CHAPTER 1

INTRODUCTION

1.1 OUTLINE OF THESIS

Quality and safety of food products have always been a significant human concern. In recent years, numerous food poisoning outbreaks, involving various pathogens and food products, and the increasing concern over the preservation of minimally processed foods have spurred growing awareness of the importance in food safety (Marth, 1998).

The burgeoning rise in rampant urbanisation has put tremendous pressure on food supply. Food safety and preservation are important to ensure that food-borne organisms do not become the bane of immunocompromised or immunodeficient individuals.

Man has used Biotechnology to make bread, wine and cheese for thousands of years. The flavours and textures of these foods are partly due to the activity of microorganisms such as yeast, bacteria and fungi. Microorganisms naturally carry out a process called fermentation in which the nutrients on which the organism grows are transformed into other chemicals eg. alcohol often with the production of gas. Not all microorganisms are useful in food production and processing. Some microbial fermentation causes food spoilage or food poisoning by producing harmful toxins.
Sterilisation, pasteurisation and packaging techniques help to prevent food spoilage. In the past few years, manufacturers have marketed a variety of food products containing germ-fighting chemical additives. (Raloff, 1997).

Investigators are exploring new alternatives - battling 'bacteria with bacteria' (Raloff, 1997). Many of these microorganisms synthesise proteins or peptides that are degraded to relatively small hydrophobic or amphipathic bioactive peptides. These peptides exhibit antibiotic, fungicidal, antiviral, haemolytic and/or tumoricidal activities by interacting with membranes and forming transmembrane channels that allow the free flow of electrolytes, metabolites and water across the phospholipid bilayer (Klaenhammer, 1993).

Lactic acid bacteria are a group of bacteria that can preserve dairy foods by producing a number of organic compounds that are antagonistic to other microorganisms. These compounds i.e., lactate, acetate, hydrogen peroxide and proteinaceous bacteriocins create an environment within the dairy food that strongly inhibits the growth of fatal pathogens such as *Listeria monocytogenes* or *Clostridium botulinum* responsible for listeriosis and botulism. (Tagg and Wannamaker, 1976).

The more recent food-preservation methods being explored involve the addition of bacteriocins to foods. It is possible to use natural milk fermentates of bacteriocin producing lactic acid bacteria, create milk-based powders, like whey or non fat dry milk that can make normal food formulations with bacteriocins. These milk-based ingredients serve to deliver the bacteriocin to food systems without the need to purify and add a US-FDA regulated additive. It is also possible to directly add bacteriocin producing starter cultures to food where lactic acid bacteria are typically used in fermented foods.
However it is necessary to determine if the bacteriocin produced is compatible with the other cultures required in the product formulations.

Nisin was the first bacteriocin derived from fermentation of a lactic acid bacterium and was approved by the US- FDA in April 1989 to prevent the growth of *Clostridium botulinum* spores in pasteurised process cheese spreads. Nisin offers processors a "clean" label as well as extending refrigerated shelf life by 14-30 days depending on the product (Morris, 1991). While most bacteriocins are produced only during the exponential growth, nisin is produced in large amounts after cells reach their stationary phase making it appropriate for foods in which lactic acid bacteria are not expected to grow after processing (Casla et al., 1996). To date nisin remains the only purified bacteriocin approved for use in food products. (McAuliffe et al., 1997). Nisin is the only bacteriocin approved by the US- FDA as "GRAS" (Generally Regarded As Safe) for use as food preservative.

Though microbes have been impregnating the food supply with bacteriocins for millennia, the vast majority of these natural, nontoxic food preservatives functioned in obscurity until about a decade ago. Lately, food scientists have been scouting out these germ killers with the aim of enlisting their help. If this may be the case then a host of newfound biopreservatives may soon be blended into commercially prepared foods or incorporated into food packaging. Some bacteriocins have been already discovered in cured meats, and others were found in cheese and milk. Bacteriocins have even been isolated from fruits, spoiled salad dressing and soybean paste (Raloff, 1997). Virtually all bacteria make at least one bacteriocin and the fact that such antimicrobial proteins are not known, reflects how few people have looked at them (Klaenhammer, 1993).
Taking all these aspects into account, this thesis has tried to focus on the isolation, screening and characterisation of lactic acid bacteria isolated from various food sources producing novel antimicrobial agents with greater efficacy, stability (pH and temperature) and a broader spectrum of activity.

1.2 OBJECTIVE OF THE PRESENT STUDY

Food, the raison d'etre of survival, and we have been facing serious setbacks in the form of its contamination and spoilage, ultimately to the loss of life, as witnessed with the case of E.coli 0157:H7.

Bacterial antagonism, though an age old technique, is now showing very promising results through the use of lactic acid bacterial strains and its food preserving action attributed to potent bacteriocins.

In order to address this issue, this study aims to attain the following goals:

1. Screening of various food sources for bacteriocin producing lactic acid bacteria, and subsequent isolation and characterisation of a bacteriocin from a lactococcal strain.

2. Observations of response of various fermentation parameters on the production of bacteriocin and biomass of the lactic acid bacterial isolate.

3. The use of bacteriocins as a preservative in enhancing the shelf life of paneer, a type of cottage cheese, popular in India.
1.3 REVIEW OF LITERATURE

The single-most important development permitting the formation of civilization was the ability to produce and store large quantities of food. The early use of biological methods for food preservation was around 6000 B.C. and fermentation was used to produce beer, bread, wine, vinegar, yogurt, cheese and butter.

In 1864 Louis Pasteur proved that microorganisms in foods were the cause of food spoilage and that heat treatment of food killed these microbes. He also observed that sealed containers helped to preserve food by preventing recontamination from atmospheric air. A major development in the distribution and storage of foods came in 1940 with the availability of low cost home refrigerators and freezers. Other developments included the artificial drying of fruits, vegetables and liquids, vacuum packaging, use of ionizing radiation, and chemical preservatives (Ray and Daeschel, 1992). Many consumers today are concerned about the synthetic chemicals used as preservatives in food and there is a growing trend towards less chemically processed foods. Untreated foods can harbour dangerous pathogens that can multiply under refrigeration and without oxygen (psychotrophs). Treatments like ionizing radiation can destroy pathogen non-chemically but may affect taste and do not protect food against post treatment contamination. A solution to this dilemma is the use of antimicrobial metabolites of fermentative microorganisms. Recent technological surveys have indicated that there is a rapidly growing awareness of the potential of peptides in various foods processing fields, particularly as antimicrobials (Ray and Daeschel, 1992).
The roots of the search and application of newer antibiotics can be traced back to the beginning of the 1900's. The search continues for the 'magic bullet' as referred to by Ehrlich due to their selective toxicity. The history of antimicrobial peptides started in 1939 when Réné Dubos demonstrated that an unidentified soil *Bacillus* produced some antibacterial compounds that could prevent pneumococcal infection in mice.

Bacterial antagonism, though an age old technique is now showing promising results through the use of lactic acid bacterial strains. The preserving action of lactic acid bacteria was attributed to their production of organic acids and hydrogen peroxide until a potent bacteriocin, nisin, was isolated from *Lactococcus lactis* subspecies *lactis*. Bacteriocins are bacteriocidal peptides produced by microorganisms. While some biologists suspect bacteriocins to be communication molecules, others suggest that they are developed as toxins against competing bacteria (Raloff, 1997). Although not exactly defined, bacteriocins differ from classical antibiotics. They are a heterogenous group of bacterial antagonists showing considerable variance in molecular weight, biochemical properties, and range of sensitive hosts and mode of action. Klaenhammer redefined them as: “Bacteriocins are proteins or protein complexes with bacteriocidal activity directed against species closely related to the producer bacterium” (Klaenhammer, 1988). However, the definition stated elicits exception such as nisin and pediocin, which exhibit broad-spectrum activity.

1.3.1 Lactic acid bacteria

Historically, food preservation by lactic acid bacteria was an empirical process in which foods were observed to undergo a change that
resulted in foods with enhanced keeping quality. The first detailed characterisation of bacteriocinogenic activity of *Lactobacilli* was reported in 1961 (De Klerk and Cotzee, 1961).

The lactic acid bacteria are non-motile rods or spherically shaped, united by an unusual constellation of metabolic and nutritional properties. The name is derived from the fact that lactic acid is produced as the result of the oxidation of glucose to two molecules of pyruvate which in turn gets converted to lactate to synthesize ATP. Lactic acid bacteria are aerotolerant anaerobes that readily grow on the surface of solid media exposed to air. However, they are unable to synthesise ATP by respiratory means, a reflection of their failure to synthesize cytochromes and other heme containing enzymes. The growth yields of lactic acid bacteria are accordingly, largely unaffected by the presence or absence of air, the fermentative dissimilation of sugars being the source of ATP under both conditions.

One consequence of the failure to synthesize heme proteins is that the lactic acid bacteria are catalase negative and hence cannot mediate the decomposition of hydrogen peroxide to water and oxygen. The absence of catalase activity readily demonstrated by the absence of oxygen formation is one of the most useful diagnostic tests for the identification of lactic acid bacteria. Another distinct physiological feature of lactic acid bacteria is their high tolerance of acid. Growth of all lactic acid bacteria continues until the pH has fallen, through fermentation, to a value of \( \sim 4.5 \). The capacity of lactic acid bacteria to produce and tolerate a relatively high concentration of lactic acid is of great selective value, since it enables them to eliminate competition from most other bacteria in environments that are rich in nutrients (Stanier et al., 1995).
As lactic acid bacteria are fastidious microorganisms they require a complex medium to grow and produce bacteriocins (De Vuyst, 1995). Even when growing on very rich media, the colonies of lactic acid bacteria always remain relatively small (at the most, a few millimeters in diameter). (Stanier et al., 1995). It was S. Orla Jensen who showed in 1920 that the lactic acid bacteria could be subdivided into two subgroups- the homo- and heterofermentors wherein the former converts glucose almost quantitatively to lactic acid, while the latter converts the same to an equimolar mixture of lactic acid, ethanol and carbon dioxide. Homofermentors follow the Embden-Meyerhof pathway to yield two moles of ATP per mole of glucose. Heterofermentors on the other hand lack an enzyme, fructose bisphosphate aldolase that mediates sugar-phosphate cleavage and hence dissimulate glucose through the oxidative pathway yielding one mole ATP per mole of glucose. Lactic acid bacteria with spherical cells are placed in three genera- Pediococcus, Lactococcus, and Leuconostoc. The homofermentors are grouped under two sub genera- Thermobacterium and Lactobacterium, whereas betabacterium are heterofermentors as given in Table 1.1 (Stanier et al., 1995).

Antimicrobial activity of lactic acid bacteria isolated from food has been the subject of intensive research due to the potential application of these bacteria as protective cultures in biological preservation. (Tagg and Wannamaker, 1976).

The major groups of antimicrobials produced by lactic acid bacteria are (Lindgren, 1993):

♦ Diacetyl, which interacts with arginine binding proteins.
♦ CO₂, which lowers cell pH and prevents growth.
♦ H₂O₂, which oxidises basic proteins.
♦ Lactoperoxidase, which oxidises SH-Groups.
♦ Bacteriocins affect membranes, DNA synthesis and protein synthesis.
♦ Organic Acids: The undissociated acid penetrates membranes.

Table 1.1 Taxonomic division of lactic acid bacteria

<table>
<thead>
<tr>
<th>Genus</th>
<th>Cell shape and arrangement</th>
<th>Mode of glucose fermentation</th>
<th>Configuration of lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactococcus</td>
<td>Spheres and chains</td>
<td>Homofermentation</td>
<td>L</td>
</tr>
<tr>
<td>Leuconostoc</td>
<td>Spheres and chains</td>
<td>Heterofermentation</td>
<td>D</td>
</tr>
<tr>
<td>Pediococcus</td>
<td>Spheres and tetrads</td>
<td>Homofermentation</td>
<td>DL</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>Rods</td>
<td>Variable among strains</td>
<td>Variable among strains</td>
</tr>
</tbody>
</table>

1.3.2 Inhibitory compounds from lactic acid bacteria

1.3.2.1 Organic acids

Lactic acid is the major metabolite produced by lactic acid bacteria and depending on the substrate and microorganism it has been reported as having good, average or poor antimicrobial activities. The US- FDA regards both lactic and acetic acids to be of 'GRAS' status. Acetic acid and its salts assert their antimicrobial activity up to pH 4.5 and the effect is due to undissociated molecules. (Doores et al., 1983). Low pH affects every aspect of
cellular metabolism, with retardation of the growth of unwanted microbes in culture media. Undissociated lactic and acetic acids penetrate the cell membranes and disturb the transmembrane potential resulting in inhibition of substrate transport and membrane bound $F_0F_1$ ATPase activity.

1.3.2.2 Hydrogen peroxide

The *Lactobacilli* have the ability to generate hydrogen peroxide during growth by different mechanisms. Accumulation of hydrogen peroxide can occur due to the lack of catalase enzyme in lactic acid bacteria resulting in anaerobic conditions. The lethal effects of hydrogen peroxide (Kandler et al., 1986) could be due to the inactivation of essential biomolecules by the superoxide anion chain reaction or through the lactoperoxidase thiocyanate to release toxic oxidation products that are detrimental to food borne pathogens (Fernandes, et al., 1987). It is more effective as a sporicide than as a bacteriocide (Baldry, et al., 1983).

1.3.2.3 Carbon dioxide

Carbon dioxide may exert its antimicrobial effect in several ways such as by rendering the environment more anaerobic, by inhibiting enzymatic decarboxylation and by disrupting the cell membrane with the accumulation of the gaseous phase in the lipid bilayer (Eklund, 1984).

1.3.2.4 Diacetyl (2,3- butanedione)

Diacetyl (2,3- butanedione) is synthesized by certain species of lactic acid bacteria from pyruvate. Diacetyl is ineffective against yeast (Jay, 1982). It
interferes with arginine utilization by reacting with arginine binding proteins of Gram negative organisms. Diacetyl is used in the manufacture of butter, which gives it its distinct flavour.

1.3.2.5 Bacteriocins

Among the many different substances known to play a role in the bacterial interactions, bacteriocins are the most specific antagonists. In general, bacteriocins of Gram positive bacteria have evolved differently from Gram negative bacteria with respect to the size and specificity of cell wall architecture (Fig. 1.1).

Fig. 1.1  Structure of the cell wall of gram-positive and gram-negative bacteria
The Gram positive bacteria possess a multilayered peptidoglycan wall without an outer membrane. This favours the penetration of small peptides into the murein network without receptor binding and specific translocation. Consequently the activity spectra of these bacteriocins are generally wider than that of colicins, an antimicrobial agent produced by *E.coli.* (Sahl, 1994). In Gram-positive bacteria, production of antibiotic peptide is a rule. The term bacteriocins are used for bacterial peptide antibiotics that are ribosomally made or derived from gene encoded precursor peptides towards which the producing strain possesses a specific self-protection mechanism (immunity). The majority of them are relatively thermo stable and have molecular masses of 2-6 kDa. These bacteriocins are different from the classical peptide antibiotics that are made through enzymatic condensation of free amino acids (Montville and Kaiser, 1993).

Hundreds of lactic acid bacterial bacteriocins fall into Klaenhammer's bacteriocin classification scheme. The bacteriocins mentioned above (Table 1.2) fall under four main categories based on structural characteristics.

1. **Class I** - lantibiotic peptides contains unusual amino acids and lanthionine rings that are introduced by posttranslational modifications. eg. Nisin

2. **Class II** - they are small heat stable non-lanthionine containing peptides eg. Pediocin PA-1
   
   Class IIa is a subgroup classified on the basis of their strong amino acid sequence similarity particularly their distinctive N-terminal part and their strong anti-listerial activity.

   Class IIb is involved in poration complexes formed by oligomers of two different proteinaceous peptides.
Table 1.2 Bacteriocins produced by Gram positive lactic acid bacteria (Ruhr and Sahl, 1985)

<table>
<thead>
<tr>
<th>Name of organism</th>
<th>Bacteriocin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactococcus lactis</em> subsp <em>lactis</em></td>
<td>Nisin</td>
</tr>
<tr>
<td><em>Lactococcus bavaricus</em></td>
<td>Bavaricin</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp <em>lactis</em></td>
<td>Lacticin</td>
</tr>
<tr>
<td>DPC 3147</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus plantarum</em> C11</td>
<td>Plantaricin</td>
</tr>
<tr>
<td><em>Lactobacillus brevis</em> V286</td>
<td>Brevicin286</td>
</tr>
<tr>
<td><em>Lactococcus helveticus</em></td>
<td>Helvicin</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp <em>cremoris</em></td>
<td>LactococcinA</td>
</tr>
<tr>
<td>LMG213</td>
<td></td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> subsp <em>lactis</em></td>
<td>NisinZ</td>
</tr>
<tr>
<td>lacticVFE1500</td>
<td></td>
</tr>
<tr>
<td><em>Lactobacillus curvatis</em></td>
<td>Curvacin</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> P13</td>
<td>Enterocin</td>
</tr>
</tbody>
</table>
The existence of a third subgroup Class IIc that has been suggested to contain thiol activated bacteriocins is uncertain.

3. Class III - These are large heat stable proteins.

4. Class IV - These are large heat labile and complex proteins that require a lipid or carbohydrate moiety for activity (Chen and Montville, 1997).

1.4 BACTERIOCINS

1.4.1 Class I (Lantibiotics)

The designation "lantibiotics" was proposed with reference to the most prominent features of these peptides, the antibiotic activity and the content of the thioether-forming amino acid lanthionine (Schnell et al., 1988). Lantibiotics belong to a small peptide group of bacteriocins although they have a broader activity spectrum than most of the bacteriocins and in that respect are more similar to antibiotics. Additionally, they differ from other types of bacteriocins because of their chemical structure. They contain unusual amino acids such as dehydroalanine, dehydrobutyryl, lanthionine and \( \beta \)-methyl lanthionine (Fig. 1.2). Individual lantibiotics may contain further unusual residues such as S-aminovinyl-cysteine or lysinoalanine. On the basis of structural features lantibiotics were distinguished as type A (e.g. nisin, subtilin, epidermin and pep5) and type B (cinnamycin, duramycin and ancovenin) lantibiotics with mersacidin and actagardine sharing properties of both groups (Jung, 1991).
Fig. 1.2 Structure of Nisin (Fowler and Gasson, 1991)

Aba - Amino butyric acid, Dha - Dehydroalanine, Ala-S-Ala - Lanthionine, Dhb - Dehydrobutyryl, Aba-S-Aba - β-Methyl Lanthionine
The lanthionine and β-methyl lanthionine residues close the single sulphur rings that are characteristic of nisin and other structurally related lantibiotics. This chemical structure depicted in Fig. 1.2 was originally determined by Gross and Morrel (1971) using classical amino acid sequencing in conjunction with cyanogen bromide cleavage of the molecule. More recently, conformation of the structure was achieved by the use of two-dimensional nuclear magnetic resonance (NMR) of nisin in aqueous solution. (Chan et al., 1989a). Attempts have been made to translate NMR derived data into a three-dimensional structure for the nisin molecule. Whilst the internal mobility of the peptide chain limited precise determination of the overall folding of the molecule, some features have been postulated. It was concluded that the amino and carboxy-terminal regions of the nisin molecule were quite flexible and the rest of the molecule consisted of an amphiphilic amino- (N-) terminal fragment composed of residues 3 to 19 joined by a flexible 'hinge' region to residues 23 to 28, which had a more rigid structure. In another study, Lian et al., 1992 calculated structures for several parts of the nisin molecule and concluded that nisin in aqueous solution was a very flexible molecule with the only defined conformational features being those imposed by the presence of the lanthionine residues. Structures were obtained for rings A, B and D/E of the nisin molecule which consist of residues 3 to 7, 8 to 11 and 23 to 28 respectively. The smallest ring B was relatively well defined; ring A was more flexible and the largest ring C exhibited a great deal of conformational flexibility.

The biosynthesis of nisin is known to involve a precursor molecule that is produced by the expression of a prenisin gene. Prenisin (Fig.1.3) contains only conventional amino acids and this molecule is converted to mature nisin by post-translational modifications. Whilst the enzymes involved in maturation of prenisin have yet to be characterised, the activities can be
Met-Ser-Thr-Lys-Asp-Phen-Asn-Leu-Asp
  |
  Leu
  |
  Val
  |
Ala-Gly-Ser-Asp-Lys-Lys-Ser-Val-Ser
  |
  Ser
  |
Pro  Ser
  |
  Arg  Ile  Leu
  |
  Ala-Thr  Cys-Lys-Thr  Gly-Cys-Asn
  |
  Ile-Thr-Ala  Pro  Gly
  |
  His-Ala
  |
Lys-Ser-Val-His-Ile-Ser-Cys  Thr-Ala

Fig. 1.3 Structure of Prenisin (Fowler and Gasson, 1991)
predicted from the structures of nisin and prenisin. The cloning and sequencing of the gene for prenisin *nisA* have confirmed the predicted amino acid sequence of the nisin precursor. The dehydroalanine and dehydrobutyrine residues of mature nisin result from the dehydration of serine and threonine residues, respectively (Fig. 1.4.).

Another aspect of nisin production is its secretion from the cell. The deoxyribonucleic acid (DNA) sequence of the prenisin gene reveals a 23-amino acid extension at the N-terminus. This region is cleaved off before mature nisin is available outside of the cell. The extra sequence of amino acids could act as a secretory leader, being involved in the export of nisin and removed during the secretory process. Another possibility is that the N-terminal extension plays a role in controlling the maturation process perhaps conferring a conformation that facilitates the specificity of ring formation. Interestingly, the DNA sequence analysis of the nisin biosynthesis gene has revealed an open reading frame, *nisT*, that encodes a protein having homology with a translocation protein that is involved in the secretion of hemolysin (Engelke et al., 1992). This suggests that a classical leader may not be involved in the secretion process. This is supported by the observation that hydrophobicity plots of the leader are atypical for a secreted protein (Buchman et al. 1988).

Whilst the primary structure of nisin A is well established, it is equally well known that nisin preparations contain a variety of distinct molecular structures. As early as 1952, Berridge et al., reported that two distinct and biologically active components of nisin could be separated by countercurrent distribution. The sequencing of nisin biosynthesis genes confirms that the established structure for nisin is the only molecular form encoded and this molecule is now named nisin A. Chan et al (1989b) recently
Fig. 1.4 Condensation reaction for the conversion of prenisin to mature nisin (Fowler and Gasson, 1991)

DHA - Dehydroalanine; DHB - Dehydrobutyryne
used high performance liquid chromatography (HPLC) purification to isolate two breakdown products of nisin and with the aid of $^1$H NMR they were able to determine their chemical structures. In one derivative the two carboxy- (C-) terminal amino acids were absent and in the other derivative this change was present together with an opening of the first lanthionine ring at residue 5. Both changes are caused by the loss of the labile dehydroalanine residue. Interestingly, the shortening of nisin A to a 32 amino acid molecule had no effect on the biological activity whereas the opening of the first lanthionine ring inactivated the nisin molecule. Whilst there are undoubtedly further variants of nisin yet to be defined, it is clear that a combination of degradation of mature nisin and incomplete modification reactions during the maturation of pre nisin could account for a considerable number of components in commercial nisin preparations.

A different type of nisin variant has recently been described in which a change to the primary amino acid sequence is present.(Graeffe et al., 1991, Muelders et al., 1991; De Vos et al., 1993 ). Nisin Z is produced by several strains of *Lactococcus lactis* subspecies *lactis* and has asparagine in place of histidine at residue 27. Nisin Z exhibits faster diffusion than nisin A and this probably reflects its different amino acid composition. DNA sequence analysis of the respective pre nisin genes revealed that a simple base substitution was responsible for the change in structure, strongly suggesting that nisin Z arose by spontaneous mutation.

### 1.4.2. Class II Bacteriocins

Class II bacteriocins (pediocin PA-1, enterocin A, curvacin A and sakacin P) are not posttranslationally modified. They contain between 30 to 60
residues and are usually positively charged at a neutral pH. Studies of a large number of class II bacteriocins have led to subgrouping of these compounds (Klaenhammer, 1993; Nes et al., 1996). One of the subgroups, class Ila, contains bacteriocins that are characterised by the presence of YGNG and CXXXXCXV sequence motifs in their N-terminal halves as well as their strong inhibitory effect on *Listeria monocytogenes*, a pathogenic gram positive microorganism (Aymerich et al., 1996). Because of their effectiveness against the food pathogen *Listeria*, class Ila bacteriocins have the potential as antimicrobial agents. Class Ila bacteriocins act by permeabilising the membrane of their target cells. The most recent studies on the mode of action of these bacteriocins indicate that antimicrobial activity does not require a specific receptor and is enhanced by a membrane potential (Chen et al., 1997). Little is known about the bacteriocin structure and unraveling the relationships between structure and function is one of the great challenges in current bacteriocin research.

### 1.5 BIOSYNTHESIS OF BACTERIOCINS FROM LACTIC ACID BACTERIA

Bacteriocins are synthesised as pre-propeptides, which are processed and externalised by dedicated transport machinery. Cleavage of leader peptides is carried out by specific peptides or by a proteolytic domain of the dedicated ABC transporter (Nes et al., 1996), which recognize highly conserved sequences in the leader peptides. In addition, the synthesis of lantibiotics, like nisin, requires posttranslational modification of selected amino acids residues prior to secretion (De Vuyst and Vandamme, 1994). In addition to structural and secretion-modification machinery genes, bacteriocin operon always includes genes for specific immunity proteins (Jack et al., 1995; Nes et al.,
which protect the producer cells from their own bacteriocins. However little is known on their mode of action. The genes nis I and nis FEG have been implicated in nisin immunity. Nis I is a 32kDa protein which is postulated to be lipid modified and extracellularly anchored to the membrane (Kuipers et al., 1993). nis F and nis E encode for an ABC transporter which shows homology with proteins implicated in resistance to subtilin and microcin B17, while predicted product of nis G is a hydrophobic protein which may interact directly with pore-forming domain of nisin in a way similar to the immunity proteins of colicins (Siegers and Entian, 1995). Immunity proteins of class II bacteriocins are usually small (51-150 amino acids) and show a low degree of homology, even when the bacteriocins are closely related or identical. This may suggest that they do not interact directly with the bacteriocins (Nes et al., 1996).

Many bacteriocin operons are regulated by a quorum sensing system (Nes et al., 1996; Kleerebezem et al., 1997). The extracellular accumulation of an induction factor (IF) is sensed by a two component signal transduction system consisting of a membrane-located histidine kinase (HK) which phosphorylates a response regulator (RR), which in turn interact with promoters of structural, biosynthetic and regulatory operons and induces gene expression. The lantibiotic nisin autoregulates its own production (Kuipers et al., 1995; De Ruyter et al., 1996a; Quiao et al., 1996).

In many class II bacteriocins, the IF, HK and RR are organized in an autoinducible regulatory operon (Nes et al., 1996) and the IF is a bacteriocin-like peptide which may have no inhibitory activity.

Induction is not the only factor affecting the expression, carbon source regulation and the amount of cell-adhered nisin have been shown to
effect nisin synthesis (De Vuyst and Vandamme, 1992; Meghrous et al., 1992) and catabolite repression has been claimed to operate in the regulation of plantaricin C production (Barcena et al., 1998). There are also a few reports of induction of bacteriocin production caused by cells and extracts of sensitive strains (Barefoot et al., 1994; Sip et al., 1998).

1.6 STRUCTURE-FUNCTION RELATIONSHIPS OF ANTIMICROBIAL PEPTIDES

Most antimicrobial peptides are highly cationic and hydrophobic. It is widely believed that they act through nonspecific binding to biological membranes, even though the exact nature of these interactions is presently unclear. High-resolution nuclear magnetic resonance (NMR) has contributed greatly to knowledge in this field, providing insight about peptide structure in aqueous solution, in organic cosolvents, and in micellar systems. Solid state NMR can provide additional information about peptide membrane binding. Despite the three-dimensional structural motifs of the various classes, they all have amphiphilic surfaces that are well suited for membrane binding. Many antimicrobial peptides bind in a membrane-parallel orientation, interacting only with one face of the bilayer. This may be sufficient for antimicrobial action. At higher concentration, peptides and phospholipids translocate to form multimeric transmembrane channels that seem to contribute to the peptides hemolytic activity. An understanding of the key features of the secondary and tertiary structures of the antimicrobial peptides and their effects on bactericidal and hemolytic activity can aid the rational design of improved analogue.
1.7 HISTORY OF NISIN

The initial discovery of nisin in the 1920s prompted evaluation of its potential use as a therapeutic agent in the areas of medical and veterinary science. Due to its rapid breakdown by digestive enzymes, instability at physiological pH (pH 7.4), and its restricted antibacterial spectrum, it was found to be unsuitable for such purposes (Hurst, 1983).

It was not until after the Second World War that the use of nisin as a food preservative was first investigated. The initial interest was its use in natural cheese as a means of preventing clostridial gas formation (Hirsch et al; 1951). A major problem was the inhibition of the cheese starter culture by nisin. In an attempt to overcome this difficulty, Lipinska (1977) 'trained' starter cultures to grow in the presence of nisin. Encouraging results were obtained but interest subsequently diminished, presumably because of the complex problem of 'training' sufficient numbers of cultures and problems of inhibiting natural ripening organisms (Fowler and Gasson, 1991). However, new developments in nisin gene technology whereby nisin production and resistance can be transferred to starter culture has led to a revival of interest in the potential use of nisin in natural cheese production (Eckner, 1991). The first use of nisin as a means of preventing clostridial spoilage of processed cheese was investigated by Mc Clintock et al., (1952). Aplin and Barrett Ltd were, at that stage in the Company's history, involved in the manufacture of processed cheese spreads. Due to the poorer quality of raw cheese available in the immediate post-war years and in early 1950's, the use of nisin as a means of controlling unacceptable spoilage in processed cheese products were investigated. Early but crude preparations of nisin were so successful that the company decided to
develop its potential further. Aplin and Barrett developed a commercial nisin concentrate known as 'Nisaplin', which possessed a high and consistent activity. Since the identification of nisin as an effective preservative in pasteurized cheese spreads, various other applications have been developed.

Nisin is produced by *Lactococcus lactis* subsp. *lactis* of dairy origin and is the only bacteriocin that is accepted as a food preservative.

1.8 CLONING AND ANALYSIS OF NISIN BIOSYNTHESIS GENES

Isolation of the genes responsible for the nisin biosynthesis was achieved by targeting the predicted gene for prenisin. The structure of prenisin could be inferred from the maturation reactions that would be needed to convert a primary translation product into mature nisin. Several groups designed gene probes on the basis of a predicted gene for prenisin and used the probe to isolate the gene, named *nis* A, from libraries of *Lactococcus lactis* subspecies *lactis* DNA (Buchman et al., 1988; Kaletta and Entian, 1989; Dodd et al., 1990). Sequence analysis confirmed the predicted structure of prenisin and subsequent sequencing of flanking DNA has revealed further details of the genes involved in the biosynthesis of mature nisin. The *nis A* gene encoding prenisin is the first gene in an operon that consists of six open reading frames. Whilst the function of these additional genes remains to be fully determined some clues have been provided by searches in DNA homology with gene sequences and data banks. The arrangement of the nisin biosynthesis gene is shown in Fig. 1.5 below.
The fact that nisin biosynthesis genes can be transferred to strains not able to produce nisin raises interesting possibilities for the construction of improved lactic starter cultures. The exploitation of nisin production during dairy fermentations has been explored but not as yet with success. One problem is the fact that starter cultures often consists of a mixture of strains and those not producing nisin would be inhibited by it. The advances in molecular genetic understanding of nisin biosynthesis raises the possibility of more sophisticated strategies to exploit this property. For example, it may probe possible to introduce genes for nisin immunity into a wide range of starter cultures, allowing their growth in combination with nisin-producing strains.

1.9 MODE OF ACTION

All bacteriocins appear to work by pore formation (Fig. 1.6). The pores were assumed to be cylindrical and based on an estimate that the channel diameter is at least one nanometer. For nisin, it was reported that it attaches itself to the plasma membrane of target cells thereby altering the membrane permeability and decreasing the proton motive force. The operon that codes for lantibiotics also carries a gene for resistance to the lantibiotic so that the cell
Fig. 1.6 Mode of action of nisin. During peptidoglycan synthesis the cell wall precursor lipid II is transferred to the outside of the membrane and becomes accessible for the lantibiotics. Nisin binds to the cell wall precursor and primarily uses it as a docking molecule for pore formation (André Guder et al, 2000).
producing the peptide is immune to the bacteriocin produced by it. The cytoplasmic membrane is the principle target since nisin treatment results in rapid, non-specific efflux of amino acids, cations, ATP and the rupture of the cell membrane with the ultimate effect being cell death. (Ruhr and Sahl, 1985). The phospholipid composition may be influential in the effectiveness of nisin (Gao et al., 1991).

The bacteriostatic or bactericidal effects of nisin are concentration dependent for both levels of nisin and the level of susceptible bacterial vegetative cells or spores. (Fowler and Gasson, 1991). Numerous studies have been carried out regarding the mode of action of nisin against susceptible vegetative cells. Fewer studies have been carried out on action against bacterial spores. The primary site of action of nisin against vegetative cells is considered to be the cytoplasmic membrane with nisin acting as a membrane-depolarizing agent in a voltage-dependent fashion (Ruhr and Sahl, 1985; Henning et al., 1986a; Jung, 1991). Henning et al., (1986a) showed that the antimicrobial effect of nisin is caused by an interaction of nisin with the phospholipid component of the cytoplasmic membrane. They demonstrated that various phospholipid and isolated cytoplasmic membranes were the sites of the inhibitory effect of nisin, that nisin caused lysis of synthetic phospholipid liposomes, and that nisin will combine with phospholipids to form a nisin-phospholipid complex. Nisin has also been shown to inhibit the synthesis of a cell wall component, murein (peptidoglycan) (Reisinger et al., 1980; Henning et al., 1986a). However, the latter workers found that microorganisms without murein in their cell walls were also sensitive to nisin, and thus it is considered that inhibition of murein synthesis is not a primary mode of action.
Cells of intact Gram-negative bacteria are generally resistant to the action of nisin. However, disruption of the Gram-negative cell wall readily made *Escherichia coli* susceptible to nisin (Kordel and Sahl, 1986). Increased permeability of the Gram negative cell wall can be achieved by the application of chelating agents, which removed magnesium ions that stabilize the lipopolysaccharide layer of the outer membrane. Using nisin in combination with the chelating agent, disodium ethylenediaminetetra-acetic acid (EDTA), achieved significant kill of *Salmonella* and other Gram-negative species of bacteria. Cells treated with EDTA or nisin alone were not killed. Kill was significantly reduced at low temperatures. The use of nisin with EDTA to allow action against Gram-negative bacteria has been patented (Blackburn *et al.*, 1989). Sub lethal injury caused by heat, freezing and exposure to organic acids can also increase the permeability of Gram-negative cell wall, making them sensitive to nisin (Kalchayanand *et al.*, 1992).

The primary action of nisin is that of disruption of the cytoplasmic membrane of susceptible Gram positive bacteria causing the efflux of small cytoplasmic compounds such as adenosine triphosphate (ATP), potassium ions and amino acids (Waites and Ogden, 1987, Bruno *et al.*, 1992, Okereke and Montville, 1992). Benz *et al.*, (1991) working with artificial black lipid membranes and using several different lipids indicated that a trans membrane voltage of above 50mV was required to induce ion permeability. It was observed that membrane conductance only occurred when the side of the membrane opposite to which nisin was applied was negative. A similar observation was made with the lantibiotic Pep 5, but with the lantibiotics subtilin, gallidermin and epidermin increase in membrane conductance occurred irrespective of which side of the membrane the voltage was applied. Dutch researchers (Abbe *et al.*, 1991, Gao *et al.*, 1991), working with
cytochrome C oxidase containing proteoliposomes prepared from *E. coli* phosphotidylethanolamine and egg phosphotidycholine, showed that nisin dissipated both the membrane potential and the pH gradient and that an increase in membrane potential (negative inside) and pH gradient (alkaline inside) across a membrane enhance the activity of nisin. They also found that the activity of nisin was influenced by the phospholipid composition of the liposomal membrane.

Sahl (1991) showed that efflux and membrane depolarization of sensitive cells exposed to nisin were much faster with energized cells (ie. cells either in the logarithmic growth phase or energized with glucose) than with starved cells. Their work indicated also that nisin and similar lantibiotic form potential dependent pores in the cytoplasmic membrane of sensitive cells, resulting in loss of ions and metabolites and eventually cell death. Concurrent studies by Benz et al., 1991, working with artificial black lipid membranes, demonstrated the formation of pores in the membrane with highly variable single-charge conductance.

The action of nisin against spores is predominantly bacteriostatic rather than bactericidal. This has important implications in its application, as a food preservative as it means that sufficient residual nisin must be maintained to provide a continued effect on any spores present. Spores damaged by heat become more sensitive to nisin (Hall, 1966). Nisin action against spores at the molecular level has been studied by Morris et al., (1984). They showed that binding of the nisin with sulphydryl groups on protein residues caused the nisin action against spores. Phospholipids were not implicated.
Despite the extensive analysis outlined above the precise biomolecular mode of action of nisin remains unclear. Important clues might be obtained if it could be determined how producer cells make themselves immune from attack by nisin. Resistance could be due to a change in phospholipid composition or arrangement in the producer cell's cytoplasmic membrane resulting in a different charge pattern compared with nisin sensitive cells. This has been advocated for gramicidin resistance in gramicidin producing *Bacillus brevis* (Vostuknutova et al., 1981). Jung (1991) puts forward an elegant theory based on the leader peptide from which the active nisin is cleaved. He proposes that the protecting effect could be provided by the leader peptide, in the vicinity of the cell membrane, so that the nisin molecule cannot exert its action on the cytoplasmic membrane of the producer organism. Interestingly mutants in which the prenisin gene *nisA* is inactive exhibit increased sensitivity to nisin. The rapidly advancing analysis of genes involved in nisin biosynthesis will also reveal the genetic basis of immunity that in turn may aid elucidation of its biochemical mechanism.

All bacteriocins deplete the electric charge stored across their susceptible membrane. Energy-driven processes in bacterial cells can be powered by this electric charge or by an energy-storing chemical known as ATP. "There's a kind of currency exchange between the two", says Montville of Rutgers University. As the bacteriocin runs down the electric charge, its embattled victim begins breaking down ATP to produce new protons and recharge the membrane. This creates a futile cycle, because eventually the cell has no more ATP to maintain its other activities and dies. Since the electrochemistry of an environment can affect the extent to which bacteriocins can form pores that allow charged molecules to leak from a cell and deplete its stored electric charge, it has now become clear why salt and acid concentrations...
affect a bacteriocin's cell-killing prowess, ie. the activity can plummet when added to salty foods and sky rocket on incorporation into acidic ones.

1.10 NISIN BIOASSAY

Among the several nisin bioassays devised, Horizontal Agar Diffusion coined by Tramer and Fowler, 1964, is the most widely used with Micrococcus flavus / Micrococcus luteus as the indicator organism. This method is effective within a range of 0.025-0.5 µg/ml. An improved agar diffusion assay using Lactobacillus sake has been developed. An interesting rapid method devised by Waites and Ogden (1987) , measures bioluminescence of the ATP released from Lactobacillus caseii following exposure to nisin. Most of the methods developed for quantification are based on its inhibitory activity to a test organism. The agar diffusion bioassay is still the most widely used. Several parameters affect the accuracy and sensitivity of this method. In addition, equal concentrations of nisin A and Z produce inhibition zones of different sizes due to the better diffusion properties of nisin Z. Immunological detection methods have also been developed. The sandwich-type enzyme-linked immunosorbant assay for nisin A, based on sheep polyclonal antibodies and developed by Falahee et al., (1990) has a detection limit of 0.5ng/ml for pure nisin and 0.2 µg/ml for nisin analysed from spiked cheese. Other methods for immunodetection of nisin involving competitive direct enzyme-linked immunosorbent assays with polyclonal and monoclonal antibodies from mice have been developed. These assays showed a detection limit of 5 to 10 ng/ml, respectively. All the immunoassays described are considerably more sensitive than the agar diffusion assay. Still, methods based on antibodies against nisin are not totally reliable due to possible cross-reactions with related compounds. Wahlström et al., in 1999 developed a better, faster and more sensitive method
of quantification. This method is based on a *Lactococcus lactis* subsp. *lactis* strain that can sense the bacteriocin nisin and transduce the signal into bioluminescence. By using this strain, a bioassay based on bioluminescence was developed for quantification of nisin for detection of nisin in milk, and for identification of nisin-producing strains. As little as 0.0125 ng of nisin per ml was detected within 3 hours by this bioluminescence assay.

### 1.11 KINETICS OF NISIN AND OTHER BACTERIOCIN PRODUCTION

Bacteriocin production in lactic acid bacteria is usually growth-associated, and occurs throughout the growth phase and ceases at the end of the exponential phase or sometimes before the end of growth (Parente et al., 1997; Lejeune et al., 1998). A decrease of bacteriocin titre usually follows. This may be attributed to adsorption on producer cells or degradation by specific or non-specific proteases. (Meghrous et al., 1992; Parente et al., 1994; Parente and Ricciardi, 1994a; De Vuyst et al., 1996; Lejeune et al., 1998, Chinachoti et al., 1997d). Since adsorption of bacteriocin to cells is maximal at pH 5.5-6.5 (Yang et al., 1992) and decreases at low pH, it is not surprising that no reduction of bacteriocin titre is sometimes observed in fermentations without pH control (De Vuyst and Vandamme, 1992).

There have been only a few attempts to model bacteriocin production. Parente and Ricciardi (1994a) and Parente et al., (1994) modeled separately the production and adsorption/degradation of enteriocin 1146 and lactococcin 140 using the following equations:
\[ \frac{dB}{dt} = Y_{B/X} \times \frac{dX}{dt} \quad \text{if } t < t' \]
\[ \frac{dB}{dt} = -k_D \times B \times X \quad \text{if } t > t' \]

where \( X \) is biomass concentration; \( B \) is bacteriocin titre; \( Y_{B/X} \) is the of bacteriocin per unit biomass produced; \( t' \) is the time at which bacteriocin production is maximum and \( k_D \) the specific bacteriocin degradation or adsorption (to the producer cells) rate. To model enterocin 1146 in batch and continuous fermentations (Parente et al., 1997), Equation (1) and (2) were combined and bacteriocin degradation was assumed to be a function of cell concentration only:

\[ \frac{dB}{dt} = Y_{B/X} \times \frac{dX}{dt} - k_0 \times X \]

Lejeune et al., (1998) modeled bacteriocin production by \textit{Lactobacillus amylovorus} DCE471 in batch cultures using a different approach:

\[ \frac{dB}{dt} = k_B \times \frac{dX}{dt} \quad \text{if } X < X' \]
\[ \frac{dB}{dt} = -k' \times B \quad \text{if } X > X' \text{ or } S = 0 \]

where \( S \) substrate concentration; \( k_B \) \((=Y_{B/X})\) is defined as the specific bacteriocin production rate; \( X' \) is the cell dry mass level at which the bacteriocin production ceases; \( k' \) is the specific bacteriocin degradation (or adsorption) rate. \( k' \) was found to be constant over a wide range of conditions while both \( X' \) and \( k_B \) significantly.

The models described by equation (1.1)-(1.5) are empirical and do not take induction into account, but this may have no consequence under practical conditions. In fact, even if the expression of \textit{nis} operons is tightly
regulated and no transcription is obtained in the absence of nisin, the level needed for full induction in *Lactococcus lactis* is very low (0.50 μg/l = 0.02 IU/ml; (De Ruyter et al., 1996b). In *Carnobacterium piscicola* LV17 the use of very low inocula (< 10^4 cfu/ml) or removal of the IF is necessary to suppress bacteriocin production (Saucier et al., 1995; Quadri et al., 1997). Basal production of the IF occurs even under these conditions and is sufficient to restore the bacteriocin production after subculturing (Quadri et al., 1997).

Even if bacteriocin production is growth associated, because of the complex interaction of many factors that affect bacteriocin production and/or adsorption, maximisation of growth does not necessarily result in maximisation of bacteriocin production. Although an approximately linear relationship was indeed obtained between bacteriocin production rate and growth rate for "some bacteriocins" in both batch and continuous fermentations (Kaiser and Montville, 1993; Parente et al., 1994; Parente and Riciardi, 1994a; de Vuyst et al., 1996; Bhugaloo-Vial et al., 1997) as predicted by equations (1)-(4), strongly nonlinear relationship has been observed for nisin in both batch (Kim et al., 1997) and continuous culture (Meghrous et al., 1992) and for plantaricin C in continuous culture (Barcena et al., 1998). Catabolite repression of bacteriocin production has been used to explain the decrease of plantaricin C production rate with increase in specific growth rates (Barcena et al., 1998). The nonlinear relationship between nisin production and growth is probably a result of the complexity of nisin biosynthesis and regulation compared to class II bacteriocins. Very contradictory statements have been observed and this may be due the difference in nature of each bacteriocin. DeVuyst and Vandamme (1992) observed an increase of nisin synthesis late in the exponential phase of batch fermentations. Since *nis* A was expressed from the beginning of the exponential phase, the late increase in nisin production was attributed to carbon...
source regulation of the expression and/or activity of prenisin-modifying genes. Kim et al., (1997) observed that, in a prt-strain in which TnNip transposon was introduced, nisin A production was growth associated but ceased when the specific growth rate was lower than 0.05 h\(^{-1}\). Nisin production was apparently a burden for the producing strain. Insertion of TnNip resulted in decreased maximum biomass and specific growth rate compared to the parent strain. However, the inhibition of growth can be relieved by increasing the nisin resistance through the introduction of nisin resistant plasmids (Kim et al., 1998).

### 1.12 FACTORS AFFECTING BACTERIOCIN PRODUCTION IN LACTIC ACID BACTERIA

#### 1.12.1 Microbial strain

A given bacteriocin can be produced by several strains or species (Bhunia et al., 1994; Jack et al., 1995; Rodriguez et al., 1995). De Vuyst (1994) screened 21 nisin-producing and 6 non-producing strains of *Lactococcus lactis* for nisin production and immunity. Nisin titres varied from 1 to 1886 IU/ml and did not correlate with the number of copies of the *nis* genes nor with their transcription or translation levels. Differences among strains were attributed to expression level and activity of maturing enzymes and, to a lesser extent, to nisin immunity. On the other hand, Quiao et al (1997) showed that nisin resistance may dramatically affect nisin production, in fact, specific nisin production of strain LAC 48, a spontaneous mutant of *Lactococcus lactis* N8, was 10 times higher than the parental strain even if expression of *nis* genes was similar in both strains. Increase in nisin resistance by introducing plasmids containing nisin immunity determinants has been recently shown to result in increased nisin titre. The final aspect in this study was the commercial
application of the novel bacteriocin in food products such as cottage cheese, faster growth and increased nisin productivity compared to the parent strains (Kim et al., 1998). A "ceiling" for bacteriocin production has been observed for nisin (Kim et al., 1997) and amylovorin L471 (de Vuyst et al., 1996; Lejeune et al., 1998). In an elegant study, Kim et al., (1997) demonstrated that the "ceiling" for nisin production is affected by both nutrient availability and nisin inhibition.

### 1.12.2 Effect of Media

Bacteriocin production is deeply affected by the type and level of the carbon, nitrogen and phosphate sources, cations, surfactants and inhibitors. Bacteriocins can be produced from media containing carbohydrate sources. Nisin Z can be produced from glucose, sucrose and xylose by *Lactococcus lactis* IO-1 (Matsusaki et al., 1996; Chinachoti et al., 1997b,d) but better results were obtained with glucose (4000 IU/ml) compared to xylose (3000 IU/ml). Glucose followed by sucrose, xylose and galactose were the best carbon sources for the production of pediocin AcH in an unbuffered medium (Biswa et al., 1991). Sucrose was found to be a better carbon source than glucose for enterocin 1146 production; fructose or lactose result in comparable levels of biomass but low levels of bacteriocin (Parente and Riccicardi, 1994b). Catabolite repression has been used to explain the production of plantaricin C at higher dilution rates (0.1-0.12/h) with sucrose and fructose compared to glucose (0.055/h) in continuous cultures (Barcena et al., 1998).

Both $Y_{B/X}$ and final bacteriocin concentration are affected by the initial carbohydrate concentration in batch fermentations at controlled pH. Using *Lactococcus lactis* subspcies *lactis* NIZO 22186 in sucrose media (de
Vuyst and Vandamme, 1992), maximum nisin titers were obtained with 30g/l sucrose. Even if more biomass was produced, $Y_{B/X}$ decreased from 19.1 -10.9 mg/g as sucrose concentration increased from 10- 40g/l. This was explained by carbon source regulation of the synthesis or activity of prenisin-modifying enzymes. Substrate and product inhibition of growth resulted in reduced enterocin 1146 production rate at high $S_o$ (Parente et al., 1997). Amylovorin L471 activity did not increase when glucose was increased from 20-60 g/l, but bacteriocin degradation started sooner. Since lactic acid bacteria are nutritionally fastidious microorganisms, growth and bacteriocin production are often limited by organic nitrogen sources rather than by the carbon substrate. Kim et al (1997) found that nisin concentration increased with increasing organic nitrogen content. However, at any given specific growth rate the specific nisin production rate decreased with the increasing amount of nitrogen source. The type of nitrogen source also affects bacteriocin production. De Vuyst and Vandamme (1993) compared the effect of organic nitrogen sources (10g/l) on nisin production in a complex medium. Best results (2500 IU/ml, with $Y_{B/X}$ 64 mg/g) were obtained with cotton- seed meal but high nisin yields (>2000 IU/ml) were also obtained with yeast extract and fish meal. The differential effect of nitrogen sources was confirmed by factorial experiments for enterocin 1146 (Parente and Hill, 1992; Parente and Ricciardi, 1994b) and lactocin D (Parente and Hill, 1992).

Anions (phosphate) and cations (Mg $^{2+}$: Ca $^{2+}$) affect the bacteriocin production, but their effect may be strain specific. Inorganic phosphate improved the nisin production in *Lactococcus lactis* subspecies *lactis* NIZO 022186 (De Vuyst and Vandamme, 1993). Best results (3500 IU/ml) were obtained in batch fermentations at pH 6.8 with 50g/l $K_2HPO_4$. Mg $^{2+}$ has been shown to increase pediocin AcH production (Biswas et al., 1991), to improve
nisin production and significantly decrease cell adhered nisin in *Lactococcus lactis* subspecies *lactis* ATCC11454 (Meghrous et al., 1992) but it did not improve nisin production with strain IO-11 (Matsusaki et al., 1996). Adding 0.1 mol/l CaCl₂ resulted in increased nisin Z concentration and specific production rate but did not affect growth or lactate production from xylose and glucose in batch fermentations at controlled pH (Matsusaki et al., 1996; Chinachoti et al., 1997b). This was explained by Ca²⁺ activation of increased immunity of the leader peptide (*nis* P) (by protecting the integrity of the cytoplasmic membrane) or by nisin Z displacement from the cell surface.

Tween 80 has been found to stimulate the production of some bacteriocins (Parente and Hill, 1992; Daba et al., 1993; Matsusaki et al., 1996). Tween simply has the effect of preventing bacteriocin adsorption on polypropylene and glass surfaces (Joosten and Nunez, 1995) thus increasing the apparent bacteriocin titers. Adding ethanol (1%v/v) improved lactocin S (Mørtvedt-Abildgaard et al., 1995) and amylovorin L471 (De Vuyst et al., 1996) production. The stimulatory effect was attributed to influences on gene expression, prevention of bacteriocin aggregation and to increased \( \frac{Y_{b/X}}{X} \) under stress conditions.

### 1.12.3 Effect of fermentation conditions

The control of pH improves the growth of lactic acid bacteria, it also results in improved bacteriocin production. However, the optimal pH for bacteriocin production is usually 5.5-6.0 (Meghrous et al., 1992; Kaiser and Montville, 1993; Parente et al., 1994; Parente and Ricciardi, 1994a; Matsusaki et al., 1996; Chinachoti et al., 1997b), often lower then the optimal pH for growth. For enterocin 1146 (Parente and Ricciardi, 1994a) the optimal pH is a
result of higher $Y_{B/X}$ and lower $k_D$. A few bacteriocins are produced only at low pH (5.0) (Biswas et al., 1991; Mørtvedt-Abildgaard et al., 1995; Barcena et al., 1998). For pediocin AcH this was attributed to the low pH needed for post-translational processing of the bacteriocin. However, this effect may be strain or species depending since pediocin Ach is produced at pH 6.0 by *Lactobacillus plantarum* WHE2 (Ennahar et al., 1996). Optimal pH may also be affected by the culture medium: nisin Z production with strain IO-1 was optimal at pH 6.0 in xylose media (Chinachoti et al., 1997b) and at pH 5.5 in glucose media (Matsusaki et al., 1996).

Growth at optimal temperature results in optimal bacteriocin production (Meghrous et al., 1992; Daba et al., 1993; Matsusaki et al., 1996; Chinachoti et al., 1997b; Lejeune et al., 1998), but temperature, stress and growth at suboptimal temperature may result in an increase of $Y_{B/X}$ (Lejeune et al., 1998).

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

Agitation and aeration affect bacteriocin production. In fermentations at pH 5.5 using glucose media with *Lactococcus lactis* IO-1 maximum nisin Z concentration (3940 IU/ml) and $Y_{B/X}$ (68.5mg/g) were obtained at 320 rpm (Chinachoti et al., 1997d) and only a small decrease of nisin concentration and yield (3410 IU/ml, $Y_{B/X}$ 68.5mg/g) was obtained at 1000 rpm. On the other
hand agitation at > 540 rpm resulted in inhibition of growth and nisin production in xylose media. Aeration (10-40 ml/min) significantly reduced nisin production, perhaps because of chemical degradation. As observed for other stress factors, fermentation in the presence of oxygen resulted in an increased $Y_{B/X}$ for amylovorin L471 (De Vuyst et al., 1996), despite lower final bacteriocin titres.

Bacteriocin production is growth associated, hence bacteriocin production rates should improve in continuous fermentation where high growth rates can be maintained. As predicted by equation (4) a linear relationship is observed between dilution rate (D) and specific enterocin 1146 production rate (Parente et al., 1997) with $0.10 < D < 0.60/h$. A similar relationship can be calculated for bavaricin MN (Kaiser and Montville, 1993) and for divercin (Bhugaloo - Vial et al., 1997). On the other hand, plantaricin C production in continuous culture is obtained only at low dilution rates (Barcena et al., 1998). A more complex pattern was observed for nisin production in continuous culture with *Lactococcus lactis* subspecies lactis ATCC11454 (Meghrous et al., 1992). In both lactose limited and non-limited cultures maximum nisin concentration, yield and specific production rate were obtained at $D = 0.25/h$ and sharply decreased at higher and lower $D$ (Meghrous et al., 1992). Both the level of adsorbed nisin to the producer cells and the specific lactose uptake rate affected nisin production.

Increase in $D$ results in a decrease of biomass and bacteriocin concentration, a high substrate concentration in the effluent, and eventually wash out of the culture. Therefore, some authors have tested continuous fermentation with cell recycle or with immobilized cell. Continuous fermentation coupled with cell recycle with *Lactococcus lactis* subspecies lactis
IFO12007 led to an increase in nisin titre and volumetric nisin productivity compared to batch fermentations (Taniguchi et al., 1994). Decrease in nisin productivity at high substrate concentration in the feed was attributed to high lactic acid concentration that inhibited growth and nisin production. Continuous culture with cell recycle using a ceramic membrane can be used to produce nisin Z at high dilution rates (Chinachoti et al., 1997c) with a slight improvement in productivity compared to batch or continuous fermentation without cell recycle. Choice of the membrane type proved to be critical since a hollow fibre polyolefin membrane completely adsorbed nisin. Retention of bacteriocin with ceramic membranes was also observed by Bhugaloo-Vial et al (1997).

In repeated batch fermentations immobilized cells produced less bacteriocin than free cells perhaps because of diffusional limitations in alginate gels. However, in continuous fermentation at high dilution rate (3/h) the titre of bacteriocin was higher (brevicin) or equal to (nisin and pediocin PO2) those obtained in batch fermentations with significantly improved productivity. Using calcium alginate immobilized cells *Cyanobacterium divergens* LV41 in a plug-flow reactor operated at D =2/h resulted in a dramatic improvement of productivity compared to batch and continuous fermentation with free cells (Bhugaloo-Vial et al.,1997; Boyaval et al., 1998).

Removal of phosphate and addition of CaCl₂ is needed to maintain Calcium- alginate gel stability in repeated batch and continuous fermentations. Adsorptions of cells on a regenerable photo- crosslinked gel (ENTG-3800) resulted in nisin titres ( upto 3000 IU/ml) and yield (2.5× 10⁶ IU /g CDW) higher than from free cells in repeated batch fermentations, with excellent support stability ( Chinachoti et al., 1997a). In continuous fermentations
immobilization allowed the use of higher $D$ compared to free cell systems and, although nisin titre was lower than with free cell, nisin productivity increased up to $3.89 \times 10^6$ IU/l/h when $D$ was increased to 0.3/h.

1.13 RECOVERY AND PURIFICATION OF BACTERIOCINS

The cationic and hydrophobic nature of bacteriocins is used for their recovery from complex fermentation broth which contain high levels of peptides (10-30g/l compared to a bacteriocin concentration 10-100 mg/l). Laboratory purification protocols usually include an ammonium sulfate precipitation step followed by various combination of ion exchange and hydrophobic interaction chromatography with a final RP-HPLC purification step. Chromatography maybe replaced by preparative isoelectric focusing (Venema et al., 1997) and immunoaffinity chromatography which allowed a one step purification of nisin A. An immuno adsorption matrix was developed by direct binding of antinisinA monoclonal antibodies to N-hydroxysuccinimide-activated sepharose. The purification procedure was rapid and reproducible and rendered much higher final yields of nisin than any other described method (Suarez et al., 1997). Although these procedures may provide excellent results in terms of yield and purification, they are unsuitable for large-scale bacteriocin recovery and purification. Several protocols based on adsorption/desorption or on phase partitioning have been developed for large-scale purification and recovery of bacteriocins.

Bacteriocins can be recovered by adsorption on producer cells at pH 6.0-6.5 followed by cell separation and desorption at pH 2.0 and 0.1 mol/l NaCl. This method was very effective for pediocin AcH, nisin, sarkacin A and leuconocin Lcm1 (Yang et al., 1992) but recovery may be limited for other
strain/ bacteriocin combinations (Daba et al., 1994). Vortex flow filtration systems may replace centrifugation (Van't Hul and Gibbons, 1996) and may be more amenable to large- scale recovery of cells. However, when bacteriocin concentration is very high, the ability of cells to adsorb nisin can be exceeded and recovery of bacteriocin with the cell fraction may only be partial. Bacteriocins can be adsorbed on hydrophobic/hydrophilic interaction columns HIC and cation exchange resins. The lantibiotics nisin and carnocin UI49 have been purified nearly to homogeneity with a simple two step protocol based on adsorption on HIC and cation exchange resins (Stoffels et al., 1993). Recovery was higher than hundred percent (attributed to removal of interferences in the bioassay due to the purification) and purification 245 and 60-fold for carnocin and nisin respectively. Chinachoti et al., (1997c) found that Sep-PakC₈ cartridge were the best adsorbent for nisin Z. Sep-PakC₈ were used in an integrated fermentation system with a batch fermentation coupled to a microfiltration. The permeate was circulated through a cartridge and returned to the fermentor column. The growth of the producer strain was improved and a higher nisin productivity was obtained.

Ingestible porous silica compounds can also be used to adsorb bacteriocins (nisin, pediocin PO2, brevicin 286 and piscicolin 126) from fermentation broth (Wan et al., 1996). Best results were obtained with MicroCel E. Adsorbed bacteriocins were still active on target organisms (Wan et al., 1996) and could be desorbed with 0.1% sodium dodecyl sulfate (Coventry et al., 1996) obtaining 110-130 fold purification. Unfortunately, removal of SDS (by cold precipitation) was only partial (60-70%).

Boyaval et al., (1998) developed a simple two step purification system based on detergent (Triton-114), phase partitioning and adsorption/
desorption on a cation exchange resin. Upon addition of two percent detergent to supernatants, divercin V41 accumulated in the detergent phase and was recovered with a purity >95% after adsorption on cation exchange resin, washing and elution with NaCl 0.7mol/l. The method was claimed to be effective in the recovery of mesenterocin Y 105 and nisin.

The methods most frequently used for isolation, concentration and purification of bacteriocins usually involve salt precipitation of bacteriocins from culture supernatant followed by various combinations of gel filtration, ion exchange chromatography and reverse phase HPLC. Yang et al., 1992; and Daba et al., 1994, have reported a similar method for the purification of pediocin involving adsorption of the bacteriocin onto the producer cells at pH 5.5 followed by extraction at pH 2.0. However the recovery of bacteriocin activity by this method did not exceed 10%. Daeschel et al., 1992 and Bower et al., 1995 have adsorbed purified nisin in phosphate buffer solution to hydrophilic and hydrophobic silicon surfaces as an approach to using nisin as a surface active in situ sanitizer. Bacteriocins were extracted from growth media by adsorption to Microcel, a porous calcium silicate produced by the hydrothermal reaction of diatomite hydrated lime and water (Coventry and Gordon, 1996).

1.14 TOXICOLOGICAL AND REGULATORY ASPECTS OF NISIN

Nisin is regarded as non-toxic for oral consumption and has been proven safe for use as a food preservative (Delves-Broughton, 1990) and in toxicity terms is comparable with sodium chloride.
The susceptibility of nisin to enzymatic degradation, as pointed out by Molitor and Sahl (1991), although presenting disadvantages for its use as a therapeutic agent, is of advantage for its use in food, as nisin is quickly digested and cannot have an effect on the intestinal flora or be absorbed into the bloodstream.

Currently, nisin is allowed in approximately 50 countries, including the USA, the UK and China. In 1988, it was affirmed by the USA Food and Drug Administration (FDA) as having 'Generally Recognized As Safe' (GRAS) status (FDA, 1988). The type of foods in which it is allowed varies from country to country, with processed cheese products and canned foods being the most common. Nisin is used against many pathogenic and food spoilage bacteria. Table 1.3 briefly depicts the bacterial sensitivity to nisin.

Presently Aplin & Barrett Co, UK, monopolises the commercial market of nisin under the brand name of Nisaplin, which is composed of nisin with stabilizers such as sodium chloride, moisture, protein (milk solids), fat, carbohydrate. Nisaplin comprises 2.5% nisin standardised to 1 million reading units per gram (RU/g). Pure nisin contains $50 \times 10^6$ RU/g, ie, 1g Nisaplin contains 25000 μg pure nisin. An international reference preparation of nisin has been established by the WHO committee on biological standardisation. The International unit is defined as 0.001mg of this preparation.
Table 1.3 Bacterial Sensitivity to nisin (Aplin & Barrett Ltd.)

<table>
<thead>
<tr>
<th>Bacillus species</th>
<th>Gram positive, aerobic and facultative anaerobes, spore formers.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. brevis</em></td>
<td>Food poisoning bacteria often associated with rice, flour and vegetables.</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>Acid tolerant. Food spoilage bacteria.</td>
</tr>
<tr>
<td><em>B. coagulans</em></td>
<td>Acid tolerant. Food spoilage bacteria.</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>Food poisoning bacteria.</td>
</tr>
<tr>
<td><em>B. macerans</em></td>
<td>Acid tolerant. Food spoilage bacteria.</td>
</tr>
<tr>
<td><em>B. subtilis</em></td>
<td>Food spoilage/ poisoning bacteria. Causative agent of &quot;rope&quot; in bread.</td>
</tr>
<tr>
<td><em>B. stearothermophilus</em></td>
<td>Food spoilage bacteria. Thermophilic. Cause of flat sour spoilage in canned foods.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Clostridium species</th>
<th>Gram positive obligate, anaerobes, Sporeformers.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cl. bifermentans</em></td>
<td>Food spoilage bacteria in canned foods.</td>
</tr>
<tr>
<td><em>Cl. botulinum</em></td>
<td>Food spoilage bacteria. Produces extremely lethal toxins.</td>
</tr>
<tr>
<td><em>Cl. butyricum</em></td>
<td>Spoilage organism, particularly dairy products including processed cheese.</td>
</tr>
<tr>
<td><em>Cl. pasteurianum</em></td>
<td>Spoilage organism for canned foods. Acid tolerant.</td>
</tr>
<tr>
<td><em>Cl. perfringens</em></td>
<td>Food poisoning bacteria.</td>
</tr>
<tr>
<td><em>Cl. putrificum</em></td>
<td>&quot;</td>
</tr>
<tr>
<td><em>Cl. sordelli</em></td>
<td>&quot;</td>
</tr>
<tr>
<td><em>Cl. sporogenes</em></td>
<td>Food spoilage bacteria. Wide variety of products, including processed cheese.</td>
</tr>
<tr>
<td><em>Cl. tertium</em></td>
<td>Food poisoning bacteria. Thermophilic.</td>
</tr>
<tr>
<td><em>Cl. thermosaccharolyticum</em></td>
<td>Spoilage organism, particularly dairy products, including processed cheese.</td>
</tr>
<tr>
<td><strong>Desulfotomaculum species</strong></td>
<td><strong>Gram variable, obligate anaerobic spore-formers.</strong> Desulfotomaculum nigreficans Thermophilic food spoilage bacteria. Causes blackening of canned foods.</td>
</tr>
<tr>
<td><strong>Enterococcus species</strong></td>
<td><strong>Gram positive, aerobic or anaerobic. Non spore forming.</strong> Varied response, depending on strain. Moderately heat-resistant spoilage organism. Varied response, depending on strain. Spoilage organism.</td>
</tr>
<tr>
<td><strong>Enterococcus faecalis</strong></td>
<td><strong>Enterococcus faecium</strong></td>
</tr>
<tr>
<td><strong>L. oenos</strong></td>
<td><strong>L. mesenteroides</strong></td>
</tr>
<tr>
<td><strong>Listeria species</strong></td>
<td><strong>Gram positive, aerobic and anaerobic. Non-spore-forming.</strong> Moderately sensitive to nisin. Food poisoning bacteria, particularly in soft cheese and pates.</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Micrococcus species</strong></td>
<td><strong>Gram positive, aerobic. Non-spore-forming</strong> Use in nisin bioassay.</td>
</tr>
<tr>
<td><strong>M. luteus</strong></td>
<td><strong>M. varians</strong></td>
</tr>
<tr>
<td><strong>Pediococcus spp</strong></td>
<td><strong>P. damnosus</strong></td>
</tr>
<tr>
<td><strong>P. pentosaceus</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Staphylococcus species</strong></td>
<td><strong>Gram positive, aerobic and anaerobic. Non-spore-forming.</strong> Varied response to nisin depending on the strain. Food poisoning bacteria.</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Sporolactobacillus species</strong></td>
<td><strong>Gram positive, aerobic and anaerobic. Spore-forming. Capable of growth at low pH.</strong></td>
</tr>
<tr>
<td><strong>Sporolactobacillus inulinus</strong></td>
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</tbody>
</table>