bacteriocins that consisted of a head-to-tail-ligated peptide. Bacteriocins that are included in this class include circularin A, microcin J25, gassericin A and AS-48 (43).

2.6.2.6 Mode of action of Class II bacteriocins

There have been several hypotheses for the mode of action for Class II bacteriocins, mainly revolving around the binding and interactions between the peptide and the cell membrane. On the whole it is agreed that the peptide forms pores in the cell wall of the target cell, cells are disrupted through the reduction of membrane potential ($\Delta\psi$) and the de-energisation of the target cell by the reduction of intracellular ATP without ATP efflux takes place. It is suggested that this loss in ATP is due to either the loss of P_i through the membrane as a result of increased permeability, or due to an increase in intercellular hydrolysis in order to correct the ion balance within the cell due because of the loss of ions from within the cell. This means that the cells use up their energy more rapidly, which leads to cell death (35). Another method suggested for bacterial inhibition is the disruption of the glucose transport system for the cell. Coupled with the disruption of the cell membrane this would also reduce the amount of ATP and lead to the de-energisation of the cell. For pediocin and leuconocin it was reported that this glucose inhibition was coupled with AMP, ADP and ATP efflux (83).

It is proposed that in order for the bacteriocins to be effective they must bind with a receptor on the cell membrane, but these receptors have not yet been identified. Possible suggestions for these receptors include the anionic phospholipid head groups found on cell membranes, which could interact with the cationic peptides. This would suggest the effect of pH on activity could be significant (61).

It is also suggested that the YGNGV sequence, which is one of the characteristics of Class IIa bacteriocins, could be the receptor and allow for the correct orientation of the peptides. This hypothesis, however, does not fully explain the activity of the peptides, as many active bacteriocins do not contain this leader sequence (24).

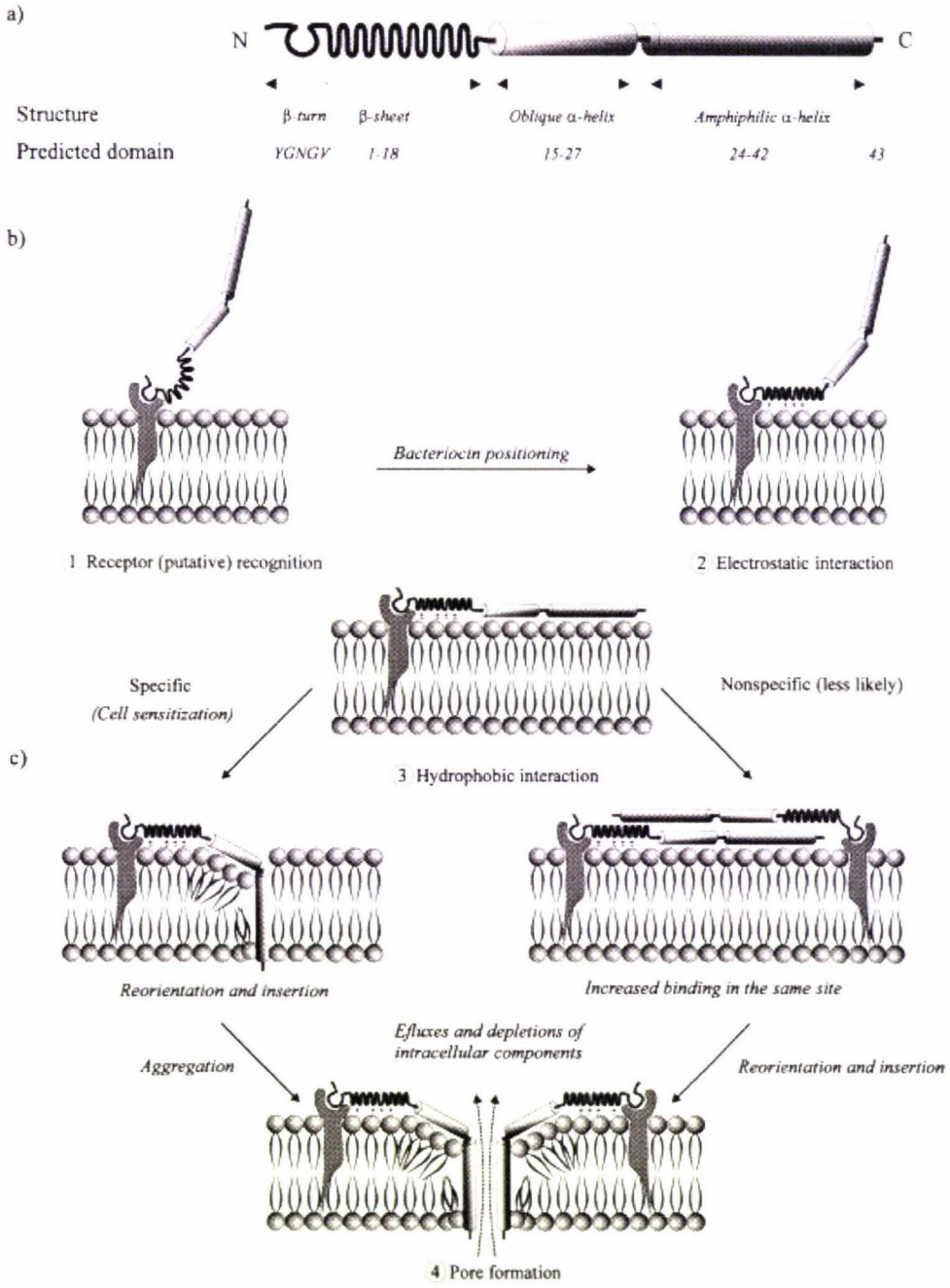


Figure 2.2 Schematic representation of the structure of a model Class-IIa bacteriocin and the predicted location domains with respect to target cell membrane. (a) bacteriocin predicted structural domains; (b) possible interactions of each domain with the membrane surface; (c) bacteriocin insertion and formation of hydrophilic pores. The hydrophobic face of the peptide is shaded dark and hydrophilic face is shaded light (24).

2.7 BACTERIOCINS FROM ENTEROCOCCI: THE ENTEROCINS

There are a wide range of enterocins isolated from *Enterococcus faecalis* and *Enterococcus faecium*, in addition to other *Enterococcus* species. This section briefly outlines the isolated bacteriocins, some of their known properties, and where possible it will also place the bacteriocins into their classes. As discussed earlier, bacteriocins can be grouped in five classes. Enterococci produce bacteriocins that fall into several of these groups, but are most common within Class II, with examples of Class IIa, IIb and IIc all being reported.

2.7.1 Class IIa bacteriocins

Class IIa bacteriocins are characterised by being a single peptide or being the dominant peptide associated with a Class IIc peptide. Examples of Class IIa bacteriocins include enterocin A, which has been isolated from *Enterococcus faecium.* Bacteria producing this bacteriocin have been isolated from a range of environments, such as fermented sausages (3), cheeses (23) and rice products (53). From N-terminal and DNA sequencing, enterocin A was shown to be part of the pediocin-like bacteriocin family and therefore part of the type Class IIa. However, enterocin was unusual as the first five residues from the N-terminal sequencing were unique to this bacteriocin (3). One of the other bacteriocins in the Class IIa family is enterocin SE-K4 produced by *Enterococcus faecalis* K-4. This bacteriocin was isolated from silage in Thailand, and shows the sequence and characteristics inherent in these bacteriocins (20). Another of the Class IIa bacteriocins is Enterocin P, purified from *E. faecium* isolated from fermented meat and secreted via the sec-dependent pathway (10).

Table 2.2 Comparison of the amino-acid sequences for a range of Class IIa bacteriocins.

	1 10	11 20	21 30	31 40	41 49
Bacteriocin 31	....ATYYGN	GLYCNKQKCW	VDWNKASREI	GKIIVNGWVQ	HGPWAPR
Carnobacteriocin BM1	....AISYGN	GVYCNKEKCW	VNKAENKQAI	TGIVIGGWAS	SLAGMGH
Divercin V41	....TKYYGN	GVYCNSKKCW	VDWGQASGCI	GQTVVGGWLG	GAIPG..KC
Enterocin A	TTHSGKYYGN	GVYCTKNKCT	VQWAKATTCI	AGMSIGGFKG	GAIPG..KC
Enterocin SE-K4	....ATYYGN	GVYCNKQKCW	VDWSRARSEI	IDRGVKAYVN	GETKVLG
Leucocin A	.....KYYGN	GVHCTKSGCS	VNWGEAFSAG	VHRLANG..G	NGFW
Mundticin	.....KYYGN	GVSCNKKGCS	VDWGKAIGII	GNNSAANLAT	GGAAGWSK

Pediocin AcH/PA-1	.....KYYGN	GVTCGKHSCS	VDWGKATTCI	INNGAMAWAT	GGHQGNHKC
Piscicolin V1a	.....KYYGN	GVSCNKNGCT	VDWSKAIGII	GNNAAANLTT	GGAAGWNKG
Sakacin A	....ARSYGN	GVYCNNKKCW	VNRGEATQSI	IGGMISGWAS	GLAGM
Sakacin P	.....KYYGN	GVHCGKHSCT	VDWGTAIGNI	GNNAAANWAT	GGNAGWN
Enterocin_P	...ATRSYGN	GVYCNNSKCW	VNWGEAKENI	AGIVISGWAS	GLAGMGH
Listeriocin 743A	.....KSYGN	GVHCNKKKCW	VDWGSAISTI	GNNSAANWAT	GGAAGWKS

Note: Enterocin A (3), Enterocin SE-K4 (40), Bacteriocin 31 (80), and Mundticin (5) are derived from *Enterococcus* sp., while Enterocin P (10) and Listeriocin 743A (41) are both sec-dependent bacteriocins. Key: Residues coloured red are conserved > 90% of the sequences. Residues coloured blue are conserved > 50% of the sequences. The alignment was prepared with the Multalin program (version 5.4.1) using the Dayhoff-8-0 settings (15) (14). Carnobacteriocin BM1 (73), Divercin V41 (58), Leucocin A (33), Pediocin AcH/PA-1 (57), Piscicolin V1a (6), Sakacin A (2), Sakacin P (79).

2.7.2 Class IIb bacteriocins

The Class IIb bacteriocins show complementary action of two peptides that are independent bacteriocins. This type of peptide has been isolated from *Enterococcus faecalis*, and the peptides were shown to be about 60% homologous to other Class IIb peptides. Testing of these peptides showed that the maximum activity occurred with a ratio of 1:1, suggesting that the mechanism of activity requires both peptides to be effective (61).

Table 2.3 Examples of Class IIb bacteriocins.

	1 10	11 20	21 30	31 40
Enterocin 1071A	ESVFSKIGNA	VGPAAYWILK	GLGNMSDVTQ	ADRINRKNH
Enterocin 1071B	GPGKWLPWLE	PAYDFVTGLF	AKGIGKEGNK	NKWKN
Lactacin FX	WGMAICGVGG	AAIGGYFGYT	HNACA	
Lactacin FA	VAGAHYLPIL	WTGVTAATGG	FGKIRK	
plNC8α	DLTTKLWSSW	GYYLGKKARW	NLKHPYVQF	
plNC8β	SVPTSVYTLG	IKILWSAYKH	RKTIEKSFNK	GFYH

Note: These bacteriocins are paired and show no particular homology between sequences or to other Class IIb bacteriocins Enterocin 1071A and B (4), Lactacin FX and FA (1), plNC8α and β (56).

2.7.3 Class IIc bacteriocins

Class IIc peptides are similar to the Class IIb peptides in that they operate synergistically with a partner peptide. However, in this case the partner peptide is more

active and is generally a Class IIa peptide. An example of this is Enterocin B, which is commonly found in conjunction with Enterocin A. Class IIc peptides show different target specificity than those of IIa and IIb, and are structurally different to their peptide partners, suggesting that they may use a different mechanism to kill sensitive cells.

Table 2.4 Examples of Class IIc bacteriocins.

	1 10	11 20	21 30	31 40	41 50	51 54
Enterocin_B	ENDHRMPNEL	NRPNNLSKGG	AKCGAAIAGG	LFGIPKGPLA	WAAGLANVYS	KCN
Carnobacteriocin A	DQ.MSDGVNY	GKGSSLSQGG	AKCGLGIVGG	LATIPSGPLG	WLAGAAGVIN	SCMK

Note: Enterocin B (8) and Carnobacteriocin A (61) are Class IIc bacteriocins and are paired with Class IIa bacteriocins Entrocin A and Carnobacteriocin B respectively, to aid in antimicrobial activity. Key: Residues coloured red are conserved > 90% of the sequences. The alignment was prepared with the Multalin program (version 5.4.1) using the Dayhoff-8-0 settings (15) (14).

2.8 CHARACTERISATION OF BACTERIOCINS

The characterisation of bacteriocins can be carried out in a number of ways. As previously mentioned, the form and function of the bacteriocins can be used to sort peptides that fall into each class of antibacterial molecules into smaller groups. Whether this is due to the effect of genetic sequencing or external conditions on the effectiveness on the peptides, it makes it easier to group similar peptides together. To that end, this section details the testing carried out on bacteriocins from *Enterococcus sp.*, and the effect conditions such as pH, enzymes and temperature have had on the effectiveness of the peptides, and what this can tell us about the molecules. These factors are important as they relate to the usefulness of the peptides in practical applications. The addition of the peptides into a processing situation means that the bacteriocins need to remain active under a range of conditions.

2.8.1 Effect of temperature on bacteriocin stability

As most of the bacteriocins from enterococci are in the class II group it is reasonable to assume that the majority of these peptides are heat stable, as this is one of the characteristics of that class. Some of these peptides include bacteriocins from *E.*

faecium that remained fully active after 30 minutes at 85°C and were still active after 30 minutes at 121°C (54). Thermo-stability also applies to bacteriocins from *E. faecalis*. Samples were shown to be active after treatment at 100°C for 30 minutes or after 60 minutes at 120°C (20). This lack of denaturation is most likely caused by the lack of a tertiary structure, which would be affected by temperature changes. It is also worth noting however that the majority of the amino acid sequences shown contain at least one disulfide bond, which would indicate some form of tertiary structure. As for storage, samples from *E. casseliflavus* were still active after 6 months of refrigeration (74).

2.8.2 Effect of pH on bacteriocin activity

The effect of pH on the peptides also relates to the application of the bacteriocin in practical terms. In some cases, bacteriocins are used in conjunction with the bacteria, as opposed to the purified peptide. This can mean a wide range of fermentation pH values as lactic acid is also produced, which lowers the pH in an uncontrolled environment. For bacteriocins from both *E. faecium* (28) and *E. faecalis* (20) the effect of pH was similar. The optimum activity for the peptides was at pH 6 with a reduction in inhibitory effect at both higher and lower pH values, within the range of pH 2-11.

2.8.3 Effect of enzymatic action on bacteriocin activity

As with temperature and pH, the presence of enzymes can affect the ability of bacteriocins to function as inhibitors. The effect of the enzymes on the peptide is related to the properties of the peptides, as enzymes require a specific site in order to be active. From experimentation a range of both positive and negative inhibition can be seen. Enzymes such as α-chymotrypsin (which cleaves peptide bonds on the carboxyl side of the aromatic side chains tyrosine, tryptophan and phenylalanine and of large hydrophobic residues such as methionine), pepsin (which digests the nitrogenous constituents of food, converting them into peptones), trypsin, (that cleaves on the carboxyl side of arginine and lysine residues), α-amylase (which hydrolyses plant starches specifically amylopectin and amylose, cleaves the internal 1-4 linkages to

create sugars) and pronase have all been shown to reduce or stop the activity of bacteriocins. Several of these are common digestive enzymes, which suggest that the activity of the inhibition is proteinaceous. In comparison, other enzymes show little or no effect on inhibition, for example α-glucosidase, (is a dietary enzyme and is used to degrade disaccharides), catalase (a ubiquitous heme protein that catalases the dismutation of hydrogen peroxide or organic peroxide into water and molecular oxygen), lipase (which cleaves fats into glycerol and fatty acids), lysozyme (cleaves the polysaccharide component of some bacterial cell walls, also hydrolyse glycosidic bonds between N-acetylhexosamine residues and other sugars), and phospholipase C (hydrolyses the phosphodiester bond linking the phosphorylated inositol unit to the acylated glycerol moiety, requires metal ion to hydrolyse phosphatidylcholine) (54) (62) (76) (77).

2.8.4 Production of bacteriocins

Optimisation of the production of bacteriocins is one of the key goals of any research into antimicrobial peptides. The excretion of the bacteriocins is affected by a variety of conditions within the fermentation media and is highly dependent on not only the environmental conditions such as temperature, substrate concentrations and oxygen level, but also how these factors can place some kind of constraints on the bacterial culture.

A number of studies have investigated the effect of 'harsh' environments on the bacteria and what affect these have on the production of the peptides. These studies also examined the overall concentration of the bacteriocins in these fermentations, together with the period of production. Investigations by Leroy *et al* 2003 and Leal-Sanchez *et al* 2002 showed that the effect of non-ideal fermentation conditions may have a positive influence on the production of bacteriocins and the length of peptide production. Because bacteriocin production provides a competitive advantage to the bacteria, in situations where the growth conditions are not ideal an increase in this production can be beneficial. The reverse is also true: in ideal growth conditions with an abundance of substrate, the bacteriocin production could be reduced and cellular energies directed more toward cellular replication.

Both studies tested environment factors such as glucose (substrate) concentration, the effect of salt concentration, fermentation temperature and inoculum size on the production of bacteriocins, with a variety of results. In several cases these papers found contradictory results regarding the effects of the conditions on bacteriocin production. This suggests that the effect of the environmental conditions is specific to each bacteria, and what maybe true for one species is probably not true for all (48) (52).

2.9 METHODS OF BACTERIOCIN PURIFICATION

There is a vast range of methods available to purify bacteriocins from fermentation media that are based on the properties of the bacteriocins themselves. The effects of pH, addition of salt or the use of resins, non-ionic, cationic or anionic, can be used in the purification methods.

2.9.1 Initial purification

The initial purification of the bacteriocins from the fermentation media is designed to reduce the working volume without the loss of any of the peptides. The use of solvents and salts are common methods of doing this.

2.9.1.1 Ammonium sulphate precipitation

The most common method of extraction is by the use of ammonium sulphate precipitation. This method can be the first step before the addition of other chemicals or can be the whole process in itself. After fermentation, the cells are removed from the broth via centrifugation. Ammonium sulphate is then added into the mix, at 40-80% saturation. Mixing is carried out at 4°C for a time ranging from thirty minutes to overnight. The resulting solution is centrifuged, the pellet is collected and re-suspended in a buffer before further processing. This method has been used on a range of bacteria, including *Enterococcus faecium* (62) (34), *Enterococcus faecalis* (55) (40) and *Lactobacillus plantarum* (39).

2.9.1.2 Non-ionic resins

Other methods of extraction include the use of non-ionic resins to remove the peptides from the solution. In this method the cell matter is also removed by centrifugation before the addition of the resin. The broth and resin are mixed and the resulting matrix is collected via centrifugation or decanting. The peptides are removed from the matrix, through the use of solvents such as ethanol and 2-propanol which allows further processing to be done. This method was used to extract the bacteriocins, enterocin L50A and L50B from *Enterococcus faecium* L50 (11).

2.9.1.3 Solvent extraction

Solvent extraction can be carried out without the use of a resin, with chloroform being used to remove bacteriocins directly from solution. Firstly, the cells are removed from the fermentation media by centrifugation, followed by the addition of a volume of chloroform equal to half the volume of the broth. This is mixed for twenty minutes before the separation of an interfacial layer, containing the bacteriocins, can be carried out. This solid layer can then be re-suspended with a buffer for testing. This method has been used to recover subtilin, pediocin, lacidin and nisin (7).

2.9.1.4 pH-dependent binding

The use of the pH depended properties of the bacteriocins is also possible. At neutral pH values bacteriocins become less soluble and can form complexes or bind with the cells in the solution. This can be used as a method of reducing the broth volume. By neutralising the pH the peptide binds to the cells allowing the separation of the media from the cells by centrifugation, this greatly reduces the working volume making processing easier. The peptides can be re-suspended from the cells by reducing the pH, which causes the bacteriocins to become soluble once more. This was used with *Enterococcus faecium* to extract Enterocin EL1 (54).

2.9.2 Final purification

In most cases final purification consists of one, two or all of the following processes, which are used until the working volume contains only the target bacteriocins.

2.9.2.1 Ion exchange chromatography

Ion exchange chromatography, also known as adsorption chromatography, uses the ionic charge of the molecule in order to bind the target to the column or resin. The non-ionic compounds are washed through the column, along with any material that has the opposite charge of the target molecule. It is also possible to bind the contaminants within the column, but this is less effective than the binding of the target which results in greater purity. In the case of bacteriocins, the peptides are cationic and any resin used will be anionic, commonly called cationic resins (71).

2.9.2.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) uses the effect of high salt concentrations on the target material to remove it from solution. Salts can be used to remove peptides and other substances from solution, through the precipitation caused by the high salt concentration. This is also called salting out. HIC uses the fact that the adsorption properties of the target are increased by the high salt concentration before precipitation occurs. Solutions with high salt concentrations are put into a column where the salt concentration assists binding of the peptides to the resin. The products can then be removed by the step-wise reduction of the salt concentration, which lowers the binding properties of the molecules to the resin in the column (70).

2.9.2.3 Reverse phase chromatography

Reverse phase chromatography is similar to HIC in that it uses the interactions between the non-polar groups on the molecules and hydrophobic ligands present on the matrix. The solution containing the peptides is placed into a column where the non-polar molecules, which include the peptides, bind to the ligands on the resin. The addition of

organic solutions elutes the bound molecules: the step-wise increase in concentration of the organic compound allows the release of the proteins with similar hydrophobic properties to be released at the same time. This allows the separation of the samples contents into the various peptides and other molecules (70).

2.10 METHODS OF LARGE SCALE BACTERIOCIN PURIFICATION

There is limited information available on the methods of large-scale production of bacteriocins from fermentation and the associated downstream processing. This is because only nisin is produced commercially on a large scale. No details of nisin's industrial fermentation process and isolation have been published in order to keep the process a trade secret (18).

A number of publications involving various procedures for large-scale industrial purification with a variety of bacteria have been established, however, such as the use of a modified salt precipitation followed by solvent extraction using *Lactobacillus amylovorin*. After fermentation in MRS media the bacterial cells are removed by centrifugation, followed by the addition of 40% ammonium sulphate to precipitate the peptides. The precipitation is followed by solvent extraction, using a 2:1 chloroform: methanol mix, to further reduce the volume before ultra-filtration. Unfortunately there is a great deal of activity lost in this process, limiting the effectiveness of this method (17).

The effect of fed-batch versus (the more common) batch fermentation was carried out using the bacteria *Propionibacterium thoenii* P127 to produce the bacteriocin propionicin PLG-1 (65). In this trial the fed-batch fermentations had a significantly increased production of the bacteriocin when compared with the batch trials. However, the trials did show that the fed-batch system suffered from almost immediate inactivation of the bacteriocin after the maximum titre had been reached, possibly caused by the presence of an inhibitor or proteolytic enzyme in the media. This paper did not discuss the problems of down stream purification and was therefore of limited use in terms of the ability to process this raw material into a usable form. This is similar to the results gathered from a continuous production method attempted using

Pediococcus acidilactici (9). With the use of immobilised cells it was possible to keep the bioreactor operating continuously for 3 months. Again this is useful but without an effective method of downstream purification it is of limited value.

2.11 CONCLUSIONS FROM LITERATURE REVIEW

Bacteriocins from lactic acid bacteria come from a large number of species and in variety of forms. Production of these extracellular peptides is predominantly to provide a competitive advantage for the bacteria in their natural environments, where competition for nutrients is high. Bacteriocin production by these bacteria can be used in food preservations, as in products like nisin or could be used in the development of medicinal applications.

The greatest interest in bacteriocins is in their use as alternatives to food preservative chemicals. Class II bacteriocins have the most potential, with the Class IIa, shows a wider and potentially stronger activity than the other classes of bacteriocins. Other advantages of these bacteriocins is that they are small (<10 kDa), heat stable, and that their producer bacteria can be advantageous in many conditions. The probiotic effect of lactic acid bacteria, along with the positive effect that they can have on fermented and cured foods, lends itself to the food industry.

The bacteriocins from *Enterococcus sp.* exhibit one of the widest ranges of bacteriocins produced by any species. The enterocins show examples in all the variations of Class II bacteriocins, from the Class IIa with the common YGNGV motif, Class IIb, the two peptide bacteriocins and Class IIc the synergistic bacteriocins with their Class IIa partners. *Enterococcus* bacteria are also commonly used in starter cultures for cheeses and other food products, suggesting that the bacterial products would be easily accepted in food stuffs.

There is a wide range of methods used for the purification of bacteriocins, most of which involve the use of the cationic nature of the peptides. However, large scale production of bacteriocins appears to be much more difficult with very few published methods available, and the methods for those bacteriocins commercially available, e.g. nisin, being protected as trade secrets.

3 MATERIALS AND METHODS

3.1 GENERAL

3.1.1 Bacteria and media

Both *Enterococcus sp.* B9510 and *Lactococcus cremoris* 2144 were sourced from Fonterra Research Centre in Palmerston North (formerly known as the Dairy Research Institute). The samples were stored and maintained at -70°C in M17 media (Difco, Becton, Dickinson and Company, USA).

3.1.1.1 Working cell cultures

Working cell cultures were produced by streaking frozen bacterial cultures onto M17 plates. Inoculated plates were incubated overnight, *L. cremoris* 2144 at 30°C and *Enterococcus* B9510 at 37°C. Single colonies were taken from these plates and incubated in M17 broth overnight at the appropriate temperature. These overnight cultures were streaked onto M17 agar and incubated to create the working cell cultures. These plates were stored at 4°C during experimental trials.

3.1.1.2 Agar plates

Soft agar was prepared with the addition of 5 g/l Select agar (GibcoBRL, Scotland) to the media. Agar plates were prepared with 15 g/l of Select agar before poring into Petri dishes.

3.1.2 Bacteriocin assay methods

Unless otherwise stated, the activity of the bacteriocins on plates was quantified by measuring the area of inhibition from the most diluted sample and multiplying that area by the dilution factor of that sample. Minimum diameter of the 'spot-on-the-lawn' method for a valid measurement was 4 mm, and measurements gave an activity in arbitrary units (au).

3.1.2.1 Spot on the lawn

This method is based on Hoover and Steenson 1993 (36), and was adopted with some variations, using *L. cremoris* 2144 as the indicator bacteria. Soft M17 agar was melted and pipetted into 5 ml aliquots before being cooled to 50°C in a water bath. The agar was inoculated with 50 µl of log phase culture, *L. cremoris* 2144, before being poured onto an M17 plate. The agar was allowed to set (30°C for 15 minutes) before 5 µl of the sample material was 'spotted' onto the 'lawn' of indicator cells using an auto-pipette, each plate contained up to ten samples. All plates also contained 5 µl of a positive control, nisin (20 mg/ml Nisaplin (Aplin and Barrett Ltd, England)) and 5 µl of a negative control, 0.5% acetic acid, or as otherwise indicated. Plates were incubated overnight at 30°C and the resulting clearances in the 'lawn' were measured and recorded.

3.1.2.2 Radial diffusion plate assay

The method of Steinberg and Lehrer 1997 (78) was used for the Minimum Inhibitory Concentration (MIC) determination of the High Performance Liquid Chromatography (HPLC) purified peptide. Bacterial cultures, in log phase, were inoculated into the underlay agar (10 ml trypticase soy broth (Becton, Dickson and Company, USA), 10 g ultra pure agarose (Sigma A-6013, USA), 1 L distilled water, pH 7.4), and allowed to set for 1hour at 37°C. Wells were made in the agar and 5 µl of sample was added to each well. Positive controls for each bacterium were also added, nisin for Gram positive (20 mg/ml Nisaplin (Aplin and Barrett Ltd, England)), polymyxin B sulphate (0.03 mg/ml) for Gram negative (Sigma P-1004, USA) and Nystatin (0.03 mg/ml) for

yeast (Sigma N-4503, USA) along with a negative control, 0.5% acetic acid. Samples were left to diffuse into the agar before the addition of the overlay agar (60 g trypticase soy broth powder, 10 g ultra pure agarose and 1l distilled water). Plates were incubated overnight at the appropriate temperature for the bacterial indicator (30°C or 37°C), and the resulting clearing zones were measured.

3.1.3 Measurement of bacteriocin activity

The activity of the samples was determined through the use of dilutions to show the minimum inhibitory concentration of each sample. Dilutions were used to differentiate samples that appeared to show similar activities. The spotting of the peptides onto the agar plate is limited by the diffusion of the peptide across the agar surface, which affects the value of the activity. In order to negate the effect of this limiting factor, dilutions were used to demonstrate the loss of activity due to concentration, as opposed to the size of the clearing zone. The activity of the peptide was defined as the greatest dilution factor that produced a zone inhibition with a diameter greater than 4 mm multiplied by the area of the inhibition. Volumes of the samples tested were typically 5 µl.

3.1.3.1 Minimum inhibitory concentration (MIC) determination

Minimum Inhibitory Concentration's (MIC) were carried out by use of a method modified from Wu and Hancock 1999 (86), using Sigma 96-well polypropylene micro titre plates (Sigma, USA). The peptide was diluted in 2-fold serial dilutions across the microtiter plate in 0.2% Bovine serum albumin (BSA, Sigma A-7030, USA) and 0.5% acetic acid. To test the peptide activity 50 µl of $2x10^5$ of the indicator cells in log phase, in M17 broth, were added to each well. Plates were incubated overnight and the wells containing bacterial growth at the end of incubation were recorded. Bacterial growth was measured visually, with the presence of a cell pellet in the well indicating bacterial growth, and therefore a negative result. The MIC was defined as the most diluted peptide concentration, which inhibited the bacterial growth. Samples were carried out in triplicate and repeated on different days. Values for MIC's were calculated by determination of the amount of active peptide in a sample, either from

crude extract or HPLC sample. The concentration present in the most dilute sample that displayed inhibition was determined and converted into to grams per litre.

3.2 FERMENTATION

Fermentations were carried out using MultiGen F-1000 fermentor (New Brunswick Scientific Co. Inc., USA), which controlled the temperature and stirring of the broth. Temperature was monitored via thermometer to ensure that the correct temperature was maintained. Bacterial cultures were taken from a working cell culture plate and inoculated into 5 ml of M17 broth (Difco: Becton, Dickson and Company, USA) with 0.5% glucose (BDH, England) and incubated overnight at 37°C, the culture was then inoculated into a 100 ml of M17 with 0.5% glucose, incubated overnight at 37°C. This inoculum was added to the fermentor, 100 ml into 900 ml of medium, with the temperature of the broth stable at 42°C, this point indicated time point zero and was the first point of collection in all fermentation trials.

Mediums used in fermentations were: M17 (Difco, USA), which was used to make GM17 (10% glucose solution) and LM17 (10% lactose solution); MRS (Difco, USA); Tryptone-Yeast extract-Tween media (TYT) which was the basis for TYT_{10} and TYT_{30}, see Table 3.1 (66); and Polypeptone-Lab lemco powder-Yeast extract media (PLY), see Table 3.2.

For experiments requiring pH control, 5 M sodium hydroxide (BDH, England) was used to neutralize any drop in pH experienced during fermentation. The pH was measured using a Horizon Ecology Company pH meter, and sodium hydroxide was introduced via a Cole-Palmer pump and Solid state Masterflex pump controller. Aeration was controlled by a Master pneumatic R55-2HW valve, and measured using a Fisher Controls Ltd 1100 air flow meter.

Table 3.1 Composition of TYT media.

Ingredient	Concentration [g/l]	
	TYT_{10}	TYT_{30}
Tryptone (BBL, USA)	10.0	2.5
Yeast extract (Oxoid, United Kingdom)	2.5	5.0
Tween 80 (Koch-Light-Laboratory, United Kingdom)	1.0	1.0
Sodium-β-glycerophosphate (BDH, England)	19.0	19.0
Glucose (BDH, England)	10.0	10.0
$MgSO_4.7H_2O$ (BDH, England)	0.25	0.25
$MnSO_4.4H_2O$ (BDH, England)	0.05	0.05

Table 3.2 Composition of PLY media.

Ingredient	Concentration [g/l]
Polypeptone (BBL, USA)	14.0
Lab lemco powder (Oxiod, United Kingdom)	7.0
Yeast extract (Oxoid, United Kingdom)	3.5
Lactose (BDH, England)	40.0
$MgSO_4.7H_2O$ (BDH, England)	0.35
Ascorbic acid (Merick, USA)	0.7

3.2.1 Design of multivariate fermentation analysis

Design of the fermentation experiments employed an empirical design method using a mixed factorial design. The factors tested were glucose concentration at 3 levels, 0.05, 0.5 and 5%, sodium chloride at 3 levels 0, 3 and 6%, and aeration at 2 levels, standard atmospheric conditions and air saturation. All media were based on M17 with no added carbon source.

Table 3.3 Possible fermentations to be investigated for the given fermentation conditions.

Fermentation	Glucose concentration (%)	Salt Concentration (%)	Aeration
1	0.05	0	Yes
2	0.05	0	No
3	**0.05**	**3**	**Yes**
4	0.05	3	No
5	0.05	6	Yes
6	**0.05**	**6**	**No**
7	**0.5**	**0**	**Yes**
8	0.5	0	No
9	0.5	3	Yes

Fermentation	Glucose concentration (%)	Salt Concentration (%)	Aeration
10	**0.5**	**3**	**No**
11	0.5	6	Yes
12	0.5	6	No
13	5	0	Yes
14	**5**	**0**	**No**
15	5	3	Yes
16	5	3	No
17	**5**	**6**	**Yes**
18	5	6	No

In Table 3.3 the full set of possible fermentations are represented, given the factors to be tested. The fermentation conditions in bold text were not carried out but could be estimated from the data gathered from the results of the other trials.

3.2.2 Collection of samples from fermentation trials

Samples were removed from the fermentation unit at hourly intervals for eight hours with a final sample taken at 24 hours. Three, one millilitre samples were taken at each time interval and placed in micro-centrifuge tubes (Quality Scientific Plastics (QSP), USA). These samples were then centrifuged at 13,000 rpm for 3 minutes in a Heraeus Sepatech Biofuge (West Germany) 13 micro centrifuge, and the resulting supernatant was filtered through a Minisart 0.45 μm filter (Sartorius, Germany) to remove any remaining cells. The cell pellet from centrifugation was resuspended in 0.5% sodium chloride solution (BDH, England). The optical density was measured with Pharmacia Biotech Ultrospec 2000 spectrophotometer at 620 nm.

3.2.3 Testing of fermentation supernatant

The filtered supernatant was tested for the concentration of glucose and L-lactate and bacteriocin activity. Bacteriocin testing was carried out using the spot-on-the-lawn technique described previously.

3.2.3.1 Substrate concentration

Glucose and lactate concentrations were measured using a YSI 2700 analyser (Yellow Spring Instruments, USA), using glucose (YSI 2365) and lactate (YSI 2329) membranes and YSI buffer 2357.

3.3 PURIFICATION

3.3.1 XAD-16 method for bacteriocin purification

The resin binding method used was based on a procedure from Cintas *et al* 2000. Fermentation media was centrifuged to remove the bacterial cell using a Sovrell Instruments RC5C centrifuge (DuPont, USA). XAD-16 resin (Superco, USA) was added to the supernatant. The resin and broth were stirred for 24 hours to allow binding of the bacteriocin. The matrix (resin and peptide combined) was collected and washed in RO water before elution. Fifty percent propan-2-ol (isopropyl alcohol) (BDH, England) in RO water was used to elute the resin. The matrix was stirred for 24 hours and the resulting solution collected for freeze drying. The dried sample was resuspended in 0.5% acetic acid (11).

3.3.2 XAD-16 bacteriocin purification, method optimisation

Tests were carried out using nisin solution, with a concentration of 5 g/L Nisaplin (Aplin & Barrett Ltd., England), giving an active concentration of 0.125 mg/ml of nisin. Samples were taken from solution, so that a loss in activity would show the absorbance of the nisin into the matrix.

3.3.2.1 Temperature for binding

Three temperatures were tested, 30°C, ambient air temperature and 4°C, for each of these, 5 g of resin was added to 100 ml of the nisin solution. Duplicate one millilitre samples were taken at 0, 0.5,1, 2, 4, 6 and 24 hours. Stirring was carried out by the use

of magnetic fleas and stirring plates. Samples were tested using MIC plates, as previously described.

3.3.2.2 Resin volume

Five shake flasks were prepared with 100 ml of nisin solution. A range of resin volumes were added to each flask, 1, 3, 5, 7, 10 g. Duplicate one millilitre samples from the shake flasks were taken at 0, 0.5, 1, 2, 4, 6 and 24 hours. Stirring was carried out by the use of magnetic fleas and stirring plates. Samples were tested using MIC method, as previously described.

3.3.2.3 Effect of pH

Nisin solution was pH adjusted using 5 M sodium hydroxide or 5 M sulphuric acid, to produce pH values of 3, 5, 7 and 9, in 100 ml samples. Duplicate one millilitre samples were taken for the activity of each sample at the adjusted pH before the addition of the resin. Five grams of XAD-16 resin was added to each sample and shaken at room temperature using an orbital incubator (Gallenkamp, UK). Samples were taken at 0, 0.5, 1, 2, 3, 4, 6 and 24 hours. Activity was measured using the MIC method, as described previously.

3.3.2.4 Elution

Resin from the pH testing was collected and washed in distilled water to remove any unabsorbed nisin from the solution. Resin was divided into two 10 g lots to test the effect of isopropyl alcohol concentration on bacteriocin elution from the resin. The first lot was eluted with successive concentrations of 10%, 30%, 50%, 70% and 90% isopropyl alcohol, while the second lot was eluted with 20%, 40%, 60%, 80% and 100%. For each elution concentration 100 ml of the solvent and water mixture was used, the resins were shaken using an orbital incubator (Gallenkamp, UK) for one hour before duplicate samples were taken.

Between each sampling, the excess liquid was removed and the resin was washed with 50 ml of distilled water. Equal volumes from each sample were freeze dried and the resulting samples were resuspended in 0.5% acetic acid. Activity was measured using the MIC method described previously.

3.3.3 pH binding method for the purification of bacteriocins

The method for binding peptides using pH variation was based on the methods specified in Eguchi *et al* 2001. Fermentation broth was grown under standard conditions for twenty four hours, and the broth then adjusted to pH 6 with 5 M sodium hydroxide and left for an hour at 4°C, to allow the peptides to bind to the cell surfaces. The broth was then centrifuged at 8,000 rpm for 30 minutes, in a Sorvall Instruments RC5C centrifuge (DuPont, USA), to isolate the cells from the broth. The cell pellet was then washed with 50 mM MES buffer (pH 6). Cells were resuspended in 50 mM MES buffer (pH 2), with 1.0 M NaCl (BDH, England) and 0.01% Brij-35, the resulting solution was stirred for one hour. The solution was again centrifuged at 15,000 rpm for 20 minutes to remove the cells. The supernatant was collected for freeze drying, before re-suspension and testing.

3.3.4 pH variation for the optimisation of bacteriocin purification

The binding of the peptides to the cells was tested at a variety of pH values. Rather than carrying out the whole separation process, a number of 100 ml samples of *Enterococcus* B9150 were grown overnight at 37°C in M17. These samples were tested for activity before pH adjustment. The samples' pH was adjusted with 5 M sodium hydroxide or 5 M sulphuric acid to the desired pH value. The tested pH values were pH 6.0, 6.5, 7.0, 7.5, 8, 8.5 and 9. Samples were then stirred using an orbital incubator (Gallenkamp, UK) for one hour at 4°C. Samples were collected to test for any loss in activity due to binding of the bacteriocin to the cells.

3.3.5 Use of ion-exchange chromatography for the purification of bacteriocins

Macro-Prep CM (Bio-Rad, USA) support is a weak cationic resin and was used to purify the crude extract. A running buffer of degassed 25 mM ammonium acetate (BDH, England) was used to separate the cationic fraction from the crude extract. 2-3 millilitres of the resuspended eluent from the XAD-16 resin was loaded into the column and allowed to run into the bed under gravity. The optical density of the buffer exiting the column was measured using a LKB Bromma 2138 UVICORDS monitor and recorded using Sekonic SS-250F recorder, set to 30 mm/hour chart speed and sensitivity of 0.2. Samples were collected using LKB Ultrorac fraction collector 7000 set to 15 minutes per tube and the LKB UVICORD II pump was set to 20 mL/hour. Once the column reached equilibrium, as indicated by optical density, the buffer was changed to degassed 10% acetic acid (BDH, England) in order to elute the bound peptides, collection and detection was carried out as described for the running buffer. The fractions from the column were collected and pooled. The acetic acid was removed by Rota-evaporation (Buchi Rotavapor R110, Switzerland) and the sample was freeze-dried (Virtis freeze dryer model 10-020, The Virtis Company, Inc. USA).

3.3.6 Gel filtration for the purification of bacteriocins from crude extract

Bio-Rad (USA) P-10 packing gel was used for size exclusion separation. Bio-Gel P10 gels are polyacrylamide beads used for high-resolution gel filtration: P10 indicates a molecular weight exclusion limit of 10,000 Da.

The running buffer for the gel column was 5% degassed acetic acid (BDH, England). Five millilitres of the eluent from XAD-16 resin was loaded onto the top of the column and allowed to run into the gel under gravity. Measurement through the column were taken using LKB Bromma 2138 UVICORDS spectrophotometer and recorded using Sekonic SS-250F recorder, set to 30 mm/hour on sensitivity 2. Samples were collected using LKB Ultrorac fraction collector 7000 set to 15 minutes per tube. The LKB UVIORD II pump was set to 20 mL/hour. The column was run for 24 hours. The fractions were pooled and acetic acid was removed using Rota-evaporation (Buchi

Rotavapor R110, Switzerland) and freeze-dried (Virtis freeze dryer model 10-020, The Virtis Company, Inc. USA).

3.4 CHARACTERISATION

Unless otherwise stated tests in the characterisation section used samples of fraction two, which was a crude peptide solution collected from the ion exchange column and re-suspended in 0.5% acetic acid.

Activity was measured using the spot-on-the-lawn technique using *L. cremoris* 2144 as the indicator bacteria.

3.4.1 Effect of temperature on bacteriocin activity

In order to test the effect of temperature on the peptide, samples were tested at three temperatures, 60°C, 80°C and 100°C. The samples were heated in a heating block (Techne Dri-block 08-3), then removed and placed in ice after 15, 30 and 60 minutes.

3.4.2 Effect of enzymes on bacteriocin activity

The effect of enzymes on the bacteriocin was determined using a modified method from Moreno *et al* 2002 (60). A range of enzymes were added to samples of fraction two (at 1 mg/ml) and incubated for two hours at 30°C or 37°C. Enzymes used were α-Catalase (Research Organics Limited, USA), Papain (BDH, England), Trypsin (BDH, England), Catalase (C-9322 Sigma, USA), Lipase (L-1754 Sigma, USA), Protease K (BDH, England), Protease I (P-4630 Sigma, USA), Protease IV (P-0384 Sigma, USA), Protease IX (P-6141 Sigma, USA), Protease XIII (P-2143 Sigma, USA), Protease XIV (P-5147 Sigma, USA), Protease (P-8811 Sigma, USA), and Pronase (Boehringer Mannhein GmbH, W.Germany). Enzyme activity was tested using BSA samples treated with each of the enzymes; the enzyme/protein mix was run through gel-electrophoresis to ensure the activity of the enzyme.

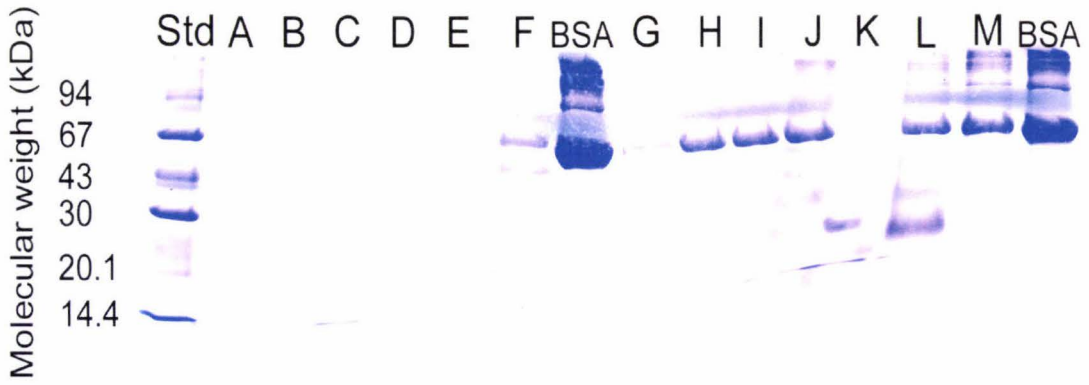


Figure 3.1 Electrophoresis gel of the effect of enzymes on BSA. Those samples with bands at 67 kDa show little enzyme activity, while those with no bands shows digestion of the BSA. A = Protease I, B = Protease XIV, C = Protease K, D = Protease, E = Pronase, F = Trypsin, G = Protease IV, H = Protease IX, I = Protease XIII, J = Catalase, K = α-Catalase, L = Papain, M = Lipase, Std = Protein standard and BSA = Undigested BSA standard.

3.4.3 Effect of pH on bacteriocin activity

The testing carried out for the effect of pH on bacteriocin activity was based on a modified method from Eguchi *et al* 2001 (20). Media was prepared over a wide range of pH values before autoclaving; this media was M17 broth with pH adjusted with 5 M sodium hydroxide or concentrated sulphuric acid to produce the required pH values. The pH values used were 1.7, 3, 4, 5, 6, 8, 9, 10 and 11. Samples of fraction two were placed into the pH adjusted media and incubated for one hour at 37°C. Samples were then plated onto M17 media inoculated with *L. cremoris* 2144. Serial dilutions were carried out both in the incubation media, i.e. pH adjusted, as well as in standard M17 media, at approximately pH 6.8. Negative control on these plates was the adjusted pH media.

3.4.4 Effect of salt concentration on bacteriocin activity

Methodology used for the effect of salt was the similar as used for pH testing, with media prepared using three sodium chloride concentrations: 100 mM, 250 mM and 500 mM. Aliquots of fraction 2 were placed into the salt adjusted media and incubated for one hour at 37°C. Samples were then plated onto M17 media inoculated with *L. cremoris* 2144. Dilutions were carried out both in the incubation media, i.e. salt

adjusted, as well as in standard M17 media, at pH 6.8 with no additional salt. Negative control on these plates was the adjusted salt media.

3.4.5 Effect of bacteriocins on a bacterial spectrum

A range of bacteria were streaked onto Brain-Heart Infusion agar (BHI) plates from frozen stock and incubated at the appropriate temperatures to produce stock plates of the bacteria. From these plates subcultures of the various bacteria were inoculated in order to test the bacterial spectrum of the bacteriocin. The MIC method, described above, was used to determine the MIC of fraction 2 over the range of different bacteria. Bacteria used were *Escherichia coli 0157*, *Staphylococcus aureus* NCTC 4163, *Salmonella enteritidis*, *Listeria monocytogene* NCTC 7973, *Listeria monocytogene* NCTC 10884, *Candida albicans* 3153A, *Streptococcus faecalis*, *Salmonella typhimurium*, *Lactobacillus plantarum*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus natto* and *Yersinia entercolitica*.

3.4.6 SDS-PAGE Gel electrophoresis of proteins

Gels were made using the recipe in Table 3.4; the chemicals were prepared and set in a Mighty Small SE 245 Duel gel casters. Samples were prepared for loading by mixing an equal amount of 2X sample buffer and sample. The sample and buffer solutions were heated for three minutes at 80°C in a Techne Dri-Block08-3 heating block, then removed and placed in ice to cool quickly. The gels were placed in Hoefer Scientific Might Small II SE250 to run; to this 250 ml of running buffer (dilute 1:5 5x electrode buffer) was added. Samples were pipetted into the wells in the gel along with a standard (LMW, 14,400-94,000, Pharmacia Biotech, USA). Gels were run using electrical controller at 20 mA for each gel. The run gels were removed and soaked in fixing solution (25% propan-2-ol (isopropyl alcohol) (BDH, England) and 10% acetic acid (BDH, England)) for one hour on rocking platform. The fixing solution was then discarded and staining solution was added. The gel was rocked in the staining solution for 1-2 hours. Stain was removed and de-staining solution (10% acetic acid) was added to rock overnight.

De-staining solution was replaced until the desired background colour was reached. Gels were scanned using a flat bed scanner to prevent damage to the results.

Table 3.4 Compounds used to prepare both peptide and protein gels.

	Stacking gel	Protein gel
RO water	6.1 ml	2.02 ml
0.5 M Tris-HCl buffer (pH 8.8)	2.5 ml	0 ml
1.5 M Tris-HCl buffer (pH 8.8)	0 ml	2.5 ml
10% SDS stock	100 µl	100 µl
30% acrylamide stock plus 0.8% Methlenebris acrylamide	1.3 ml	5.3 ml
Temed (UltraPURE, USA)	10 µl	5 µl
10% ammonium persulphate (UltraPURE, USA)	50 µl	50 µl

2X Sample Buffer: 25 ml 0.5 M Tris-HCl buffer (United States Biochemical Corp. USA), 20 ml glycerol (BDH, England), 40 ml 10% Sodium Dodecylsulfate (SDS) (Serva, Germany), 10 ml β-mercaptoethanol (Merck, Germany) and 5 ml 0.05% bromophenol blue (BDH, England).

5X Electrode Buffer: 15 g Tris-HCl, 72 g glycine (Sigma, USA) and 5 g SDS in 1 l. of RO water. Adjust pH to 8.3. Diluted to1:5 in RO water before use.

Staining solution 1.25 g Coomassie brilliant blue R-250 (Bio-Rad, USA), 242 ml RO water, 242 ml methanol (Merck, Germany), 46 ml acetic acid (BDH, England).

3.4.6.1 Activity gel for the determination of the size of the active fraction from fraction two

Activity gel was based on a method from Moreno *et al* 2002. The gel was prepared as previously described. Half of the gel was prepared normally, while a mirror of the samples was prepared on the other half of the gel, but did not contain the 2X buffer. After the gel had been run the gel was cut in half, the stained half was treated as normal (samples with the 2X buffer), while the unstained gel was washed in R.O. water for 24 hours, the wash water was regularly replaced. After washing the gel was placed in a Petri dish and covered with 20 ml of liquid M17 soft agar inoculated with a log culture

of *L. cremoris* 2144. The plate was incubated at 30°C overnight and the area of inhibition was compared to the stained gel (60).

3.4.7 Protein concentration determination

3.4.7.1 Low protein concentrations

The *dot*METRIC protein assay (Geno Technology, Inc, USA) method was used for protein from HPLC samples. Protein samples were diluted in the dilution buffer, before being pipetted onto test strips. The strips were then placed in Fixer-A, and incubated for 2 minutes. Strips were then shaken in Developer-B and incubated for two to four minutes to allow the results to become visible. Protein concentration was compared to the *dot*METRIC scale to determine protein concentration. Tests were carried out in duplicate to ensure the results.

Figure 3.2 Protein dotMETRIC measurement. Both spots represent protein concentrations provided by the dotMETRIC method, samples of 4 µl and 5 µl from the same sample were taken and the ratio taken to indicate a repeatable test. In this case protein concentration was 0.029 µg/µl.

3.4.7.2 High protein concentrations

This method was based on that developed by Tsaffir and Zvi (1996) (89) which uses linearisation of the Bradford method by plotting Abs590 nm/Abs450 nm against the protein concentration. Dilutions of Bovine serum albumin (BSA) (Sigma A-7030, USA) standard, in duplicates, were used to construct a standard protein curve with BSA samples containing 0, 2.5, 5, 7.5 and 10 µg of BSA. The Protein assay reagent (BioRad, USA) was added to each of the BSA standards or peptide samples. Absorbance of the BSA standards and of the samples was measured at 450nm and 590 nm using Pharmica Biotech Ultrospec spectrophotometer. From plotting Abs590/Abs450 against mass of

BSA (μg) a standard curve was created (Figure 3.3). The amount of protein present in the samples was determined by positioning the Abs590/Abs450 value for the unknown sample onto the graph and extrapolating the protein concentration. Protein concentration of the original sample was determined by multiplying the dilution factor of the sample by its position on the standard curve.

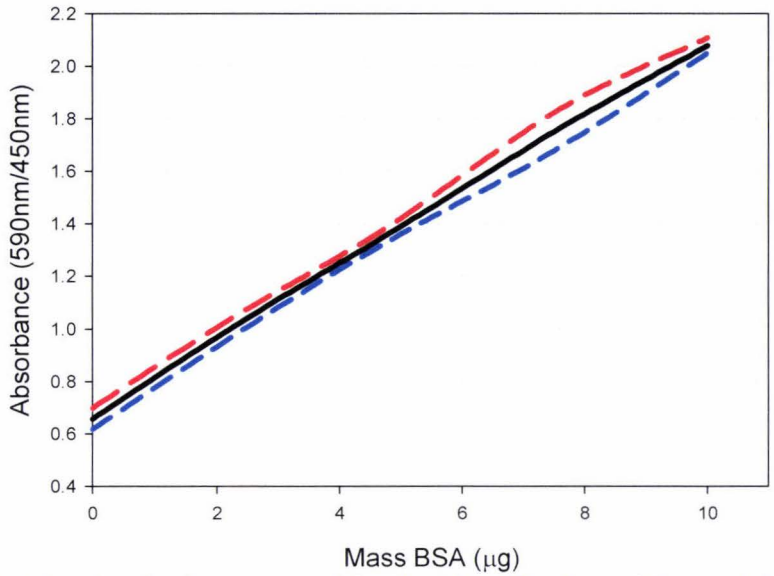


Figure 3.3 Protein standard curve used for the identification of the protein concentration in fraction 2 samples. Protein replicates 1 (--), 2 (--), average (—). Equation of the line of best fit was y = 0.142x + 0.673.

3.4.8 High Performance Liquid Chromatography (HPLC)

HPLC was used to separate fraction two into its component parts, and to isolate the active fractions. The separation was carried out on a Dionex HPLC system with a Phenomenex Jupiter Proteo column. Acetonitrile (Merck, Germany) buffers were used to produce gradients to created the separation of the peptides, Buffer A was 5% acetonitrile with 0.1% trifluoroacetic acid (TFA) (BDH, England), in RO water, while buffer B was 95% acetonitrile with 0.1% TFA in RO water. Detection of the peptides was carried out using a UV system at 215 nm and 230 nm. Fractions were collected, placed in a thermostat vacuum oven (Townson and Mercer Ltd, Croydo, England) for one hour at 40°C before freeze drying.

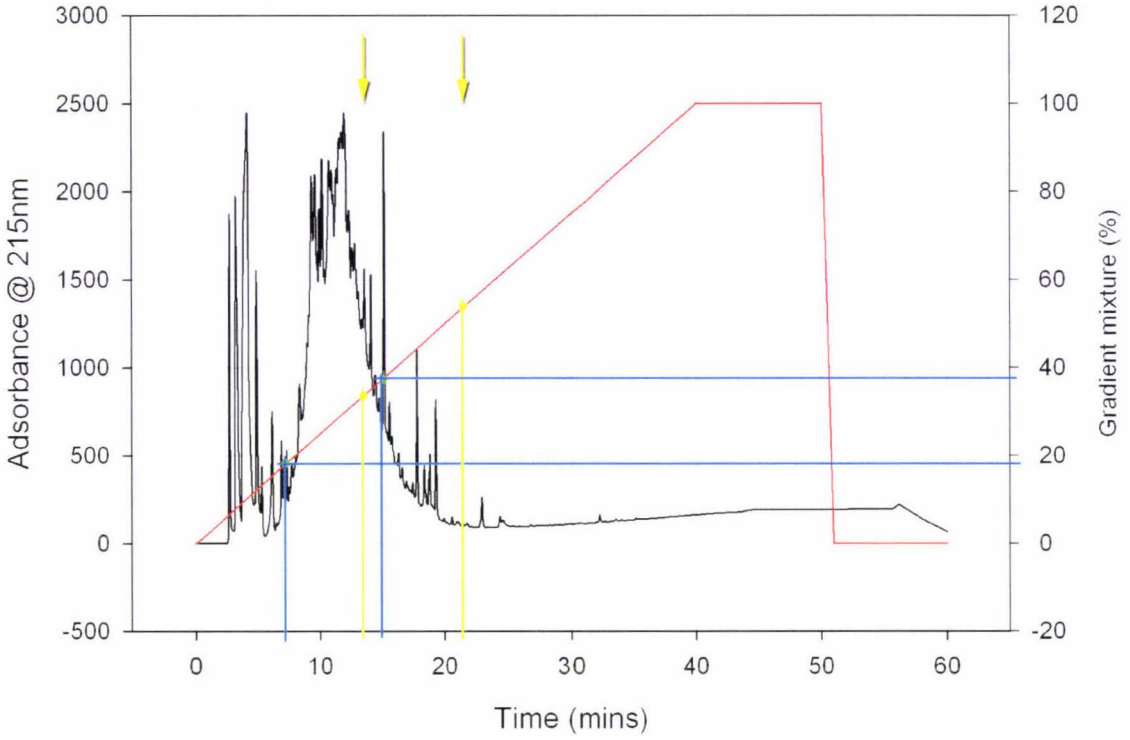


Figure 3.4 Example of Fraction two HPLC graph. Gradient of 0-100 (red line) was used in order to define the region which contains the active fraction. The yellow arrows indicate the area containing the active fraction; the blue lines indicated the region of this activity modified by the void volume and lag time within the column, giving the gradient range where elution of the active portion occurred.

3.4.9 Mass spectrometry

Dried HPLC samples were sent to Protein Analysis Facility at The University of Auckland. Mass spectrometry was carried out using Voyager-DE PRO MALDI-TOF determination with Applied Biosystems Voyager system 6233 software. Acquisition mass range was measured between 500 and 5000 Da and the calibration matrix used was a-Cyano-4-hydroxycinnamic acid.

Samples were also sent to the Protein Microchemistry Facility, Department of Biochemistry, University of Otago, for MALDI-TOF-MS. Samples were prepared by mixing peptide samples (0.5 µl, from 50 µl total volume HPLC fractions) and matrix (0.5 µl of 10 mg ml^{-1} α–cyano-4-hydroxycinnamic acid in 0.1% TFA, 60% acetonitrile) directly on a stainless steel slide, and left to dry at room temperature. Mass data was collected, at near threshold laser fluencies in the positive ion mode, with a linear

instrument (Finnigan Lasermat 2000, from Thermo Bioanalysis). Where internal calibration was used, this was added to the sample/matrix mixture as indicated.

3.4.10 N-terminal sequencing

N-terminal sequencing was also carried out by the Protein Analysis Facility at the University of Auckland; an automated Edman degradation system using an Applied Biosystems Procise Sequencer.

Samples sequenced at Otago University used an automated Edman peptide sequencing, carried out on a glass fibre disk using a Procise 492 Protein Sequencer (Applied Biosystems) with pulsed liquid trifluoroacetic acid delivery.

3.4.11 Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS)

Samples to be tested using LC-MS-MS were sent to the University of New South Wales, Bioanalytical Mass Spectroscopy facility. Peptides at this facility were separated by nano-LC using an Ultimate HPLC and Famos auto sampler system (LC-Packings, Amsterdam, Netherlands). Samples (5 µl) were concentrated and desalted onto a micro C18 pre-column (500 µm x 2 mm, Michrom Bioresources, Auburn, CA) with H_2O:CH_3CN (98:2, 0.1 % formic acid) at 20 µl/minute. After a four minute wash the pre-column was switched (Switchos, LC Packings) into line with a fritless analytical nano column manufactured according to Gatlin (29) containing C18 RP silica. Peptides were eluted using a linear gradient of H_2O:CH_3CN (95:5, 0.1 % formic acid) to H2O:CH3CN (40:60, 0.1 % formic acid) at 200 nl/minute over 30 minutes. The column outlet was positioned approximately 1 cm from the orifice of an API QStar Pulsar i hybrid tandem mass spectrometer (Applied Biosystems, Foster City CA). High voltage (2300 V) was applied through to a low volume tee (Upchurch Scientific). Positive ions were generated by electrospray and the QStar operated in information dependent acquisition mode (IDA). A Tof MS survey scan was acquired (m/z 350-1700, 0.75 s). The 2 largest multiply charged ions (counts > 15) were sequentially selected by Q1 for MS-MS analysis. Nitrogen was used as collision gas and an optimum collision energy

chosen (based on charge state and mass). Tandem mass spectra were accumulated for 2.5 s (m/z 50-2000). A processing script generated data suitable for submission to the database search program (Mascot, Matrix Science). High scores indicate a likely match. Spectra were interpreted also manually and possible amino acids sequences derived from the fragmentation ions.

4 FERMENTATION

4.1 INTRODUCTION

The development of a fermentation process is the first step required in large scale applications producing products that use biological fermentation as their primary method of production, such as bacteriocins. For bacteriocins, fermentation has the ability to produce the maximum amount of the peptide before downstream refinement isolates the active fraction, maximising the amount of material available for later work. In order to create the greatest benefit to any increase in fermentation size it is first necessary to optimise the fermentation conditions at a bench top scale. This provides the greatest amount of peptide for downstream processing and eventually as a sellable product. To that end this section looks to build on the work previously undertaken in the development and optimisation of the fermentation media and environmental conditions for the production of bacteriocins of *Enterococcus faecalis* B9510.

The origin of this work stems from a group of projects working on isolates of lactic acid bacteria provided by the Fonterra Research Centre in Palmerston North (FRC). The *E. faecalis* B9510 strain was judged to be the most promising strain from 25 bacterial strains tested from a selection that included strains of *Pediococcus*, *Lactobacillus* and other enterococci (unpublished). *E. faecalis* B9510 was selected due to its wide range and strength of inhibition against other Gram positive bacteria. It was also found that *E. faecalis* B9510 also inhibited a range of Gram negative food pathogens, unusual for what is suspected to be a type II bacteriocin. This activity against both Gram positive and negative bacteria was the driving force behind this additional further research.

The majority of the work carried out prior to this investigation focused on the effects of a variety of carbon sources and concentrations of these substrates. In Figure 4.1 it can be seen that the M17 media with glucose (GM17) produced the highest bacteriocin activity. High activity was also present in the fermentations using M17 with lactose

(LM17) and TYT_{10}. However in fermentations using MRS, PLY and TYT_{30} no bacteriocin activity was observed.

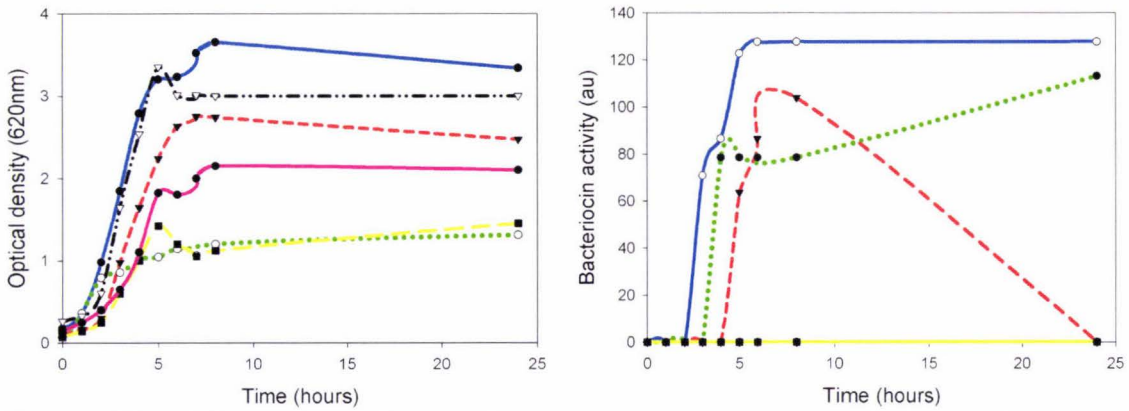


Figure 4.1 Effect of media on the cell growth and bacteriocin production of *Enterococcus* B9510. LM17 (···), GM17 (—), TYT_{10} (--), TYT_{30} (-···-), MRS (—) and PLY (–), (based on data from Zimmermann, T. 2002 unpublished) (88).

Cellular growth appeared to fall into three groups when related to the media. The lowest optical densities were observed using LM17 and PLY (lactose media), while the observed optical densities for GM17 and TYT_{30} were approximately three times higher (glucose mediums), MRS and TYT_{10} fell in between these extremes. There was however no distinct relationship between cellular production and bacteriocin production. TYT_{30} and GM17 produced the highest cellular growth, however, only GM17 produced bacteriocins in detectable quantities. Similar observations could be made for PLY and LM17; both grew poorly but LM17 produced high levels of bacteriocin activity. These experiments formed the basis of the optimisation of the growth media and the physiology of the bacteria.

In the following section of work, initial testing carried out was on the effect of temperature and pH on bacteriocin production. Previous fermentations used an uncontrolled pH, resulting in a drop in the pH through the duration of fermentations, caused by the bacteria's production of lactic acid. This gradient had the potential to affect the activity and production of the peptides; as bacteriocin production could be pH sensitive or dependent on cell growth, pH change may have an effect on biomass production. The second series of fermentation tests were multivariate tests and concentrated on salt concentration, glucose (carbon source) concentration in addition to testing the effect of aeration on the fermentation media. This was done in order to see if

these conditions effected the growth of the bacteria, substrate consumption and most importantly bacteriocin production when compared with standard conditions.

The main goal of these experiments was to maximize the peptide production in a manner that was both stable and efficient. The addition of extra processing steps or chemicals which resulted in only small improvements in the volume or activity of the peptide produced, but added extra expense or complication to the process would not be beneficial.

The secondary goal of these experiments was to see the effect that the interactions between each of the variables had on the production of the peptide. The interactions between possible growth factors had not previously been examined. It was possible that the interactions between environmental factors had an effect on the bacteriocin production, just as great as the individual effects of each variable. This experimentation was used to judge if these interactions needed to be investigated in any future work investigating the optimal growth conditions for this bacterium.

4.2 RESULTS

4.2.1 Initial testing into the production of bacteriocins from *Enterococcus* B9510

4.2.1.1 Effect of temperature on cellular and bacteriocin production from *Enterococcus* B9510

Maximum growth rate and bacteriocin activity were shown at 42°C (Figure 4.2 B). Growth and activity are still present below this temperature, but at 47°C there was no detectable bacteriocin production, suggesting that the bacteria were sensitive to fermentation temperatures higher than 42°C. This sensitivity to higher temperatures could also be observed in the maximum optical densities for the fermentations. The total cell growth for the fermentations at 30°C, 37°C and 42°C were very similar, while at 47°C growth observed was only about two thirds that of the other samples (Figure 4.2 A).

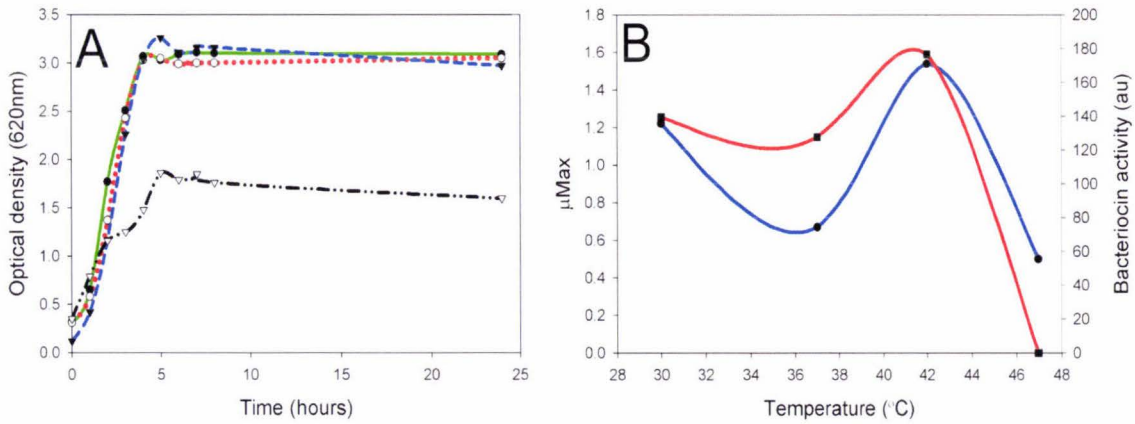


Figure 4.2 Effect of temperature on the growth rates of the bacteria and the detectable bacteriocin production in the fermentation broth. A. 30°C (—), 37°C (···), 42°C (--) and 47°C (-···-). B. µMax (—), Maximum bacteriocin activity (—), (based on Gazin, V. 2001 unpublished) (30).

Testing for the optimum fermentation temperature was carried out in conjunction with German exchange student V. Gazin (30).

4.2.2 Effect of pH on the production of bacteriocins

The greatest biomass produced during fermentation trials was measured in the fermentations with pH values within the range of 5 to 6, this can be seen in Figure 4.3. The cellular growth observed in the uncontrolled fermentation, also in Figure 4.3,

showed poor growth, similar to those produced at pH 4 and 7. These results show that the optimum conditions for cellular production of *Enterococcus* B9510 was within the range of pH 5 and 6.

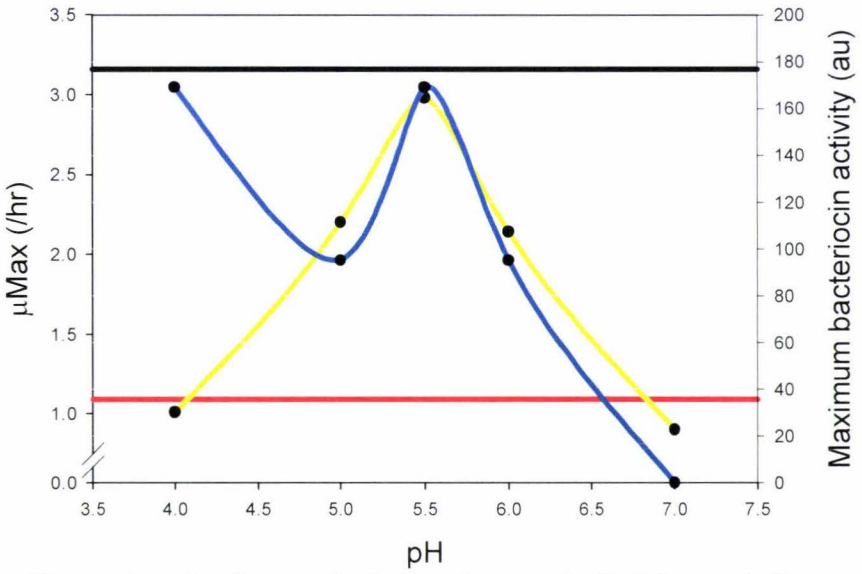


Figure 4.3 µMax values for the controlled and uncontrolled fermentations. µMax (—), µMax for uncontrolled pH fermentation (—), Bacteriocin activity (—), Maximum bacteriocin activity for uncontrolled pH (—).

The link between overall cell mass and the maximum cellular growth rate (µMax) was also important, from the fermentation trials the highest µMax value, Figure 4.3 was seen at pH 5.5, with decreasing values for any increase or decrease in pH.

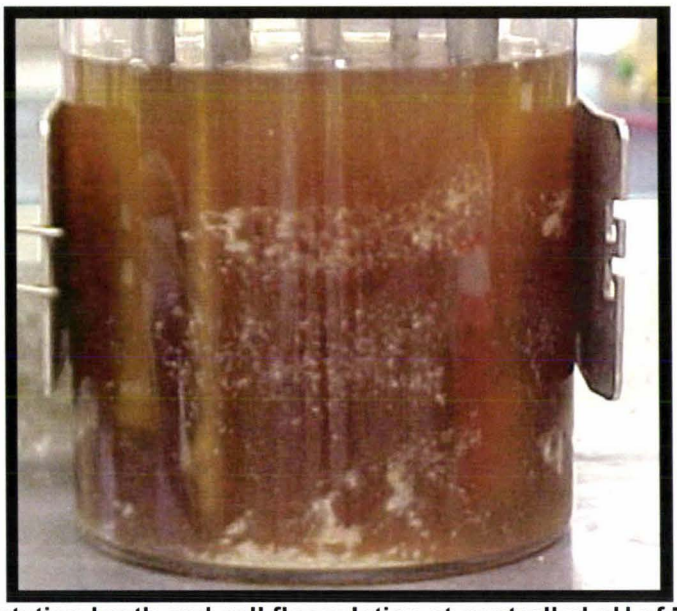


Figure 4.4 Fermentation broth and cell flocculation at controlled pH of 7.

Results for fermentation at pH 7 could have been affected by flocculation that occurred during this trial. Growth rates and bacteriocin activity may have been misrepresented by the clumping of the cells, as can be seen in Figure 4.4.

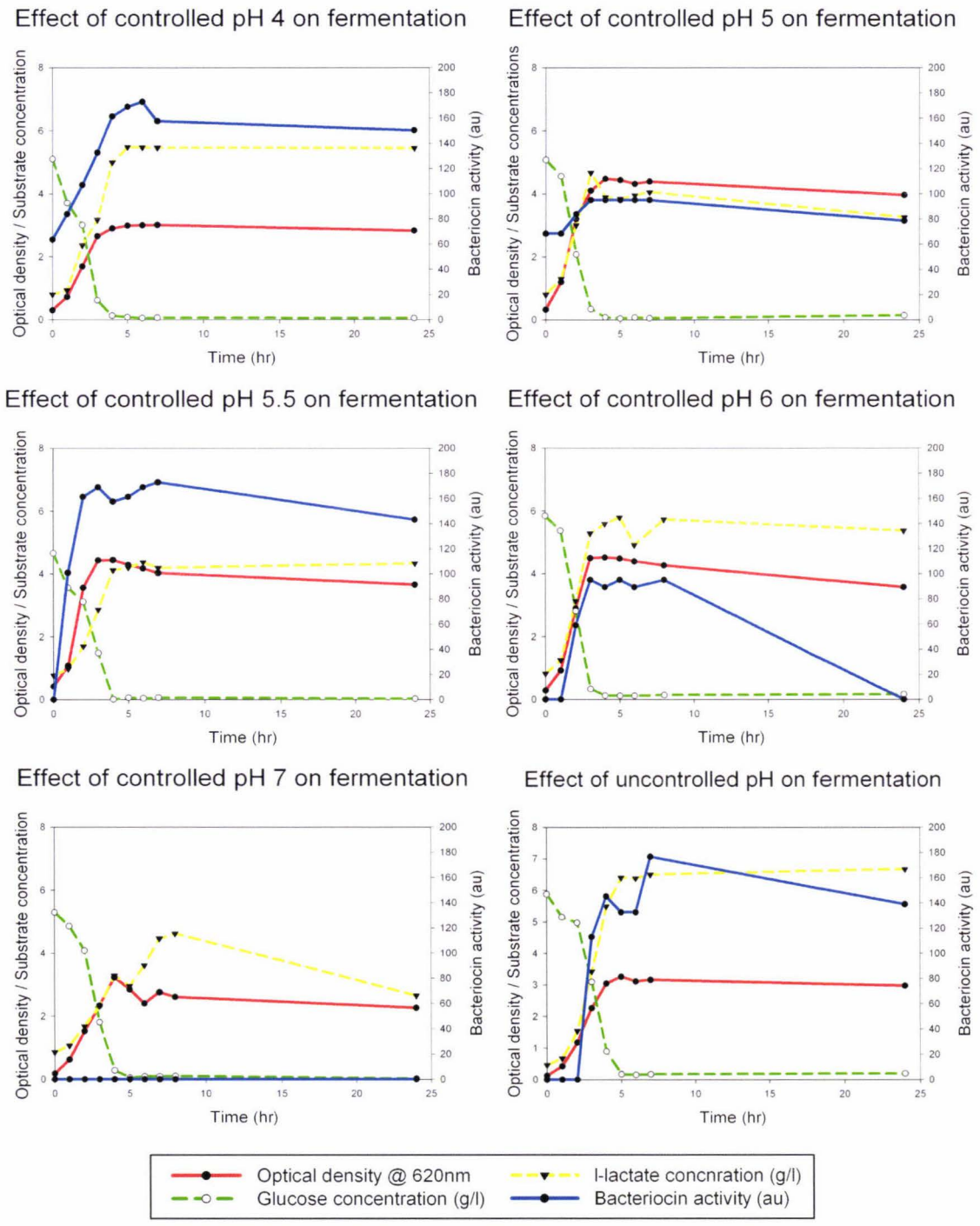


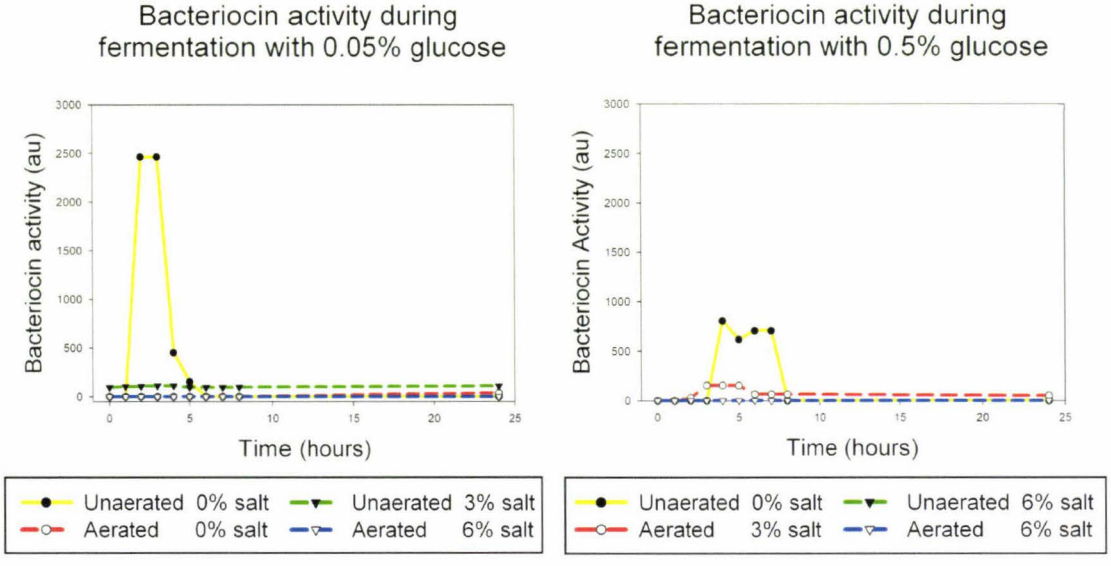
Figure 4.5 Fermentation trials for each of the controlled pH values as well as uncontrolled pH fermentation. Biomass of the bacteria (—), consumption of glucose (--), production of l-lactate (··) and the activity of the bacteriocin activity (—).

From this data it was possible to stipulate that the results of this investigation produced fermentation conditions that optimised bacterial growth and bacteriocin production using M17 with 0.5% glucose. The data also suggested that the bacterial stock was thermophilic with an optimally grown rate and production of the peptide at 42°C. The difference between the maximum controlled bacteriocin activities at pH 5.5 was not significantly different to that of the uncontrolled fermentation, as can be seen in Figure 4.3. However, in order to better control fermentation, a controlled pH of 5.5 was used in fermentations from this point.

4.2.3 Effects of multi-factor fermentation on bacteriocin production from *Enterococcus* B9510

4.2.3.1 Effect of glucose on bacteriocin production

In Figure 4.6 it can be seen that the highest bacteriocin activity was produced with 0.05% glucose in an unaerated fermentation with no additional salt. However, for each of the glucose levels (0.05%, 0.5% and 5%) the highest activity was found in all the samples with the lowest salt concentration, indicating that salt inhibited the activity of the antimicrobial peptide. Also from Figure 4.6, the glucose concentration appears to reduce/inhibit the maximum bacteriocin production, as the maximum activity reduces as the glucose concentration increases.



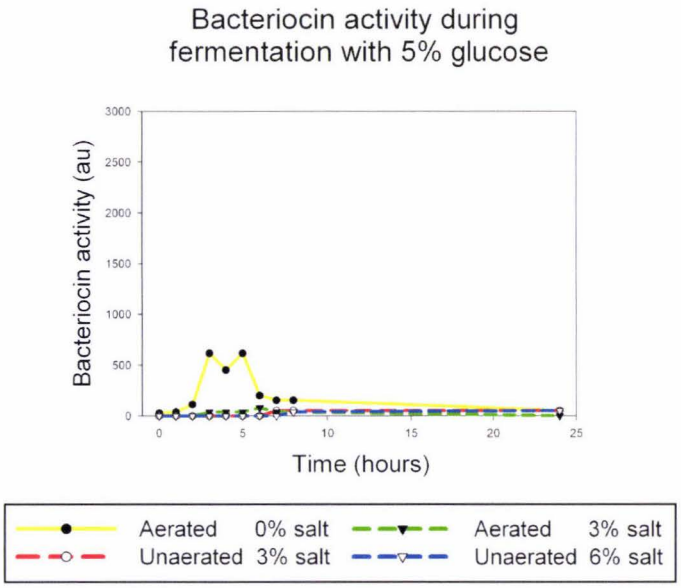


Figure 4.6 Bacteriocin activity, separated based on the initial concentration of glucose in the broth. Each graph shows the four different conditions that each of the glucose concentrations were tested under.

4.2.3.2 Effect of sodium chloride concentrations on bacteriocin production

Salt appeared to have a negative effect on the activity of the bacteriocin, illustrated in Figure 4.7. The samples produced from fermentations with no additional salt all produced higher bacteriocin activity when compared to those carried out under similar glucose and aeration condition. The exception to this was the sample with 0.05% glucose and aeration, which could be due to the effect of aeration on the bacteria. It seems quite clear that as the salt concentration increased the bacteriocin activity decreased, with only one sample tested at 6% salt showing any activity at all. This positive activity at such a high salt concentration may have been the result of the high glucose concentration, and the aeration in that sample that caused a prolonged lag phase, producing conditions conducive for the production of bacteriocins.

In terms of bacterial growth, μMax values do appear to be affected by salt concentration as the highest salt concentrations tendered to reduce the μMax values when compared with lower salt concentrations also shown in Figure 4.8. This reduction in growth may be a contributing factor in the production of the peptides.

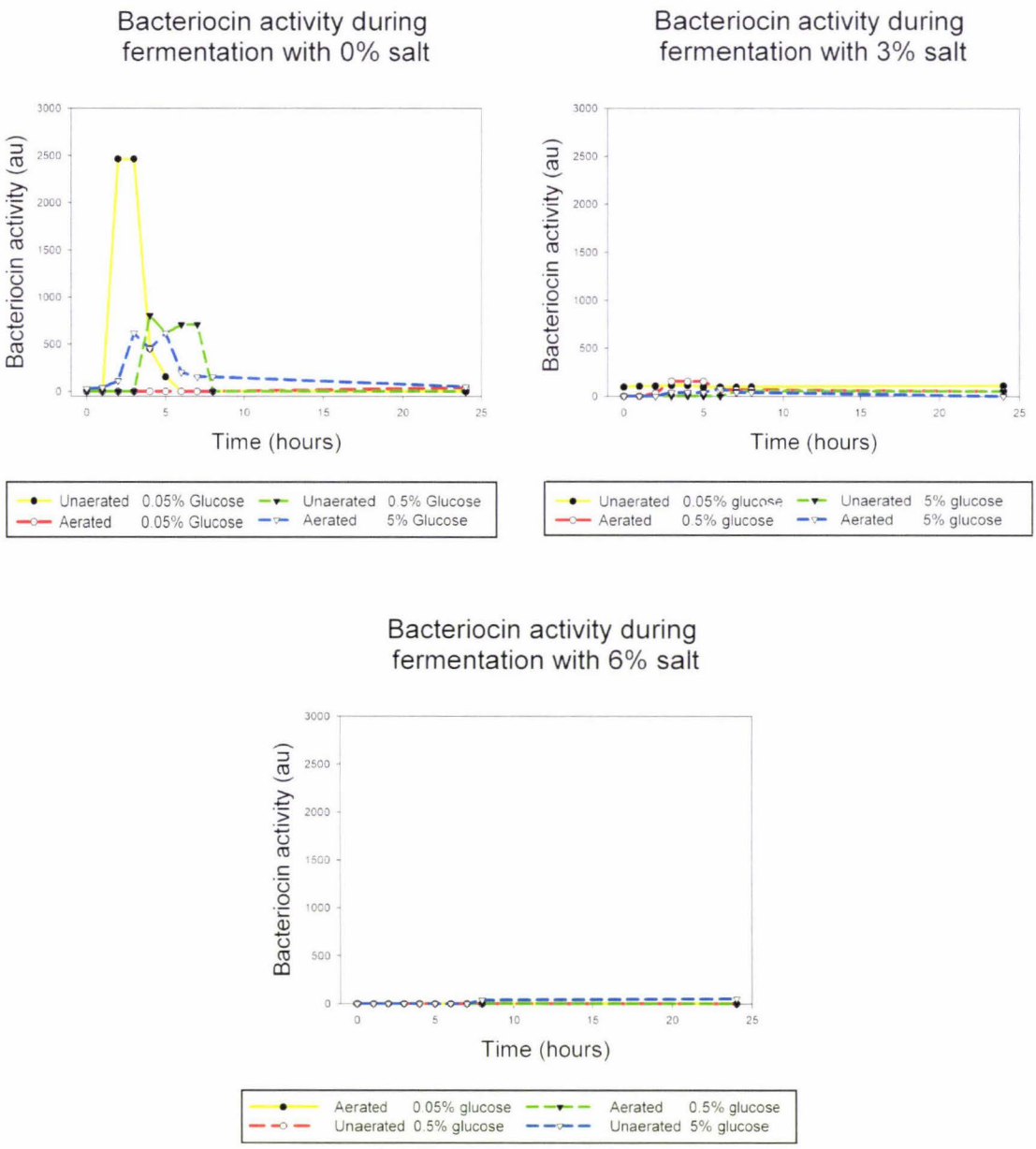


Figure 4.7 Bacteriocin activity separated based on the initial concentration of salt in the broth. Each graph shows the four different conditions that each of the salt values was tested under.

4.2.3.3 Effect of aeration on bacteriocin production

The effect of aeration appears to have some effect on both the cellular growth rate, and bacteriocin production. The aerated samples exhibited a small increase in μMax over the unaerated samples, as can be seen in Figure 4.8, and in biomass production Table 4.1. This increase in cellular growth could be due to better mixing, with an increase in contact between the substrates in the vessel and the cells, although stirring in all of the fermentations was such that there would have been no stagnation.

Table 4.1 Biomass of *Enterococcus faecalis* B9510 produced in various fermentations.

Initial salt concentration (%)	Initial Glucose concentration (%)	Aerated fermentation biomass ($minute^{-1}$)		Unaerated fermentation biomass ($minute^{-1}$)	
		24 hours	max.	24 hours	max.
0	0.05	2	2	1.64	1.96
3	5	9.44	9.44	6.3	6.3
6	0.5	1.55	2.11	1.35	1.48

Note: The only change in condition was the aeration of the broth.

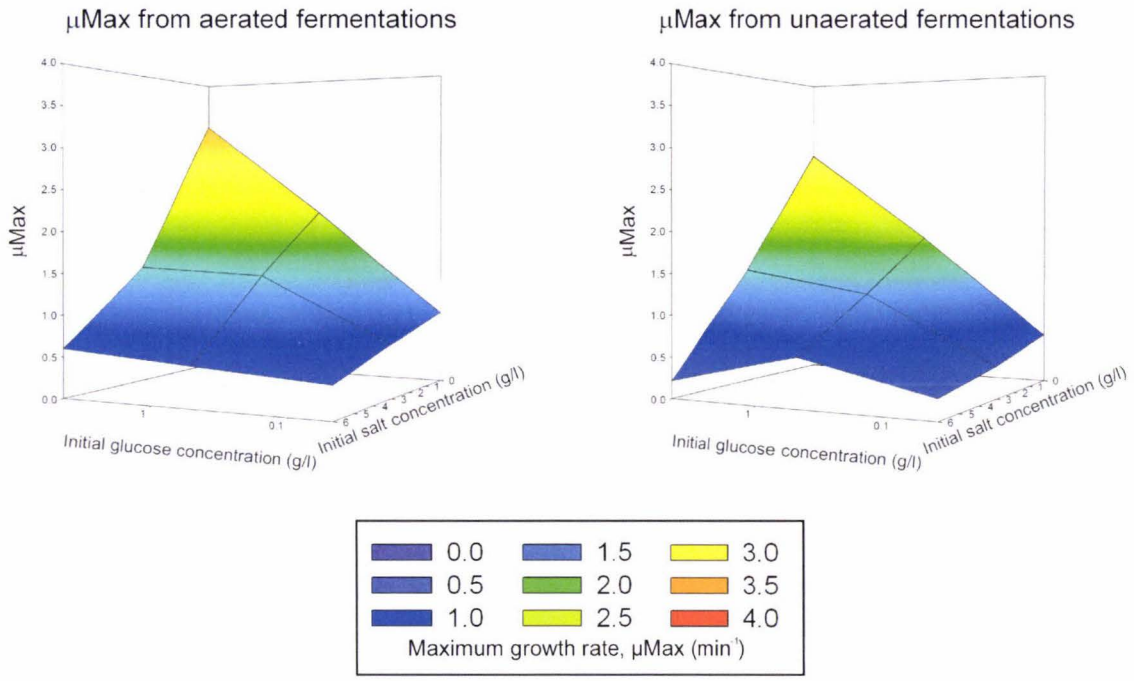


Figure 4.8 3D representation of µMax from the aerated and unaerated the fermentation trials. This was compared to the initial glucose and salt concentrations within the broth.

Biomass was reduced by the addition of salt, however, this effect was reduced with aeration. There was a pronounced difference in the biomass of the bacteria produced in direct comparison; and having the same glucose and salt concentration, aeration seems to produce a greater biomass of solid. This was most apparent in the 3% salt and 5% glucose fermentations where the volume of glucose allows for the production of biomass so the difference was more pronounced. However, in all three cases the amount of biomass produced in the unaerated fermentation was between 87-66% that of the aerated fermentation at 24 hours.

Bacteriocin production was greater in the un-aerated fermentations rather than the aerated fermentation, the exception being the fermentations with the highest glucose concentrations. The variation in the bacteriocin production amongst the differing fermentations condition is most likely related to the differing effect of stress on the bacteria in the range of fermentation conditions. This stress could cause a reduction in the ability of the bacteria to use the glucose present and produce the bacteriocins. A possible alternate explanation could be that the changes in the biochemical pathways between aerobic and anaerobic growth may be a factor in the production of the precursors for bacteriocin expression.

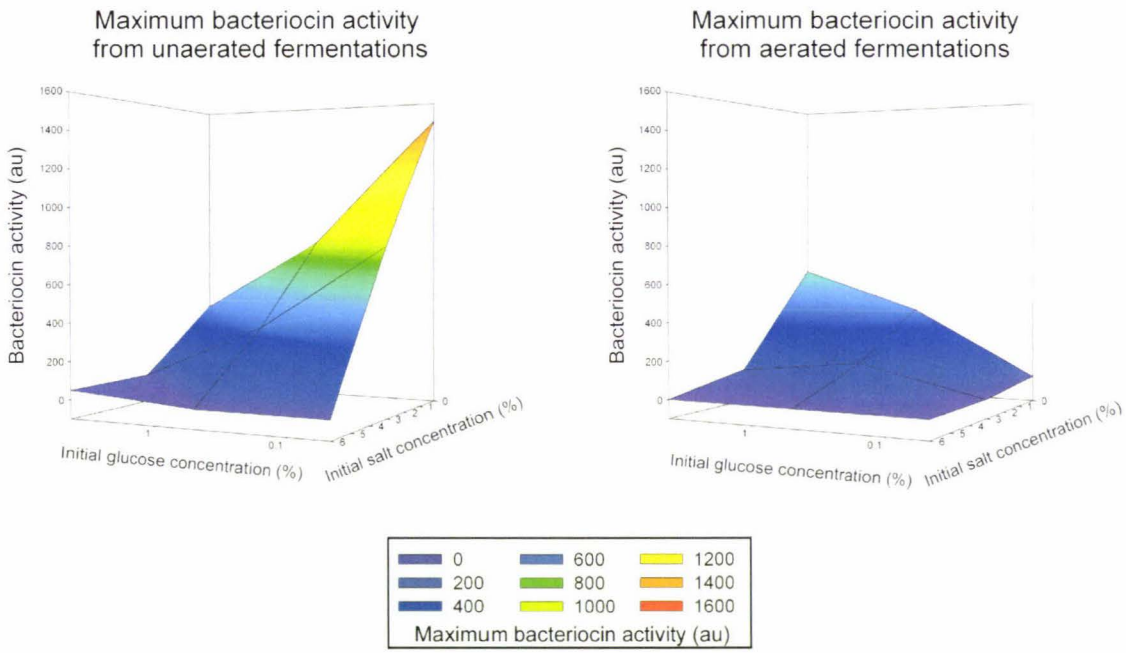


Figure 4.9 3D representation of the effect of aeration on the maximum activity of bacteriocins during fermentation. This was compared to the initial glucose and salt concentrations within the broth.

4.2.3.4 Effect of exponential growth on the production of bacteriocins

From the fermentation data it can be seen that the fermentations with the shortest periods of the exponential growth tended to have the highest production of bacteriocins. Table 4.2 shows an apparent relationship between the length of the exponential growth phase and the maximum bacteriocin production from the fermentations. The length of this period appears to depend mostly on the salt and glucose concentrations present,

while the aeration may reduce the period slightly. Salt and glucose both appear to have a negative effect on the growth rate. Increases in salt and glucose increase the time frame in which the bacteria experience exponential growth, and perhaps because of this a decrease in activity.

Table 4.2 Time at which the end of the exponential growth phase was reached in each fermentation, and the maximum bacteriocin activity from that fermentation.

Fermentation conditions	0.05%, 0%, N	0.05%, 0%, Y	0.05%, 3%, N	0.05%, 6%, Y
Exponential growth (hours)	3	3	3	6
Bacteriocin activity (au)	2463	38	110	0

Fermentation conditions	0.5%, 0%, N	0.5%, 3%, Y	0.5%, 6%, N	0.5%, 6%, Y
Exponential growth (hours)	4	5	8	7
Bacteriocin activity (au)	804	154	0	0

Fermentation conditions	5.0%, 0%, Y	5.0%, 3%, N	5.0%, 3%, Y	5.0%, 6%, N
Exponential growth (hours)	6	8	8	8
Bacteriocin activity (au)	616	50	79	50

Note: The fermentation conditions describe the glucose concentration (0.05, 0.5 or 5%), salt concentration (0, 3 or 6%) and whether or not the broth was aerated (Yes or No).

4.3 DISCUSSION

4.3.1 Effect of media, temperature and pH on growth and bacteriocin production

Several things were observed in the media trials, most important of which was the lack of any apparent pattern between the growth rates of the bacteria and the production/activity of the antimicrobial agents in the broth. Investigation into the media suggests that *Enterococcus* B9510 grew poorly in lactose mediums, both LM17 and PLY displayed low growth rates using lactose as the carbon substrate. The results from the glucose media suggest that it may be the ratio of carbon and nitrogen that is a larger factor than carbon alone, for both growth and antimicrobial production. This was suggested by the higher activity produced in both M17 mediums when compared to the TYT mediums. The TYT contained twice as much sugar as any of the other media, but this extra substrate did not equate to greater cellular growth or bacteriocin production. This put forwards the idea that the balance of nutrients in the media was the reason for the high production as opposed to the concentration of one chemical. One other observation that is worth noting is the difference in the stability of the activity between the M17 and TYT_{10} samples during the stationary phase of bacterial growth. While good activity was shown by both media, activity of the TYT_{10} dropped to zero in the stationary phase, while activity remained approximately the same in the M17 sample. The reason for this variation is unknown; however, it demonstrates another advantage offered by M17 over other mediums.

From the four temperatures tested it was obvious that the growth of the *Enterococcus* was quite robust between 30°C and 42°C, with little difference in growth and only a relatively small change in antimicrobial activity. However, at 47°C the significant drop in cell growth and bacteriocin activity showed that the bacteria was susceptible to temperatures above 42°C. The effect of high fermentation temperatures was similar to that reported by Eguchi *et al* 2001 (20) .

The testing on temperature and media conditions used uncontrolled pH conditions, meaning that the pH of the broth would drop from around pH 6.5 (set in the broth to a pH of 3 or 4), due to the production of lactic acid. Results for the testing of pH values showed that the optimal pH for μMax in this trial was pH 5.5, the same as observed in

Cho *et al* 1996 for *Pediococcus acidilactici* (9). Literature suggests that μMax values can be greatly varied: the maximum growth rate for *Enterococcus faecium* RZS C5 was observed at pH 7.5 (50), well outside the range of optimal growth range for most *Enterococci*. The low μMax value for the uncontrolled fermentation was most likely affected by the changing pH of the fermentation environment. The change in pH from near neutral at inoculation through to a final value of around 3.5 would have produced an initially beneficial pH, between 5 and 6, which was later inhibited in the fermentation by the production of lactic acid. This may have in turn affected both the cell production and maximum growth rate. These values were contradictory to the conventional values used in literature; when testing non-pH specific environmental conditions pH values of 6.5 and 7 are most commonly used to stabilise the fermentation (51) (34). This was supported by the trials carried out by Parente and Ricciardi 1994, that showed maximum cell growth for *Enterococcus faecium* DPC1146 at pH 6.5 (67).

Bacteriocin activity was shown to be highest at pH 5.5, but this level of activity was also replicated at the lowest pH tested, pH 4, where the biomass production and the μMax values were significantly lower. The unexpectedly high activity at pH 4 could be explained by an increase in activity of the bacteriocins at a lower pH values, but this conclusion will need further investigation. As Class II bacteriocins are cationic it was possible that pH conditions could affect the activity of the peptides in broth. For example at near neutral or basic pH values the peptides may become inhibited or inactivated due to the effect of the charges on the cells surface or of the peptide itself, causing a drop in apparent activity and at acidic pH's the relative activity increased. Alternatively, the bacteriocins may possibly have bound to the cells at these near neutral pH's, as suggested in Eguchi *et al*. 2001 and Lyon *et al*. 1995 (54). This would explain the reduction in activity at pH 6 and lack of any activity at pH 7; and the increase at pH 4, due to the release of these bound peptides. Bacteriocin activity for the uncontrolled fermentation produced values similar to those observed for pH 5.5 and pH 4. This activity may gain the benefit of high peptide production at pH 5.5, and a possible increase in relative activity due to the lower pH level at the end of the uncontrolled fermentation.

One criticism of this work was that tests were carried out on an empirical basis, changing only one factor at a time. This did not take into consideration the effects of

multiple variables on the system and their possible interactions. The interactions between factors could have a significant influence on the production of the peptide, possibly as large as each individual element, so future testing in this area will be important. A closer examination of interactions was also suggested by the choice of the media and the possible effect of the carbon-nitrogen ratio in the media, where the M17 media performed better than the TYT media despite much less sugar.

The effect of the different pH values will be revisited later when the investigation deals with the effect of pH on the amount of bound and unbound peptides that are present in the broth (Chapter 4) and the effect of pH on the activity of the bacteriocin (Chapter 5). Both these tests could have an influence on how the results from this section are interpreted.

4.3.2 Multi factor analysis of fermentation conditions on bacteriocin production and cell growth

The multi-factor fermentation media experiments were designed to test the effect of a variety of physiological factors, and to investigate which of these were significant to the production of the bacteriocin. The test was also designed to see if the interaction between these factors was significant to the production of bacteriocins.

The carbon substrate in fermentation was vital as it provides the 'food' from which the bacteria can replicate. Glucose is the simplest of sugars and can be easily absorbed by the bacteria to provide the fastest growth rate, as seen in Figure 4.1. It is used preferentially when more than one carbon substrate is present. From the data from the multivariate analysis, glucose appeared to have had little effect of the production of bacteriocins, when compared to the effect of salt. However, glucose did have a significant effect on the biomass and μMax in each of the fermentations. When examining the fermentations where salt was constant for each fermentation (as seen in Table 4.2), bacteriocin activity was similar. The only exception to this was the lowest glucose concentrations without salt, where aeration appeared to exert a large influence on the results. It was suggested that very small volumes of glucose may limit the ability of the bacteria to produce secondary products like bacteriocins, or may cause an increased bacteriocin production to produce a competitive advantage for the bacteria.

This effect may be reversed in samples with relatively large concentrations of glucose in the fermentation broth, as the need for a competitive advantage would be reduced, neither of options could be conclusively proved from the present data. This result would also fall in line with the findings of the media study were the TYT media with twice as much sugar produced no extra bacteriocins.

As was expected, μMax values were highest in the fermentations with the highest glucose concentration, as shown in Figure 4.8. This was unsurprising as the cellular growth was not limited by the carbon substrate within the broth, and cell production was almost constant for the length of the trial for those fermentations containing the highest glucose concentrations.

Salt appears to have a negative effect on both production of bacteriocins, and cell growth in terms of μMax. An increase in salt concentration in the fermentation broth reduced the bacteriocin activity in the collected samples. Fermentations with the highest salt concentrations showed little or no activity while those with no salt generally showed the highest average activity. The addition of salt was investigated as it was found to be a significant factor for the bacteriocin production from *Lactobacillus plantarum* produced on MRS media, and was more significant than glucose concentration or the initial inoculum size for that bacterium (48). This was also found to be true in this research, but as a negative relationship rather than a positive one. Sodium chloride was also found to enhance the bacteriocin production of *Enterococcus faecium* grown on MRS: while high levels reduced activity, moderate levels (20-40g/l) increased the recoverable bacteriocin activity. Additional salt prolonged bacteriocin production and produced a more stabilised activity over time. While there was no explanation for this result it can be speculated that the salt interferes with a bacteriocin regulating factor (52), or effectively reduces the amount of bacteriocin that can be absorbed by the producer cells.

The effect of aeration on the fermentations seems to have little influence on production of biomass (Table 4.1), μMax (Figure 4.8) but may have inhibited bacteriocin production (Figure 4.9). Small variations in biomass and μMax could be explained by more complete mixing under aerated conditions, but the variation was relatively small. The one exception to this was the effect of aeration on bacteriocin production with 0.05% glucose. While aerated, the trial produced minimal bacteriocin activity, whereas

the corresponding unaerated sample produced the highest activity of all the trials. One explanation is that the high relative activity of the unaerated sample may be caused by the low glucose concentrations. It is suggested that stress on the bacteria can cause an increase in bacteriocin production in order to provide a competitive advantage (16), (52), while the addition of aeration disrupts the bacteria in such a way that it prevents further production. In Leal-Sanchez *et al* 2002 the effect of aeration was found in initial testing not to be significant, but in later testing anaerobic conditions were chosen over aerobic ones as oxidative stress decreased maximum bacteriocin activity by 75% at 80% air saturation (16). This was due to either enhanced production or a reduction in inactivation. Inactivation may be caused by the oxidation of methionine residues sometimes present in Type II bacteriocins, which could destabilise the bacteriocin, or the presence of sulphite bridges, which may affect activity. The presence of oxygen, however, had no measurable effect on *E. faecium* RZS C5 (52).

It was commonly held that the bacteriocins are produced in the exponential phase of growth (47), but it may actually be that the length of this period may affect the quantity of bacteriocins produced. Table 4.2 indicates that this may well be the case, with those fermentations reaching stationary phase between 3-5 hours producing a much higher average activity than those taking 6+ hours to reach the stationary phase. This could relate to the preferable conditions under which these fermentations were carried out, with the idea that favourable growth cellular growth conditions increase bacteriocin production. The other option is that the limited substrates and harsh conditions which meant only a short amount of cellular growth stimulated the peptide production while conditions were favourable.

A further factor for consideration was that fermentations with only 0.05% glucose saw level of the carbon substrate as the limiting factor. Rapid growth at low glucose concentration with high bacteriocin activity suggests that the possibility for a continuous fermentation exists, if a viable method of removing the bacteriocins could be found.

4.4 CONCLUSIONS

Results from the fermentation studies showed no constant distinguishable relationship between the growth rates of the bacteria and the production of bacteriocins. There are multiple examples of samples that showed good bacterial growth and no bacteriocin activity. example: TYT media. and samples that show relatively poor growth and inhibit indicator bacteria, example LM17. The effect of media showed that while good growth rates can be achieved this does not always relate to the production of bacteriocins. However. in the multivariate analysis and in the testing in the effect of pH. there does appear to be some relationship. The pH test showed similar trends in μMax and activity. discounting pH 4. and the 3D representations of μMax and the bacteriocin activity also showed similar patterns. for most values. What the factors are that effect bacteriocin production and activity are unclear. but it appears that they maybe linked to cellular growth. although other unknown factors also appear to have some influence.

In support of the bacteriocin production being part of the exponential growth phase was the observation that in the multifactor trial. the highest bacteriocins levels were observed. in the most part, to be in those samples that ended the exponential growth phase the quickest. This suggests that the rate of cellular growth may also be a factor when considering the levels of bacteriocin production from fermentation.

The best growth temperature for the bacteria and the production of bacteriocins was shown to be at 42°C in uncontrolled pH conditions. Good activity was also shown at 30°C and 37°C. while the bacteria preformed poorly. in terms of cellular growth and bacteriocin activity at 47°C.

Optimum pH for growth and antimicrobial activity was found to be pH 5.5 with both lower growth rates and reducing antimicrobial activity. with increases or decreases in pH. At pH 4 bacteriocin activity was found to be similar to that at pH 5.5. but also displayed poor cellular growth. It is supposed that this increase is possibly due to the effect of the pH on the bacteriocin rather than an increase in cellular production. which gives a false level of activity.

Salt appeared to have the greatest effect on bacteriocin production of the element tested in the multi-factor fermentations. Increases in salt levels decreased the activity of the samples tested from the fermentation broth. with poor inhibition demonstrated by the

indicator bacteria. Glucose appears to have little effect on activity but a large effect on bacterial growth, with biomass levels increasing with increasing levels of glucose. Aeration had little effect on the production of bacteriocins but may have increased the cellular growth of the fermentations containing higher salt and glucose concentrations, allowing for better mixing of the substrates.

Key factors for maximisation of bacteriocin production by *Enterococcus* B9510 were:

- uncontrolled pH
- 42 °C
- No/Low salt
- No aeration

5 PURIFICATION

5.1 INTRODUCTION

Research into the purification of bio-actives from fermentation media is an important step in the development of products after bacterial fermentations. In some applications, purified bacteriocins from *Enterococcus faecalis* are required before they can be used in the food industry. The objective was to produce a product with the purity and concentration required for a particular need. Since the use of the peptides in such a diluted form after fermentation is generally not desirable, the goal of purification was to remove the excess chemicals, and biomass, along with any other by products of the fermentation process and concentrate the bacteriocins.

This chapter deals with a variety of methods for the purification of bacteriocins from the fermentation media, based on the properties inherent to Type II bacteriocins. Further downstream purification was also considered, and a range of methods were tested for the removal of the non-bacteriocin peptides from the resultant mixture.

The purification of bacteriocins is of vital importance for the further development of antimicrobial products from fermentation broth. Previous attempts at isolating these peptides have demonstrated that not all the described methods work with each peptide (45). This could be due to a number of factors; the polarity of the peptide, sequence length, tertiary structure, or other unknown factors. Common extraction methods such as ammonium sulphate precipitation and solvent extraction have previously been used to isolate the bacteriocin from the broth with little success. Without an effective purification method the possible applications for the bacteriocin become very limited, due to the volumes required, and the limit activity.

5.2 RESULTS

5.2.1 Purification using XAD-16 resin

Experimental work was carried out to develop a method to isolate bacteriocins produced by *Enterococcus faecalis* B9510 using the non-ionic resin XAD-16 from M17 media. Tests were carried out using commercial grade nisin as a model bacteriocin for the study of the effects of temperature, time and resin concentration on the removal of the peptides from a nisin solution. The XAD-16 method was a variant of the method shown in Cintas *et al* 2000 and uses the hydrophobic properties of the peptides to bind the active material to the resin for easy removal from the fermented broth. In order to optimize this process for *E. faecalis* B9510 a series of experiments were used based on the standard protocol but included a range of time and solvent concentrations to provide the optimum results (11).

5.2.1.1 Effect of temperature on binding of antimicrobial peptides to resin

Figure 5.1 shows that the absorbance of the nisin was highest and most stable at 4°C. Samples at ambient temperatures and 30°C showed similar results, but did not reach the same level of absorbance. This suggested that the optimum temperature from those tested was 4°C.

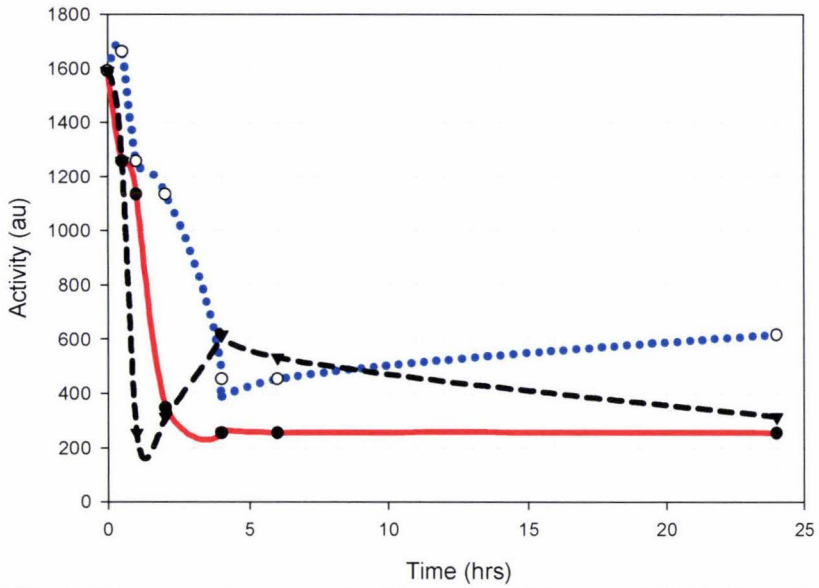


Figure 5.1 Effect of temperatures on the binding efficiency of XAD-16 resin to nisin. 4°C (—), Ambient temperature (···), 30°C (--).

After this experimentation was carried out, it was found that the resin was unrecoverable. Since one of the important factors when using resins is its recovery and reusability, the cost of using resins would be prohibitive if a new one was required for each extraction. The resin destruction was most likely due to the mechanical action of the magnetic stirrer on the resin, grinding it into a powder. The use of shake flasks was introduced to replace the magnetic stirrers. The experiment was repeated using the new method of mixing at ambient temperature and at 4°C. These values were used as the availability of shaking equipment was limited. The new test was carried out in the same way as the magnetic stirring method with samples removed at the same time intervals.

Figure 5.2 shows that the extraction of the nisin from solution by the resin was more effective at ambient temperature than at 4°C. Similar levels of absorbance were observed when a comparison between the two methods, the shake flask and magnetic stirring, for the ambient temperature, both having a minimum residual activity of around 400 au.

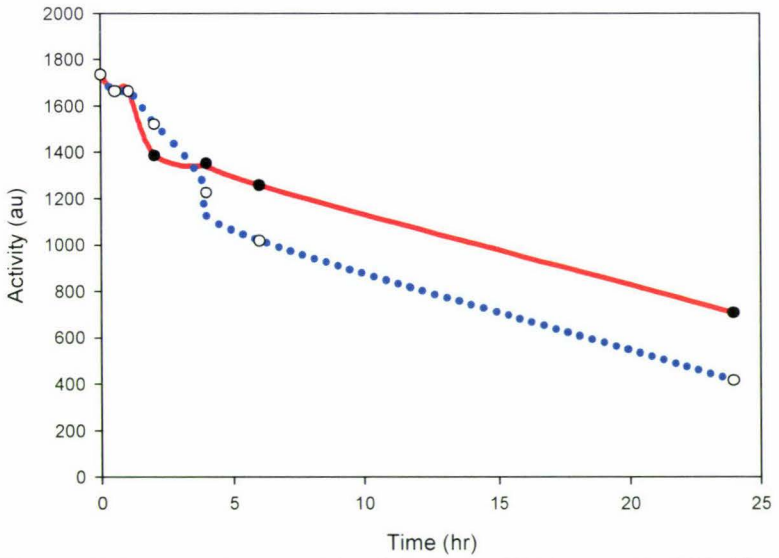


Figure 5.2 Effect of temperature on the binding efficiency of XAD-16 resin for shake flasks. 4°C (—), Ambient temperature (···).

5.2.1.2 Effect of pH on binding of nisin on resin

Figure 5.3 shows that the effect of pH does not appear to affect the binding efficiency of the resin. All the tested values appear to drop to similar levels within the first three hours, but this may be dependent on the initial activity shown. The initial activity was most likely a factor of the reaction of the antimicrobial activity to pH within each solution.

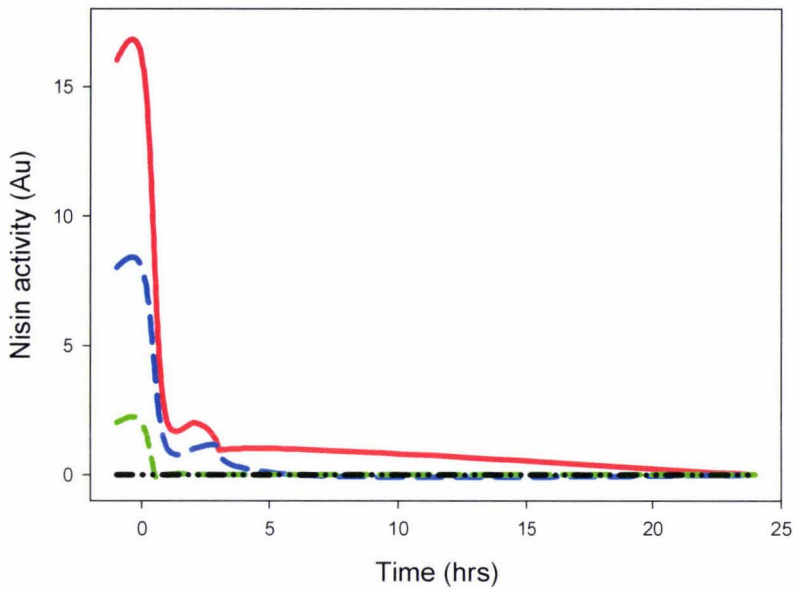


Figure 5.3 Effect of pH values on the binding efficiency of nisin onto XAD-16. pH 3 (—), pH 5 (--), pH 7 (--), pH 9 (-···-).

5.2.1.3 Effect of resin volume on binding efficiency of antimicrobial activity

In Figure 5.4 it was obvious that an increase in the resin volume increased the rate of absorbance. However, for this concentration of peptide the values for 5, 7 and 10 were all very similar from around 5 hours onwards.

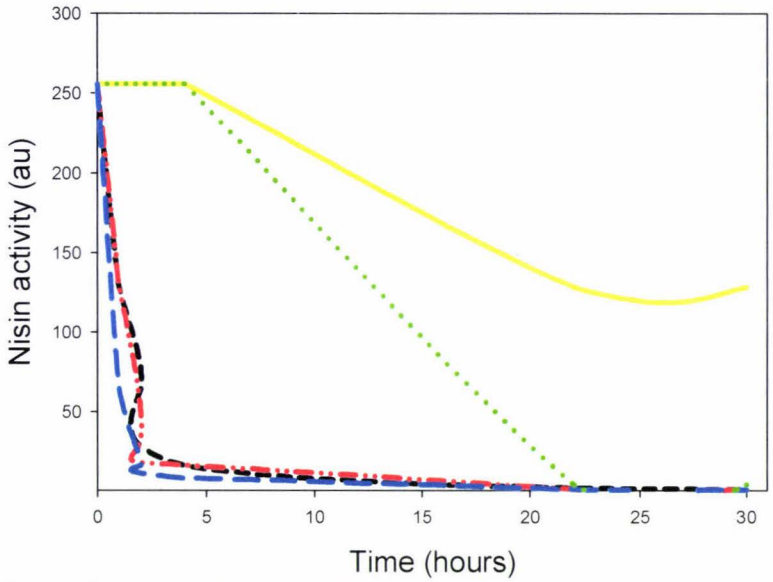


Figure 5.4 Effect of resin volume on the removal of nisin from test solutions. Resin volume (g), 1 (—), 3 (···), 5 (--), 7 (-···-), 10 (--).

5.2.1.4 Elution

Testing of the wash water from the resin showed no activity, which suggests that, the peptide/resin matrix was stable enough so that the peptide was not eluted under all conditions. This ensured that a wash phase would not decrease the active material bound.

From the different elution conditions (shown in Figure 5.5), it was shown that the majority of the bioactive was released between 50-60% isopropanol. Total elution occurred at 80% isopropanol with no extra active material present at 90-100% isopropanol or from the regeneration of the resin in 100% methanol. The results also showed that small amounts of isopropanol, 10-20%, were able to elute the peptide from the resin. Only a small volume of the total amount of active material was released at this stage but it does limit the materials that can be used to wash the resin matrix.

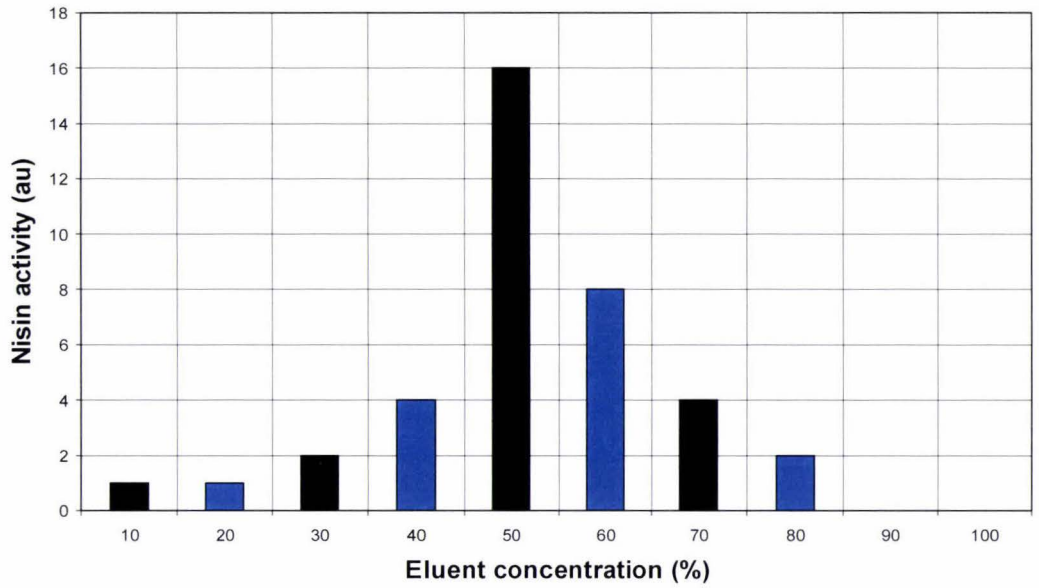


Figure 5.5 Effect of various eluent concentrations on the elution of nisin from the XAD-16 matrix.

5.2.1.5 Use of XAD-16 resin with *Enterococcus* and M17

After fermentation in M17, the *Enterococcus* cells were removed via centrifugation. Resin was then added to the broth which was placed in a conical flask, and this mixture was allowed to mix for 24 hours. The resin was then removed and washed in distilled water, to remove any unbound material. Elution was carried out with 50% isopropanol and water. The eluent was then freeze dried and the resulting powder re-suspended in 0.5% acetic acid to produce the active sample. Testing of the fermentation broth after the removal of the resin and wash water showed no signs of bacteriocin activity. This suggests that the resin method was capable of removing high proportions of the peptides from the fermentation broth, and that it was stable enough to be washed without fear of peptide loss.

Table 5.1 Summary of purification from fermentation broth to resin eluent.

Purification step	Volume (ml)	Activity (au/ml)	Total Activity (au)	Activity recovered (%)
Fermentation broth	1000	38.5	38500	100
Resin eluent	10	615	6150	16

5.2.2 Purification of bacteriocins by pH variation

5.2.2.1 Determination of the optimal binding pH value for binding bacteriocins

The lack of activity in the supernatant, in Table 5.2, where it was expected that the isolated bacteriocins should have been collected shows that this particular method was unsuccessful in isolating the activity from the fermented broth. This is also demonstrated by the lack of any drop in activity from the fermented broth to the pH adjusted broth, suggesting that the bacteriocins were not binding to the cells, which would explain why there was no activity in the final extraction.

Table 5.2 Bacteriocin activities at different stages of the pH binding experiment.

Sample	Trial 1 activity (pH6) (mm)	Trial 2 activity (pH6) (mm)
Fermented broth	15	14
pH Adjusted broth	15	14
Cell wash	0	0
Supernatant	0	0

In order to test the effect of varying the pH at the cell binding phase, a series of experiments were carried out. A range of pH values were used from 6 to 9; pH 9 was chosen as an upper limit as in Kelly *et al* 1996 and Eguchi *et al* 2001 since high pH values were shown to reduce bacteriocin activity, or even inactivate bacteriocin at high enough pH values (42) (20). A value of pH 6 was used as the low level since the fermented broth was typically in the region of 4.5 to 5.5, meaning that if the peptides were to bind at those values then the bacteriocins would already be attached to the cells before pH adjustment. Testing was carried out to measure any significant reduction in the activity of the fermented broth before and after the pH adjustment, to see if the peptides were binding at different values than those published.

Table 5.3 Activity of fermented broth compared to the pH adjusted broth with the cells removed.

pH	Fermented broth activity (mm)	Adjusted broth activity (mm)
6.0	11	12
6.5	11	9
7.0	12	11
7.5	12	13
8.0	12	11
8.5	11	12
9.0	11	11

The data in Table 5.3 appears to indicate that the binding of the peptides to the cells was not taking place. Whether this was due to properties of the cell wall or the peptides was unclear, but it was clear that the interactions between the two had not produced the expected, or desired, result. This suggests that this technique would not be useful for the isolation of bacteriocins from *E. faecalis*.

5.2.3 Use of Ion exchange chromatography in the separation of bacteriocins

Figure 5.6 shows the isolation of the hydrophobic fraction from the XAD-16 resin elution. The initial peak (Fraction i) from the Macro-Prep CM column in the most part contained no bactericidal activity, as the fraction containing the antimicrobial activity was bound to the column. The second peak (Fraction ii) on the detector was produced when the base line on the detector had flattened, and the running buffer was changed to 10% acetic acid. This changed the binding properties of the resin allowing the peptides to be released.

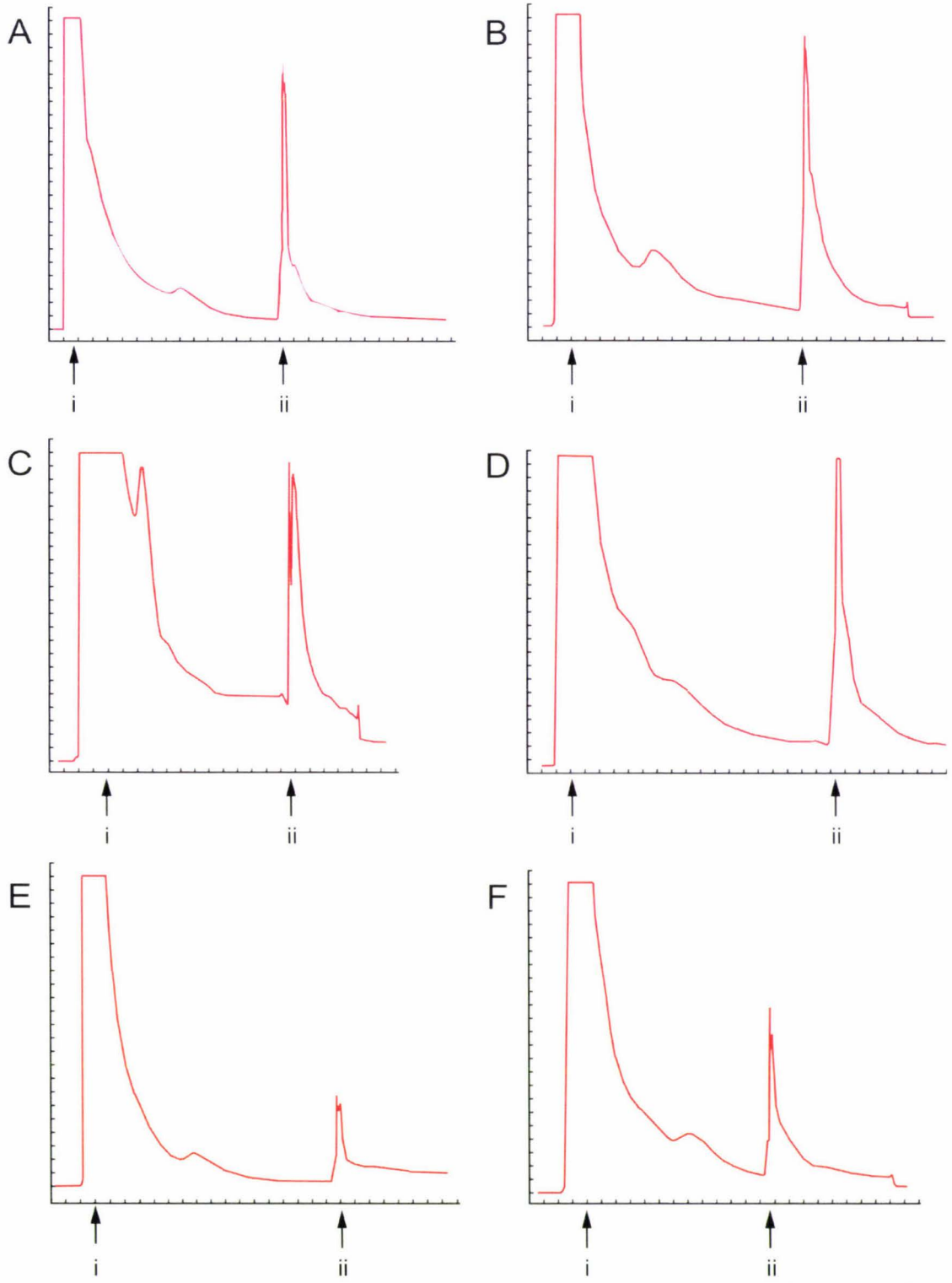


Figure 5.6 Examples of ion-exchange (Macro-Prep CM) graphs from eluent of the XAD-16 resin. Figures A, B, C and D are examples of graphs provide from the ion-exchange of the eluent of the XAD-16 resin. The initial peak (i) was the material from the eluent that contains no cationic charge and passes through the column with the ammonium acetate buffer, this sometimes contains active material due to the practice of overloading the column in order to maximise the collection of the active peptide. The second fraction (ii) was the active fraction and was eluted from the column with 10% acetic acid. Graphs E and F show the elution of fraction one (i) from previous ion-exchange runs for a second time, in order to remove any bacteriocins still present. As can be seen, the active peak (ii) was smaller in these two graphs than in the first four.

It was common procedure to overload the column, which resulted in Fraction i often containing active material. This was removed by running the fraction through the column a second time. Examples of these re-run samples are shown in E and F in Figure 5.6 where the second peak was much smaller compared to A, B, C and D.

Table 5.4 Summary of purification from resin eluent to ion exchange fraction 2.

Purification step	Volume (ml)	Activity (au/ml)	Total Activity (au)	Activity recovered (%)
Resin eluent	10	615	6150	100
Fraction 2	3	706	2118	34

The loss in activity between the peptide solution eluted and the ionic fraction from the ion-exchange column (shown in Table 5.4) is significant. The recovery of only about one third of the activity is a concern: but the loss of this material is not easily identified. Both fractions from the column were tested for activity, and on the occasions where activity was present in Fraction (i) the sample was freeze dried and run though the column a second time. This loss in activity is coupled with an almost total removal of the pigment from the resin eluent, which was a very dark brown colour. This is important, as the removal of the pigment makes further purification less problematic. In terms of areas where loss of the peptide may have occurred, the major source is most likely in the freeze drying process, where the powdered sample may be sucked into the chamber when the pressure is released, despite the care taken in this step. Other possible sources were through permanent binding to the column, losses when transferring from the fractionation test tubes to the round-bottom flasks, or inactivation of the peptide due to an unknown interaction of the chemicals used and the temperatures used to purify the sample.

5.2.4 Separation of bacteriocin using gel filtration chromatography

Gel filtration was another method used to isolate the peptide. The gel used size mediated separation to isolate proteins and peptides of different sizes, in order to create better separation. In this process the peptide solution from the XAD-16 resin eluent was collected and a sample was run thought a Biorad P-10 column.

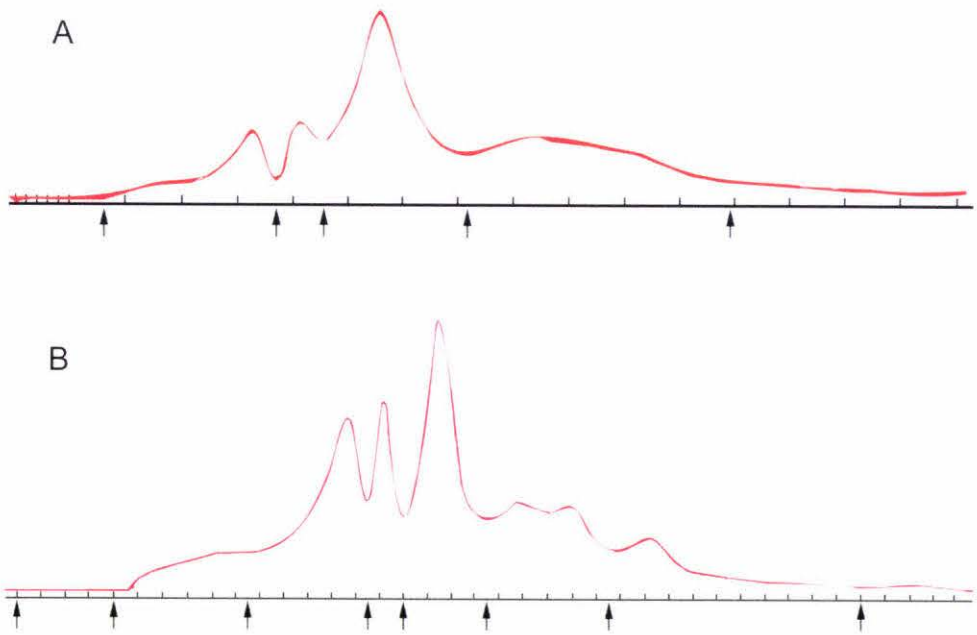


Figure 5.7 Examples of peaks displayed by the P-10 gel. The above shows replicated trials of the peaks produced in the separation of Fraction 2 from the ion exchange column using the P-10 gel filtration column. The arrows indicate the points where the fractions were pooled for activity testing.

Table 5.5 Summary of purification from ion exchange fraction 2 to P-10 gel active fraction.

Purification step	Volume (ml)	Activity (au/ml)	Total Activity (au)	Activity recovered (%)
Ion Exchange (F2)	3	615	1845	100
Active P-10 fraction	0.5	143	71.6	4

The almost total loss of activity from the gel filtration method meant that this method of isolation was abandoned after two attempts. The exact nature of the loss in activity was unknown, as all the fractions were gathered and freeze dried before testing for activity. The presence of the activated fraction in only the first two peaks, admittedly at very low activities, was surprising since the small size of the peptide should have led to elution of the activity fraction much later than observed. The early elution of the activity may have been due to aggregation of the bacteriocin, and the loss of activity due to precipitation on the column in the 5% acetic acid. Collection of the washing buffers and solvents may have recovered some of the lost activity.

5.2.5 RP-HPLC separation of active bacteriocin peptides

From the ion exchange column, further purification was carried out using High Performance Liquid Chromatography (HPLC). This was used to separate peptides according to the hydrophobicity of the molecule, and was an accurate and reliable method by which to separate individual proteins for analysis.

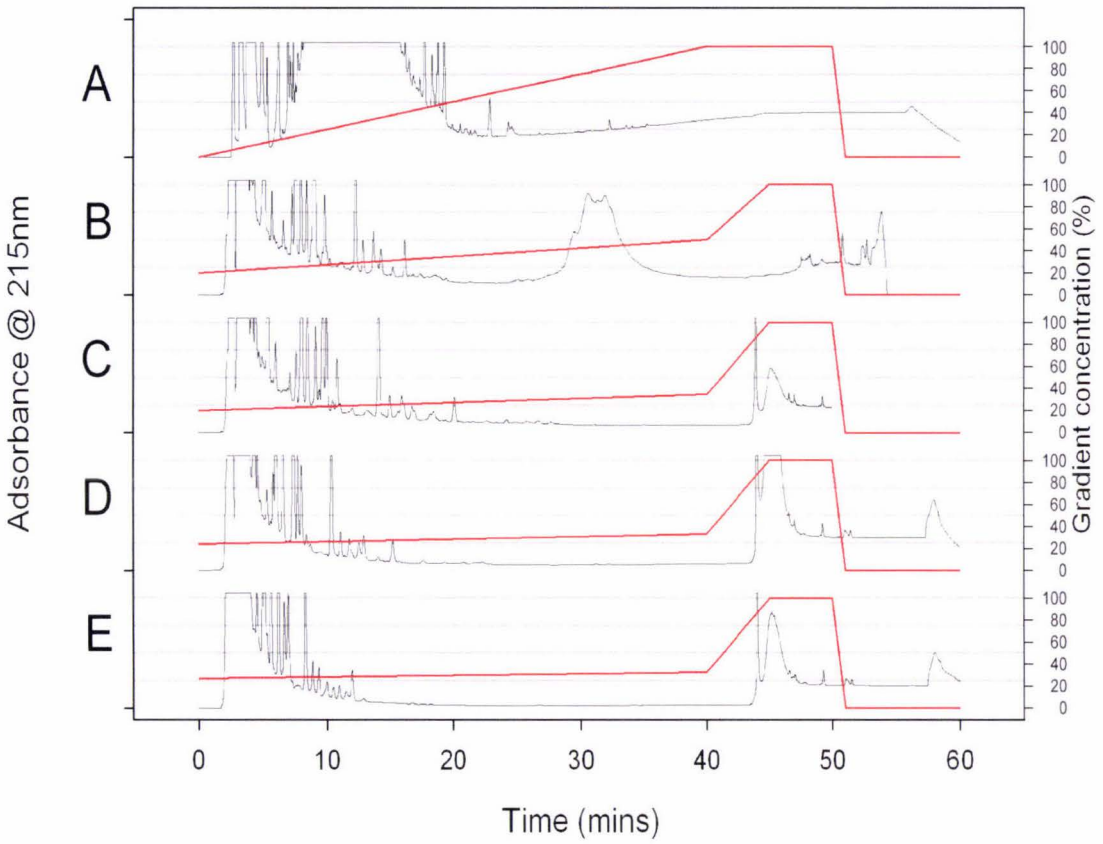


Figure 5.8 Sequence of HPLC runs. (A) The initial run with a gradient of buffer A running from 0-100%, (B) 20-50%, (C) 25-40%, and (D) 27-33%. Buffer A consists of 5% acetonitrile and 0.1% TFA while buffer B is 95% acetonitrile and 0.1% TFA. Graphs are displayed within the range of 0-500 mAU.

The peaks of each run were collected and tested in order to produce the next gradient. A final gradient of 27-33% acetonitrile was used, as it produces the active peaks in such a way that they could easily be collected without interference from other proteins. Samples of these were collected and tested for molecular weight and N-terminal sequencing, discussed in Chapter 6.

HPLC also allowed the determination of the active portion from Fraction ii of the ion exchange column. Using the Chromeleon software of the HPLC, the fraction of active

sample was determined to be less than 7% of the relative area for the whole run. From this data we can use the total protein concentration in the sample to determine the active protein concentration. Figure 5.9 shows the active samples and where they fall in relative to the rest of the peptides.

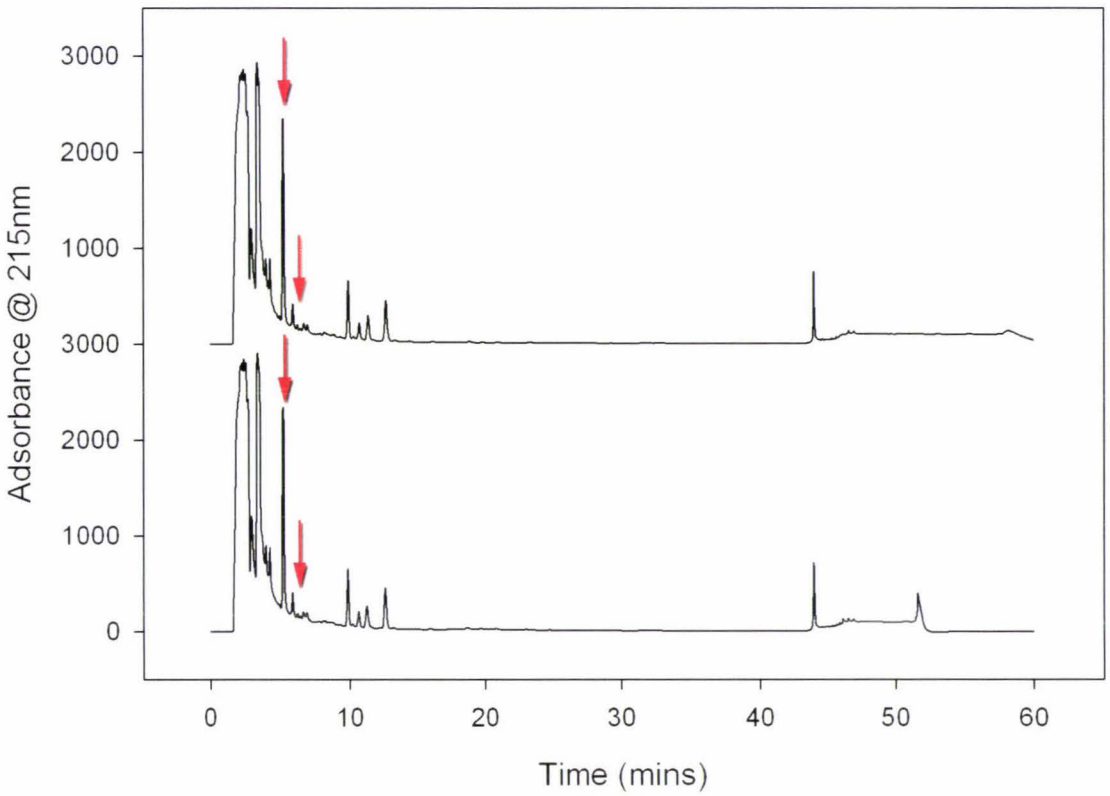


Figure 5.9 Two HPLC runs with a gradient of 27-33% acetonitrile. The arrows indicate the active peaks after plate assays.

5.2.6 Four hour fermentation sample

The majority of the work carried out for purification and characterisation used fermentation broth, where the fermentations lasted 24 hours, in order to gather cellular growth and bacteriocin activity over that period. From the fermentation data, maximum bacteriocin activity was shown under various fermentation conditions at the end of the exponential growth phase. For the best performed fermentations this was typically around four hours, from the initial inoculation. The investigation into the peptides isolated showed a slight variation to those from the 24 hour fermentations when using HPLC, after purification with XAD-16 resin and ion-exchange chromatography.

5.2.6.1 HPLC results

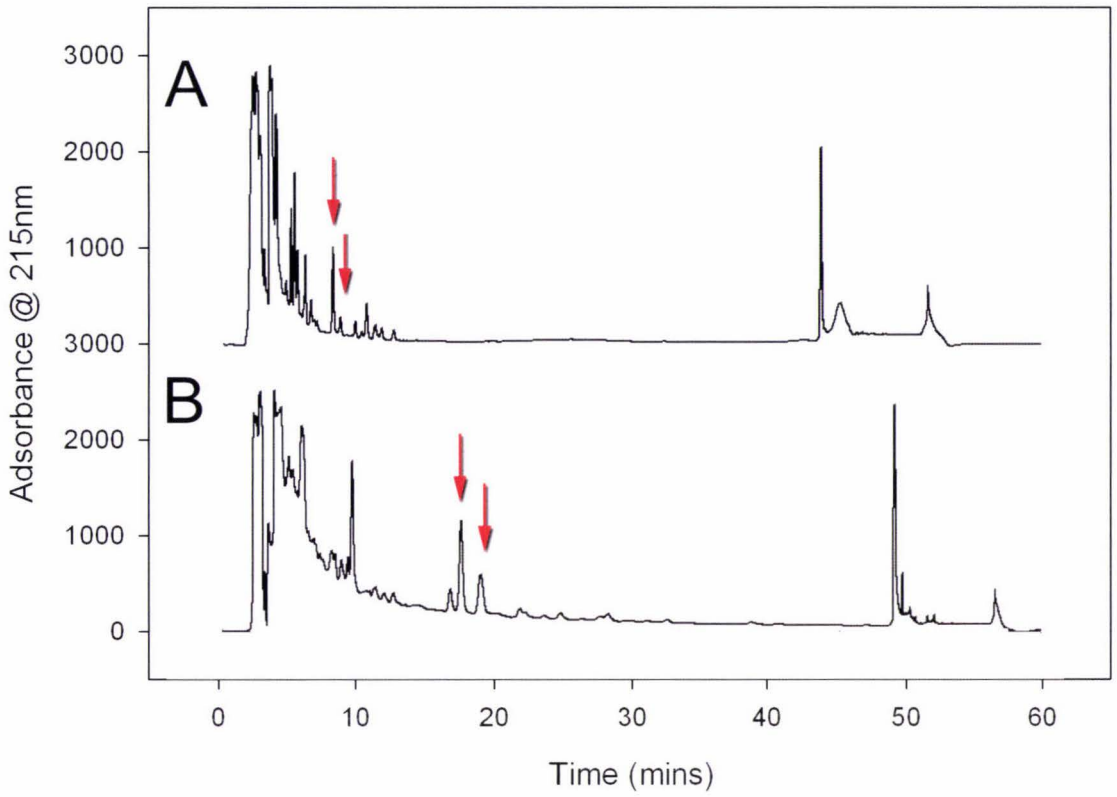


Figure 5.10 HPLC graphs of active material from ion-exchange column. Graph A shows a 24 hour fermentation sample, with an acetonitrile gradient of 27-33%. Graph B shown a sample from a four hour fermentation, with an acetonitrile gradient of 20-25% with a 5 minute holding period at the start of the run at 20%. The arrows indicate the active peaks after plate assays.

The HPLC gradient for the four hour fermentation was found to be slightly different from that of the 24 hour samples. The active fraction was eluted very early when run over the 27-33% gradient used for the 24 hour fermentation samples. A gradient of 20-25% was used instead, and was found to be an effective replacement for the new sample, an additional 5 minute buffering time was also shown to be an effective way of separating the peptides of interest. Figure 5.11 represents the plate assay carried out from the collection of the HPLC peaks of a standard run. From the Plate assay it was obvious that peak 16 showed the major activity for the bacterial indicators, *Escherichia coli* and *Staphylococcus aureus*.

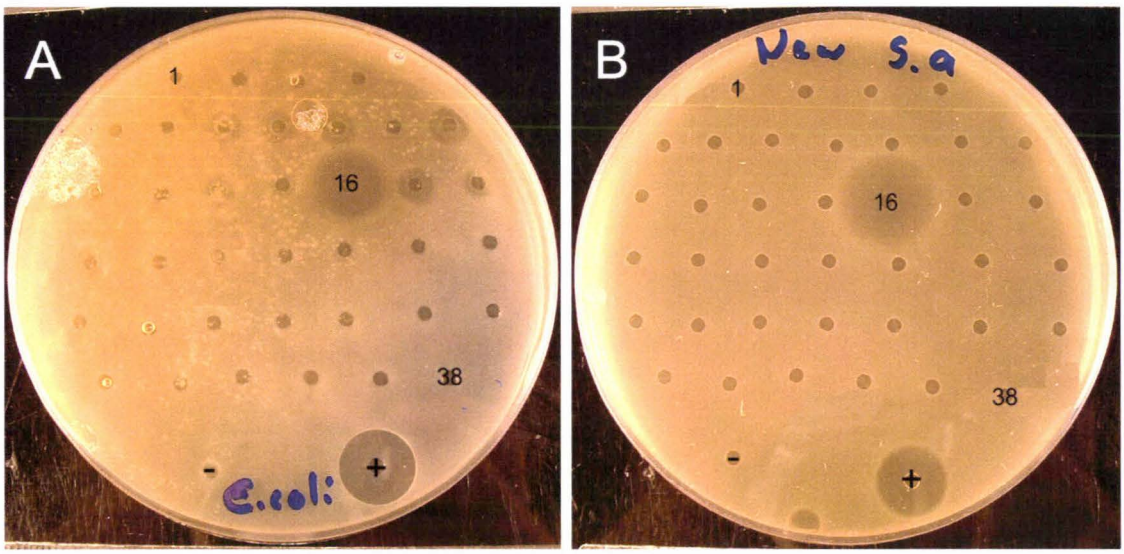


Figure 5.11 Activity plate from the HPLC peaks. Plate A was inoculated with *E. coli*, with activity 8, 9, 11, 16 and 17, positive control (+) polymyxin and negative control (-) 0.5% acetic acid. Plate B was inoculated with *S. aureus*, with activity only exhibited at peak 16, positive control (+) nisin and negative control (-) 0.5% acetic acid.

5.3 DISCUSSION

5.3.1 Effect of temperature, stirring, pH and resin volume on the binding of bacteriocins to XAD-16 resin

A comparison of the effect of shaking as opposed to the use of magnetic stirring shows a reduction in binding efficiency in the shaken samples, shown in Figure 5.1 and Figure 5.2. This reduction in efficiency may be partially due to an increased surface area in the stirred flasks, due to the resin being ground down by the magnetic stirrer and the glass surface of the flask, effectively increasing the of resin surface area available. The shake flask graph (Figure 5.2) suggests that the ambient conditions provided more efficient binding, although the values had not been stabilised. This suggests that a greater volume of resin would be required to absorb the peptides, or perhaps a longer contact time. While the activity in the shake flasks had not reached equilibrium, the samples taken at ambient temperature had reached an equivalent level to that shown for the magnetic stirring, suggesting a level close to equilibrium, given the same test conditions. Better binding was demonstrated at ambient temperature, this is advantageous as it means that the binding could be carried out on the bench top and the requirement for any excess cooling is reduced, a factor when looking at large scale processing.

The effect of temperature on the binding properties of the resin was unclear from Cintas, *et al* 2000, where the binding was carried out at 4°C. The use of low temperatures for binding, given the fact that no specific benefit to binding could be assumed, suggests that the low temperatures were used to protect the bacteriocins. This seems unnecessary as the peptides are generally stable at high temperatures and have a good storage lifetime. The investigation into temperature was undertaken to see if the binding properties of the resin were affected by the temperature of the media. The use of magnetic stirrers and magnetic fleas as a method of stirring was an obvious error in experimental design. While mixing was important in order to maximize the contact of the resin and the peptides, the destruction of the resin would produce elution problems as well as an unnecessary expense, of having to replace the resin.

The biggest factor revealed by the testing of the effect of pH on the binding efficiency of the resin, was the effect of the pH values on the activity of the nisin. Results showed

that there was a reduction in the bacteriocin activity present in the samples over time, indicating that the peptide was either being bound to the resin or becoming inactivated. Significant reductions in activity were observed in the initial samples, before the addition of the resin, from the low pH samples through to the high pH samples. This demonstrated the inactivation of the nisin at higher pH values. This inactivation made it difficult to surmise the effect of pH on binding efficiency, but pH did have a significant effect on nisin activity.

From Figure 5.4, it appears that five grams of the resin was sufficient to effectively recover the activity from a 200 ml sample of nisin, with an approximately similar level of activity. This would equate to 25 g/l of fermentation broth. The analysis of the minimum amount of resin used was important, as it maximises the removal of the peptides in solution without increasing the capital cost of resin purchase. The excessive use of resin in a larger scale extraction could become an added expense in terms of extra processing cost requirements for larger resin masses for elution and regeneration.

5.3.2 Elution of the resin

A 50% solution of isopropanol was found to elute the vast majority nisin from the resin. A second elution at 80% was found to remove any remaining peptide still bound to the resin, but this second elution provided a much lower activity than the 50% elution. When using the fermentation broth and peptides from *Enterococcus* B9510 a similar pattern was shown. In a practical application, the necessity for a second elution becomes apparent. While the resin is in the fermentation broth it binds all the hydrophobic components present, this includes not only the bacteriocins but also some coloured factors in the M17 media, as the XAD-16 resin is designed to absorb organic substances of low to medium molecular weight. During elution different concentrations of isopropanol remove different amounts of bacteriocins, as seen in Figure 5.5, and also the pigmentation. Because of this the 50% elution is used as it removes the majority of the peptide and only a portion of the associated pigmentation, ensuring fewer pigment problems downstream.

The XAD-16 method provided a good method of concentrating the active fraction from the majority of the fermentation broth. The apparent loss activity may be distorted by

the large change in volumes, with the resuspended sample being only 0.1% of the fermentation volume. It is expected however that some of the losses were due to the many steps involved in the purification process and the inherent losses associated with each step.

5.3.3 Effect of the pH method on binding bacteriocins from fermentation broth

The second extraction method tested was that based on pH binding method described in Eguchi *et al* 2001 and Lyon *et al* 1995. Briefly, the method uses the cationic characteristics of the peptide in order to reduce to the volume on non-active material. Similar to a cationic resin method, the pH binding method uses the charge of the bacteriocin to separate the active molecules. This method works by altering the pH of the fermented broth after fermentation, in order to get the bacteriocins to bind to the cells within the medium. Typical values for pH adjustment stated were approximately neutral pH values of 6 or 7. The cells can easily be removed from the broth via centrifugation, which greatly reduces the working volume containing the active peptides. The resulting cell and peptide matrix can then be washed to remove excess broth. Finally, the cells are pH adjusted to an acidic pH to release the peptides, typically at around a pH value of 2 (20) (54).

This method is advantageous as it quickly provides a significant reduction in volume, making the resulting matrix much simpler to handle. It was preferable over the method requiring a resin as it requires fewer processing steps and does not require additional materials.

Tests carried out using this method were unsuccessful. Initial testing followed the method in Eguchi *et al* 2001, and did not provide positive results. Samples were taken from the fermentation broth before pH adjustment, after cell (and in theory peptide) removal, the cell wash and finally the substrate. As Table 5.2 shows, the use of pH 6 to bind the peptides to the cells appears to be the limiting factor of the method, as there was no significant variation between the activity of the fermented broth and the supernatant after the cells had been removed. This makes sense when coupled with the lack of any activity in the final supernatant, suggesting that the peptide did not bind to

the cells under these conditions. Further testing into this method was carried out with an increased range of pH values. This further investigation only showed a reduction in activity in the fermentation broth with the cells removed at pH values of 9.5 and 10. At these highly basic levels, however, there was a fear that the pH was inactivating the bacteriocin as opposed to the peptides binding to the cells (85). The effect of pH on activity will be further tested in Chapter 6, so the relationship between the pH and bacteriocin can be quantified.

One of the disadvantages of this method was that it used high levels of salt in the purification process, adding extra steps in the downstream purification of the eluent. Salt levels were reduced by the addition of a dialysis step before freeze drying. The dialysis step added another delay into an already long process, and without the removal of the majority of the salt the dried product could not be tested for antibacterial activity. The high salt concentrations could lead to the inhibition of the indicator bacteria, with or without the presence of the bacteriocins.

5.3.4 Downstream purification, Ion-exchange chromatography, P-10 gel chromatography and High Performance Liquid Chromatography

Through the use of an ion exchange column the bacteriocin was separated and collected for testing. The resulting suspension from the initial XAD-16 extraction was placed into the column and washed through with ammonium acetate; this allows the non-cationic material to pass through the column without binding. The active fraction was collected from the elution of the resin with 10% acetic acid. The collected fraction exhibited good activity against the test strains showing that this was a good method for purification. The column also removed a great deal of the pigment that was part of the XAD-16 eluent. The use of the highly coloured fraction could prove to be difficult for practical applications, as it would likely stain any product it was introduced to. The activity of the eluent from the ion-exchange column was similar to the activity demonstrated by the XAD-16 resin eluent, however, a drop in the volume collected from the column leads to a reduction in the recoverable activity of the peptide. This loss in activity could have been due to losses during freeze drying, collection with the

fractionator or due to the need for three or four runs thought the column in order to process all of the eluent from the XAD-16 resin.

The gel column (P-10) is a method of fractionating a sample based on the size of the proteins and peptides within a sample. The size of the active peptide from both the SDS-PAGE gel (not shown) and from molecular weight determination, described later, shows that the active peptide was very small, less than 5 kDa. This would indicate that the peptide should come out in the latter part of the run through the gel column. However, the active fraction was only found in the early fractions, Fractions One and Two. SDS-page gels run from the fractions from the column (not shown) also confirmed that the peptides were very small, suggesting that another property was also being selected for from the P-10 gel, or that the P-10 gel was not suitable for the separation of this peptide. The activity displayed from the eluent of the column was also a very small percentage of the total activity from the sample placed into the column (4%), while some losses are expected the recovered activity from this was so low that it could not easily be explained by sample losses. The P-10 gel method was used only twice due to the large amount of sample used in each trial and loss of activity encountered both times it was attempted.

The use of High Performance Liquid Chromatography was a reliable method for peptide purification provided a good source of pure sample for the testing of MIC's against a range of bacteria. This technique also provided samples for the sequence analysis carried out in Chapter 6. There was a slight variation in the gradients used for the 24 hour and 4 hour fermentations suggesting that there is a difference in the hydrophobicity in the samples, possibly due to the activity of ions present in the broth which may cause a change to the charge of the peptide, however this is pure speculation and the actual reason for these different gradients is unknown.

5.4 CONCLUSIONS

The resin binding method was found to be an effective way of isolating the active fraction from the fermentation broth, inoculated with *Enterococcus* B9510. The testing carried out in order to identify the optimal conditions for binding bacteriocins, using nisin, showed that there was little effect on binding efficiency of the resin, in this solution, over a range of temperatures, between 4°C and 30°C.

The resin was destroyed when used in conjunction with magnetic stirring, as the resin was ground into a powder by the kinetic action between the stirrer and the flask. Because of this shaking was employed to mix the resin and solution, this was shown to show no significant difference in binding efficacy.

The effect of pH on binding appeared to be limited; however, the effect of pH on the activity of nisin was not limited. At high pH values, above pH 7, the antimicrobial effect of the nisin was reduced. This was shown by a drop in the ability of the peptide to inhibit the indicator bacteria in the control samples.

Resin volumes of 50, 35 and 25 g/l were shown to demonstrate similar levels of nisin activity adsorption, at approximately equivalent activity levels to those shown in the fermentation broth of *Enterococcus* B9510. A volume of 25 g/l was selected for further use as it was the smallest volume that showed complete binding of the sample activity.

Samples were eluted with 50% isopropanol, as this was found to be the best concentration to maximise bacteriocin elution and minimise the elution of the pigment, which also bound to the resin, most likely from the fermentation media, M17. A second elution at 80% isopropanol was also carried out which contained a small amount of activity but a much larger proportion of the pigment.

The use of the pH method to isolate the bacteriocin from solution did not provide a viable method for the extraction of bacteriocins from the fermentation broth. A wide range of pH values were tested in order to bind the active fraction to the cells in the fermentation broth, however, none of the tested values proved a successful method for the isolation of the antimicrobial fraction in the broth.

The ion-exchange column was found to be an effective method of reducing the amount of pigment within the sample; however this method did cause the loss of the large proportion of the relative activity from the eluent of the XAD-16 resin. Similar activities were demonstrated in both the resin eluent and the ion-exchange column eluent; however, the volumes of column eluent were approximately one fifth that went into the column.

The P-10 gel column was found to be a poor method for isolating the active fraction from the XAD-16 resin eluent. The column did not behave a predicted, as the activity was isolated in the first fractions collected from the column instead of near the end of the run as would be expected due to the size of the active peptide. This coupled with the large lost of antimicrobial activity, when compared to the resin eluent; cause the P-10 to be abandoned as a method to isolate the bacteriocins.

High Performance Liquid Chromatography was an accurate and effective way of isolating the active fraction from the samples eluted from the ion-exchange column. The effect of the different fermentation times, 24 and 4 hours, had an effect on the gradient used in the column; the reason for this change is unknown.

6 CHARACTERISATION

6.1 INTRODUCTION

This chapter deals with characterization of the peptide, covering a range of properties from the effect of heating the peptide, discovering which enzyme will digest the amino acids, and the effect of the media in which the peptide was present. Factors such as pH and salt concentration can affect the range of possible applications that the bacteriocin may be used for, and potentially limit its further usefulness.

This chapter also deals with the taking of HPLC samples, which are essentially pure, having the molecular weight determined and the amino acid sequence characterised. This will allow a comparison between the size of the peptide and other bacteriocins, and also allow the identification of the properties that the specific sequence confers on the peptide.

6.2 RESULTS

6.2.1 Characterisation of bacteriocins from *Enterococcus* B9510

6.2.1.1 SDS-page gel and activity determination of purified bacteriocin

Figure 6.1 shows the SDS-page gel used to identify the approximate size of the peptide that displayed antimicrobial activity. The sample fraction in gel A was used to identify the position of the peptide when compared against a molecular weight marker. The band produced was smaller than the lowest indicator standard of 14.4 kDa. This was unsurprising as typical molecular weights for Type II bacteriocins are between 2-5 kDa. The overlaid gel, indicated as B, showed the activity present in the band and showed the isolation of the activity into one area.

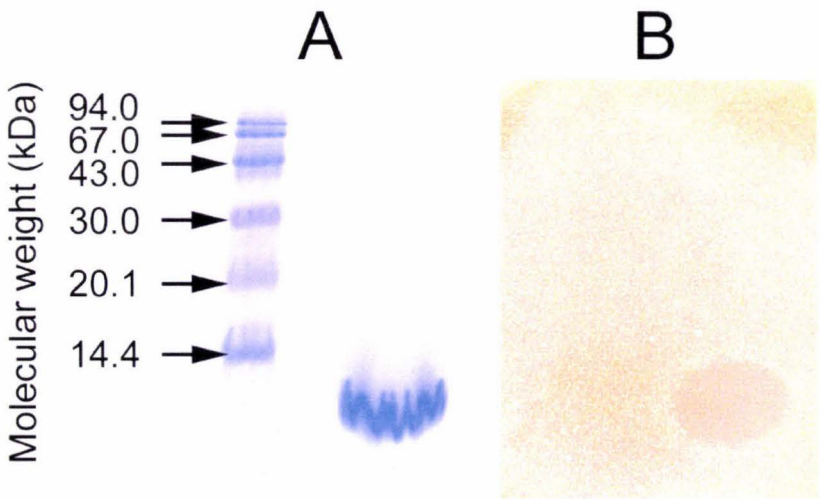


Figure 6.1 SDS-PAGE Electrophoresis and activity testing of purified bacteriocin from *Enterococcus*. B9510. Panel A shows a standard protein gel run with fraction 2 from the ion-exchange column along with a sample of protein standards, (weights are measured in kDa). Panel B is the other half of the protein gel run without stain and overlaid with *L. cremoris* 2144 to indicate bacterial inhibition. The inhibition zone in B matches the stained area in A indicating the inhibitive quality of the fraction.

6.2.1.2 Effect of temperature over time on bacteriocin activity

Figure 6.2 shows the antimicrobial activity of fraction 2 after heating. The activity was unaffected by heating at 60°C, even after an hour. The higher temperatures did affect the activity; drops in activity were shown at 60 minutes for the 80°C sample and at 30 minutes for the sample heated at 100°C. A sample was also autoclaved (121°C for 15 minutes). The sample did not produce a defined clearing, which could be measured, but did appear to show some inhibition to the bacteria (results not shown).

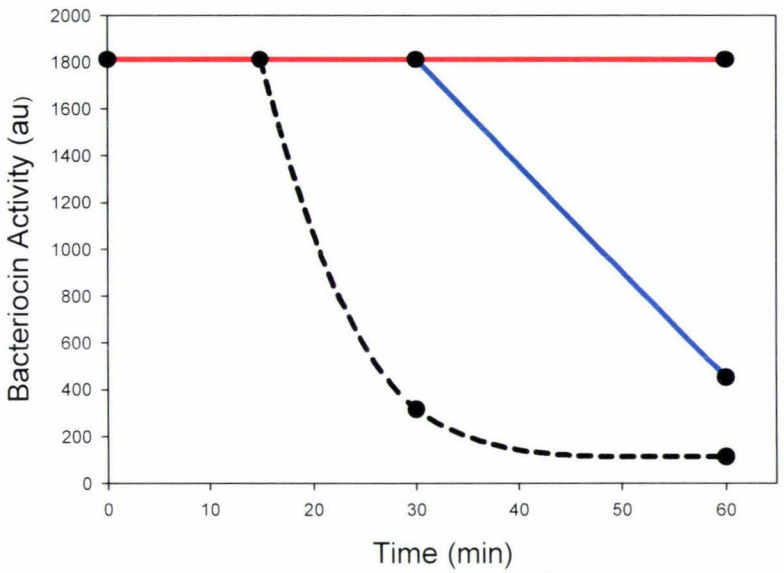


Figure 6.2 Effect of heat on the activity of the peptide. 60°C (—), 80°C (—), 100°C (--).

6.2.1.3 Effect of pH on bacteriocin activity

The effect of pH on the bacteriocin activity is displayed in Figure 6.3, with a marked increase in activity at pH 3. Apart from this spike in activity, the other pH values appear to have little effect on the activity of the bacteriocin. Between pH 4 and 8 there seems to be no effect on activity. If we ignore pH 3, there is some loss in activity in more basic pH values, and a slight increase in activity in acidic pH values. Samples diluted in order to standardise the pH tested seem to indicate that the effect of the pH interactions was reversible, with a more uniform activity over the range of values.

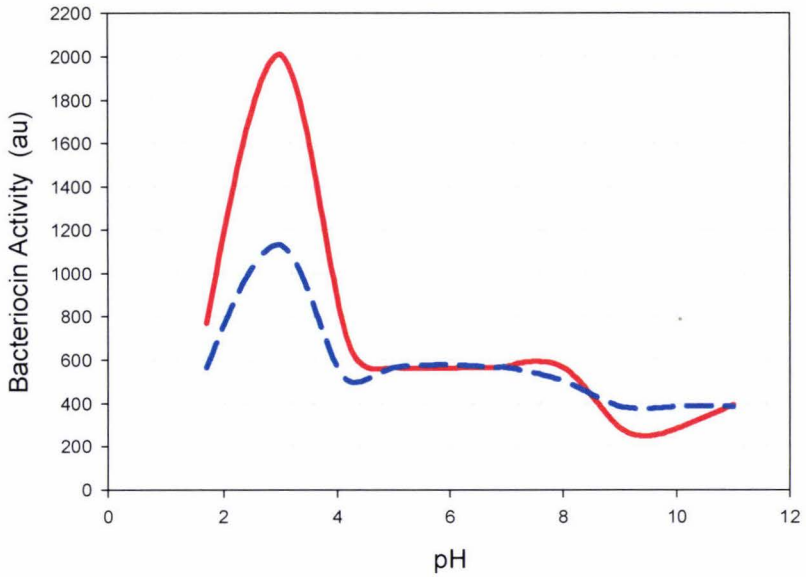


Figure 6.3 Effect of pH on the activity of bacteriocins from *Enterococcus faecalis* B9510. Red line indicates samples that were diluted in media exhibiting the same pH as was present in the incubation media. The Blue line indicates samples that were diluted in standard media with an unaltered pH (standard M17 pH 6.8), so that the results indicate any permanent effect on the activity.

6.2.1.4 Effect of sodium chloride on the bacteriocin activity

Low concentrations of salt appeared to aid the activity of the bacteriocin, with higher activity observed in Figure 6.4 for the sample tested with 100 mM of salt present. Samples tested at 250 mM also showed higher activity than those samples with no additional salt. At 500 mM, however, activity was negatively affected with an observed activity lower than the unaltered sample.

In the samples that were diluted in media contain no additional salt a loss in activity was demonstrated. This decrease in activity was observed in both the 100 and 250 mM samples, when compared to samples diluted in media containing the same salt content. The reductions in the salt concentration produced activity levels, similar to or lower that the control sample, which had no added salt. The exception to this was the 500 mM sample, which produced a higher activity with a diluted salt concentration. This is partially expected, as the dilution would return the salt level to that similar to the 100 mM sample.

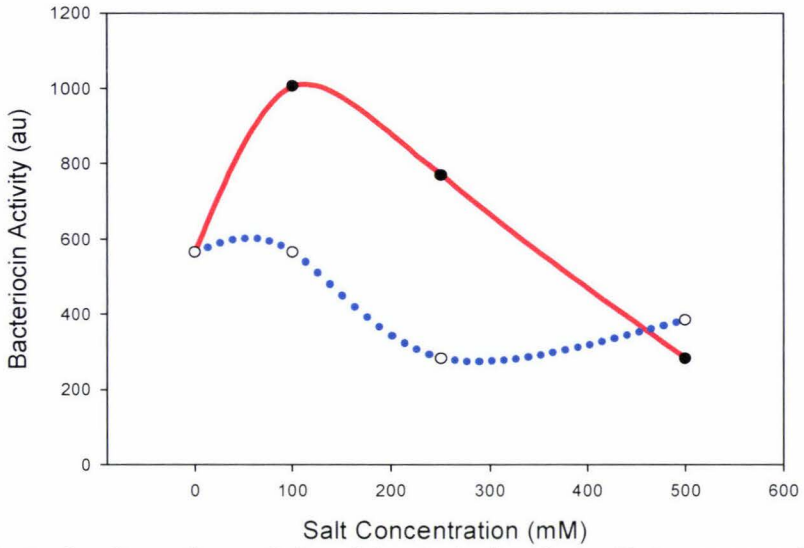


Figure 6.4 Effect of salt on the activity of bacteriocins from *Enterococcus* B9510. Red line indicates samples that were diluted in media containing the same concentration of salt as was present in the incubation media. The Blue line indicates samples that were diluted in media contain no additional salt, so that the results indicate the activity without the presence of salt.

6.2.1.5 Effect of enzymatic action on bacteriocin activity

Table 6.1 shows the effect of enzymes on the activity of antimicrobial samples. Enzymes that affect the protein bond disrupted activity, while those that affected other bonds did not appear to be effective. Figure 3.1 shows the effect of the enzymes on bovine serum albumin, a protein that would be digested by the proteolytic enzymes. Several of the proteolytic enzymes did not demonstrate any activity in this test and were therefore discounted, as inactivation of the bacteriocin could not be expected.

Table 6.1 Effect of enzymatic digestion on the activity of the bacteriocin.

Enzyme	Activity
α-Chymotrypsin	+
Trypsin	-
Catalase	+
Lipase	+
Protease	-
Pronase	-
Proteinase K	-
Protease Type I	-
Protease Type IV	-
Protease Type XIV	-

Note: + indicates bacterial inhibition still present, - indicates no bacterial inhibition against the test bacteria. Results from Protease IX and XIII, and Papain were discarded as they did not digest the BSA standard as active enzymes of this type should have done.

6.2.1.6 Spectrum of bacterial inhibition (MIC)

The range of activity shown in the MIC's is unusual as the peptide exhibits activity against both Gram negative and Gram positive bacteria, as well as some activity against the yeast *Candida albicans*.

Table 6.2 Minimum inhibitory concentration of antimicrobial peptide from *Enterococcus* B9510.

Bacteria tested	MIC of ion exchange fraction 2 (µg/ml)	MIC of HPLC purified sample (µg/ml)
Escherichia coli 0157	8.0	3.6
Salmonella entritidis	8.0	3.5
Salmonella typhimurium	17.1	-
Yersinia entercolitica	10.7	-
Bacillus cereus	17.1	-
Bacillus natto	8.5	-
Bacillus subtilis	5.3	-
Lactobacillus plantarum	10.7	-
Listeria monocytogenes NCTC 7973	8.5	6.6
Listeria monocytogenes NCTC 10884	8.5	6.6
Staphylococcus aureus NCTC 4163	5.3	3.1
Streptococcus faecalis	5.3	-
Candida albicans 3153A	21.4	25

Note: Protein concentration in the ion exchange sample was found to be 1.22 mg/ml, of this 7% was deemed to be active peptide from HPLC analysis.

6.2.2 Molecular weight determination, N-terminal sequencing

6.2.2.1 Molecular weight determination

Samples sent to Auckland University from the 24 hour fermentations came back with the active fraction demonstrating a molecular weight around 1.308 kDa (Figure 6.5). Results from the University of Otago showed fractions with a molecular weight of 1.348 kDa and a second fraction with a weight of 1.067 kDa (data not shown). Molecular weight for the two active peaks detected in the four hour fermentations from University of New South Wales. Bioanalytical Mass spectroscopy facility were 1.227 and 1.270 kDa (Graph A and B Figure 6.7).

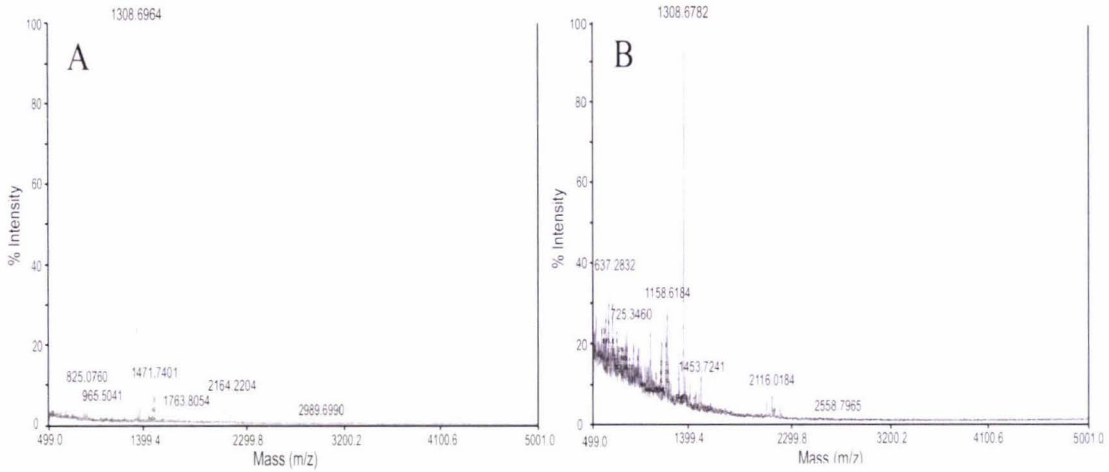


Figure 6.5 Molecular weight of the active peptide from HPLC samples of fraction two. Graph A is from June 2003 and B from September 2003.

6.2.2.2 N-terminal sequencing

Results for N-terminal sequencing did not provide a reliable sequence. Multiple attempts were made to sequence purified samples without success. Typical samples were first tested for molecular weight to ensure that the samples were 'clean' (as shown in Figure 6.5A), but even these clean samples did not provide good sequence data.

Table 6.3 Amino acid sequences reported from active peptide samples.

Date	Method	1	2	3	4	5	6	7	8	9	10	11
11/2004	1			P	V	Y	F	F	P			
11/2004	2	L/V/A	M/K/H/R	P/G/Q/H	F/Q/P	Y/P	G/H	V/Q	I	M	F	
05/2004	2	L/V/A	R/K/H/M	P/G/H/Q	F/Q/P	Y/P	G/H	V/Q	I/M/P	M	F	F
05/2004	2	L	M/K	P	P/Q	T/Y	H/G	K	M	Y	F	
03/2004	3	A	M/R	F	P/R	F	G/R	V	R	G		
10/2003	3		A/P/V/L	P/A	P/A	P/A						
		L/A	**M**	**P**	**P**	**Y**	**G**	**V/Q**	**I/M/P**	**M**	**F**	**F**

Note: Method 1 is LC-MS-MS carried out by the University of New South Wales, method 2 is N-terminal sequencing carried out by Otago University and method 3 is N-terminal sequencing carried out by Auckland University. Some residues were removed for space reasons; those residues removed did not demonstrate any homology to other peptides in proposed positions.

6.2.2.3 Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS)

The results from the LC-MS-MS were also inconclusive in providing a defined amino acid sequence. The results provided some sequence data, for a small part of the peptide (Figure 6.6), but did not produce the whole sequence. The result provided is the mostly likely given the MS data, but is not necessarily correct. The proposed sequence as described in Figure 6.6 was PVYFFP.

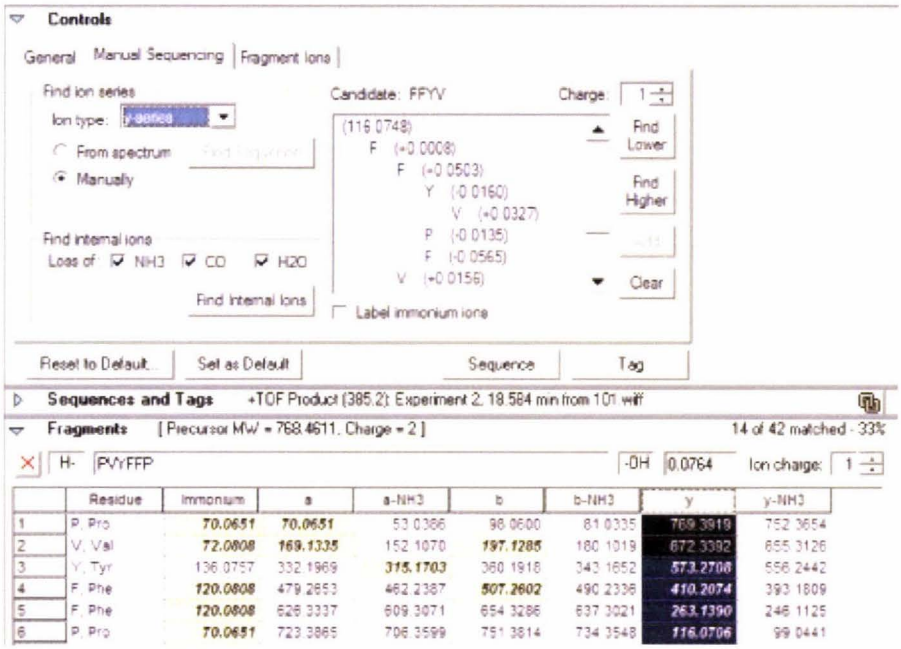


Figure 6.6 LC-MS-MS result for partial sequence of active peptide from *Enterococcus* B9510, Proposed sequence PVYFFP.

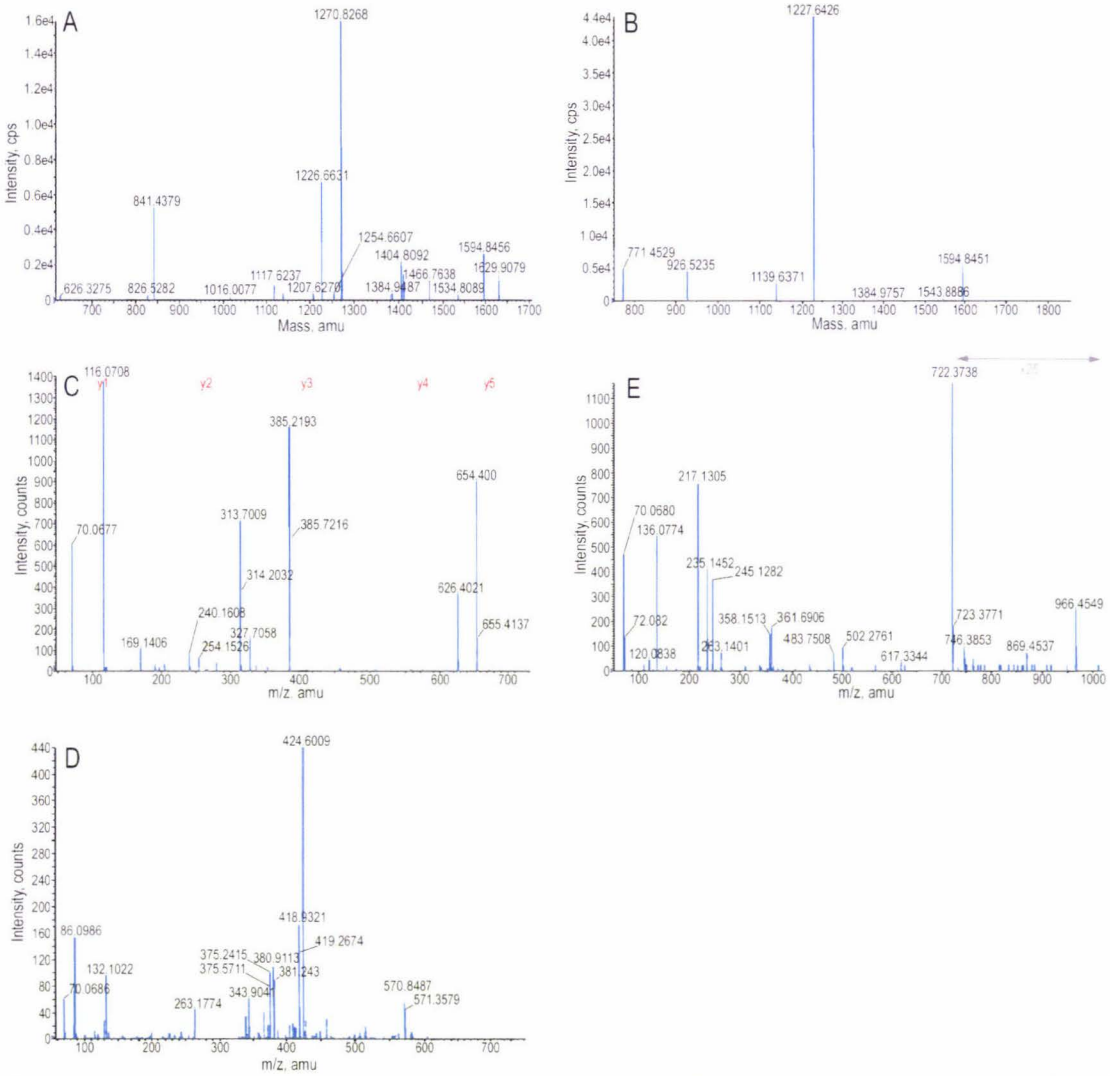


Figure 6.7 Mass spectrometry data for active HPLC fractions from November 2004. Figure A and B represent Mass spectroscopy data for the 2 active HPLC peaks. C, D and E represent the LC-MS data from the dominant MS peaks.

6.3 DISCUSSION

6.3.1 Effect of temperature, pH, salt and enzymes on antimicrobial activity.

The effect of temperature on the activity of the peptide, shown in Figure 6.2, demonstrated that the activity of the crude peptide isolate (fraction 2) was stable for an hour at 60°C, with no loss of activity. The other temperatures tested showed losses in activity over time. However, even at 100°C for an hour, total inactivation of the peptide did not occur. An autoclaved sample, 121°C for 15 minutes, showed partial inhibition of the indicator bacteria when plated, but did not produce a clear zone of inhibition. This suggests that the activity was still present but at a concentration too low to greatly inhibit the bacteria. These results are typical for the effect of heat treatment on bacteriocins. Results from Kelly *et al* 1996 (42), Villani *et al* 1993 (82), and Park *et al* 2003 (69), show positive activity up to and including 100°C for 30 minutes and a loss of activity after autoclaving, similar results to those reported here. The stability of the peptide shows that one of the major characteristics of a Class II bacteriocin was exhibited by the peptide, being highly heat stable. This gives further support to the theory that the bacteriocin isolated here was a Class II bacteriocin.

Previous testing carried out by Knox, M 2001 (45) indicated that the presence of acid did not negatively affect the activity of the bacteriocins. This work further detailed the effect of a range of pH values on the bacteriocin activity. From Figure 6.3 the activity of the peptide can clearly be seen to be most active at a pH value of 3. The activity of the sample was relatively stable between pH 5 and 8 and demonstrated a loss of activity as the pH increased above 9. Comparisons of activity relating to pH in literature were difficult to find, the majority using either a positive or negative response to the effect of pH. Papers indicated that bacteriocins are relatively pH stable. Only two examples demonstrated where a pH value caused the inactivation of the peptide: in Losteinkit *et al* 2001 (53) pH values of 3.5 and 4 were found to inactivate bacteriocin N15 from *Enterococcus faecium*, and in Kelly *et al* 1996, pH 12 (a value not tested in this investigation) was found to inactivate plantaricin KW30 from *Lactobacillus plantarum*.

The effect of salt on the activity of the bacteriocins appears to be similar to some of the observations noted during the fermentation trials. Low concentrations of salt appear to aid in the activity of the bacteriocin. In this experiment a concentration of around 100 mM appeared to produce the optimum activity. An increase in salt concentration, however, reduced the

activity demonstrated by the samples. Samples tested in dilutions that did not contain the same salt concentrates as the test valves, i.e. tested without salt present to demonstrate any permanent effect on the peptides, it appears that low salt levels, 100 mM, had no permanent effect on the activity. Levels at 250 mM, however, showed much less activity than samples diluted in the presence of salt. On the whole it appeared that the levels of sodium chloride had only a moderate effect on the permanent inhibitory effect of the peptides.

The enzymatic testing of the peptide showed that it was susceptible to the enzymes that affect the peptide bond, specifically the proteases, while the enzymes that did not affect peptide bonds were of limited effect in inhibiting the antimicrobial response, as shown in Table 6.1. This was expected, as the activity in the samples is supposed to come from a peptide/protein source, which would be digested by these enzymes.

6.3.2 Spectrum of antimicrobial activity

The range of activity demonstrated by the active fraction produced from *Enterococcus* B9510 is unusual, as the peptide appears to affect both Gram positive and Gram negative bacteria. Since bacteriocins are typically active against a range of similar bacteria, bacteriocins produced mostly from Gram positive bacteria will generally only affect other Gram positive bacteria. There are numerous references to the inhibition of Gram positive bacteria by bacteriocins in literature, most of which demonstrate a high affinity for the inhibition of bacteria of the same variety as that of the producer strain (4) (26) (28) (54). However, there are very few references to the inhibition of Gram negative bacteria, such as *E. coli* or *Salmonella*, by bacteriocins produced by Gram positive bacteria. This study showed both *E. coli* and *Salmonella* were found to be inhibited by the antimicrobial properties of the purified fermentation broth. The reason for this range of activity is unclear, however the results support those of Knox 2001 (45), which also showed positive activity against Gram negative bacteria from this bacteriocin.

There were very few examples of bacteriocin MIC's with which to compare the values found in this study. The most likely reason for the variation between the two MIC's values for the liquid and solid media is the effect of the antimicrobial activity in liquid culture and solid media systems. Pinchuk *el al* 2001 suggested that the underestimation of MIC's determined in solid medium could be caused by the physicochemical instability of the antibiotic in the

solid media, which may also relate to bacteriocin activity (72). Another example of this variation between liquid and solid media activities is used as common selection method for screening bacteria for bacteriocin activity. Bacteria are grown on solid media for the initial screening and then in liquid culture, to reduce the number of selected bacteria, as fewer bacteria show activity in the liquid cultures (49) (81) (13). The possibility also exists that the protein concentrations calculated or the percentage of active protein in the samples may also have affected these results.

6.3.3 Peptide sequencing and identification

The molecular weight of the active fraction was tested at three different facilities, each of which showed some variation in the size of the active peptide, ranging from 1.27 kDa to 1.35 kDa. The different equipment used for the size identification between the facilities may have caused some of this variation. Another factor in the variation could be the time at which fermentation was halted for each sample. The effect of the fermentation time on the HPLC gradient required to isolate the active fraction has previously been mentioned, and the cause of this variation may also be the reason for the size discrepancies between the samples.

The peptide sequence provided the most difficult and ultimately unsuccessful testing undertaken during this project. Multiple attempts were made to provide sequence data, using both N-terminal sequencing and LC-MS-MS. Sequences provided from N-terminal sequencing often came back with multiple residues for each position in the sequence. The lack of defined resides in each position made comparison of the sequence very difficult and provided no conclusive sequence homology with any know peptides or proteins. The problems with N-terminal sequencing could be linked to several possible reasons, firstly the presence of sequence blockers in the sample, which prevent amino acid identification. Secondly there are amino acids that can make sequencing difficult due to their physical properties, as in the case of some of the modified amino acids. A third possible source of difficulty could have been due to the presence of sequences of very similar size being isolated together, interfering with the identification. For the LC-MS-MS technique, the size of the peptide was a limiting factor, this method uses the sizes of know sequences to identify fragments, based on enzyme digestion, and interprets the amino acid sequence based on the

molecular weight of these fragments. In the case of a novel peptide this sequence data may not be available and would make identification difficult.

The lack of a whole sequence makes the identification of this peptide very difficult. The small fragment, which was identified via LC-MS-MS, produced a wide range of similar sequences over a range of microorganisms, when investigated using amino acid databases. A sequence of 5 amino acids was too small to provide any statistically significant results. The consensus sequence, shown in Table 6.3, was used to search for any similar sequences using the BLAST network service, at http://au.expasy.org/tools/blast, however no similar sequence could be found within a significant level of confidence (95%), and those sequences that were identified, but outside the confidence interval, did not relate to any known antimicrobial peptides.

6.4 CONCLUSIONS

Tests showed that the antimicrobial activity of the sample was unaffected at 60°C for at least an hour, and retained activity even when subjected to 100°C for an hour, demonstrating the heat stability commonly seen in type II bacteriocins.

The effect of pH showed an optimal activity at pH 3, but showed activity for all the values tested. A constant inhibition was demonstrated within the range of pH 5-8, while a reduction in activity was observed at pH values above 9.

Low levels of salt, 100 mM, increased the activity of the peptides, but an increase in salt concentrations reduced the effectiveness of the bacteriocin, where salt was present in the sample. In the samples where the salt had been removed before testing there was a slight reduction in antimicrobial activity in the samples treated with the highest salt concentrations, suggesting that the salt had some small permanent effect on activity.

The effect of enzymes on activity showed that those enzymes, which acted upon peptide bonds, were effective at inactivating the antimicrobial activity of the sample. Those enzymes, which affect other systems, were shown not effective at inactivating the bacteriocin.

Testing against a variety of bacteria showed that the peptide demonstrated activity against both Gram positive and Gram negative food pathogenic bacteria.

Mass determination of the active fraction showed that the peptide was approximately 1.3 kDa in size. Attempts to sequence the peptide were overall unsuccessful; however, a consensus sequence of LMPPYGVIMFF, which has a predicted molecular weight of 1.314 kDa, was compiled from several sequencing attempts. This sequence showed no significant homology to any known sequence, when tested for using the BLAST network service.

7 CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

7.1 CONCLUSIONS

Results from the fermentations study showed no constant distinguishable relationship between the growth rates of the bacteria and the production of bacteriocins. The effect of media showed that while good growth rates could be achieved this did not always relate to the production of bacteriocins. Results from the multivariate analysis and the effect of pH on the system showed that there did, however, appear to be a relationship between growth and antimicrobial activity. The pH test showed similar trends in μMax and activity, and the 3D representations of μMax and the bacteriocin activity also showed similar patterns. It appeared that there maybe a link between bacteriocin production and cellular growth, although other unknown factors also appear to have some influence on this relationship. The highest bacteriocins levels observed in the multivariate testing were shown to be those samples with the shortest exponential growth phase, enhancing the idea that the rate of cellular growth was a factor when considering the levels of bacteriocin production from fermentation.

The best growth temperature for the bacteria and the production of bacteriocins was shown to be at 42°C in uncontrolled pH conditions, although 30°C and 37°C were also effective.

Optimum pH for growth and antimicrobial activity was found at pH 5.5. At pH 4 bacteriocin activity was found to be similar to that at pH 5.5, but displayed poor cellular growth.

In the multi-factor fermentations salt appeared to have the greatest effect on bacteriocin production. Increases in salt levels in the fermentation broth decreased the activity of the samples. Glucose appeared to have little effect on antimicrobial activity but had a large effect positive effect on bacterial growth. Aeration had little effect on the production of bacteriocins but may have increased the cellular growth of the fermentations containing higher salt and glucose concentrations.

The resin binding method was found to be an effective way of isolating the active fraction from the fermentation broth, inoculated with *Enterococcus* B9510. Testing showed that there was little effect on binding efficiency of the resin, over a range of temperatures, between 4°C and 30°C. The effect of pH on binding appeared to be limited; however, pH did appear to affect the activity of the test bacteriocin, limiting the usefulness of these tests.

A volume of 25 g/l was selected for the isolation of the bacteriocin from *Enterococcus* in solution, as it was the smallest volume that showed complete binding of the sample activity, at an approximately equivalent activity level to those shown in the fermentation broth.

Samples were eluted from the XAD-16 resin with 50% isopropanol, as this was found to be the best concentration to maximise bacteriocin elution and minimise the elution of the pigment which was also bound to the resin. A second elution at 80% isopropanol was also carried out which contained a small amount of activity but a much larger proportion of the pigment.

The use of the pH method to isolate the bacteriocin from solution did not provide a viable method for the extraction of bacteriocins from the fermentation broth.

Ion-exchange chromatography was found to be an effective method for reducing the amount of pigment within the sample. The P-10 gel column was found to be a poor method of isolating the active fraction from the XAD-16 resin eluent and was abandoned as a method to isolate the bacteriocins. High Performance Liquid Chromatography was an accurate and effective way of isolating the active fraction from the samples eluted from the ion-exchange column.

The effect of the different fermentation times, 24 and 4 hours, had an effect on the gradient used in the column; the reason for this change was unknown.

Tests showed that the antimicrobial activity of the sample was unaffected at 60°C for at least an hour, and samples retained activity even when subjected to 100°C for an hour.

The effect of pH showed an optimal activity at pH 3, and showed activity for all the values tested, pH 2-10.

Low levels of salt, 100 mM, increased the antimicrobial activity of the peptides, however, as salt concentrations increased the effectiveness of the bacteriocin was reduced.

The effect of enzymes on activity showed that those enzymes which acted upon peptide bonds were able to inactivate the antimicrobial activity of the sample. Those enzymes which affect other systems did not show the same inactivation.

Testing against a variety of bacteria showed that the peptide demonstrated activity against both Gram positive and Gram negative food pathogenic bacteria.

Mass determination of the active fraction showed that the peptide had a molecular weight of approximately 1.3 kDa. Attempts to sequence the peptide were overall unsuccessful; however, a consensus sequence of LMPPYGVIMFF, which had a molecular weight of 1.314 kDa, was compiled from several sequencing attempts. This sequence showed no significant homology to any known sequence, when tested for using the BLAST network service.

7.2 RECOMMENDATIONS FOR FUTURE RESEARCH

7.2.1 Fermentation

7.2.1.1 Effect of the carbon/nitrogen ratio

Testing of different media sources showed that the effect of the carbon source on the production of bacteriocins exhibited some influence, with glucose showing the most influence in producing activity, in M17. However, in the TYT media, which contained the highest levels of glucose, poor activity was produced. From this it was supposed that the effect of the carbon/nitrogen ratio might play a larger influence into the production of bacteriocins than the individual substrates in the media. Using a standard media and influencing the ratio between carbon and nitrogen may provide a better and more cost effective medium in which to grow the bacteria. The media used extensively in this study, M17, was an effective medium, but it was expensive, and could be a limiting factor in any large scale production.

7.2.1.2 Investigation into the effect of oxygen on bacteriocin production

In the multivariate analysis it was shown that aeration had an influence on the production of the bacteriocins, however, this was not explored fully. Investigation into the limitation of the dissolved oxygen in the system could show that oxygen supply, or lack thereof, was a contributed to the production of bacteriocins. This may be linked to the pathways associated with anaerobic growth and the related stress on the bacteria.

7.2.1.3 Investigation into continuous or batch-fed fermentation

The fermentations in the multi-factor analysis showed high bacteriocin production, in the sample that was unaerated, contained no salt and 0.05% glucose. With such low levels of glucose present it was unlikely that the other nutrients within the system, i.e. nitrogen, would have been fully utilised. This could lead into the possibility for a continuous or more likely a fed-batch system, where the bacteriocins are removed, and glucose is added to utilise the nutrients in the media, while maintaining at optimum level of production. If effective this could provide high levels of active peptides, while utilising the available media, and allowing for longer production runs.

7.2.2 Purification

7.2.2.1 Optimisation of purification

While the current method described in this document was effective at recovering the antimicrobial activity from the fermentation broth, large losses were incurred during most of the purification steps. In order for this to become a more viable method improvements into the processing steps would be necessary to maximise recovery of the antimicrobial activity. The need for this would be determined on the application for which the peptides were intended, in some cases the desired product may result in less purification steps and a cruder final product, decreasing processing cost and increase the amount of peptide being utilised.

7.2.2.2 Pigmentation removal

One of the major reasons for the purification methods used was in order to remove the pigment, which was produced from the resin eluent, most likely from the organic fraction in the media. The colour of the crude extract from the XAD-16 resin is a dark brown colour and would most likely be unacceptable as a commercial product due to this colouration. If this pigment could be removed then the purification possibilities may increase, methods such as freeze drying into a powder form, at an earlier stage may become an option and the colour removal could possibly reduce the processing steps and therefore the loss of activity shown in other techniques.

7.2.3 Characterisation

7.2.3.1 Further investigation into spectrum of activity

The range of antimicrobial activity demonstrated by the bacteriocin was large; however, the test range of bacteria was relatively small. Testing of a wider range of bacteria, and a more comprehensive number of varieties would be useful in determining the spectrum of activity and the usefulness of the bacteriocin.

7.2.3.2 Peptide identification

The most unsuccessful part of this project was in the identification of the peptide, from its amino acid sequence. Without this data it was not possible to relate this peptide to any other types of bacteriocins or bioactive peptides. Due to the unusual spectrum of activity demonstrated by the peptide, and the small size shown from mass spectrometry, it would be of interest to see how the sequence related to other amino acid sequences.

8 REFERENCES

1. Allison G., Fremaux C., and Klaenhammer T. 1994. Expansion of bacteriocin activity and host range upon complementation of two peptides encoded within lactacin F operon. Journal of Bacteriology 176:2235-2241.

2. Axelsson T., Holck A., Birkeland S., Aukrust T., and Blom H. 1993. Cloning and nucleotide sequence of a gene from *Lactobacillus sake* Lb706 necessary for sakacin A production and immunity. Applied and Environmental Microbiology 59:2868-2875.

3. Aymerich T., Holo H., Havarstein L., Hugas M., Garriga M., and Nes I. 1996. Biochemical and genetic characterisation of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the Pediocin family of bacteriocins. Applied and Environmental Microbiology 62:1676-1682.

4. Balla E., Dicks L., Du Toit M., van der Merwe M., and Holzapfel W. 2000. Characterisation and cloning of the genes encoding enterocin 1071A and enterocin 1071B, two antimicrobial peptides produced by *Enterococcus faecalis* BFE 1071. Applied and Environmental Microbiology 66:1298-1304.

5. Bennik M., Vanloo B., Brasseur R., Gorris L., and Smid E. 1998. A novel bacteriocin with YGNGV motif from vegetable-associated *Enterococcus mundtii*: full characterisation and interaction with target organisms. Biochemistry and Biophysics 1373:47-58.

6. Bhugaloo-Vial P., Dousset X., Metivier A., Sorokine O., Anglade P., Boyaval P., and Marion D. 1996. Purification and amino acid sequences of piscicoins V1a and V1b, two class IIa bacteriocins secreted by *Carnobacterium piscicola* V1 that displays significantly different levels of specific inhibitory activity. Applied and Environmental Microbiology 59:3577-3584.

7. Burianek L. and Yousef A. 2000. Solvent extraction of Bacteriocins from liquid cultures. Letters in Applied Microbiology 31:193-197.

8. Casaus P. , Nilsen T., Cintas L., Nes I., Hernandez P., and Holo H. 1997. Entercin B, a new bacteriocin from *Enterococcus faecium* T136 which can act synergistically with enterocin A. Microbiology 143:2287-2294.

9. Cho H., Yousef A., and Yang S. 1996. Continuous production of pediocin by immobilised *Pediococcus acidlactici* in a packed-bed bioreactor. Applied Microbiology and Biotechnology 45:589-594.

10. Cintas L., Casaus P., Havarstein L., Hernandez P., and Nes I. 1997. Biochemical and genetic characterisation of Enterocin P, a novel *sec*-dependent bacteriocin from *Enterococcus faecium* P13 with a broad antimicrobial spectrum. Applied and Environmental Microbiology 63:4321-4330.

11. Cintas L., Casaus P., Herranz C., Havarstein L., Nes I., Hernandez P., and Holo H. 2000. Biochemical and Genetic Evidence that *Enterococcus faecium* L50 Produces Enterocins L50A and L50B, the *sec*-Dependent Enterocin P, and a Novel Bacteriocin Secreted without an N-Terminal Extension Termed Enterocin Q. Journal of Bacteriology 182:6806-6814.

12. Cintas L., Casaus P., Holo H., Hernandez P., Nes I., and Havarstein L. 1998. Enterocins L50A and L50B, two novel bacteriocins from *Enterococcus faecium* L50, are related to *Staphlococcal hemolysis*. Journal of Bacteriology 180:1988-1994.

13. Cintas, M., Rodriguez J., Fernadez M., Sletten K., Nes I., P. Hernandez, and Holo H. 1995. Isolation and characterisation of Pediocin L50, a new bacteriocin from *Pedicoccus acidlactici* with a broad inhibitory spectrum. Applied and Environmental Microbiology 61:2643-2648.

14. Corpet, F. 1988. Multiple sequence alignment with hierarchical clustering. Nucleic Acids Research 16:10881-10890.

15. Dayhoff, M., R. Schwartz, and B. Orcutt. 1979. Atlas of Protein and Sequence Structure, National Biomedical Research Foundation, Washington D.C.; USA.

16. De Vuyst L., Callewaert R., and Crabbe K. 1996. Primary metobolite kinetics of bacteriocin biosysthesis by *Lactobacillus amylovorus* and evidence for stimulation of bacteriocin production under unfavourable conditions. 142:817-827.

17. De Vuyst L., Callewaert R., and Pot B. 1996. Characterisation of Antagonistic Activity of *Lactobacillus amylovorus* DCE 471 and Large Scale Isolation of its Bacteriocin Amylovorin L471. Systematic and Applied Microbiology 19:9-20.

18. De Vuyst L. and Vandamme E. 1994. Nisin, a lantibiotic produced by *Lactococcus lastis subsp. lactis*: properties, fermentation, biosynthesis and applications, De Vuyst L. and Vandamme E. (ed.), Bacteriocins of Lactic Acid Bacteria: Microbiology, Genetics and Applications, Blackie Academic and Professional, London.

19. Dillon V. and Board R. 1994. Natural Antimicrobial Systems and Food Preservation, CAB International, Wallingford.

20. Eguchi T., Kaminaka K., Shima J., Kawamoto S., Mori K., Choi S-H., Doi S., and Ogata S. 2001. Isolation and Characterisation of Enterocin SE-K4 produced by thermophilic Enterococci, *Enterococcus faecalis* K-4. Bioscience, Biotechnology and Biochemistry 65:247-253.

21. Eijsink V., Axelsson L., Diep D., Havarstein L., Holo H., and Nes I. 2002. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie van Leeuwenhoek 81:639-654.

22. Ennahar S., Aoude-Werner D., Assobhei O., and Hasselmann C. 1998. Antilisterial activity of enterocin 81, a bacteriocin produced by *Enterococcus faecium* WHE 81 isolated from cheese. Journal of Applied Microbiology 85:521-526.

23. Ennahar S., Asou Y., Zendo T., Sonomoto K., and Ishizaki A. 2001. Biochemical and Genetic Evidence for Production of Enterocins A and B by *Enterococcus faecium* WHE 81. International Journal of Food Microbiology 70:291-301.

24. Ennahar S., Sashihara T., Sonomoto K., and Ishizaki A. 2000. Class IIa Bacteriocins: biosynthesis, structure and activity. FEMS Microbiological Reviews 24:85-104.

25. Fisher, M.C. 1998. Control of methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus* in hospitalized children. Pediatric Infectious Disease Journal 17:825-826.

26. Foulquie Moreno M., Callewaert R., Devreese B., Van Beeumen J., and De Vuyst L. 2003. Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. Journal of Applied Microbiology 94:214-229.

27. Franz C., Abriouel H., and Holzapfel W. 2002. Biotechnology in Food Production of *Enterococci* and Food. Australasian Biotechnology 12:31-37.

28. Franz C., Schillinger U., and Holzapfel W. 1996. Production and characterisation of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. International Journal of Food Microbiology 29:255-270.

29. Gatlin C., Kleemann G., Hay L., Link G., and Yates J. 1998. Protein Identification at the low femtomole level from silver-stained gels using a new fritless electrospray interface for liquid chromotography microspray and nanospray mass spectrometry. Analytical Biochemistry 236:93-101.

30. Gazin, V. Influence of Growth Conditions on Bacteriocin Production by Thermophilic Enterococci: *Enterococcus faecalis* B9510. 2002.

31. Guder, A., I. Wiedemann, and H.-G. Sahl. 2000. Posttranslationally Modified Bacteriocins - The Lantibiotics. Biopolymers 55:62-73.

32. Hammes W. 1998. Safety assessment of lactic acid bacteria and probiotics. Monatsschrift Kinderheilkunde 146:S31-S38

33. Hastings J., Sailer M., Johnson K., Roy K., Vederas J., and Stiles M. 1991. Characterisation of leucocin A-UAL 187 and cloning of the bacteriocin gene *Leuconostoc gelidum*. Journal of Bacteriology 173:7491-7500.

34. Herranz C., Casaus P., Mukhopadhyay S., Martinez J., Rodriguez J., Nes I., Hernandez P., and Cintas L. 2001. *Enterococcus faecium* P21: A strain occurring naturally in dry-fermented sausages producing the class II bacteriocins enterocin A and enterocin B. Food Microbiology 18:115-131.

35. Herranz, C., Y. Chen, H.-J. Chung, M. Cintas, P. Hernandez, T. Montville, and M. Chikindas. 2001. Enterocin P selectively dissipates the membrane potential of *Enterococcus faecium* T136. Applied and Environmental Microbiology 67:1689-1692.

36. Hoover, D. and L. Steenson. 1993. Bacteriocins from lactic acid bacteria, Academic Press, San Diego, USA.

37. Jack R., Tagg J., and Bibek R. 1995. Bacteriocins of Gram-Positive Bacteria. Microbiological Reviews 59:171-200.

38. James R., Lazdunski C., and Pattus F. 1992. Bacteriocins, Microcins and Lantibiotics, Springer-Verlag, Barcelona.

39. Jimenez-Diaz R., Ruiz-Barba J., Holo H., Cathcart D., Nes I., Sletten K., and Warner P. 1995. Purification and Partial Amino Acid Sequence of Plantaricin S, a Bacteriocin Produced by *Lactobacillus plantarum* LPCO10, the Activity of Which Depends on the Complementary Action of Two Peptides. Applied and Environmental Microbiology 61:4459-4463.

40. Joosten H., Nunez M., Devereese B., van Beeumen J., and Marugg J. 1996. Purification and Characterisation of Enterocin 4, a Bacteriocin Produced by *Enterococcus faecalis* INIA 4. Applied and Environmental Microbiology 62:4220-4223.

41. Kalmokoff, M., S. Banerjee, T. Cyr, M. Hefford, and T. Gleeson. 2001. Identification of a new plasmid-encoded *sec*-dependent bacteriocin produced by *Listeria innocua* 743. Applied and Environmental Microbiology 67:4041-4047.

42. Kelly W., Asmundson R., and Huang C. 1996. Characterisation of plantaricin KW30, a bacteriocin produced by *Lactobacillus plantarum*. Journal of Applied Bacteriology 81:657-662.

43. Kemperman, R., A. Kuipers, H. Karsens, Nauta A., O. Kuipers, and J. Kok. Identification and Characterisation of two novel Clostridial Bacteriocins, Circularin A and Closticin 574. Applied and Environmental Microbiology 69:1589-1597.

44. Klaenhammer, T. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiological Reviews 12:39-86.

45. Knox M. 2001. Bacteriocins from *Lactobacillus* and *Enterococcus*. Report of 4th year research project, Massey University, Palmerston North 1-26 unpublished.

46. Kuntz, R.L., P.G. Hartel, D.G. Godfrey, J.L. McDonald, K.W. Gates, and W.I. Segars. 2003. Targeted sampling protocol as prelude to bacterial source tracking with *Enterococcus faecalis*. Journal of Enviromental Quality 32:2311-2318.

47. Laukova, A. and M. Marekova. 2001. Production of bacteriocins by different enterococcal isolates. Folia Microbiologica 46:49-52.

48. Leal-Sanchez M., Jimenez-Diaz R., Maldonado-Barragan A., Garrido-Fernandez A., and Ruiz-Barba J. 2002. Optimisation of Bacteriocin Production by Batch Fermentation of *Lactobacillus plantarum* LPCO10. Applied and Environmental Microbiology 68:4465-4471.

49. Lee, H., C. Park, Y. Joo, Kim S., Yoon J., Park Y., Hwang I., Ahn J., and Mheen T. 1999. : Identification and characterization of bacteriocin-producing lactic acid bacteria isolated from Kimchi. Journal of Microbiology and Biotechnology 9:282-291.

50. Leroy F. and De Vuyst L. 2002. Bacteriocin production by *Enterococcus faecium* RZS C5 is cell density limited and occurs in the very early growth phase. International Journal of Food Microbiology 72:155-164.

51. Leroy F. , Moreno M., and De Vuyst L. 2003. *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. International Journal of Food Microbiology 88:235-240.

52. Leroy F., Vankrunkelsven S., De Greef J., and De Vuyst L. 2003. The stimulation effect of a harsh environment on the bacteriocin activity of *Enterococcus faecium* RZS C5 and dependency on the environmental stress factor used. International Journal of Food Microbiology 83:27-38.

53. Losteinkit C., Uchiyama K., Ochi S., Takaoka T., Nagahisa K., and Shioya S. 2001. Characterisation of Bacteriocin N15 produced by *Enterococcus faecium* N15 and cloning of related genes. Journal of Bioscience and Bioengineering 91:390-395.

54. Lyon W., Olson D., and Murano E. 1995. Isolation and Purification of Enterocin EL1, a Bacteriocin Produced by a Strain of *Enterococcus faecium*. Journal of Food Protection 58:890-898.

55. Maisnier-Patin S., Forni E., and Richard J. 1996. Purification, partial characterisation and mode of action of enterococcin EFS2, an antilisterial bacteriocin produced by a strain of *Enterococcus faecalis* isolated from a cheese. International Journal of Food Microbiology 30:255-270.

56. Maldonado A., Ruiz-Barba J., and Jimenez-Diaz R. 2003. Purification and genetic characterization of Plantaricin NC8, a novel coculture-inducible two-peptide bacteriocin from *Lactobacillus plantarum* NC8. Applied and Environmental Microbiology 69:383-389.

57. Marugg J., Gonzalez C., Kunka B., Ledebor A., Pucci M., Toonen M., Walker S., Zoetmulder L., and Vandenbergh P. 1992. Cloning, expression and nucleotide sequence of genes involved in production of pediocin PA-1, and bacteriocin from *Pediococcus acidilactici PAC 1.0*. Applied and Environmental Microbiology 58:2360-2367.

58. Metivier A., Pilet M., Dousset X., Sorokine O., Anglade P., Zagorec M., Piard J., Marion D., Cnatiempo Y., and Fremaux C. 1998. Divercin V41 a new bacteriocin with two disulphide bonds produced by *Carnobacterium divergens* V41: primary structure and genomic organisation. Microbiology 144:2837-2844.

59. Montville T. and Chen Y. 1998. Mechanistic action of Pediocin and Nisin: Recent progress and unresolved questions. Applied Microbiology and Biotechnology 50:511-519.

60. Moreno M., Leisner J., Tee L., Ley C., Radu S., Rusul G., Vancanneyt M., and De Vuyst L. 2002. Microbial analysis of Malaysian tempeh, and characterisation of two bacteriocins produced by isolates of *Enterococcus faecium*. Journal of Applied Microbiology 92:147-157.

61. Nes I. and Holo H. 2000. Class 2 Antimicrobial Peptides from Lactic Acid Bacteria. Biopolymers (peptide science) 55:50-61.

62. Ohmomo S., Murata S., Katayama S., Nitisinprasart S., Kobayashi M., Nakajima T., Yajima M., and Nakanishi. 2000. Purification and some characteristics of enterocin ON-157, a bacteriocin produced by *Enterococcus faecium* NIAI 157. Journal of Applied Microbiology 88:81-89.

63. Olasupo, N.A., U. Schillinger, C.M.A.P. Franz, and W.H. Holzapfel. 1994. Bacteriocin production by *Enterococcus faecium* NA01 from Wara - A fermented skimmed milk product from West-Africa. Letters in Applied Microbiology 19:438-441.

64. Ostrowsky, B. and G.M. Eliopoulos. 1999. Enterococcal infections: Resistance mechanisms, treatment strategies, and hospital issues . Journal of Intensive Care Medicine 14:62-73.

65. Paik H. and Glatz B. 1997. Enhanced Bacteriocin Production by *Propionibacterium thoenii* in Fed-Batch Fermentation. Journal of Food Protection 60:1529-1533.

66. Parente E. and Hill C. 1992. A comparison of factors affecting the production of two bacteriocins from lactic acid bacteria. Journal of Applied Bacteriology 73:290-298.

67. Parente E. and Ricciardi A. 1994. Influence on pH on the production of enterocin 1146 during batch fermentation. Letters in Applied Microbiology 19:12-15.

68. Parente E. and Ricciardi A. 1999. Production, recovery and purification of bacteriocins from lactic acid bacteria. Applied Microbiology and Biotechnology 52:628-638.

69. Park, S.H., K. Itoh, and T. Fujisawa. 2003. Characteristics and identification of enterocins produced by *Enterococcus faecium* JCM 5804. Journal of Applied Microbiology 95:294-300.

70. Pharmacia Bioprocess Technology. 1993. Hydrophobic Interaction chromatography: Principles and methods, Vastra Aros Tryckeri, Sweden.

71. Pharmacia Biotech. 1993. Ion Exchange Chromatography: Principals and methods, Vastra Aros tryckeri, Sweden.

72. Pinchuk, I., P. Bressollier, B. Verneuil, B. Fenet, I. Sorokulova, F. Megraud, and M. Urdaci . 2001. In vitro anti-Helicobacter pylori activity of the probiotic strain *Bacillus subtilis* 3 is due to secretion of antibiotics . Antimicrobial Agents and Chemotherapy 45:3156-3161.

73. Quadri L., Sailer M., Roy K., Vederas J., and Stiles M. 1994. Chemical and genetic characterisation of bacteriocins produced by *Carnobacterium piscicola*. Journal of Biological Chemistry 269:12204-12211.

74. Sabia C., Manicardi P., Messi P., de Niederhausern S., and Bondi M. 2002. Enterocin 416K1, an antilisterial bacteriocin produced by *Enterococcus casslifavus* IM 416K1 isolated from Italian sausages. International Journal of Food Microbiology 75:163-170.

75. Sarantinopoulos P., Leroy F., Leontopoulou E., Georgalaki M., Kalantzopoulos G., Tsakalidou E., and De Vuyst L. 2002. Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. International Journal of Food Microbiology 72:125-136.

76. Sinnott, M. 1998. Comprehensive Biological Catalysis, A mechanistic reference. Volume I Reactions of Electrophilic Carbon, Phosphorus and Sulphur, Academic Press, Harcourt Brace and Company, London.

77. Sinnott, M. 1998. Comprehensive Biological Catalysis, A mechanistic reference. Volume III Radical Reactions and Oxidation/Reduction, Academic Press, Harcourt Brace and Company, London.

78. Steinberg, D. and R. Lehrer. 1997. Designer assays for antimicrobial peptides, p.169-186. In W. Shafer (ed.), Methods in Molecular Biology, Vol 78: Antimicrobial peptide protocols, Humana Press Inc., Totowa, NJ.

79. Tichaczek P., Vogel R., and Hammes W. 1994. Cloning and sequencing of sakP encoding sakacin P, the bacteriocin produced by *Lactobacillus sake* LTH 673. Microbiology 140:361-367.

80. Tomita H., Fujimoto S., Tanimoto K., and Ike Y. 1996. Cloning and genetic organisation of the bacteriocin 31 determinant encoded on the *enterococcus faecalis* pheromone-responsive conjugative plasmid pYI17. Journal of Bacteriology 178:3585-3593.

81. Villani, F., O. Pepe, G. Moschetti, G. Salzano, Parente E., and S. Coppola. 1995. Antagonistic activity of lactic acid bacteria isolated from natural whey starters for Water-Buffalo mozzarella cheese manufacture. Italian journal of food science 7:221-234.

82. Villani, F., G. Salzano, E. Sorrention, O. Pepe, P. Marino and S. Coppola. 1993. Enterocin 226NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes*. Journal of Applied Bacteriology 74:380-387.

83. Waite, B. and R. Hutkins. 1998. Bacteriocins inhibit glucose PEP:PTS activity in *Listeria monocytogenes* by induced efflux of interacellular metabolites. Journal of Applied Microbiology 85:287-292.

84. Wheeler, A.L., P.G. Hartel, D.G. Godfrey, J.L. Hill, and W.I. Segars. 2002. Potential of *Enterococcus faecalis* as a human fecal indicator for microbial source tracking. Journal of Enviromental Quality 31:1286-1293.

85. Wong, K. Purification of enterocins from *Enterococcus faecalis* B9510. 2003.

86. Wu, M. and R. Handcock. 1999. Improved derivatives of bactenecin, a cyclic dodecameric antimicrobial cationic peptide. Antimicrobial Agents and Chemotherapy 43:1274-1276.

87. Zendo, T., M. Fukao, K. Ueda, T. Higuchi, J. Nakayama, and K. Sonomoto. 2003. Identification of the lantibiotic nisin Q, a new natural nisin variant produced by *Lactococcus lactis* 61-14 isolated from a river in Japan. Bioscience, Biotechnology and Biochemistry 67:1616-1619.

88. Zimmermann T. Comparison of growth and bacteriocin production of *Enterococcus sp.* B9510 in different complex media and partial bacteriocin purification. 1-31. 2002.

89. Zor, T. and Z. Selinger. 1996. Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. Analytical Biochemistry 236:302-308.

APPENDIX A1

A1.1 BACTERIAL IDENTIFICATION

A1.1.1 Polymerase Chain Reaction (PCR)

PCR was carried out to produce sufficient rRNA to identify the bacteria. Identification of the bacteria was based on the latest 16s rRNA sequence homology. *Enterococcus* B9510 showed high homology, up to 97.96%, to various *Enterococcus faecalis* strains. The closest match was that of rRNA submitted by Jun Shima, National Food Research Institute, in Japan, and used by Eguchi, T. *et al* 2001 in the paper "Isolation and characterization of bacteriocin K4 produced by thermophilic enterococci" (20).

A1.1.1.1 Method of PCR amplification

PCR was carried out by the Auckland DNA Sequencing Facility at the University of Auckland, and was based on a technique by Woo, T. *et al* 1992[1]. Samples for PCR were prepared from a single colony, resuspended in 100µl of water, and boiled in a water bath of 5 minutes. The PCR reaction was carried out using 1.5-2 µl of the supernatant after the boiled solution was centrifuged for 15 seconds. The amplification program used in this case was: hold at 94°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 94°C for 30 seconds, final cycle 72°C for 5 minutes.

The primers used were:

HDA1 (21-mer) 5' ACT CCT ACG GGA GGC AGC AGT 3'

HDA4 (22-mer) 5' GGA CTA CC(G/T) GGG TAT CTA ATC C 3

[1] Woo, T.H.S., A.F. Cheng and J.M. Ling. 1992. An application of a simple method for the preparation of bacterial DNA. BioTechniques 13(5):696-698

The amplification reactions for the sequence were combined and purified using a Boehringer High Pure PCR product purification kit. Purified DNA was eluted in 50 µl of elution buffer for sequencing. The final sequence is shown in Figure A1.1.

```
ggacgaacgc tggcggcgtg cctaatacat gcaagtcgaa cgcttctttc ctcccgagtg      60
cttgcactca attggaaaga ggagtggcgg acgggtgagt aacacgtggg taacctaccc     120
atcagagggg gataacactt ggaaacaggt gctaataccg cataacagtt tatgccgcat     180
ggcataagag tgaaaggcgc tttcgggtgt cgctgatgga tggacccgcg gtgcattagc     240
tagttggtga ggtaacggct caccaaggcc acgatgcata gccgacctga gagggtgatc     300
ggccacactg ggactgagac acggcccaga ctcctacggg aggcagcagt agggaatctt     360
cggcaatgga cgaaagtctg accgagcaac gccgcgtgag tgaagaaggt tttcggatcg     420
taaaactctg ttgttagaga agaacaagga cgttagtaac tgaacgtccc ctgacggtat     480
ctaaccagaa agccacggct aactacgtgc cagcagccgc ggtaatacgt aggtggcaag     540
cgttgtccgg atttattggg cgtaaagcga gcgcaggcgg tttcttaagt ctgatgtgaa     600
agcccccggc tcaaccgggg agggtcattg gaaactggga gacttgagtg cagaagagga     660
gagtggaatt ccatgtgtag cggtgaaatg cgtagatata tggaggaaca ccagtggcga     720
aggcggctct ctggtctgta actgacgctg aggctcgaaa gcgtggggag caaacaggat     780
tagataccct ggtagtccac gccgtaaacg atgagtgcta agtgttggag ggtttccgcc     840
cttcagtgct gcagcaaacg cattaagcac tccgcctggg gagtacgacc gcaaggttga     900
aactcaaagg aattgacggg ggcccgcaca agcggtggag catgtggttt aattcgaagc     960
aacgcgaaga accttaccag gtcttgacat cctttgacca ctctagagat agagctttcc    1020
cttcggggac aaagtgacag gtggtgcatg gttgtcgtca gctcgtgtcg tgagatgttg    1080
ggttaagtcc cgcaacgagc gcaaccctta ttgttagttg ccatcattta gttgggcact    1140
ctagcgagac tgccggtgac aaaccggagg aaggtgggga tgacgtcaaa tcatcatgcc    1200
ccttatgacc tgggctacac acgtgctaca atgggaagta caacgagtcg ctagaccgcg    1260
aggtcatgca aatctcttaa agcttctctc agttcggatt gcaggctgca actcgcctgc    1320
atgaagccgg aatcgctagt aatcgcggat cagcacgccg cggtgaatac gttcccgggc    1380
cttgtacaca ccgcccgtca caccacgaga gtttgtaaca cccgaagtcg gtgaggtaac    1440
ctttttggag ccagccgcct aaggtgggat agatgattgg ggtgaagtcg taacaaggta    1500
gccgtatcgg aaggtggg                                                  1518
```

Figure A1.1 rRNA sequence of *Enterococcus* B9510. Sequence shows 97% homology to known *Enterococcus faecalis* strains.