ANTIBACTERIAL ACTIVITY OF *LACTOBACILLUS CASEI* AGAINST *STAPHYLOCOCCUS AUREUS* AND ISOLATION AND PURIFICATION OF BACTERIOCIN FROM *LACTOBACILLUS CASEI*

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Central Department of Biotechnology
Tribhuvan University
Kirtipur, Kathmandu, Nepal

By
Susmita Bhattarai

Supervisors
Dr. Rajani Malla
Dr. Anil Kumar Anal

Exam Roll No: 082/068
Registration No: 5-2-37-763-2007

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List of abbreviations

CFU/ml = colony forming units per ml

µl = microlitre

µm = micrometer

MSA = Mannitol Salt Agar

MRS = de Man, Rogosa and Sharpe Agar

NB = Nutrient Broth

OD = Optical Density

SD = Standard Deviation

◦c = Degree Celsius

hr = Hour

g = Gram

kDa = Kilodalton

MIC = Minimum Inhibitory Concentration

MBC = Minumum Bactericidal Concentration

LAB = Lactic Acid Bacteria

SDS-PAGE = Sodium dodecyl polyacrylamide gel electrophoresis

FBD = Food Borne Diseases

SE = Staphylococcal Enterotoxin

SFD = Staphylococcal Food Borne Dieases

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ABSTRACT

Antimicrobials are the substances which show inhibitory activity against test organisms. These substances are produced by microorganism against other microorganisms. In the present study antimicrobial activity of *Lactobacillus casei* (*L. casei*) is studied against *Staphylococcus aureus* (*S. aureus*). The probiotic bacteria *L. casei* was co-cultured with *S. aureus* and it was observed that *L. casei* was able to inhibit the growth of *S. aureus* when co-cultured in milk. The inhibition was observed as the log cfu/ml of the *S. aureus* which was decreased from 6 log cfu/ml to 3.6 log cfu/ml when cultured for 48 hours. As when cultured individually there was no decrease in log cfu/ml of *S. aureus.* Also the pH of the co-culture was decreased from 6.34 to 5.47. And also the lactose content was found to be decreased as the incubation period increased. It decreased from 77.9 mg/ml to 60.3 mg/ml concentration.

In this research the bacteriocin which was isolated partially purified and studied for its activity. The ammonium sulphate precipitation was carried out and found that 70% saturation of ammonium sulphate gave the best inhibitory activity. The inhibitory activity shown was 800 AU/ml after dialysis. The total amount of protein after dialysis was estimated to be 12.45 mg/ 100ml and 30% recovery was made. The protein was active at pH between 4 to 9 and also active between temperature 70- 90 ◦c when exposed to heat for 10 minutes. The MIC of the bacteriocin against *S. aureus* was found to 75 mg/ml and MBC to be 150mg/ml. The molecular weight of the partially purified bacteriocin by SDS-PAGE was found to be 36 kDa. The Antimicrobial activity of *L. casei* against *S. aureus* was observed in this study and also the bacteriocin isolated was showing the inhibitory activity against test organism *S. aureus*.

Keywords: Antibacterial, *Staphylococcus. aureus*, *Lactobacillus. casei*, Bacteriocin

CHAPTER 1

INTRODUCTION

1.1 General background

These days' research studies are focused on producing safe food stuffs as consumers are aware of their health. Although producing safe and quality food without alteration of its basic features has become a challenge. It's because microbial growth in food destroys the food by changing the color, texture, taste and can cause serious health problems if there is the production of the toxins by the microbes.

Microbial contamination in food is the issue that leads to food preservation. Chemical food preservatives are widely used in maintaining the food quality but these are not safe as per the health concerns. So, the preservatives should be non harmful even though in low amount and that's why natural preservatives can be the alternative for this. Natural food preservatives also known as biopreservatives are searched now a days, so that can be used in daily food stuffs. Natural substances that can maintain the quality of the food can be isolated from microorganisms as well. Probiotic bacteria are microorganisms which are used against the harmful bacteria.

Probiotic bacteria are those bacteria which when introduced inside the body can show some measurable human health benefits. Probiotics have preventative as well as a curative effect on several types of diarrhoea of different aetiologies Probiotic bacteria produce different antimicrobial agents and also other peptides which are beneficial to the gut. The metabolically produced functionary are said to improve immune system, wipe off the useless bacteria and help in different internal metabolisms. Probiotic bacteria interact with the intestinal micro biota and intestinal epithelial cells by producing different bioactive metabolites. It is thought that the intestinal micro biota not only effects the environment of the gut but has the capacity to influence as a whole metabolic homeostasis of humans. Bacteriocins are among the antimicrobial substances produced by probiotic bacteria. Bacteriocins are peptides that are ribosomally synthesized by bacteria which can be used as biopreservative in food stuffs so that the shelf life is prolonged. Also the bacteriocins are non toxic as such are safe for the health issues.

Lactobacilli are mostly used genera for the probiotics. They have many antimicrobial compounds that act against other organisms. They also release the bacteriocins as their

defenses which are proteinous in nature. They can be isolated and purified and can be used in food stuffs and medical fields after studying their properties.

Non-thermal treatments are attracting interest of the food industry due to their capability of assuring the quality and safety of food. Consumer demand today is for natural and minimally processed foods, with a fresh appearance and taste, ease-to eat and high safety. As a result, research and development of new products is leading to the reduction or even displacement of heat treatments and traditional preservatives by treatments capable of assuring the sensory and nutritional properties of the product without reducing food safety. Non-thermal preservation methods are thus of growing interest as alternative treatments, especially high intensity pulsed electric fields (HIPEF), high pressure (HP) and the addition of natural antimicrobial substances. (Lopez and Belloso, 2008)

Lactic acid bacteria have the preservative effect because of the active metabolites produced by them having antimicrobial properties such as organic acids, which are lactic and acetic acid. These acids decrease the pH of the media and show the antibacterial activity. But other mechanisms as well are involved in killing and inhibiting the pathogenic microorganisms. Among which the action of bacteriocin and its production by LAB is ganing attention which can be developed in the form of biopreservatives. Although LAB are the main source of bacteriocin producing bacteria and bacteriocin breakthroughs, few have been studied as potentially applicable in dairy products. Therefore, many bacteriocins have not been fully characterized yet and, consequently, they are not extensively used in food industry.

LAB have been used for centuries in the fermentation of food, partly due to the fact that they can prevent the growth of spoilage and pathogenic microorganisms. They produce bacteriocins, the lantibiotics, so named because they are post-translationally modified to contain amino acids such as thioether bridges of lanthionine and 3- methyllanthionine or dehydroalanine. Lantibiotics are ribosomally synthesized bacteriocins that target a broad range of Gram-positive bacteria.

The major difference between bacteriocins and antibiotics is that bacteriocins restrict their activity to strains of species related to the producing species and particularly to strains of the same species, antibiotics on the other hand have a wider activity spectrum and even if their activity is restricted this does not show any preferential effect on closely related strains. In addition, bacteriocins are ribosomally synthesized and produced during the primary phase of growth, though antibiotics are usually secondary metabolites. Nisin is the only bacteriocin that has been officially employed in the food industry and its use has been approved worldwide. Bacteriocins can be applied on a purified or on a crude form or through the use of a product

previously fermented with a bacteriocin producing strain as an ingredient in food processing or incorporated through a bacteriocin producing strain (Zacharof and ovitt, 2012).

Bacteriocins can also be used to improve food quality and sensory properties, for example increasing the rate of proteolysis or in the prevention of gas blowing defect in cheese. Another application of bacteriocins is bioactive packaging, a process that can protect the food from external contaminants. The increased consumption of foods containing additives formulated with chemical preservatives and consumer concerns have created a higher demand for more natural and minimally processed foods, therefore, there is a high interest in naturally produced antimicrobial agents that do not produce adverse effects. This interest and also the potential applications in health care sectors have attracted the interest of academia and industry resulting in increased numbers of published research on bacteriocin production, purification, genetics, and applications.

Staphylococcal food-borne disease (SFD) is one of the most common food-borne diseases worldwide resulting from the contamination of food by preformed *S. aureus* enterotoxins. It is one of the most common causes of reported food-borne diseases. Although several Staphylococcal enterotoxins (SEs) have been identified, SEA, a highly heat-stable SE, is the most common cause of SFD worldwide. Outbreak investigations have found that improper food handling practices in the retail industry account for the majority of SFD outbreaks. Presence of pathogens in food products imposes potential hazard for consumers and causes grave economic loss and loss in human productivity via food-borne disease.

A typical FBD caused by *S. aureus* has a rapid onset following ingestion of contaminated food (usually 3– 5 hours).This is due to the production of one or more toxins by the bacteria during growth at permissive temperatures. However, the incubation period of SFD depends on amount of toxin ingested. Very small dose of SEs can cause SFD. For example, one report indicated that approximately 0.5 ng/mL concentration of SEs contaminated with chocolate milk caused a large outbreak. Various types of foods serve as an optimum growth medium for *S. aureus.* Foods that have been frequently implicated in SFD are meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, especially cream-filled pastries and cakes, and sandwich fillings. Foods implicated with SFD vary from country to country, particularly due to variation in consumption and food habits. If food is prepared in a central location and widely distributed, SFD outbreaks can have grave consequences impacting thousands of people. (Kadariya et al., 2014)

1.2 Statement of problem

The application of bacteriocin and other natural additives for quality food products are widely under research now days. They are regarded as safe than other chemically synthesized things. Also the antimicrobial agents can be used as control against the harmful microorganisms that will lead to lesser contamination which will eventually lead to inhibition of microbial spoilage in foods. Hence this research is also intended to find if either bacteria or bacteriocins can be used against *S. aureus* and decrease the microbial contamination caused by it. *S. aureus* is an important pathogen due to combination of "toxin-mediated virulence, invasiveness, and antibiotic resistance." Solution to this problem is to be researched .

1.3 Scope of study

Biopreservation systems such as bacteriocinogenic LAB cultures and/or their bacteriocin have received increasing attention, and new approaches to control pathogenic and spoilage microorganisms. Probiotic bacteria itself or antimicrobial agents produced by it, is being used as natural preservatives and food additives. Despite the several methods other than bacteriocin are employed for the preservation of food/beverages, an increasingly, health conscious public may seek to avoid foods that have undergone extensive processing or which contain chemical preservatives. Thus, the products of probiotic bacteria can be featured for their action. Also the probiotic culture and its extracts can be antimicrobial to *S. aureus* so that it can be applicable further for controlling the food borne diseases caused by it.

1.4 Objectives

General Objectives:

- To study the Antimicrobial activity of *L. casei* and its bacteriocin against *S. aureus* then isolation and characterization of the bacteriocin.

Specific Objectives:

- To co-culture *L.casei* and *S.aureus* in milk.
    - Study the changes in growth of the *L.casei* and *S.aureus* and determine antimicrobial activity.
    - Study the changes in pH as per the co- culture continues.
    - Study the changes in the lactose content of the co-culture media.

- To isolate the bacteriocin from *L.casei* by Ammonium sulphate precipitation method and to purify bacteriocin by dialysis method

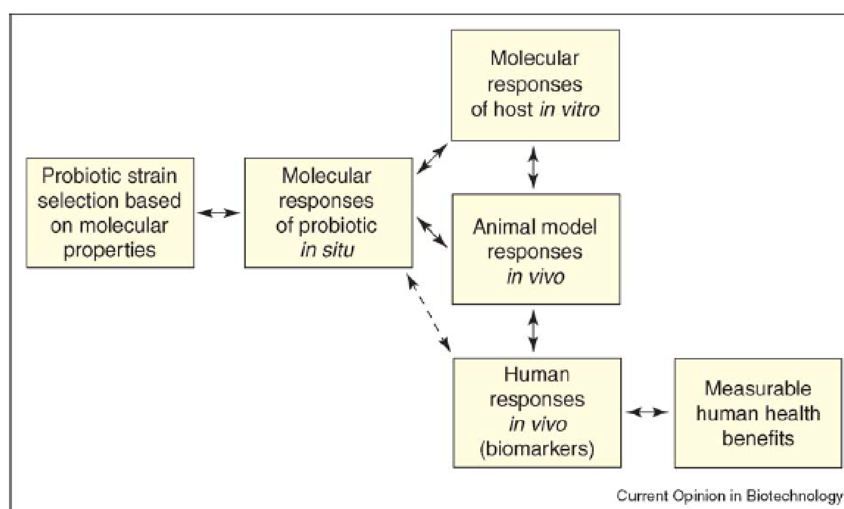
- To characterize the bacteriocin obtained from *L.casei*.

CHAPTER 2

LITERATURE REVIEW

2.1 Probiotics:

Probiotics are generally defined as the microbial cells that are live and are supposed to benefit the host by balancing the microbes in intestinal wall. The World Health Organization's 2001 definition of probiotics is "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host". So it is to be considered that probiotics should be lively when administered. Also the probiotic effect of a microorganism can be concluded only when there is healthy interaction between microbiota and the host. The molecular host microbe interaction model can be presented as follows which is given by Macro et al., 2006.



Schematic representation of bi-directional, reciprocal molecular approaches to elucidate the molecular mechanisms underlying probiotic function in relation to human health.

Figure 2.1: Probiotic relation to human health. (Macro et al., 2006)

The term probiotic was derived from the Greek, meaning “for life.” The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) have stated that there is adequate scientific evidence to indicate that there is potential for probiotic foods to provide health benefits and that specific strains are safe for human use. Many health care professionals such as holistic practitioners, naturopaths, chiropractors, and herbalists routinely use products perceived to contain lactobacilli, bifidobacteria, and other possible probiotics. Several factors are now leading many physicians to examine probiotics and other

alternatives to pharmaceutical remedies. These include the surging levels of multidrug resistance among pathogenic organisms, particularly in hospitals, the increasing demands of consumers for natural substitutes for drugs, and the emergence of scientific and clinical evidence showing the efficacy and effectiveness of some probiotic strains. Evaluation of probiotics is necessary before using in food stuffs according to guidelines as given below by WHO and FAO (Reid et. al., 2003).

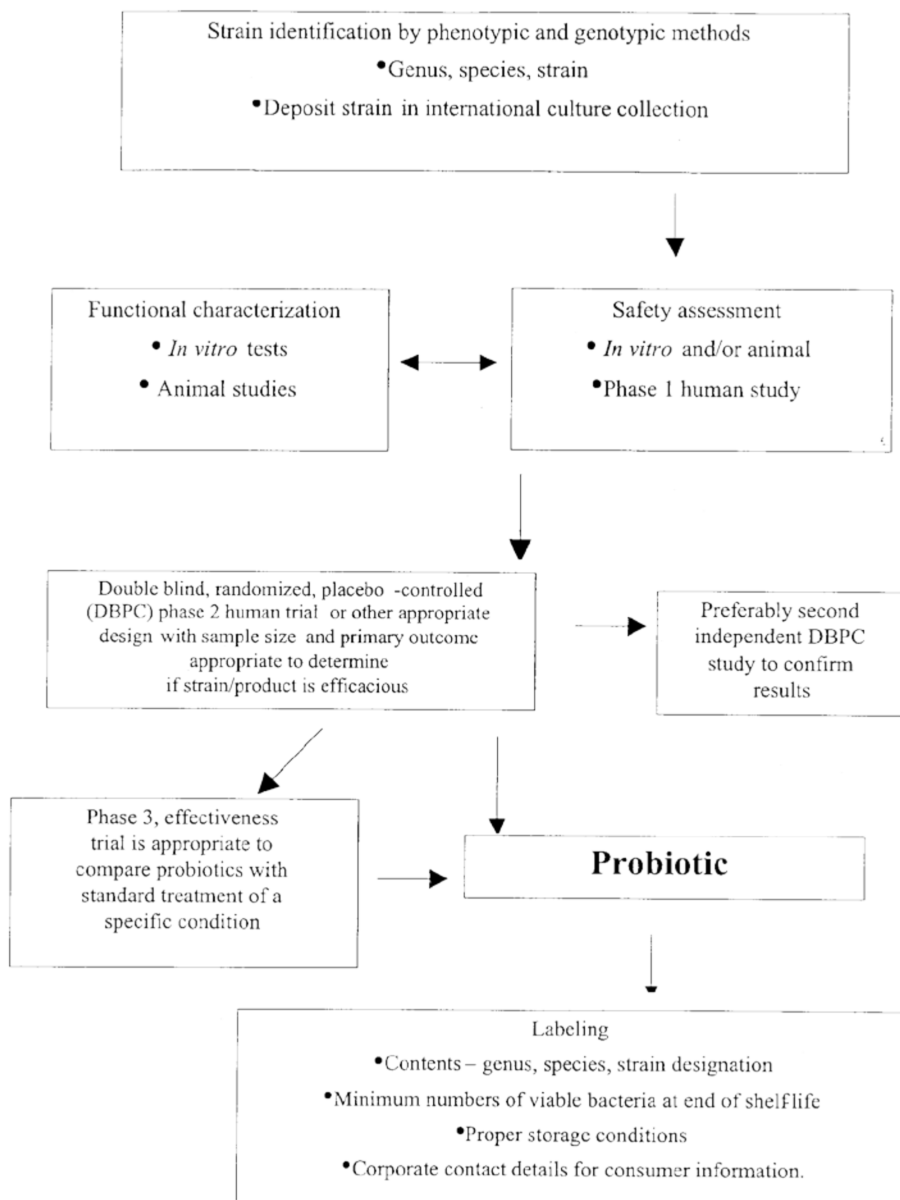


Figure 2.2: Guidelines given by WHO and FAO (Reid et. al., 2003)

There are various probiotic bacteria that are included as healthy bacteria but these days the widely used ones are of genera Lactobacillus and Bifidobacterium. They are considered as safe

by qualified experts or it can be said that they have low pathogenic potential as compared to others.Also the genera of Enterococcus spp., Streptococcus spp and Bacillus spp are considered as probiotics.

Although there are several dozen, in many cases related, microbial strains that are claimed to have probiotic activity, representatives of only a handful of species dominate the market or have been used in multiple clinical trials as shown in the table below. These include strains of Lactobacillus casei, Lactobacillus johnsonii, Lactobacillus rhamnosus and Lactobacillus plantarum, which are all of human origin and are known under defined brand names. By contrast, the Bifidobacterium strain BB12, which is used in most studies under the name B. lactis BB12, is marketed under a variety of labels. (Saxelin et al.,2005).

m

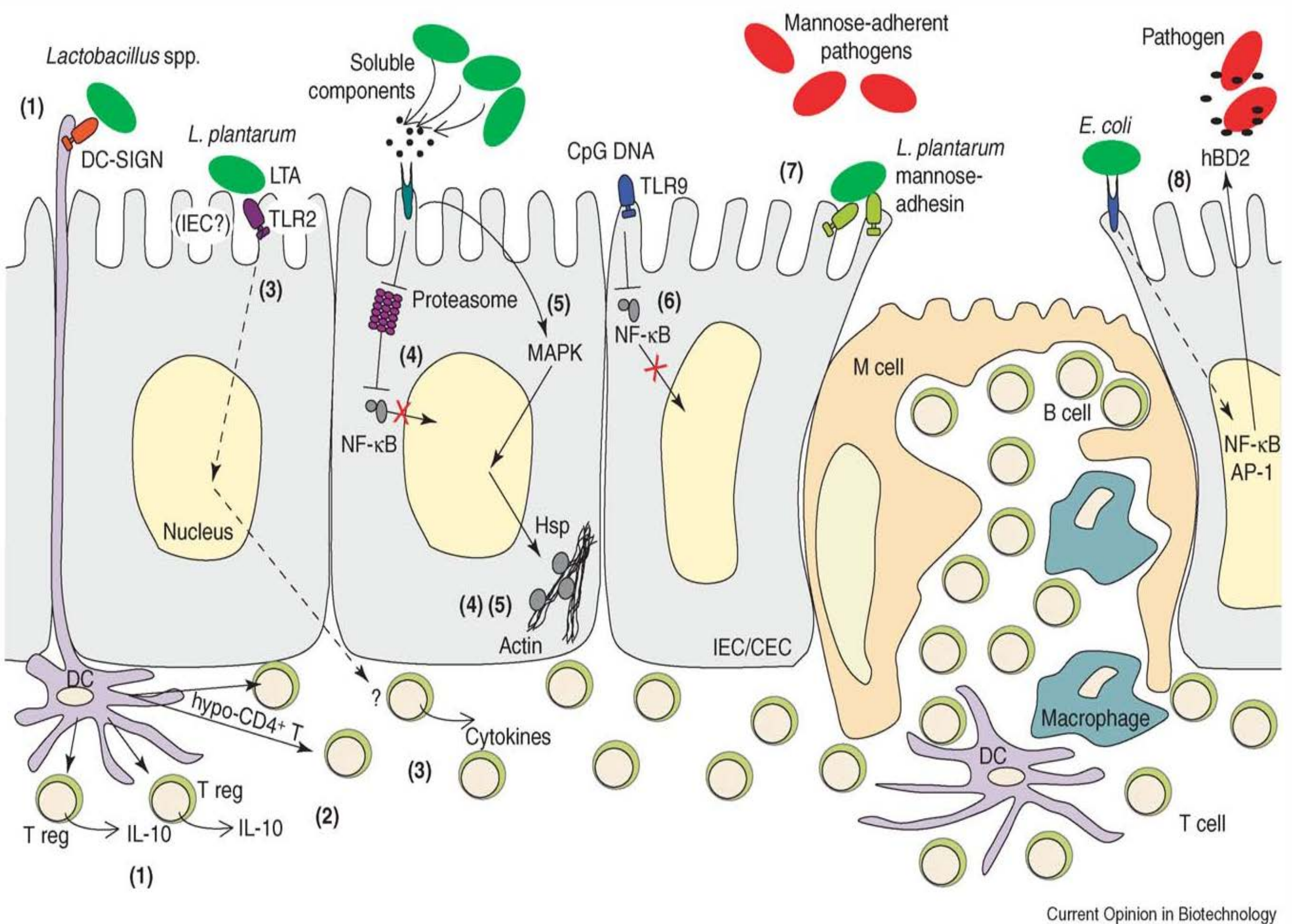
Commonly used species of probiotics, their strain designations and commercial products.

Species	Strain	Commercial brand name(s)
Lactobacillus casei	DN114001	Actimel®
Lactobacillus casei	Shirota	Yakult®
Lactobacillus plantarum	299v	ProViva®
Lactobacillus rhamnosus	GG	Actifit Plus®, Gefilus®, LGG®, Onaka He GG!®, Vifit® etc.
Lactobacillus johnsonii	La1	LC1®
Bifidobacterium lactis	BB12	Various brand names

Figure 2.3: Commonly used species of Probiotics (Saxelin et al., 2005)

Probiotic bacteria are commonly used in different food items which can be dairy product or non dairy product. The dairy products which contain probiotic bacteria are yoghurts, cheese, milk, kefir, etc. And the non dairy products that include probiotic bacteria are meat, herbs, fruits juices, soy products etc.

Probiotic bacteria when studied in vitro and in vivo then only can be regarded as safe if there are good results. The functioning of probiotic is researched with human intestinal cells to know the interactions. Modulation of host cell function by probiotics: in vitro studies can be given as, probiotics are known to beneficially modulate several host cell functions, the most prevalent of which are immune responses and intestinal barrier integrity. The mechanistic events underlying these effects are now beginning to be understood from in vitro studies of host intestinal epithelial or immune cell responses to probiotic strains. A summary of these developments is provided in figure below. (Marco et al., 2006)



Bacterial and host effector molecules with potential probiotic effects. Lactobacillus strains are able to induce IL-10-producing, regulatory T cells (T reg) through DC-SIGN interaction (1). They can also induce hyporesponsive CD4+ T-cell populations after DC interaction (2). LTA composition is responsible for the differential modulation of cytokine production (3). Modulation of inflammatory responses by inactivation of the NF-kB signaling pathway is achieved through proteasome inhibition after IEC recognition of soluble probiotic components (4) or after recognition of bacterial motifs (e.g. CpG DNA by TLR9 receptors) (6). The induction of Hsps (either via 4 or 5) stabilizing the actin cytoskeleton would strengthen the mucosal barrier. Pathogen attachment and growth could be counteracted by strains possessing mannose adhesins (7) or by induction of hBD2 in IECs (8). M cell, an epithelial cell specialized in antigen uptake and transport.

Figure 2.4 : Immune modulation by Probiotic (Macro et al., 2006)

In human trials probiotic strains have been associated with the reduction of fecal mutagenicity or fecal enzymatic activities involved in mutagen or carcinogen activation. Reduction of faecal enzyme activities has also been shown after *L. gasseri* strain ADH and *L. casei* strain Shirota consumption in humans. *L. casei* strain Shirota consumption has further proved beneficial for some cancer patients by reducing the recurrence of superficial bladder cancer and by prolonging survival and relapse-free interval in cervical cancer. However, although there is evidence that probiotic bacteria may show antimutagenic and nticarcinogenic

properties in vitro and in animal models, the possible role of probiotics in the cancer prevention in humans still remains highly controversial. (Saarela et al., 2000)

Eventhough there are various claims of probiotic bacteria in different diseases still there are a lot of controversy regarding using them. The probiotic strains can only be delivered to consumers when they are able to be manufactured industrially and can survive and retain activity even after frozen or freeze dried along with the food stuffs. The use of probiotics in general clinical practice is not far away, given that products such as VSL#3 are already being used. Molecular tools will continue to be used to understand and manipulate lactic acid bacteria with a view to producing vaccines and new and improved probiotic products. The critical step in wider application will be to make products available that are safe and clinically proven in a specific formulation easily accessible to physicians and consumers. (Reid et al., 2003)

2.2 Lactic acid bacteria

Lactic acid bacteria(LAB) are lactose utilizing bacteria as they use lactose and other sugar and give lactic acid as their metabolic end product which increases the pH of the medium. So, they are gram positive acid tolerant bacteria which can be cocci or bacilli.The main genera that comprise the LAB are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*. The two main probiotics that have been studied extensively are of genera *Lactobacillus* and *Bifidobacterium. Lactobacillus*are gram positive microaerophilic rod shaped bacteria.

According to metabolism, *Lactobacillus* species can be divided into three groups:

- Obligately homo fermentative (Group I) including:
    - *L. acidophilus*, *L. delbrueckii*, *L. helveticus*, *L. salivarius*
- Facultatively hetero fermentative (Group II) including:
    - *L. casei*, *L. curvatus*, *L. plantarum*, *L. sakei*
- Obligately hetero fermentative (Group III) including:
    - *L. brevis*, *L. buchneri*, *L. fermentum*, *L. reuteri*

(wikipedia)

L. casei are mesophilic gram positive, rod shaped non-motile, non-sporing bacteria. As given by Microbewiki they are found in various environments such as fermented dairy products, reproductive system and intestinal tracts of humans and animals. It can be classified as,

Domain: Bacteria Kingdom
Bacteria Phylum: Firmicutes
Class: Bacilli
Order: Lactobacillales
Family: Lactobacillaceae
Genus: *Lactobacillus*
Species: *Lactobacillus Casei*

The application of *L. casei* in biotechnology is various due to their beneficial effects which are increase in immune system, decrease risk of bladder cancer and reduced cholesterol level. They can be effective against other pathogenic bacterias as well such as the study done by Cats, et al 2003, L. casei inhibits H. pylori growth when cultured In vitro and the culture of *L.casei* is effective when grown in milk rather than in DeMan–Rogosa–Sharpe medium. When studied by Takeda and Okumura, 2007, it was observed that the daily intake of *Lactobacillus casei* strain Shirota provides a positive effect on NK-cell activity so that it is helpful for increasing the immune system.

Lactobacillus produces various types of by-products that act as bacterial defense system. As given by Riley,(2009), the defense system of microbes include antibiotics, lysozymes, metabolic products as lactic acid, different types of proteins that can be exotoxins and bacteriocins. But as per the choice as microbial weapons bacteriocins are used as they have natural abundance and its diversity. Because of the bacteriocins produced by LAB, they are effective against pathogenic bacterias. Also, they have industrial importance as they are considered as generally regarded as safe (GRAS) so that can be used in food items.

2.2.1 Lactic Acid Bacteria and Their Antimicrobial compounds:

Lactic acid bacteria are industrially important organisms recognized for their fermentative ability as well as their health and nutritional benefits. Antimicrobial metabolites produced during the fermentation by LAB act as inhibitory to other organisms. These include many organic acids such as lactic, acetic and propionic acids produced as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms. Acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intracellular pH and inhibiting a variety of metabolic functions. (Rattanachaikunsopon et al.2010)

Table2.1: Bacteriocins of lactic acid bacteria and their main characteristics (Parada et al 2007)

Producing species	Bacteriocin	Spectrum of action	Characteristics
Lactococcus lactis subsp. lactis	Nisin	Gram-positive bacteria	Class I lantibiotic, 3,5 kDa, 34 amino-acids, commercially available
	Lacticin 3147	*Clostridium sp* *Listeria monocytogenes* *Staphylococcus aureus* *Streptococcus dysgalactiae* *Enterococcus faecalis* *Propionibacterium acne* *Streptococcus mutans*	Class I two-component lantibiotic, 4,2 kDa, heat-stable, active under acid and physiological pH
Lactococcus lactis subsp. cremoris	Lactococcin B	*Lactobacillus*	Class II bacteriocin, approx. 5 kDa, narrow spectrum of action
Lactobacillus acidophilus	Acidocin CH5	Gram-positive bacteria *Lactobacillus*	Class II bacteriocin, forms high molecular weight aggregates
	Lactacin F	*Lactobacillus fermentum* *Enterococcus faecalis* *Lactobacillus delbrueckii* *Lactobacillus helveticus*	Class II bacteriocin, 6,3 kDa, 57 amino-acids, heat-stable at 121° C for 15 minutes
	Lactacin B	*Lactobacillus debrweckii* *Lactobacillus helveticus* *Lactobacillus.bulgaricus.* *Lactococcus lactis.*	Class III bacteriocin, 6,3 kDa, heat-stable, detected only in cultures maintained between pH 5.0 to 6.0
Lactobacillus amylovorus	Lactobin A	*Lactobacillus acidophilus* *Lactobacillus delbrueckii*	Class II bacteriocin, 4,8 kDa, 50 amino-acids, narrow spectrum of activity
Lactobacillus casei	Lactocin 705	*Listeria monocytogenes* *Lactobacillus plantarum*	Class II two-component bacteriocin (33 amino-acids each component), 3,4 kDa,
Leuconostoc gelidum	Leucocin A	*Lactobacillus* *Enterococcus faecalis* *Listeria monocytogenes*	Class II bacteriocin, 3,9 kDa, 37 amino-acids, stable at low pH values, even after heating (100°C for 20 min)
Leuconostoc mesenteroides	Mesentericin Y105	*Enterococcus faecalis* *Listeria monocytogenes*	Class II bacteriocin, 3,8 kDa, 37 amino-acid residues, heat stable (60°C for 120 min at pH 4.5)
Pediococcus acidilactici	Pediocin F	Gram-positive bacteria	Class II bacteriocin, 4,5 kDa, sensitive to proteolytic enzymes, resistant to heat and organic solvents, active under a wide range of pH
	Pediocin PA-1	Listeria monocytogenes	Class II bacteriocin, 4,6 kDa, 44 amino-acids,
	Pediocin AcH	Gram-positive and Gram-negative bacteria under stressing situations	Class II bacteriocin, 4,6 kDa, 44 amino-acids, broad spectrum of action
Pediococus pentosaceous	Pediocin A	*Lactobacillus* *Lactococcus* *Leuconostoc* *Pediococcus* *Staphylococcus* *Enterococcus* *Listeria* *Clostridium*	Class II bacteriocin, 2,7 kDa, sensitive to proteolytic enzymes and heat stable (10 min 100°C)
Enterococcus faecium	Enterocin A	*Listeria monocytogenes* *Pediococcus*	Class II bacteriocin, 4,8 kDa, 47 amino-acid residues, heat-stable
Lactobacillus sake	Lactocin S	*Lactobacillus* *Leuconostoc* *Pediococcus*	Class I bacteriocin, 3,7 kDa, active between pH of 4,5 and 7,5
	Sakacin P	*Listeria monocytogenes*	Class II bacteriocin, 4,4 kDa, heat-stable
Lactobacillus curvatus	Curvacin A	*Listeria monocytogenes* *Enterococcus faecalis*	Class II bacteriocin, 4,3 kDa
Lactobacillus helveticus	Helveticin J	*Lactobacillus bulgaricus* *Lactococcus lactis*	Class III bacteriocin, 37 kDa, narrow spectrum of action, sensitive to proteolytic enzymes, reduction of activity after 100° C for 30 min

In addition to acids, starter strains can produce a range of other antimicrobial metabolites such as ethanol from the heterofermentative pathway, H2O2 produced during aerobic growth and diacetyl which is generated from excess pyruvate coming from citrate. In particular, H2O2 can have a strong oxidizing effect on membrane lipids and cellular proteins and is produced using such enzymes as the flavo protein oxidoreductases NADH peroxidase, NADH oxidase and α-glycerophosphate oxidase. Obviously, each antimicrobial compound produced during fermentation provides an additional hurdle for pathogens and spoilage bacteria to overcome before they can survive and/or proliferate in a food or beverage, from time of manufacture to time of consumption. Lactic acid bacteria and their products give fermented foods distinctive flavors, textures, and aromas while preventing spoilage, extending shelf-life, and inhibiting pathogenic organisms. (Rattanachaikunsopon et al.2010)

2.3 Bacteriocins:

According to Riley, (2009) bacteriocin family are diverse group of bacterial defenses. Their evolutionary history is still under study. Bacteria produce a wide array of microbial defense systems. A species of bacteria may produce many different kinds of bacteriocins. The bacteriocins produced by gram positive bacteria are generally cationic, amphiphilic, membrane-permeabilizing peptides. As the bacteriocins by them are not lethal to the producing cells they act upon other pathogenic bacteria.

The bacteriocins comprise a subgroup within the far larger body of natural commercial food preservatives. They are produced by bacteria and possess antibiotic properties, but are normally not termed antibiotics in order to avoid confusion and concern with therapeutic antibiotics that can potentially illicit allergic reactions in humans. Bacteriocins also differ from most therapeutic antibiotics in being proteinaceous agents that are rapidly digested by proteases in the human digestive tract and they have been defined as “extracellularly released primary or modified products of bacterial ribosomal synthesis, which have bacteriocidal activity on a relatively narrow spectrum of strains of the same or closely related species”. The increasing interest in such antimicrobial peptides as natural food additive candidates, has stimulated the isolation and characterization of many novel peptides from bacteria that have traditionally been used by humans in food applications (Deraz et al 2005).

LAB produced bacteriocins are well characterized as they have been used in fermentation and milk and meat preservation. Based on their primary structure, molecular mass, heat stability and molecular organization, bacteriocins produced by LAB can be subdivided into four classes. As given by to Riley, (2009) there are four classes of LAB bacteriocins, Class I are the bacteriocins that are modified known as lantibiotics which are ribosomally synthesized and can act against a broad range of Gram-positive bacteria. They are of three type, Type A are the one with 2-5 kDA such as lacticin. They kill by membrane polarization. Type B are of around 2 kDa such as mersacidin which kill by interfering the cellular enzymatic reactions. Type C are made up of two lantibiotic peptide and they act as antimicrobial.

Class II bacteriocins are minimally modified and heat-stableof less than 10 kDa. They are divided into two sub groups. Class IIa consists of *Listeria*-active peptide which have conserved N- terminal sequence of YGNGVXaaC and act by forming pores in cytoplasmic membrane such as, pediocinAcH,leucocin A and sakacin A. Class IIb are lacticin F and lactococcin G composed of two different proteins and act by forming pores in the target bacteria.

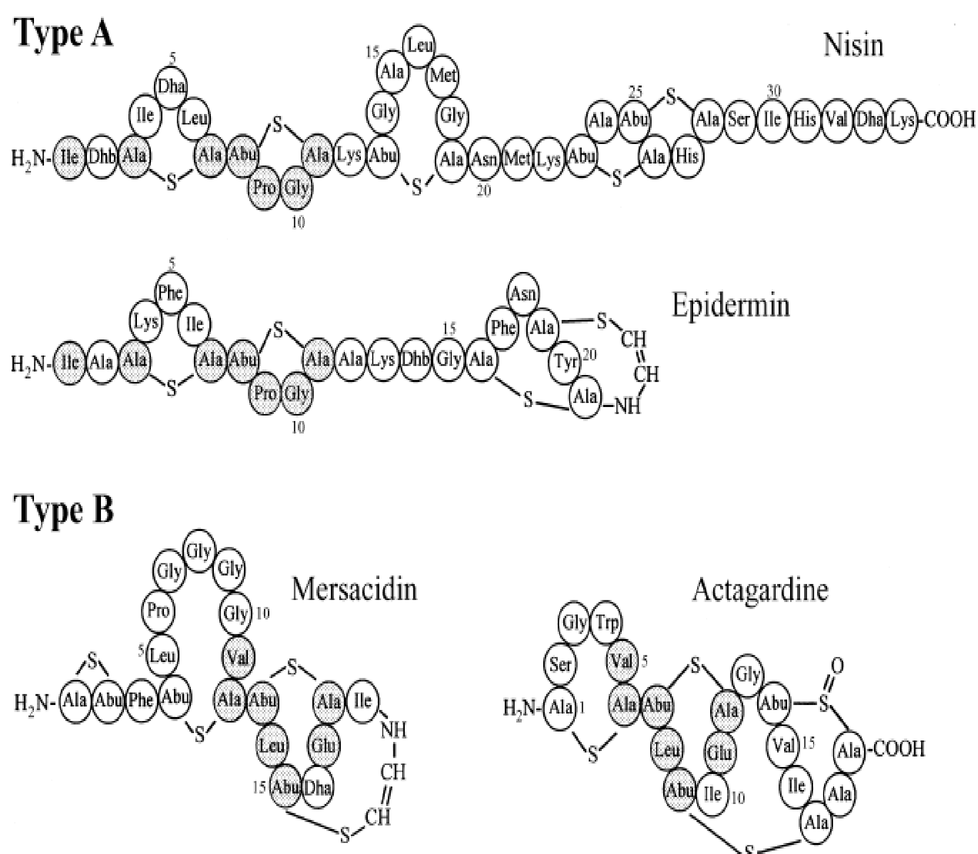


Figure 2.5: Amino acid sequence of type A and type B lantibiotics. (Brotz and Sahl, 2000).

Class III are heat labile large proteins, such as helveticins J and lactacin B. Class IV bacteriocins are those which needs lipid or carbohydrate moieties for their action, so are complex as compared to other bacteriocins. They are the least studied one whereas the Class I bacteriocins are the mostly studied one in probiotic research.(Riley ,2009)

Table 2.2: Classification and general characteristics of bacteriocins (Bodaszewska-Lubas M. et al., 2012)

Bactriocin classes	Bacteriocin subclasses	Molecular mass	Characteristics of class/subclass	Bacteriocin
Class I	A	<5 kDa	lantibiotics	nisin [16,43]
	B			marsacydin alametycin [16,42]
Class II	IIa	<10 kDa	pediocin-like bacteriocins	sakacin A, sakacin P [30]
	IIb		two-peptide bacteriocins	lactacin F [30]
	IIc		sec-dependent bactriocins	carno-bacteriocin A [30]
Class III		>30 kDa	heat-labile protein bacteriocins	lactococcin B [31]
Class IV		large protein	mixture of protein(s), lipid(s) and carbohydrate(s) in bacteriocin molecule	leucocin S, mesenterocin 52 [31]

The action of bacteriocins may be of broad range or can be of narrow range limited upto their own species. Nisin is a bacteriocin produced by *Lactococcuslactis* and is the widely studied bacteriocin being active against wide range of gram positive targets.Nisin is active against most of the enteropathogens. As per Blay, 2007, Nisins Aand Z had similar antimicrobial activity spectra so, inhibited all Gram-positive intestinal bacteria at different levels (except Streptococcus salivarius). Nisin has been used as food preservative for over 50 years and it has been reported that there is no significant bacterial resistance against it.(Nagalakshmi, 2013)

Production of bacteriocins in Gram-positive bacteria is generally associated with the shift from log phase to stationary phase. For example, nisin production begins during mid-log phase and increases to a maximum as the cells enter stationary phase. The regulation of expression is not cell cycle-dependent, *per se*, but rather culture density-dependent. It has been demonstrated that nisin A acts as a protein pheromone in regulating its own expression, which

is controlled by a two-component signal transduction system typical of many quorum-sensing systems. The genes involved are *nisR* (the response regulator) and *nisK* (the sensor kinase). Gram-positive bacteriocins in general and lantibioticsin particular require many more genes for their production than do those of Gram-negative bacteria. The nisin gene cluster includes genes for the prepeptide (*nisA*), enzymes for modifying amino acids (*nisB, nisC*), cleavage of the leader peptide (*nisP*), secretion (*nisT*), immunity (*nisI, nisFEG*), and regulation of expression (*nisR, nisK*). These gene clusters are most often encoded on plasmids but are occasionally found on the chromosome. Several Gram-positive bacteriocins, including nisin, are located on transposons. (Riley ,2009)

Bacteriocins are gaining interest because of their wide antibacterial spectrum with feasible application in foods, such as meat and fish products, fruits and vegetables, cereals and beverages. Nisin is one of the widely studied and used bacteriocin. Nisin is a highly surface-active molecule that can bind to different compounds, such as fatty acids of phospholipids; this feature makes it suitable for adsorption to solid surfaces and killing bacterial cells that subsequently adhere. Therefore, nisin adsorption may represent a promising advance in the development of active packaging, where the classical protective function of packaging is supported by the antimicrobial action of nisin.

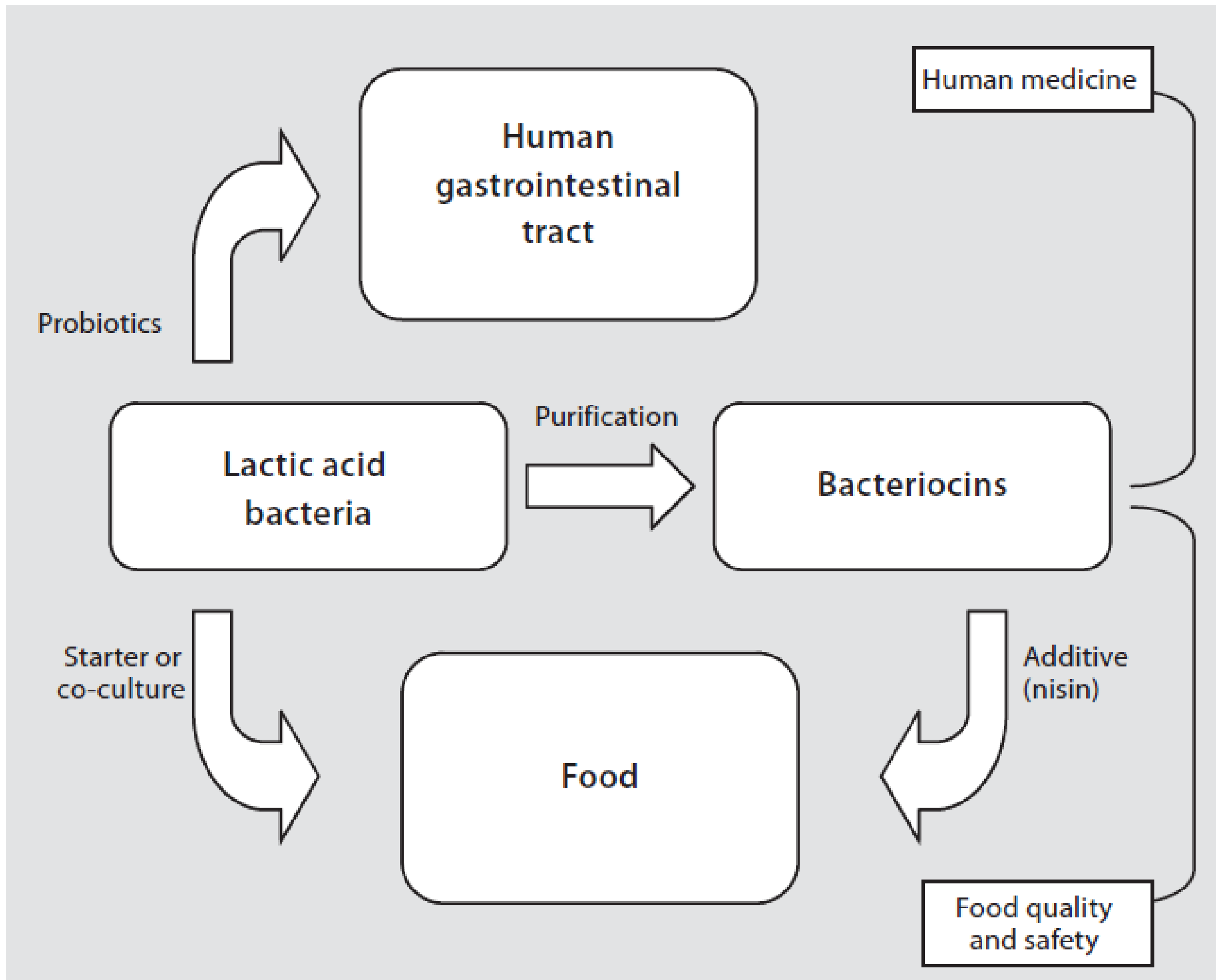


Figure 2.6: Overview of the application potential of bacteriocin production by LAB in food quality and safety and in medicine, emphasizing their role as food ingredient and in the human gastrointestinal tract, respectively (Vuyst and Leroy, 2007).

Also the spread of antibiotic resistance leads to the demand of novel antibiotics or antimicrobials. So, the existing classes of antimicrobials are to be studied deeply in molecular level to find better and safer antimicrobials. The molecular mechanisms of their killing bacteria are to be characterized further as until now only less has been done. One example is the lantibiotics where recent investigations have led to unexpected results. It became evident that representatives of different classes of lantibiotics share the same molecular target, although this interaction leads to different antibacterial effects. In one particular case, two different types of mechanisms of action appear to reside in same molecule and these combine to

produce high-potency antimicrobial activity. Nisin is the most well known lantibiotics which is being used in food and dairy industries for more than 30 years. (Brotz and Sahl, 2000).

Bacteriocins produced by LAB have the potential to cover a very broad field of application, including both the food industry and the medical sector. Concerning their use in food, bacteriocin-producing starter or co-cultures have been successfully applied in pilot-scale experiments (cheese, fermented sausage, sourdough, etc.), yielding food quality and food safety advantages. The current bottleneck hampering widespread industrial practice seems to be market implementation rather than scientific evidence or proof-of-concept. With respect to medical applications, antimicrobials produced by probiotic LAB might play a role during in vivo interactions occurring in the human gastrointestinal tract, hence contributing to gut health. (Vuyst and Leroy, 2007)

2.3.1 Mode of action of bacteriocin:

Bacteriocins can be defined as bacterially produced, small, heat-stable peptides that are active against other bacteria and to which the producer has a specific immunity mechanism. The bacteriocins that will probably have the most immediate potential in food applications will be those produced by food-grade LAB, as they are more likely to meet with regulatory approval owing to their origin, and they can be readily introduced into fermented foods without any concentration or purification. Different classes of bacteriocins have different modes of action.

In general, the elongated amphiphilic cationic lantibiotics (for example, nisin) are active through the formation of pores, leading to the dissipation of membrane potential and the efflux of small metabolites from sensitive cells. By contrast, the globular lantibiotics (for example, mersacidin) were originally defined as those lantibiotics that act through enzyme inhibition. The majority of class II bacteriocins are active (also in the nanomolar range) by inducing membrane permeabilization and the subsequent leakage of molecules from target bacteria. The two-peptide bacteriocins require the combined activity of both peptides with a mechanism of action that again involves the dissipation of membrane potential, the leakage of ions and/or a decrease in intracellular ATP concentrations. Bacteriolysins (formerly class III bacteriocins) are large, heat-labile antimicrobial proteins. They have a domain-type structure, in which different domains have functions for translocation, receptor binding, and lethal activity.Their mechanism of action is distinct from that of bacteriocins as they function through the lysis of sensitive cells by catalyzing cell-wall hydrolysis. (Cotter et al, 2005)

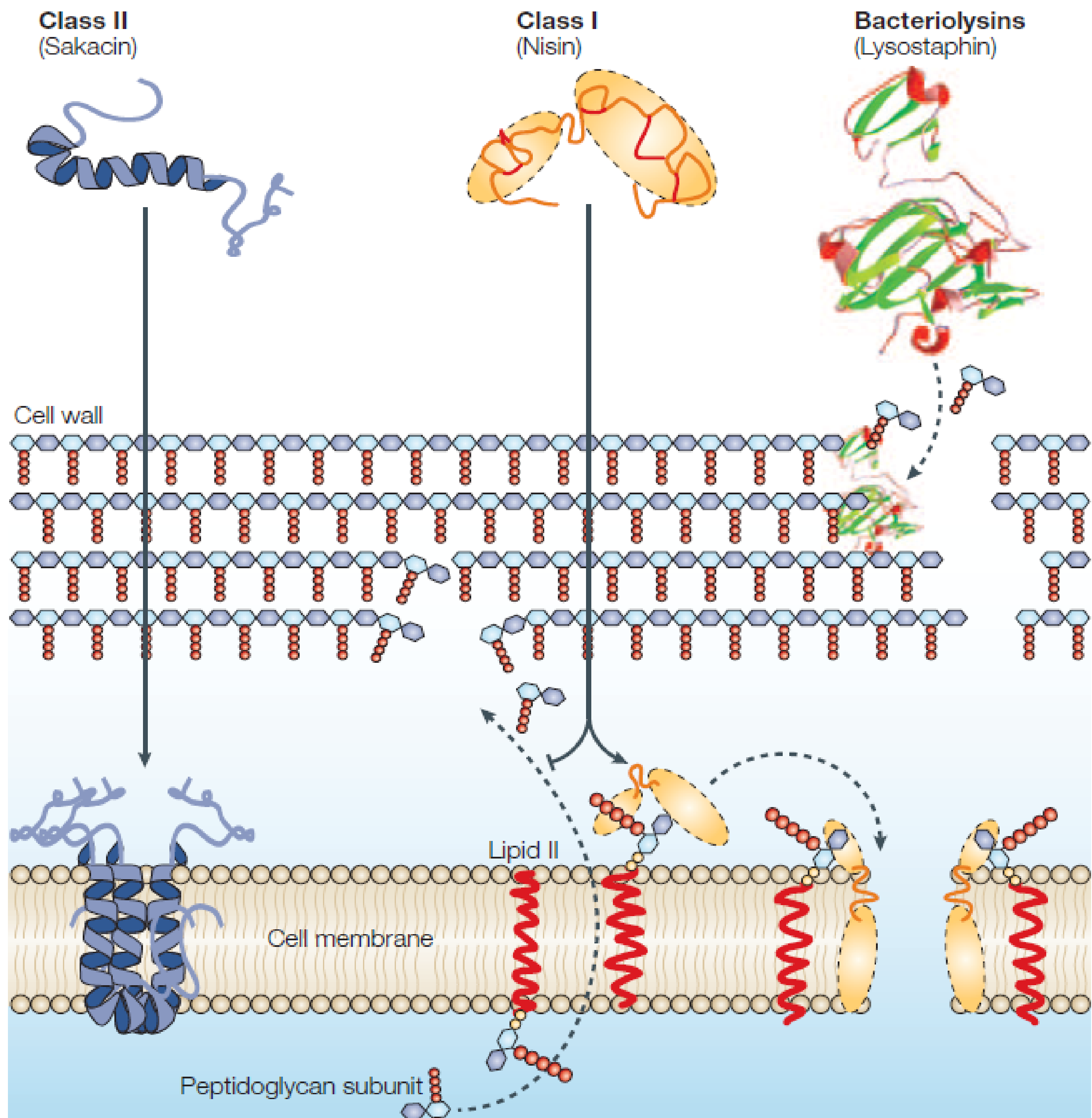


Figure 2. 7: Mode of action of lactic acid bacteria bacteriocins. Lactic acid bacteria (LAB) bacteriocins can be grouped on the basis of structure, but also on the basis of mode of action. Some members of the class I (or lantibiotic) bacteriocins, such as nisin, have been shown to have a dual mode of action. They can bind to lipid II, the main transporter of peptidoglycan subunits from the cytoplasm to the cell wall, and therefore prevent correct cell wall synthesis, leading to cell death. Furthermore, they can use lipid II as a docking molecule to initiate a process of membrane insertion and pore formation that leads to rapid cell death. A two-peptide antibiotic, such as lacticin 3147, can have these dual activities distributed across two peptides, whereas mersacidin has only the lipid-II-binding activity, but does not form pores. In general, the class II peptides have an amphiphilic helical structure, which allows them to insert into the membrane of the target cell, leading to depolarisation and death. Large bacteriolytic proteins (here called bacteriolysins, formerly class III bacteriocins), such as lysostaphin, can function

directly on the cell wall of Gram-positive targets, leading to death and lysis of the target cell. (Cotter et al, 2005)

2.3.2 Gene location of bacteriocins:

Bacteriocins may be either chromosomally or plasmidencoded. Plantaricin 423 is plasmid encoded, while plantaricin ST31 is chromosomally determined. Plasmids associated with bacteriocin production vary considerably in size. Some plasmids are known to carry the genetic determinants for several bacteriocins. Where more than one bacteriocin is produced, the bacteriocins can be plasmid (carnobacteriocin B2) and chromosomally (carnobacteriocin BM1) encoded. (Todorov, 2009). The widespread phenomenon of bacteriocin production among LAB is undoubtedly partly due to the fact that the relevant genes are often associated with transferable elements such as conjugative transposons or plasmids. (Cotter et al, 2005)

2.4 *Staphylococcus aureus*

Staphylococcus aureus are gram positive facultative anaerobic cocci bacteria which is capable of secreting several exotoxins. They are normal inhabitant of skin and mucous membrane. *Staphylococcus aureus* nasal carriage is established constantly in 20%-40% of healthy human population and intermittently in 60% and only 10%-20% of people are non-carriers (Andersen et al, 2012). They can grow at low water activity (approx.0.86) and the salt content can be upto 14%. It can be classified as,

Domain: Bacteria Kingdom
Bacteria Phylum: Firmicutes
Class: Cocci
Order: Bacillales
Family: Staphylococcaceae
Genus: Staphylococcus
Species: *Staphylococcus aureus*

As given by microbewiki *S.aureus* are the most commom cause of the *staphylococcal* infections. Staphylococcal food poisoning is caused by contamination. *Staphylococcus aureus* contamination is now a day's one of the upcoming issue in medical field. *S. aureus* can cause a range of illnesses, from minor skin infections such as pimples, impetigo, boils, cellulitis

abscesses and also life-threatening diseases such as pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacterimia and sepsis. Its incidence ranges from skin, soft tissue, respiratory, bone, joint, endovascular to wound infection. It is still one of the five most common causes of nosocomial infections and is often the cause of postsurgical wound infections. *S. aureus* causes economical loss as they are responsible for mastitis in cows, goats and sheep.(Microbewiki)

SFD is one of the most common FBD and is of major concern in public health programs worldwide. The onset of SFD is abrupt. The conclusive diagnostic criteria of SFD are based upon the detection of staphylococcal enterotoxins in food, or recovery of at least 10^5 *S. aureus* g−1 from food remnants Symptoms include hypersalivation, nausea, vomiting, and abdominal cramping with or without diarrhea. If significant fluid is lost, physical examination may reveal signs of dehydration and hypotension. Abdominal cramps, nausea, and vomiting are the most common. Although SFD is generally self-limiting and resolves within 24–48 hours of onset, it can be severe, especially in infants, elderly, and immunecompromised patients. Antibiotics are not used for therapy. (Kadariya et al., 2014)

Mostly the food intoxication by *S. aureus* is due to bad hygiene practices either in house or in industries. Foods that are involved in food borne intoxication by *S. aureus* are:

- dairy products;
- meat and meat products;
- poultry meat and egg products;
- egg salads, fish, poultry meat, potatoes and pasta;
- pastry like cream and custard cake.

2.4.1 *Staphylococcus aureus* contamination in milk

In the present study milk is taken as the substrate for co-culture as there are many cases reported for the comtamination of *S. aureus* in milk. Milk is easily contaminated by *S. aureus* because of the low hygiene during handling, processing and transportation. For example massive *S. aureus* food poisioning occurred in japan in 2000 after drinking milk, thousands of people felt ill with vomiting, nausea and diarrhoea (Food safety file: *S. aureus,* 2008). As the *S. aureus* can grow well in milk contamination becomes easy. The composition of the cow's milk is 87.2% water, 3.7% fat, 3.5% protein, 4.9% lactose and o.7% Ash. (Milk and dairy products in human nutrition, FAO, 2013)

S. aureus does not form spores but can cause contamination of food products during food preparation and processing. *S. aureus* can grow in a wide range of temperatures (7◦Cto 48.5◦C; optimum 30 to 37◦C), pH (4.2 to 9.3; optimum 7 to 7.5), and sodium chloride concentration up to 15% NaCl. *S. aureus* is recurrent in food poisoning in dairy products as the traditional dairy products are still sold by small vendors and they are not much aware of the consequences bad hygiene. According to Fooladi et. al , 2010 the enterotoxins of *S. aureus* are of 18 serological types. The type SEA and SEB are two of the most important enterotoxin that causes gastroenteritis. Also it was found that 32% of all dairy products were contaminated with *S. aureus* and contained SEA and SEB genes. In a research carried out by Oliveira et al, 2011, it was observed that the pasteurized milk also had as much counts of *S. aureus* as compared to the raw milk. There was no differences between the averages of the two and it can be accepted that the raw milk can have high counts as there is not much care taken. But high counts in pasteurized milk shows that there is in adequate hygiene during the operation and processing and also the *S. aureus is* found in the nasal cavity of human beings.

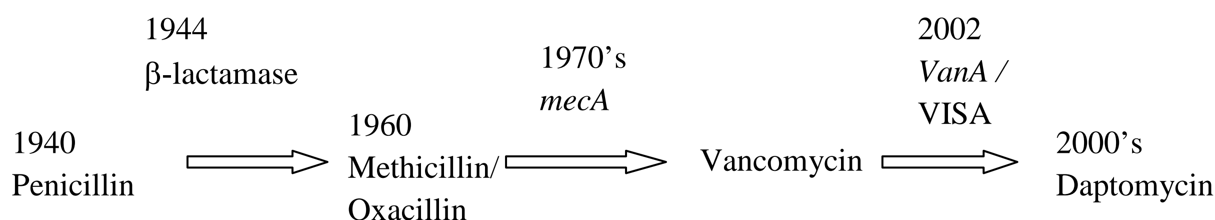
Staphylococcal enterotoxins are heat-stable exoproteins consisting from 236 to 296 aminoacids with a molecular mass of 25-35 kDa. Upon hydrolysis, 18 amino acids are present, mostly aspartic acid, glutamic acid, lysine and tyrosine. For the majority of SEs, an isolectric point of pH 5.7-8.6 is considered. Enterotoxins are resistant to proteolytic enzymes, such as trypsin, chymotrypsin, rennin and papain, but at pH of about 2, they are sensitive to pepsin.

S. aureus is a common causative agent of bovine mastitis in dairy herds. A study conducted in Minnesota to estimate the heard prevalence of *S. aureus* from bulk tank milk found that heard prevalence of MSSA and MRSA was 84% and 4%, respectively. Other studies estimated that the prevalence of *S. aureus* in bulk milk tank was 31% in Pennsylvania and 35% in cow milk samples in Louisiana. Studies from Argentina, Brazil, Ireland, and Turkey have documented the presence of staphylococcal enterotoxin genes and production of SEs by *S. aureus* of bovine origin. The udders with clinical and subclinical staphylococcal mastitis can contribute to the contamination of milk by *S. aureus* via direct excretion of the organisms in the milk with large fluctuations in counts ranging from zero to 10^8 CFU/mL. (Kadariya et al., 2014)

Natural niche of this organism is the mammalian skin, hull, and mucous membranes. The infected mammary glands of cows and other milk producing animals are the most important reservoirs. In the case of a contaminated udder, *S. aureus* is excreted into the milk during milking. In well drawn milk, its counts are from 100 CFU/ml to 200 CFU/ml, in the case of a contaminated udder, the counts may increase up to 104 CFU/ml and even to 108 CFU/ml. During the cheese manufacture, especially in the case of a slower or insufficient acidification of lump cheese, *S. aureus* can also be found in the final products. Poor performance of hygienic

precautions can lead to the contamination of thermally treated milk, and this is why it can be found in cheeses made either from raw or pasteurised milk. (Medvedova et al., 2009)

The emergence of antibiotic resistant forms of pathogenic *S. aureus* is a worldwide problem in clinical medicine. S.aureus is hetero resistance bacteria. Methicillin resistant *Staphylococcus aureus* is one of the threat to the medicine field. The antibiotic resistance of *S. aureus* has arised as follows:



As most of the antibiotics have acquired resistance against the *S. aureus* now there is the search of bacteriocins that can be effect against the pathogens. So, bacteriocins from LAB can act against the pathogens and help to decrease the contamination of pathogenic bacterias. According to Wysocki et al, 2010 when the mixture of LAB were co-cultured with MRSA , they were vulnerable to the antimicrobial action of LAB which shows that LAB can be bactericidal against pathogenic microorganism. In the study carried out by Anas et al, 2008, *S. aureus* was strongly inhibited by *Lactobacillus plantarum* and the inhibition is due to the inhibiting substance such as bacteriocin. The LAB isolated from goat's milk was effective against *S. aureus* as the crude supernatant of LAB when tested there was upto 90% death rate.

According to Charlier et al, 2009, there are various factors that influence the interactions between *S. aureus* and lactic acid bacteria which leads to inhibition of the growth of *S. aureus* such as the acidification and organic acid production, production of hydrogen peroxide, production of bacteriocins and nutritional competition. But the molecular approaches for the mechanisms involved in the inhibition of the *S. aureus* are yet to be studied using gene expression arrays. Although acidification plays an important role in *S. aureus* survival, we demonstrated here that acidification is not involved in the early *L. lactis* antagonistic potential against S. aureus growth in milk and that low-acidifying *L. lactis* strains efficiently inhibit S.

aureus growth even in milk at initial contamination levels as high as 10^3 cfu/mL. This is particularly relevant since a trend in semi-soft and soft cheese technology is to use low-acidifying LAB starters so that ripening starts faster. Numerous studies explored the effect of bacteriocin producing *L. lactis* strains on different spoiling or pathogenic bacteria in milk products. In contrast, bacteriocin-independent inhibition is poorly documented. Apart from bacteriocin production or acidification, the involvement of nutrient-related phenomena in the *S. aureus* growth inhibition was never clearly demonstrated in milk (Charlier et al, 2008). Using non-antibiotic formulations to prevent bovine mastitis can reduce the need of using antibiotics in treatment of this disease, so the problem of the emergence of antibiotic resistance pathogens can to a great extent be solved. (Soleimani et al., 2010)

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 *Lactobacillus casei* TISTR 1463
3.1.2 *Staphylococcus aureus* TISTR 029

3.2 Chemicals

3.2.1 MRS agar (Himedia, India)
3.2.3 Nutrient broth (Himedia, India)
3.2.4 Glycerol
3.2.5 Ammonium sulphate
3.2.6 DNS (3,5-Dinitrosalicylic acid**)**
3.2.7 40% Acrylamide/Bis solution (BIO-RAD,USA)
3.2.8 10X TRis/Glycine/SDS (BIO-RAD,USA)
3.2.9 Coomassie Blue G-250
3.2.10 SDS
3.2.11 Tris Hcl
3.2.12 Tetramethylethylenediamine (TEMED)
3.2.13 Laemmlli sample buffer (BIO-RAD,USA)
3.2.14 SDS-PAGE molecular weight standards (BIO-RAD,USA)
3.2.15 Lactose
3.2.16 MHA agar (Himedia, India)
3.2.17 Bardford reagent (BIO-RAD,USA)

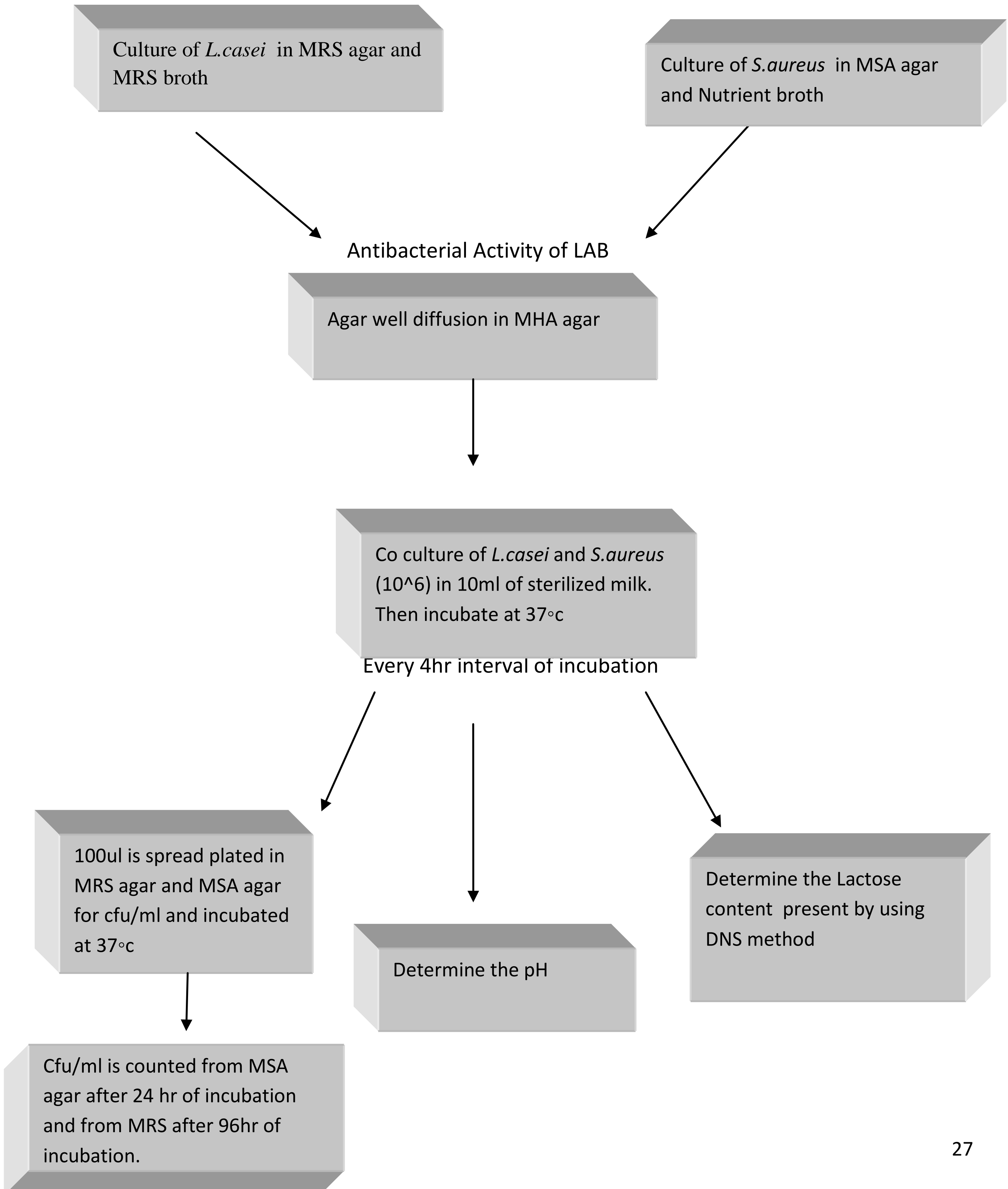
3.3 Equipments

3.3.1 Plastic petri plates
3.3.2 Glasswares
3.3.3 Microscope
3.3.4 Magnetic stirrer and magnetic bar

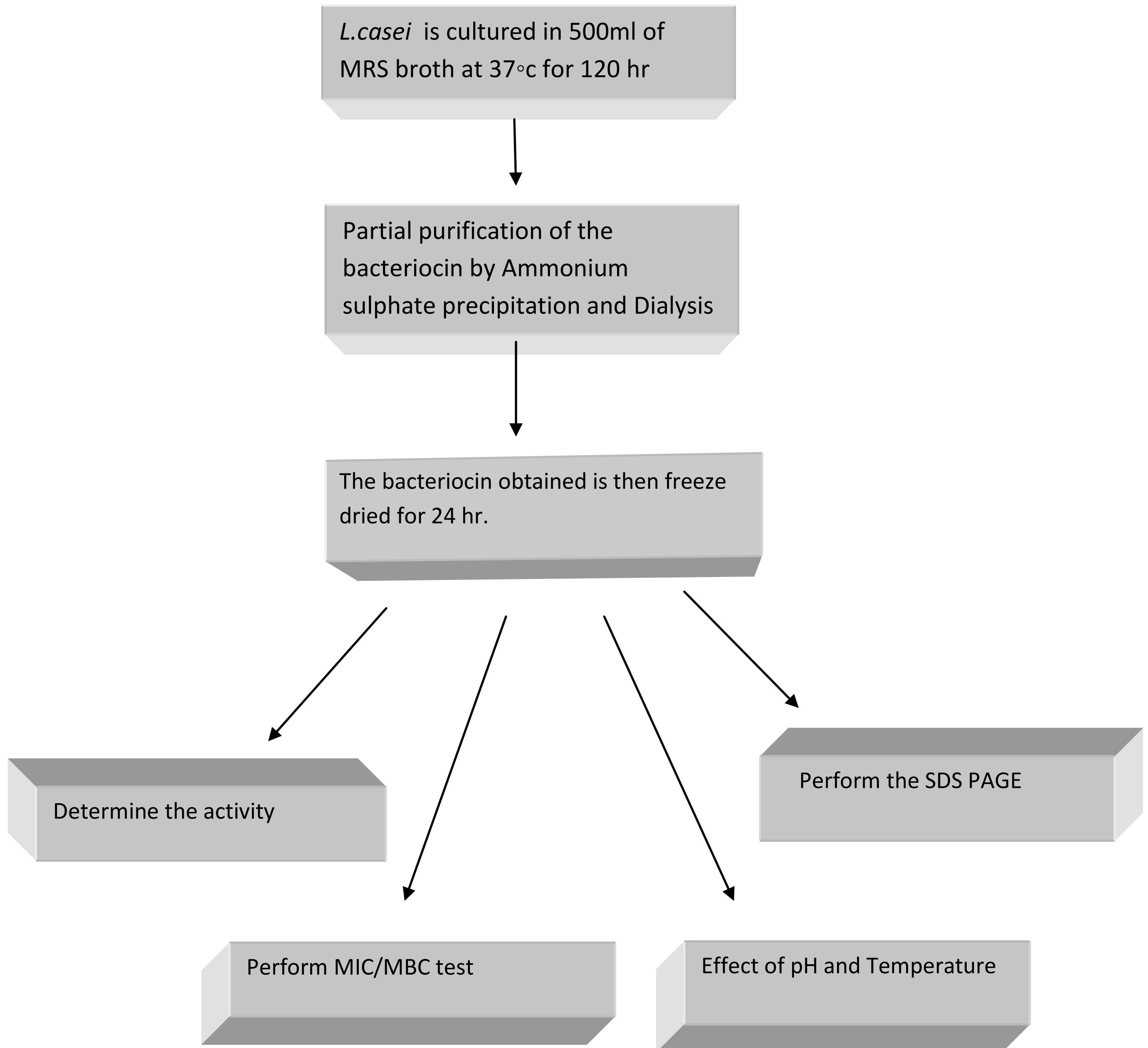
3.3.5 Autoclave
3.3.6 Vortex mixer
3.3.7 Laminar flow
3.3.8 Centrifuge
3.3.9 Incubator
3.3.10 Hot air oven
3.3.11 Balance
3.3.12 pH meter
3.3.13 Dialysis membrane 1kD MWCO (Sigma-Aldrich)
3.3.14 UV Spectrophotometer
3.3.15 Colony counter
3.3.16 Water bath
3.3.17 Freeze dryer
3.3.18 Micropipette
3.3.19 Desiccator

Flow-chart of overall experiment:

- **Co-culture of *L. casei* And *S.aureus***



- **Isolation And Partial Purification of bacteriocin from L.casei and Characterization of the bacteriocin:**



3.4 Methodology

3.4.1 Revival of the cultures of bacteria:

L. casei obtained from the TISTR (Thailand Institute of Scientific and Technological Research) lab is revived by culturing in the MRS agar and broth media. In the same way *S. aureus* obtained from the lab is revived by culturing in the MSA agar and Nutrient broth media. And before use all the culture are subcultured in 10ml medium. The inoculum used was 1% (v/v).

3.4.2 Antibacterial activity of *L. casei* against *S. aurues*:

The antibacterial activity was carried out by Agar well diffusion method in MHA agar. The culture of *L. casei* was maintained in MRS medium and was cultured for 3days and the culture of *S. aureus* was maintained in Nutrient broth. The culture of MRS was then centrifuged for 10 minutes at 10,000 rpm. The indicator strain i.e. *S.aureus* was swabbed in MHA agar plate and with the borer well of 5mm diameter was made. Now, the 100ul of the supernatant was placed in each well. The plates were left for diffusion for 30 minutes and then the plates were incubated at 37◦c for 24 hr. The antimicrobial activity was observed by formation of inhibition zone around the well.

3.4.3 Co-culture of *L. casei* and *S. aureus* in milk:

Co-culture of *L. casei* and *S. aureus* was carried out in sterilized plain milk. Also for control the individual culture of the organisms was carried out in sterilized plain milk. 10ml of the sterilized was inoculated with 10^6cfu/ml of *L. casei* and *S. aureus* for the co-culture. Whereas for the individual culture 10^6cfu/ml of each organisms were cultured in different set of sterilized milk. The cultures were then incubated at 37◦c up to 48hours. But in every four hour interval the samples were withdrawn to determine cfu/ml, pH, and the lactose content. The number of organisms were determined by measuring the OD at 600nm. 10^6cfu/ml of organisms were obtained in 0.018 absorbance.

3.4.4 Determination of cfu/ml:

The colony forming units of the test organisms (*S.aureus*) were carried out to determine their growth scenario in the co-culture condition. To determine cfu/ml of *L. casei* MRS agar plates were used and for the *S. aureus* MSA plates were used. After every 4hr interval of the incubation sample were drawn and spread plated to determine cfu/ml. 100µl of the sample was plated in solid medium. Then, the plates were incubated at 37◦c. The MRS plates were incubated for 4 days whereas the MSA plates were incubated for 24 hours. After incubation the plates with colonies were counted using the colony counter. The cfu/ml of the individual culture was also determined as above. Every test was carried out in triplicates.

3.4.5 Determination of pH of co-culture:

The change in pH was observed to see if there is any relation between pH and the co-culture of the organisms. The pH of the sample taken after every 4hour interval of the co-culture was determined using the pH meter. The change in pH of the sample was observed by plotting pH vs hours of incubation.

3.4.6 Determination of lactose content by DNS method:

The lactose content in the co-culture was determined in every 4 hour interval by the DNS method. 3,5-Dinitrosalicylic acid reacts with the reducing sugars i.e. the carbonyl groups to give the color. The absorbance is measured at 540nm. In this method 1ml of sample solution was taken in test tube and 2ml of DNS solution was added to it. Then, 7ml of water was added to the tube. The tube was heated for five minutes in boiling water and left for cooling. After cooling the absorbance was taken at 540nm and the OD was noted. The concentration of the lactose in the sample was calculated using the standard curve. The standard curve was prepared by using the lactose as standard. The concentration of lactose prepared were 0mg/ml, 0.1mg/ml, 0.3mg/ml, 0.5mg/ml, 0.8mg/ml and 1mg/ml. To each of the above concentration 2ml of DNS and 7ml of water was added. The above process described above was carried out here as well and the standard curve was plotted as Absorbance vs concentration.

3.4.7 Isolation and the purification of bacteriocin from *L.casei*:

L.casei was cultured in 500ml of MRS broth with 1% inoculum and incubated for 120hr at 37◦c with constant shaking. The inoculum was prepared by culturing 1 colony in 20ml of MRS

broth and incubated overnight at 37◦c. After the incubation the 100ml of culture was taken and centrifuged for 15 minutes at 10,000 rpm. Now, the supernatant was transferred in a beaker and the pellet was discarded. The supernatant was then precipitated with ammonium sulphate with constant stirring at 0◦c. This process was carried out for every different concentration of ammonium sulphate from 40% to 90%.

Optimization of the ammonium sulphate precipitation was done according to the protocol given in A Manual of Biochemistry Protocols. As given below,

Table 3.1: The amount of ammonium sulphate required for a solution to give desired Final saturation at 0 ◦C

Initial concentration of ammonium sulfate	Percentage saturation at 0°																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	Solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

The precipitated solution was left with stirring for 2 hours so that the protein can completely precipitate after final addition of ammonium sulphate. Then the solution was centrifuged at 10,000 rpm for 30 minutes. After centrifugation pellet is taken and the supernatant was discarded. The pellet was dissolved in 5ml of 0.1M phosphate buffer of pH 7.

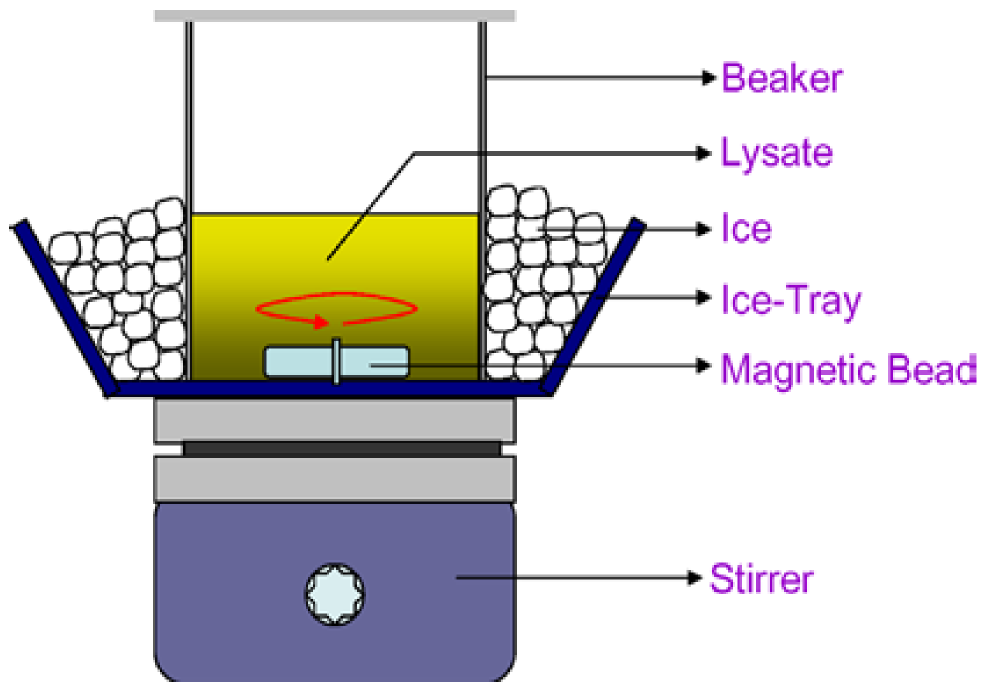
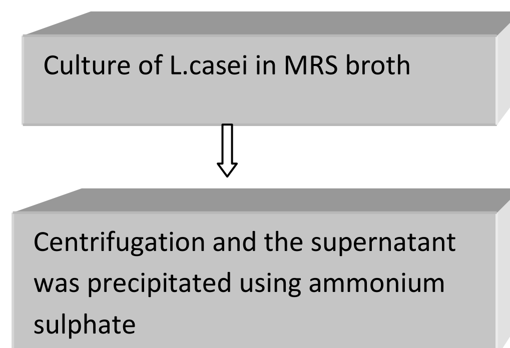


Figure 3.1: Protein Precipitation using ammonium sulfate.

The dissolved pellet solution was used for the dialysis. The dialysis was carried out at 4◦c for 12 hours with constant stirring using 1,000 MWCO dialysis bag. The dialysis was done against the same buffer with 100X volume.



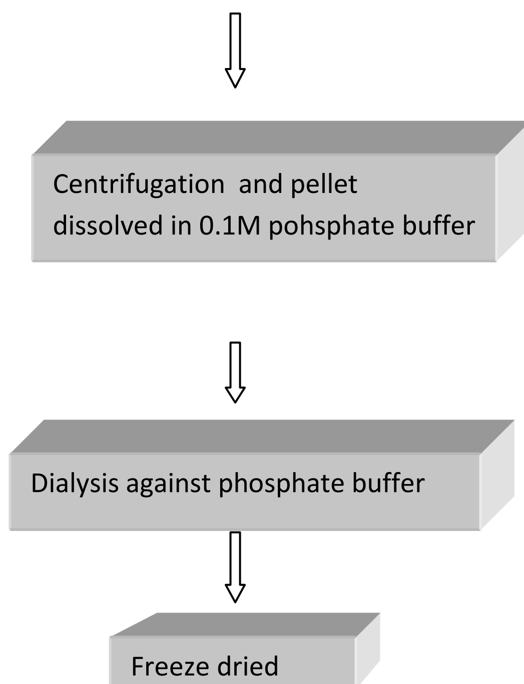


Figure 3.2: Flow- chart of partial purification of bacteriocin

3.4.8 Freeze-drying:

The dialysed solution was then filtered using 0.2 µm pore size filters and then the filtered solution was used for freeze drying. The solution obtained after dialysis was kept in freezer overnight and then taken for vaccum freeze-drying which was carried out for 24 hours or until when extract was in powdered form. Now this extract of bacteriocin was kept in refrigerator until use. The extract was dissolved in 0.1M phosphate buffer with pH 7 while using for its characterization.

3.4.9 Determination of inhibitory activity of bacteriocin

The inhibitory activity was determined and expressed in AU/ml (Arbitrary unit) tested by two fold serial dilutions. AU is defined as the reciprocal of the highest dilution producing a clear zone of growth inhibition of the indicator strain. It is calculated as

AU/ml = (1000/10)D

where D was the dilution factor.

Here, the bacteriocin containing solution was taken and two fold serial dilution was carried out. The *S. aureus* was swabbed in MHA agar plate and the well was made with a borer. The diluted solution was kept in the wells then left for 30 minutes for diffusion. Then the plates were incubated at 37◦c for 24hr.

3.4.10 Estimation of the Protein content by Bradford Assay method:

Coomassie Brillant Blue G-250 dye binds selectively to arginine and aromatic residues in the protein or peptides. So, the concentration of dye that reacts gives the protein concentration. The absorbance is measured at 595 nm. The dye is first of all diluted to 1:4 and filtered by using Whatman filter paper to make working dye solution. 100ul of sample was into the testtube and 5ml of working dye solution was added. Then it was left for 15 mins and the absorbance was taken at 595 nm. The OD value was used to find the protein concentration using the standard curve. The standard curve was prepared by using standard BSA solution. The1mg/ml stock solution of BSA was prepared by dissolving 0.02 g of BSA in 20ml of distilled water. Then the diluted solution was also prepared as 1mg/ml, 0.8mg/ml, 0.6mg/ml, 0.4mg/ml, and 0.2mg/ml. Then, 5 testubes with 5ml of working dye solution was taken and added 0.1ml of each concentration of BSA. Now, the tubes were vortexed and let for 15mins then, absorbance was taken. The standard curve was prepared by plotting the OD value vs concentration.

3.4.11 Effect of pH on bacteriocin:

5ml of the bacteriocin solution was maintained at various pH values. The pH values of 4, 6, 7, 9 were used wich was maintained by the use of NaOH or HCl. They were incubated for 1 hour and then tested for their activity. Then, the test organism was swabbed in the MHA agar and wells of 5mm were made with the borer. After that 100µl of the various pH solution were kept in the wells and left for 30 minutes for diffusion. Now, the plates were incubated at 37◦c for 24 hours and the zone of inhibition was noted.

3.4.12 Effect of temperature on bacteriocin:

5ml of bacteriocin solution was taken and heated at 70, 80, 90,100, 121 ◦C for 15minutes in the water bath. Then the treated samples were tested for the antimicrobial activity as done for the pH as above using agar well diffusion method.

3.4.13 Determination of MIC/MBC:

MIC and MBC was carried out by the serial dilution method .The minimum inhibitory concentration (MIC) is the highest diluted concentration at which there is no visible growth of the indicator strain or the lowest concentration that inhibits the growth. Minimum bactericidal concentration (MBC) is defined as the highest diluted concentration that kills 99.9% of the indicator strain.

To determine MIC/MBC two fold serial dilution of the bacteriocin solution was carried out. Here, 10 test tubes were taken and numbered in sequence. 2ml of the bacteriocin solution was added to the first tube and 1ml of sterile nutrient broth was added to all the other tubes. Then 1ml from first tube was transferred to the second tube and the content is mixed. Then 1ml from second tube was transferred to the third tube and the process is carried out till the ninth tube. 1ml is discarded from ninth tube. Now, 10^6 /ml number of test organisms were inoculated in every tube from 2-10. Tube number 1 and 10 are considered as control. Then, all the tubes were incubated at 37◦c for 24 hours. After incubation the tubes were observed and the tube with highest dilution having no visible growth was noted as MIC value. Now, for the MBC value 200µl from tubes with no visible growth was spread plated in solid agar medium. Then the plates were incubated for 24 hours at 37◦c. After incubation the plate having no growth with highest dilution was recorded as the MBC value. All the test were carried out in three replications.

3.4.14 Estimation of the molecular weight of bacteriocin by SDS-PAGE:

Tris-glycine SDS polyacrylamide gel electrophoresis was carried out to find the weight of unknown bacteriocin. It was carried out in 12% gel which was maintained by mixing acrylamide and bis acrylamide in proportion. SDS is a reducing agent and it forms SDS polypeptide complexes which migrate through the gel according to the molecular weight of polypeptide. When known markers are used along the weight of the polypeptide can be estimated. SDS- PAGE requires the protein to be denatured to their constituent polypeptide chains, so that it is limited in the information it can provide. ß-mercaptoethanol was used for

denaturing the protein present in the solution. The molecular weight of the denatured protein is estimated by this process.

The SDS-PAGE was carried out by first preparing the resolving gel and stacking gel. 5% stacking gel and 12% resolving gel was prepared. For the preparation of sample laemmli sample buffer by mixing 5µl sample, 4.75µl 2X laemmli sample buffer and 0.25µl ß-mercaptoethanol. The samples were heated at 90◦c for 5 minutes before heating. Then the samples were loaded and gel was run until the loading dye reaches the end of the gel. The electric potential created between the electrodes helps to move the protein from cathode to anode (Sambrook and Russell, 2001). After that the gel was stained with coomassie blue for 30 minutes and then destained for 2hours. Then the gel was visualized. The molecular weight was determined by comparing the bands with the marker bands.

The bacteriocin band was determined by using the bioassay method. In this method the SDS-PAGE gel after running was laid in the lawn of *S.aureus* over the MSA plate. Then the plates were incubated overnight. The area around where the inhibition zone was formed was noted then compared with the bands of the SDS-PAGE to determine the band of the bacteriocin.

CHAPTER 4

RESULTS

4.1 Antimicrobial Activity:

The antimicrobial property of *L. casei* was tested against *S. aureus* was carried by agar well diffusion method. This was done to know if *L. casei* has antimicrobial activity against *S. aureus*. The formation of inhibition zone around the culture shows that *L. casei* is effective against *S. aureus.* 96 hours culture supernatant was used to test its activity against *S. aureus*. The zone of inhibition formed were found to be of average size of 15.6 mm.

Table 4.1: Zone of Inhibition formed by *L. casei* culture supernatant

Replication	zone of inhibition(mm)			Average
R1	15	16	15	15.33
R2	16.5	15	16	15.83
R3	16	15.5	16	15.83

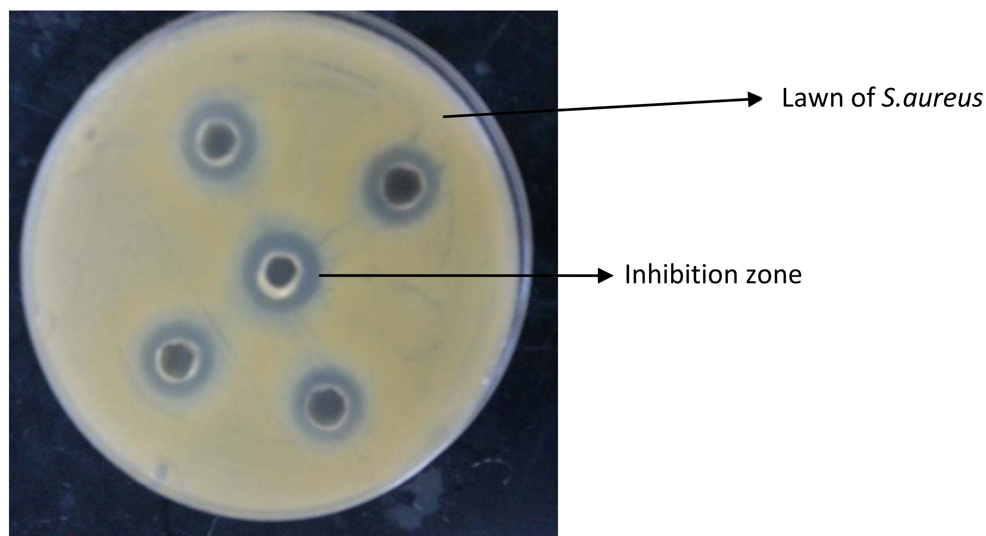


Figure 4.1: Zone of inhibition formed by *L. casei* Culture Supernatant. All the five wells contained culture supernatant of *L.casei.*

4.2 Co-culture of *L. casei* and *S. aureus* in milk:

The co-culture of *L. casei* and *S. aureus* was carried out in milk. Milk was taken as substrate for culturing to study the changes when cultured. The co-culture was done for 48 hours after incubation and every four hour interval tests were performed. 10^6cells/ml were inoculated initially. Simultaneously, the individual culture of *L. casei* and *S. aureus* was also done separately as controls. The changes in cfu/ml, change in pH and the change in Lactose content was studied.

- Change in colony forming units:

There was smooth growth of *S. aureus* when individually cultured in milk. The cfu/ml was increased with the increasing incubation time. Also the growth of *L. casei* was increased with the incubation time which can be shown below. But in co-culture medium the log cfu/ml of the *S. aureus* was decreasing as the hours of incubation was increasing. The log cfu/ml was decreased sharply after 28 hours of incubation. As a whole there was decrease of log cfu/ml from 6 to 3.8 after 48 hours of incubation. While the growth of the *L. casei* was not much affected. The decrease in log cfu/ml of *S. aureus* shows that there is the antimicrobial activity of *L. casei* against *S. aureus* when cultured in milk.

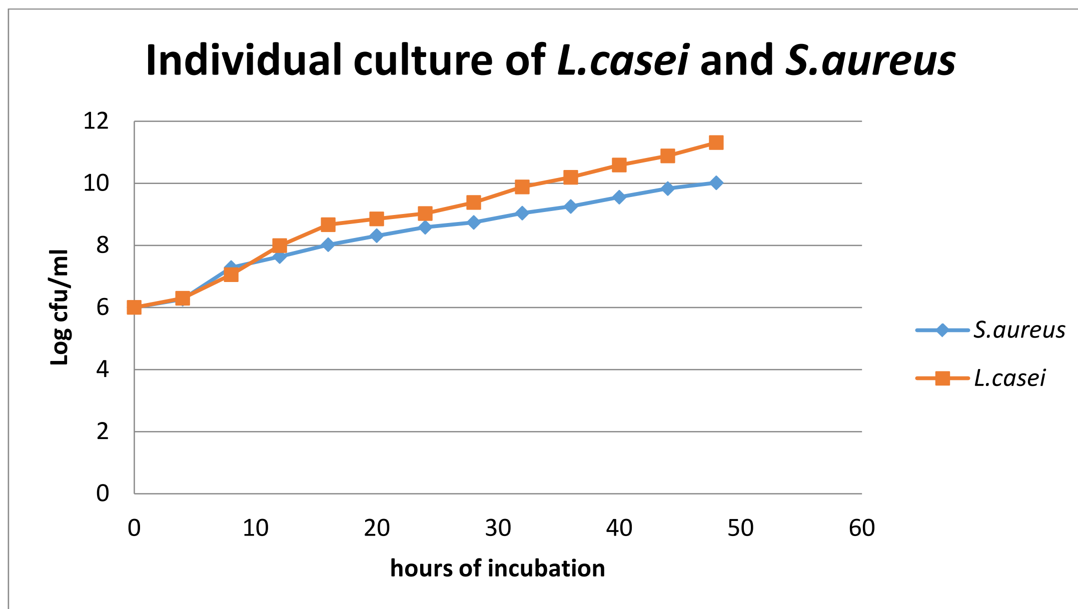


Figure 4.2: Individual culture growth curve of *L. casei* and *S. aureus* in milk. The colonies were counted from MRS agar for *L.casei* whereas MSA agar was used for *S. aureus*.

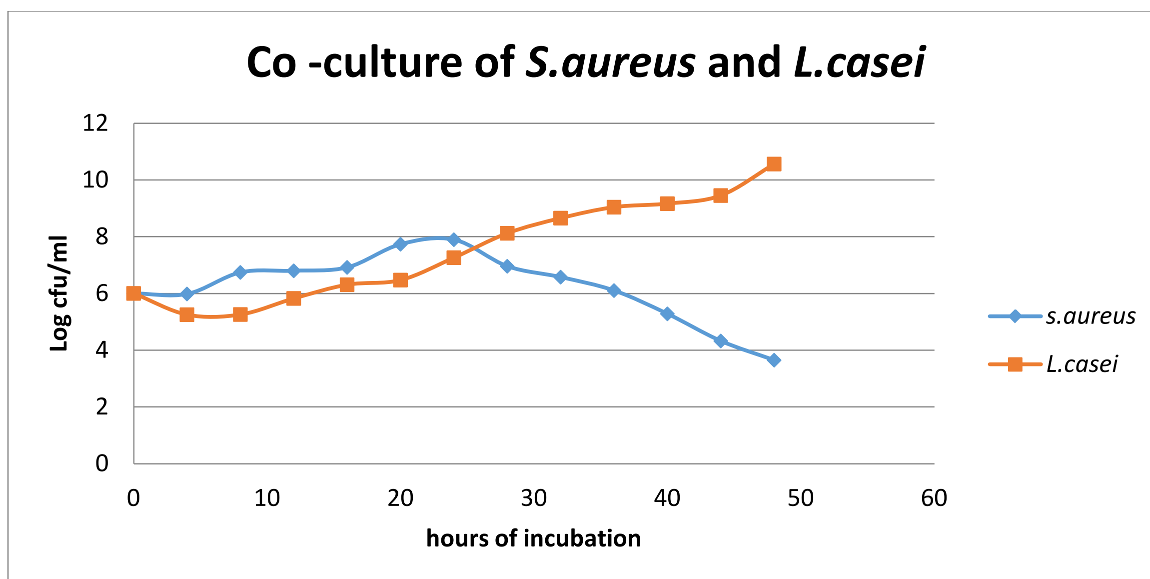


Figure 4.3: Growth of *L. casei* and *S. aureus* when co-cultured in milk. The graph shows that there was decrease in the cfu/ml of the *S.aureus* when co-cultured with *L.casei.*

Hence, the % inhibition was determined by using the following formula:

$$\% \text{ Inhibition} = \frac{(\log \text{CFU/ml in control}) - (\log \text{CFU/ml in co-incubation culture})}{(\log \text{CFU/ml in control})} \times 100$$

The % inhibition was found to be 64%. The co-culture of *S. aureus* and *L.casei* in sterilized milk showed that the 64% of the test organism *S. aureus* was found to be inhibited within the 48 hours of continuous co-culture. According to Soleimani et al,2010 as well the % inhibition of lactobacilli varied from 68- 72%.

- Change in pH:

  The change in pH of the co-cultured medium i.e. milk was noted every 4 hour interval. Initially the pH of the milk was found to be 6.34 after sterilization and as the incubation hour increased the pH was found to be decreased. So, the final pH after 48 hours of incubation was 5.47. The decrease in pH of the medium was because of the lactic acid produced as the lactose was degraded.

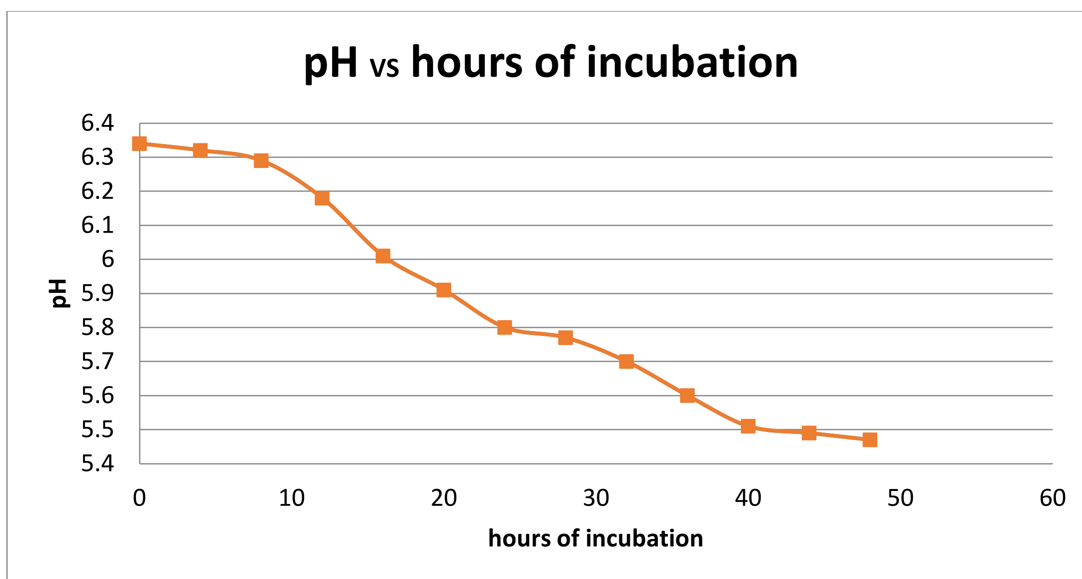


Figure 4.4: Change in pH when *L.casei* and *S.aureus* co-cultured. The pH decreased with increased time of incubation because of the production of metabolic products like lactic acid.

- Estimation of lactose content of co-culture medium:

  The lactose content of the co-cultured medium was estimated by the DNS method. In this method the reducing sugar is measured spectrophotometrically at 540 nm. Lactose is also the reducing sugar whose concentration was estimated by comparing with the standard curve. The Standard curve was prepared of the standard lactose solution. The concentration of lactose was found to be decreased as the incubation of co-culture increased. The initial concentration of lactose was found to be 77.9mg/ml and it was decreased to 60.3 mg/ml after 48 hours of incubation.

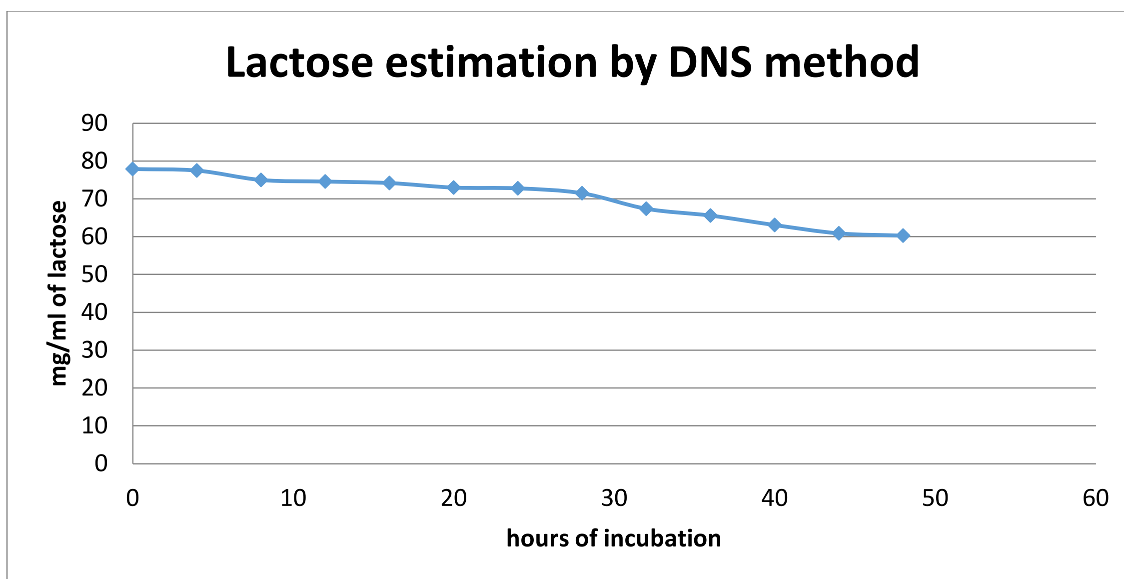


Figure 4.5 Lactose concentration in co-culture medium. The lactose content was decreased with increased time of incubation as lactose was utilized by the microorganism.

4.3 Optimization of Antimicrobial Culture:

The culture of *Lactobacillus casei* in MRS was incubated for different incubation period to find antimicrobial activity. The inhibitory activity was done by agar well diffusion method using *S.aureus* as test organism.The antimicrobial test of the cell free supernatant was carried out in every 24 hours of incubation. It was found that the maximum antibacterial activity was found at 120 hrs of culture incubation time as there was highest zone of inhibition which was 17.8 mm. Before and after this incubation period there were smaller zone of inhibition as compared to 120 hrs of incubation at 37◦c. This was done to optimize the hours of incubation at which there was maximum antimicrobials production. It was done in three replications and average value was taken.

Figure 4.6: Zone of inhibition in MHA plates. Incubation time was differed in the culture of *L.casei* and the zone of inhibition of each was measured and 120 hr incubated culture gave the highest zone of inhibition as compared to others.

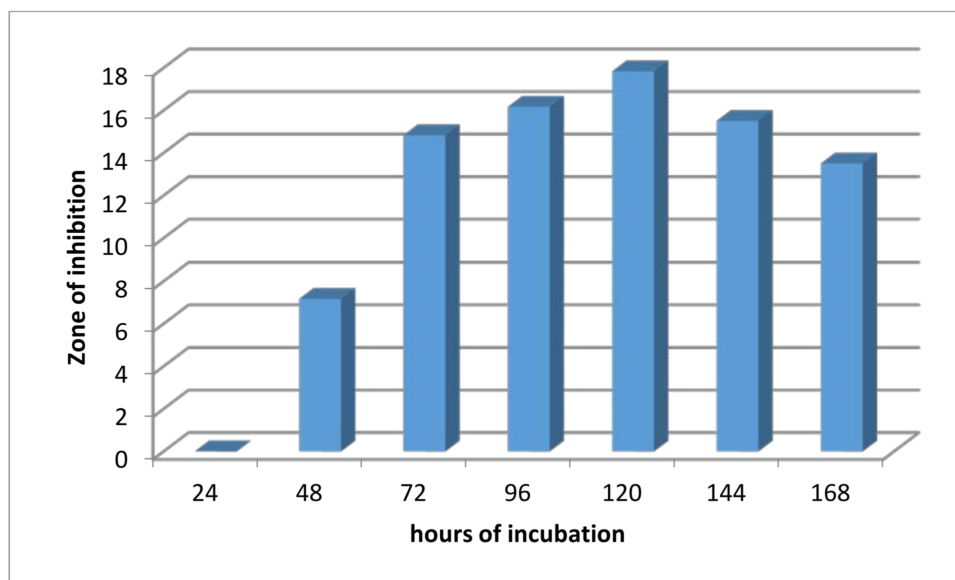


Figure 4.7 : zone of inhibition at different hour of incubation. The average zone of inhibition from triplicates was measured and plotted against incubation time. The average zone of inhibition of the 120hr culture was highest with 17.8mm.

4.4 Protein precipitation by ammonium sulphate:

The Bacteriocin in the cell free culture supernatant was precipitated by using the various saturation of the Ammonium sulphate. The culture of *L.casei* grown for 120 hrs was used for precipitation after centrifugation. The precipitation was carried out using the 40%, 50%,60% , 70% 80% and 90% saturation concentration of ammonium sulphate. The pellet obtained after precipitation followed by centrifugation was dissolved in 0.1M phosphate buffer of pH 7. The agar well diffusion test of the final solution was done to know the best concentration that gives highest inhibitory activity. After agar well diffusion it was found that the 70% saturation concentration of ammonium sulphate gave the highest zone of inhibition of 9 mm. 80% precipitation gave zone of inhibition of 5.8 mm. And there was very less zone of inhibition of 60% saturation concentration whereas all other concentrations did not give any zone of inhibition.

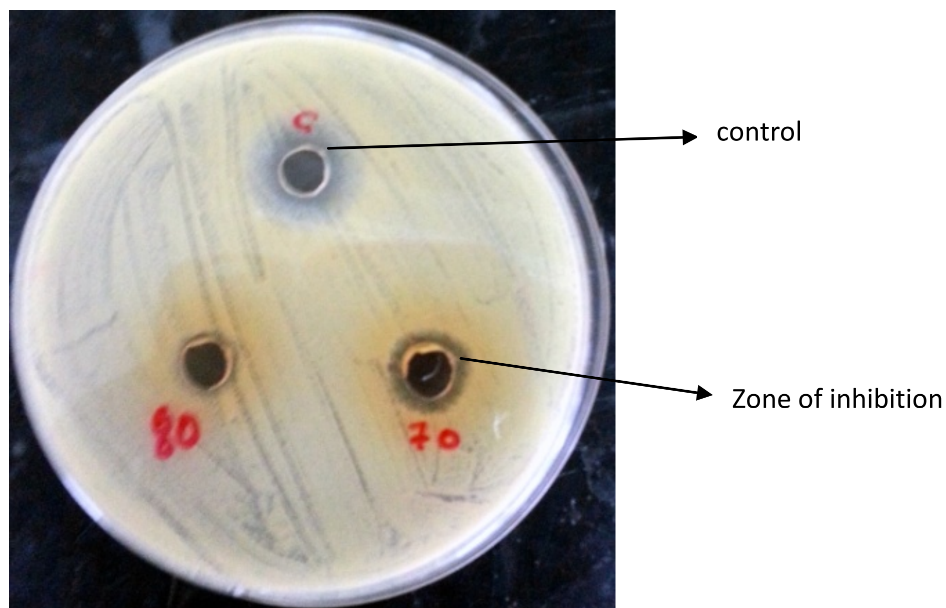


Figure 4.8: Zone of inhibition after precipitation. The samples precipitated with 70% concentration of ammonium sulphate gave the highest zone of inhibition. Whereas 80% concentration gave negligible zone of inhibition.

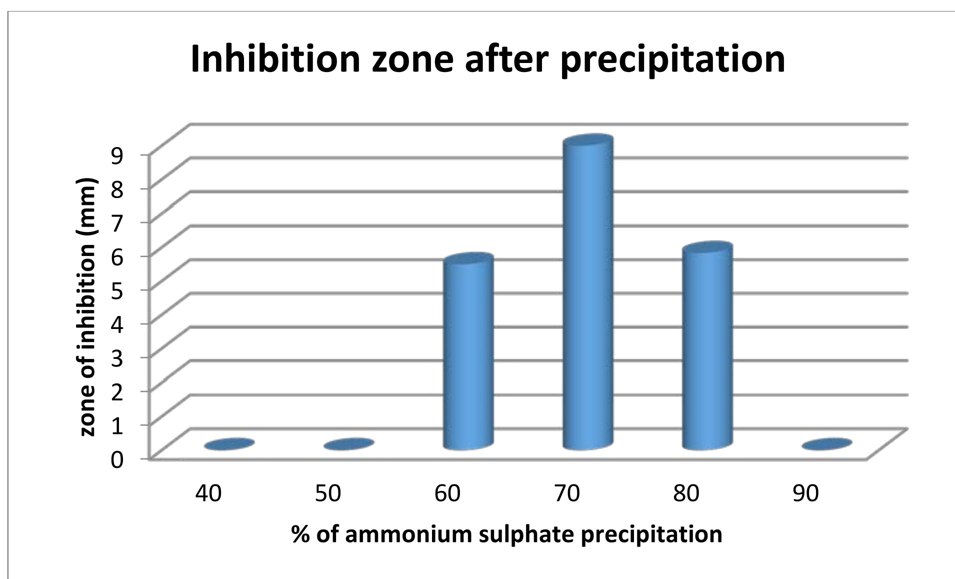


Figure 4.9: Inhibition zone by different concentrations of ammonium sulphate. The average zone of inhibition of three replication were measured and plotted.

4.5 Partial purification by dialysis and freeze-drying:

Dialysis was done for the pellet obtained after ammonium sulphate precipitation. The pellet obtained was dissolved in ph 7 0.1M Phosphate buffer and then dialysed against the same buffer for 12hr with 100X volume at 4◦C. During the dialysis the smaller molecules are dialysed out of the dialysis membrane so the protein can be purified. The dialysis membrane used was of MWCO 1000 kDa so the ions of ammonium sulphate were dialysed out in this process giving ammonium sulphate free bacteriocin. After the completion of dialysis the solution in dialysis membrane was syringe filtered through 0.22µm of filter paper so that all the contaminants during the process were filtered and the clear solution was obtained. The solution obtained after the dialysis was used for the estimation of protein and freeze-drying.

The dialysed bacteriocin is partially purified which can be used for its characterization but convert it to the powdered form the bacteriocin solution was freeze-dried by the freeze dryer for 24hours. Powder form of the bacteriocin was obtained which was used for characterization of the bacteriocin such as for MIC/MBC and SDS-PAGE. From 5ml of solution dried 0.8mg of powder was obtained.

4.6 Protein estimation

The protein estimation was carried out by Bradford assay method using BSA standard curve. The protein content was estimated in the cell free supernatant of the culture of *L.casei* and the bacteriocin solution obtained after dialysis. Bradford reagents react with the protein to give the coloration which was measured at 595nm. The protein was found to be 0.41 mg/ml in culture supernatant and 2.49mg/ml in solution after precipitation and dialysis. The total protein after dialysis was found to be 12.45 where as the total protein in culture supernatant was 41. The partially purified solution of bacteriocin contained less protein as the proteins only precipitated at 70% were present in there. In others studies as well it was found that the total protein after dialysis was less as all the proteins from the supernatant are not precipitated. Therefore 30% recovery of the protein was made.

Table 4.2 Protein estimation in different solution

S/N	Sample	Volume (ml)	OD	Dilution factor	Protein (mg/ml)	Total protein
1	Culture supernatant	100	0.451	1	0.41	41
2	After ammonium sulphate precipitation and dialysis	5	0.287	10	2.49	12.45

4.7 Bactoriocin activity

The inhibitory activity of the bacteriocin was measured by serial two fold dilution method. The diluted bacteriocin solution was used for the inhibitory activity by agar well diffusion method using *S.aureus* as test organism . The inhibitory activity is expressed in AU/ml (arbitrary unit). The inhibitory activity of the crude culture cell free supernatant was found to be 1600 Au/ml whereas the inhibitory activity of the solution after dialysis was found to be 800 AU/ml. The inhibitory activity after dialysis is less as compared to crude sample because the crude sample has many other antimicrobials which show inhibition to the test organism. And the dialysed solution only contains the bacteriocin which acts as antimicrobial.

Table 4.3 Inhibitory activity of different samples

S/N	Sample	Inhibitory activity (AU/ml)
1	Culture Supernatant	1600
2	After dialysis	800

4.8 MIC/MBC test

The freeze dried sample of bacteriocin was used for performing MIC/MBC test by using tube dilution method. The bacteriocin found effective against *S.aureus* will have no visible growth in the tubes. The third tube of the serial dilution had no visible growth which means that the concentration of the baceriocin in the third tube was the MIC value. So, the MIC value was 75 ng/ml of the partially purified bacteriocin. Then the tubes with no visible growth were plated in MSA plates. And it was observed that there were some colonies in the plates having the solution from third tube whereas the plates plated from second tubes had no colonies. This gives that the second tube concentration of bacteriocin was the MBC value which was 150 ng/ml. The MIC and MBC value of the bacteriocin was high because the bacteriocin was only partially purified and the pellet obtained also contains other proteins as well which will add the weight. If the bacteriocin was more purified than definitely the value could have decreased.

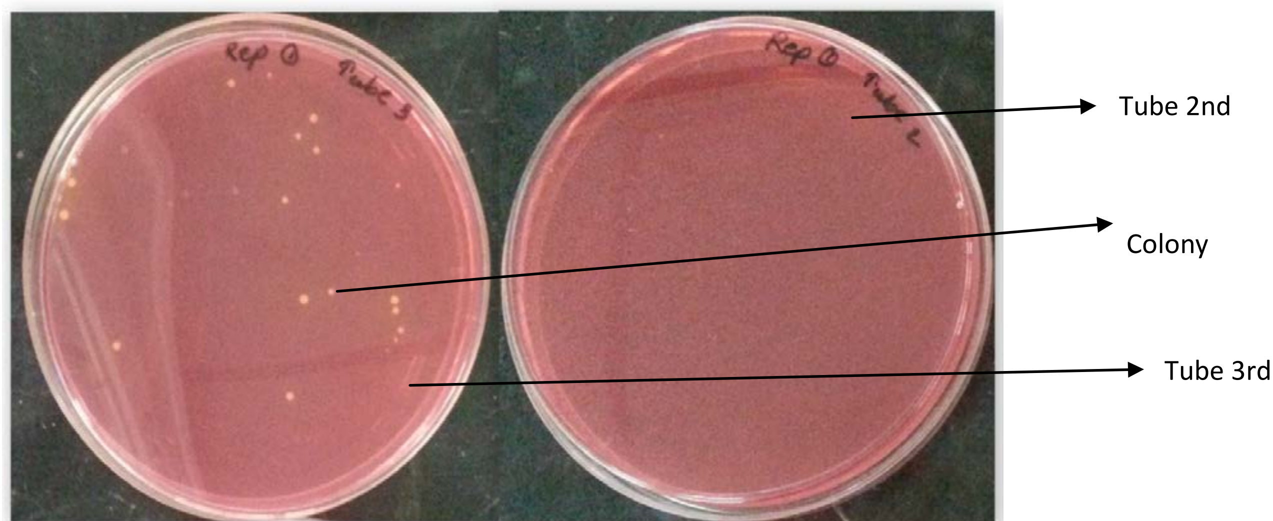


Figure 4.10: Plates showing colonies. Right side plate had concentration of bacteriocin of 75ng/ml (MBC) and left side plate had concentration of bacteriocin of 150ng/ml (MIC).

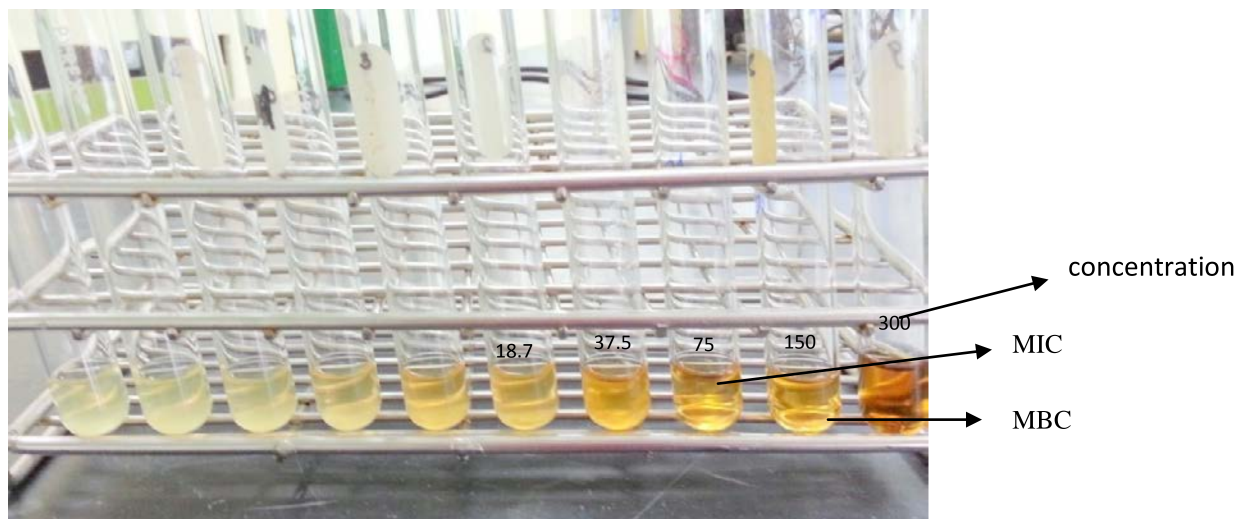


Figure 4.11: Tubes showing MIC/MBC. Concentration of the bacteriocin is given in each tube in ng/ml.

4.9 Estimation of the molecular weight of bacteriocin:

The molecular weight of the partially purified bacteriocin was estimated by Tris-glycine SDS-PAGE. The samples loaded in the wells of the gel are moved along the electric potential between the electrodes. SDS forms complexes with the peptides and this complex migrate through the gel according to the molecular size. The molecular weight of the peptide was estimated as compared to the molecular ladder which was also run along with the sample. The molecular weight of the partially purified bacteriocin was found to be 36 kDa as there was effective band found at 36kDa area according to the molecular marker. The molecular weight of the bacteriocin was higher than the class I and class II bacteriocin so the bacteriocin can be of class III bacteriocin as it has higher molecular weight and it is heat labile. Also, Gautam and Sharma, 2009, had determined the molecular weight of bacteriocin produced from *L. brevis* to be 93.74 kDa. And also they said that the molecular weight of *L. helviticus* 481 and *L. brevis* SD27 had molecular weight of 37 and 52 kDa respectively which can be characterized as class III and class IV bacteriocin.

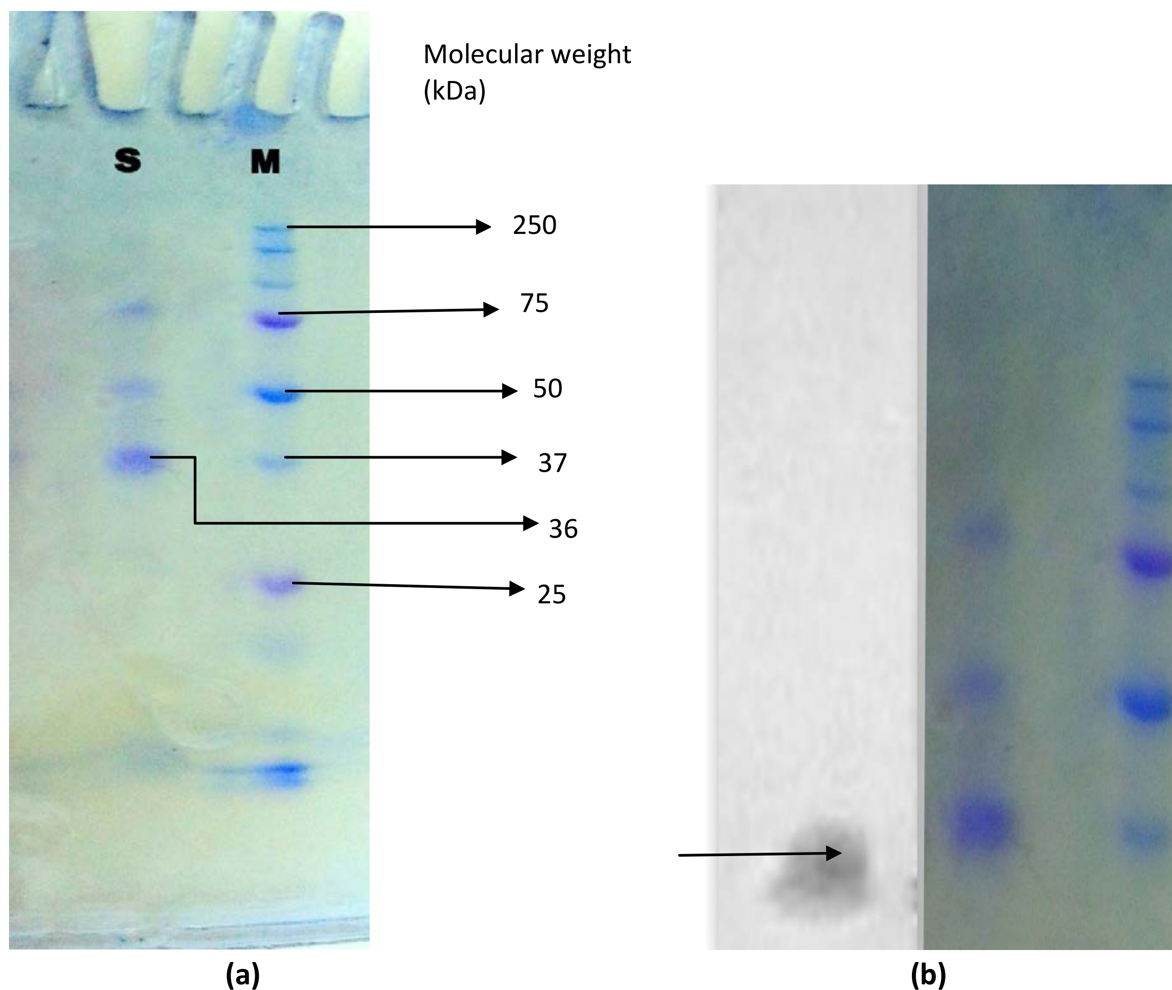


Figure 4.12: (a) SDS-PAGE showing marker and sample (S- Sample, M- Marker). **(b)** Bioassay showing the band that has the bacteriocin, the band that gives the inhibition effect is the bacteriocin which is shown with the arrow. 36 kDa was the molecular weight of the bacteriocin that gives inhibition.

4.10 Effect of pH and Temperature on bacteriocin:

The bacteriocin was exposed to various pH and temperature and the antimicrobial activity against *S.aureus* was observed. pH is essential for the activity of a bacteriocin. The bacteriocin isolated was dissolved in different pH phosphate buffer and the activity was determined by measuring the zone of inhibition by agar well diffusion method. It was found that bacteriocin obtained was effective between pH range from 4-9. But the maximum activity was obtained at pH 7. The activity was lower as the alkalinity of the buffer was increased so it can be said that the bacteriocin was more active when acidic than when alkaline. Still the

bacteriocin activity is shown with both at alkaline and acidic environment so can be used as preservatives with both kinds of food stuffs. But usually the bacteriocin acitivity is higher at neutral pH according to others as well. (Yusuf et. al., 2012) (Messens et al., 2002)

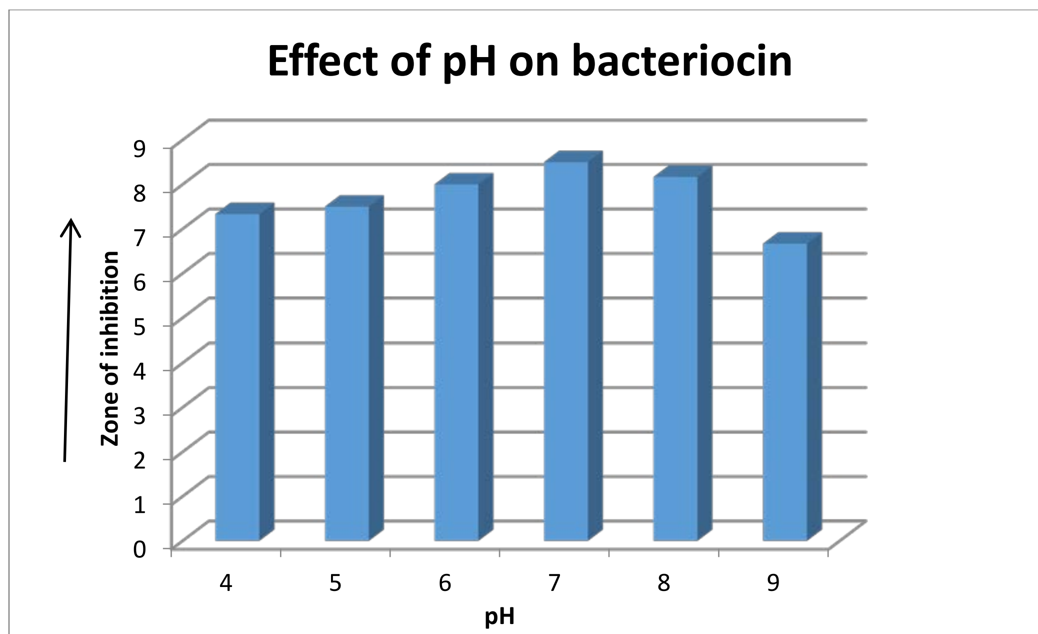


Figure 4.13: Effect of pH on Bacteriocin. Bacteriocin was effective at both acidic and alkaline environment. pH 7 was the optimum pH for the activity of the bacteriocin.

The bacteriocin activity was observed at different temperature by heating for 10 minutes. The temperature as well affects the activity of bacteriocin. The bacteriocin when heated it was not found active at every temperature. The activity was found to be decreased as the temperature increased. The heat treatment was done from 70◦c to 120◦c. And the bacteriocin gave higher inhibitory activity at lower temperature. At 70◦c the inhibitory activity was good and there was no inhibitory activity at 120◦c. So, the bacteriocin can be considered as heat labile bacteriocin. The effect of temperature shows if the bacteriocin is heat labile or not and how much temperature it can tolerate and can give the inhibition reaction. If the bacteriocin is heat labile it can be used only for the food products with low temperature. According to other literatures as well some bacteriocins from lactobacilli are heat labile so, can be degraded at high temperature. But still they have good inhibitory activity at medium and low temperatures.

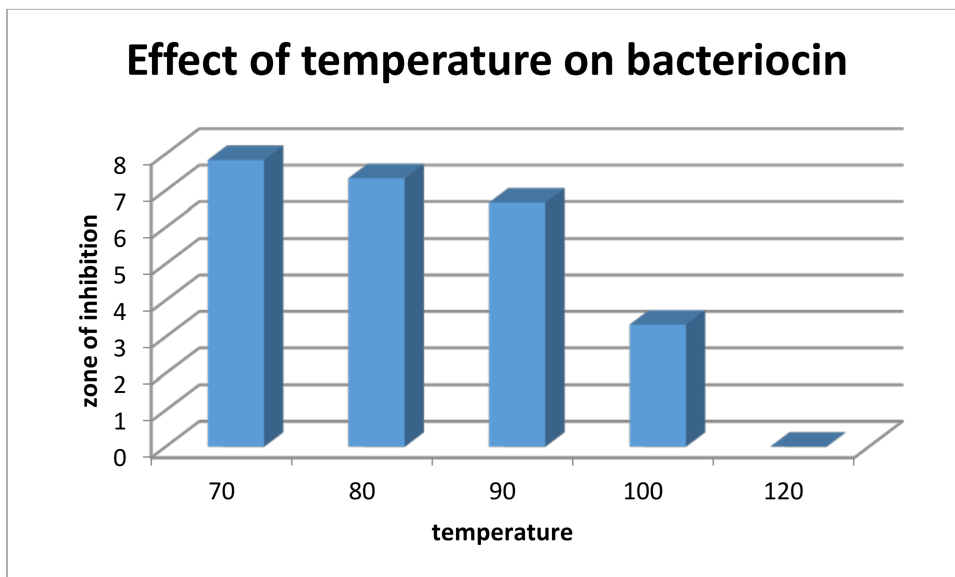


Figure 4.14: Effect of temperature on bacteriocin. The bacteriocin had antimicrobial activity upto the temperature of 100◦C. Whereas above that temperature it lost its activity.

CHAPTER 5

DISCUSSION

Antimicrobial activity:

The antimicrobial activity of the *Lactobacillus casei* was determined by the method of agar well diffusion and it was found to be inhibitory against test organism *Staphylococcus aureus.* The zone of inhibition was observed when the culture supernatant of the *L. casei* was used for the test. The inhibitory activity was because of the antimicrobial substances produced by the probiotic bacteria. The antimicrobial substances were released in the culture supernatant so they were extracellular. The antimicrobial substances which were active for the inhibitory effect to the growth of *S. aureus* can be the organic acids, hydrogen peroxide, diacetyl, oxidases and bacteriocins. These all show the combined effect against the test organism.

Co-culture of *Lactobacillus casei* and *Staphylococcus aureus* in milk:

The sterilized plain milk was used as substrate for the co-culture of *L. casei* and *S. aureus.* The milk was used for the experiment so that the probability of using *L. casei* in milk as preservative as well as probiotic. If *L. casei* is effective against *S. aureus* when cultured in milk it can be used as the preventive measures for decreasing the risk of effect of *S. aureus* food poisioning. The plain milk when sterilized was used for co-culturing the microorganisms. The co-culturing was done continuously for 48 hours to study and observe the changes. In every four hour interval the changes such as cfu/ml of the organisms was determined, the change in pH and the change in lactose content was determined. It was found that the number of colonies was decreasing of *S. aureus.* There was distinct decrease in cfu/ml of *S. aureus* after 28 hours of incubation. This is because of the antimicrobial compounds produced by *L. casei* which were effective and responsible for growth reduction of *S. aureus*. As compared to the individual culture of S. aureus in milk in co-culture there was 64% inhibition after 48 hours of continuous culture. This gives that the 64% of the *S. aureus* was inhibited when co-cultured, so there was 64% decrease in the number of microorganisms as compared to the control individual culture. It was seen that the cfu/ml of *S. aureus* was constantly increasing when cultured individually which shows that it grows well enough in milk so has high chance of contamination. Here we can observed that if *L. casei* can be used in milk there will be less health risk caused by *S. aureus* as its number will be controlled by *L. casei* in case of contamination. But there was not much

effect in the growth rate of the *L. casei* when cultured individually or co-cultured. This shows that there was no effective inhibition in the growth of the L. casei caused by S. aureus and the metabolites produced by it. There was decrease in the pH of the co-culture medium due to the production of lactic acid by *L. casei*. The pH was decreased from 6.34 to 5.57 in 48 hours of incubation. The decrease in pH shows that there was metabolic change of lactose in milk to lactic acid. The change in pH was observed and determined so that the chance milk fermentation can be predicted. From the change in pH and observing the substrate it can be predicted that the texture of milk will not be change as the pH was not decreased sharply and the optimum pH for growth of L. casei was also the same. So, there will be no more decrease in the pH of the substrate. If the pH decreases then there will be chances of change in texture of milk. Also the lactose content was determined of the co-cultured substrate and was found that lactose content was decreased from 77.9mg/ml to 60.3mg/ml. the lactose in the medium was used up by *L. casei* and converted to lactic acid. The concentration was determined as compared to the standard graph prepared by standard lactose concentration. The decreased lactose content shows that *L. casei* can be in cases where lactose should be decreased. Also the optimization can lead to develop products for lactose intolerance as the lactose is utilized by *L. casei* for its metabolism.

Optimization of Antimicrobial culture:

The optimization of the culture is important as to determine the best culture to isolate antimicrobials from the culture supernatant. The antimicrobials produced by *L. casei* should be obtained in right time in order to get effective inhibitory activity of the culture supernatant. The culture supernatant was used for agar well diffusion and the maximum inhibition zone was observed. The culture supernatant of different time period of incubation was used for observing antimicrobial activity. The maximum inhibition was observed in 120 hr incubated culture supernatant. It means the concentration of antimicrobials was highest at this time, so in order to isolate antimicrobials this incubation period would be the best. This may be because most of the antimicrobials including bacteriocins are produced in the stationary phase of the growth of a microorganism. As most of the literature suggests the bacteriocins are produced mostly during the stationary phase and early death phase as bacteriocins are the defense system used by the organism for its own. Also others antimicrobials are also more concentrated as the growth of the organism is increased. So further 120hr incubated culture was used for the study of the bacteriocin from *L. casei*.

Protein precipitation by ammonium sulphate:

The bacteriocins produced by microorganisms are peptides or proteins which should be isolated in order to study it. There are various methods given for the isolation of the protein but the commonly used method is the precipitation method followed by dialysis. In the present work ammonium sulphate precipitation method was followed in order to get the bacteriocin from culture supernatant of *L. casei*. To optimize the concentration of the ammonium sulphate that precipitates bacteriocin different concentration of ammonium sulphate was used and the precipitate was observed for its antimicrobial activity. The antimicrobial activity now shown will be only due to the bacteriocin as only bacteriocin is precipitated and other antimicrobials are left behind. As in the experiment the maximum concentrated pellet that showed the inhibitory activity against *S. aureus* was obtained at 70% concentration of ammonium sulphate. The pellet obtained at this concentration showed maximum zone of inhibition against the test organism which may be due to maximum bacteriocin precipitated at this concentration of ammonium sulphate. The other concentration 60% and 80% showed less inhibition zone because of less concentration of bacteriocin precipitated at this concentration. Whereas, other concentration showed no inhibitory activity as there was no bacteriocin precipitated or in negligible amount which was not effective against the test organism. So effective amount of ammonium sulphate concentration is required in order to get the desired protein.

Partial purification by dialysis and freeze-drying:

The purification of the obtained protein is necessary in order to further study about it. In the present study the partial purification of the bacteriocin was done by the method of dialysis. The dialysis is the method which helps to get rid of the ions from the sample. The bacteriocin in the present study as well is contaminated of the ammonium sulphate ions used during its precipitation. The dialysis was carried out with the dialysis tube with 1000 MWCO which leaches out the ions and the peptides are retained in the dialysis tube. The dialysis was carried out at 4 ◦C for 12hrs with continuous stirring so that most of the ammonium sulphate ions are leached out. The dialysed solution is almost pure as the ions are diffused out from the tube. The solution obtained then is to be converted to solid form in order to study its characters. So, freeze-drying method was used for this process. The vaccum drying method gives the extracts of *L. casei* the solid form. The freezing drying was carried out 12 hours to get the powered form of the extract. The dialysed solution was first filtered through 0.22 µm filter to get rid of the contamination during the process of precipitation and dialysis. The freeze-dried sample now will be free of microbial contamination and can be used further for its characterization.

Protein estimation:

The amount of protein present in the processed sample is determined by the method of Bradford assay taking BSA as standard. The protein is to be estimated in order to find the concentration of the protein in the sample before and after processing. The protein precipitated will be mostly bacteriocin but there will be some contamination of other protein as well as the sample is only partially purified and not specifically purified for the bacteriocin. Further purification by different chromatographic method is possible to get the pure form of bacteriocin. The protein estimated in the solution after dialysis was found to be 12.45 mg/ml. The recovered protein was 30% as it is the protein that is precipitated at 70% concentration of ammonium sulphate and there are other protein as well which are not precipitated so the recovery will be obviously less. If the sample is more purified than the recovery will lesser but the purity will be increased as the contamination of other proteins in the bacteriocin solution will be decreased. Then the exact concentration of the bacteriocin in the culture supernatant can be determined.

Bacteriocin activity:

The study of the inhibitory activity of the bacteriocin against the S. aureus is determined to observe how effective the bacteriocin against the test organism is. The low arbitrary unit suggests that the bacteriocin obtained needs lower concentration in order to inhibit the test organism and higher shows higher concentration needed for antimicrobial activity. In the present study the bacteriocin obtained had 800 AU/ml inhibitory activity. The inhibitory activity of the culture supernatant was 50% higher than that of the bacteriocin solution. It may be because of the other antimicrobials present in the culture supernatant but they are absent in the bacteriocin solution.

MIC/MBC test:

MIC/MBC test is determined to find out the effective concentration that can inhibit the number of the undesired microorganisms. It was carried out by tube dilution method in the present experiment. The extract obtained after vaccum drying was used to find MIC/MBC. The MIC value was found to be 75ng/ml and the MBC value was 150ng/ml. At 75ng/ml concentration of the bacteriocin solution there was no visible growth so this concentration of partially purified bacteriocin is able to inhibit the growth of *S. aureus.* But for 99.9 % inhibition of the *S. aureus* 150ng/ml was required. The concentration of MIC and MBC can be decreased with the further purification of the bacteriocin as contaminated proteins which are other than bacteriocin will be eliminated.

Estimation of the molecular weight of bacteriocin:

The estimation of the molecular weight of the protein helps to classify the bacteriocin as well as determine the weight of the bacteriocin. The molecular weight of the bacteriocin was determined by SDS-PAGE. The distinctive band obtained when compared to the marker gives the tentative molecular weight of the protein. The protein when denatured travels along the gel to give a band according to its molecular size. The molecular weight of the bacteriocin obtained from *L. casei* was found to be 36 kDa. According to the molecular weight the bacteriocin can be of class III bacteriocin. As the bacteriocins of class III are of molecular weight more than 10 kDa and are heat labile. The further amino acid analysis of the bacteriocin will help to characterize it more efficiently.

Effect of pH and Temperature on bacteriocin:

The bacteriocin activity at different pH and temperature defines how effective is the bacteriocin against test organism at different conditions. It gives the range of the activity spectrum of the bacteriocin. We can determine the optimum pH and temperature at which the bacteriocin gives better activity. The bacteriocin obtained here had optimum pH of 7 and optimum temperature was found to be 70 ◦C so, that the maximum inhibitory activity of the bacteriocin was found at these conditions. Varying with the change in temperature and pH the activity of the bacteriocin varies. The neutral pH was found to be best for the activity of the bacteriocin. The bacteriocin was heat labile as at higher temperature it did not show any activity.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions:

- *L. casei* showed antibacterial activity against *S.aureus* when co-cultured in milk.
- *L. casei* when co-cultured in milk was able to decrease the growth of *S.aureus* and there was the decrease in pH and lactose content along with.
- Bacteriocin was isolated from *L. casei* which was also found to be effective against the *S .aureus.*
- The bacteriocin produced was precipitated with 70% concentration of ammonium sulphate and was partially purified.
- Bacteriocin isolated was purified and its inhibitory activity against *S. aureus* was found to be 800AU/ml
- The activity of bacteriocin was not much affected by pH change but it was more active at 5-7 pH.
- The bacteriocin isolated was found to be approximately of 36 kDa molecular weight. So it can be classified as classIII bacteriocin when classified according to the molecular weight.
- The activity of bacteriocin was affected by high temperature and it was more active at low temperature so it is heat labile bacteriocin.
- MIC/MBC value of the partially purified bacteriocin was calculated and it was found that 75ng/ml was MIC value and 150ng/ml was MBC value. The values were higher because of the impurity present in the bacteriocin solution.

6.2 Recommendations:

- *L. casei* can be used as antibacterial against the pathogenic *S. aureus* (TISTR 029).
- *L. casei* can be used as probiotic and also can be used in the milk products. It can be taken as biopreservative in milk and products from milk and fermented milk.
- The bacteriocin from *L. casei* can act as biopreservative in food products when more purified.
- The bacteriocin if purified more can be more active as inhibitory when used against *S. aureus* in dairy products and as well as non- dairy products.
- Further amino acid analysis of the bacteriocin can be done to find out its characteristics.

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APPENDIX:A

1. Preparation of DNS (100ml):

1gm of 3, 5-Dinitrosalicylic acid is dissolved in 50ml of water to which 20ml of 2N NaOH was added . After completely dispersing DNS in water 30gm of sodium potassium tartrate was added then, the final volume is made to 100ml with deionized water.

2. Preparation of Phosphate buffer:

pH 7, 0.1M phosphate buffer at 25◦C (1000 ml)

1M K_2HPO_4	61.5 ml
1M KH2PO4	38.5 ml
Water	900 ml

3. Preparation of SDS-PAGE Solutions:

Composition of 5ml 5% Stacking gel:

	Volume
Water	2.975 ml
0.5 M Tris HCl pH6.8	1.25 ml
10% (w/v) SDS	0.05 ml
Acrylamide/bis acrylamide (30%/0.8% w/v)	0.67 ml
10% w/v Ammonium persulphate	0.05 ml
TEMED	0.005 ml

Composition of 10 ml 12 % Resolving gel:

	Volume
Water	3.2 ml
1.5 M Tris HCl pH8.8	4 ml
10% (w/v) SDS	2.6 ml
Acrylamide/bis acrylamide (30%/0.8% w/v)	0.1 ml
10% w/v Ammonium persulphate	100 µl
TEMED	10 µl

Gel fix solution: (100ml)

50% Methanol	50ml
10% Acetic acid	10ml
Distilled water	40ml

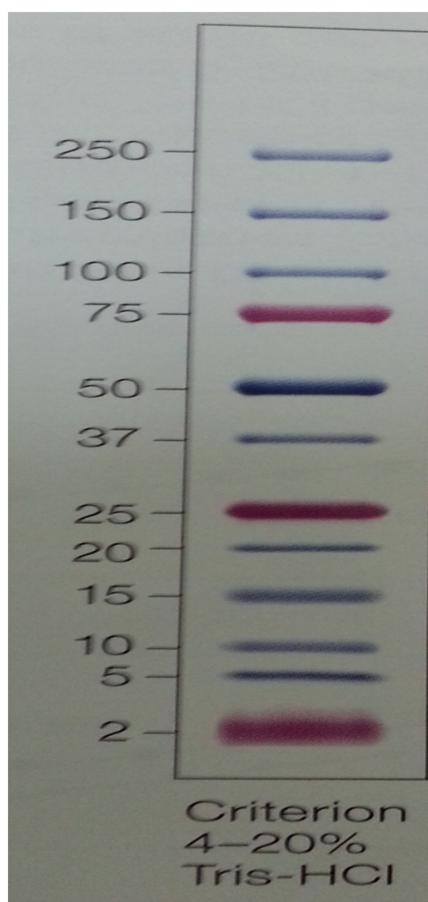
Staining solution:

Chemicals	Volume (100ml)
Coomassie Brilliant Blue	1 ml
40% Methanol	40 ml
10% Acetic acid	10 ml
Distilled water	49 ml

Destaining solution:

Chemicals	Volume (100ml)
10% Methanol	10 ml
7% Acetic acid	7 ml
Distilled water	83 ml

4. Molecular weight strandards (BIO-RAD):



APPENDIX: B

- **Co-culture of *L. casei* and *S. aureus*:**

- **Cfu/ml of individual culture of *S. aureus* and *L.casei***

S.aureus

S/N	Hours of incubation	Log cfu/ml			Average	SD
		R1	R2	R3		
0	0	6	6	6	6	0
1	4	6.39	6.4	6	6.26333	0.22810
2	8	7.2	7.31	7.34	7.28333	0.07371
3	12	7.3	7.76	7.85	7.63666	0.29501
4	16	8.07	8.01	7.98	8.02	0.04582
5	20	8.1	8.43	8.4	8.31	0.18248
6	24	8.49	8.66	8.59	8.58	0.08544
7	28	8.73	8.79	8.69	8.73666	0.05033
8	32	8.78	9.34	8.99	9.03666	0.28290
9	36	9.08	9.5	9.17	9.25	0.22113
10	40	9.53	9.62	9.5	9.55	0.06245
11	44	9.81	9.76	9.91	9.82666	0.07637
12	48	10.04	9.98	10.02	10.0133	0.03055

L.casei

S/N	Hours of incubation	Log cfu/ml			Average	SD
		R1	R2	R3		
0	0	6	6	6	6	0
1	4	6.38	5.99	6.51	6.29333	0.27061
2	8	7.25	6.95	6.96	7.05333	0.17039
3	12	8.15	7.89	7.93	7.99	0.14
4	16	8.53	8.66	8.8	8.66333	0.13503
5	20	8.89	8.9	8.76	8.85	0.07810
6	24	9.03	9.05	8.99	9.02333	0.03055
7	28	9.23	9.41	9.5	9.38	0.13747
8	32	9.83	9.89	9.92	9.88	0.04582
9	36	10.19	10.26	10.12	10.19	0.07
10	40	10.59	10.74	10.43	10.5866	0.15502
11	44	10.63	10.99	11.02	10.88	0.21702
12	48	11.35	11.32	11.26	11.31	0.04582

- **Cfu/ml of co-culture of *S. aureus* and *L.casei***

S. aureus

S/N	Hours of incubation	Log Cfu/ml			Average	SD
		R1	R2	R3		
1	4	5.76	5.79	6.39	5.98	0.3553

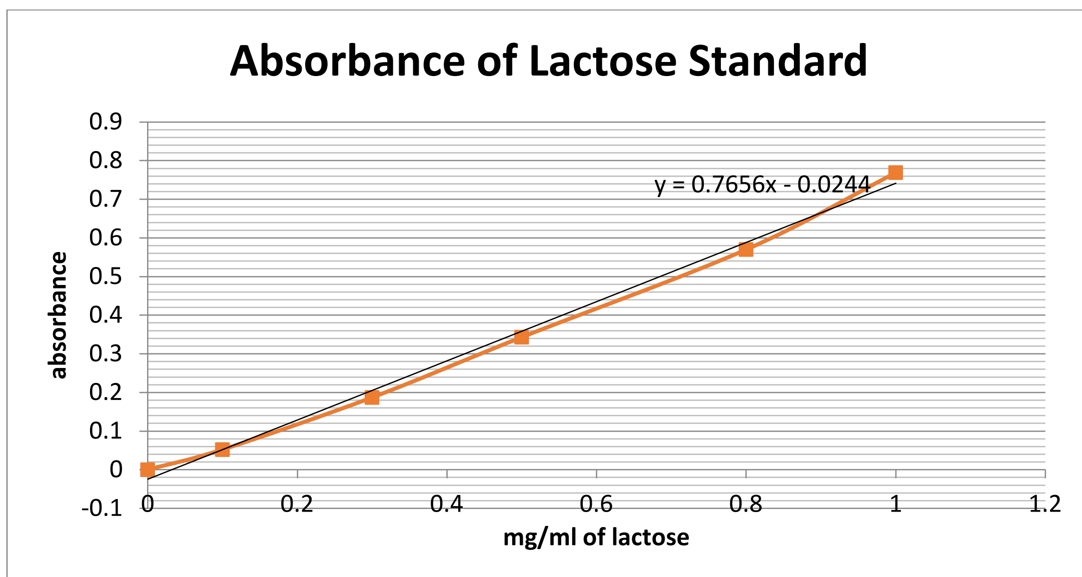
2	8	6.61	7.07	6.54	6.74	0.2879
3	12	6.8	6.7	6.89	6.7966	0.0950
4	16	6.76	6.49	7.5	6.9166	0.5229
5	20	7.8	7.9	7.5	7.7333	0.2081
6	24	7.95	7.93	7.8	7.8933	0.0814
7	28	6.79	7.14	6.94	6.9566	0.1755
8	32	6.5	6.81	6.41	6.5733	0.2098
9	36	5.7	6.2	6.4	6.1	0.3605
10	40	5.34	5.7	4.8	5.28	0.4529
11	44	4.5	4.57	3.9	4.3233	0.3682
12	48	3.64	3.79	3.51	3.6466	0.1401

L. casei

		Log cfu/ml			Average	SD
S/N	Hours of incubation	R1	R2	R3		
0	0	6	6	6	6	0
1	4	5.04	5.62	5.09	5.25	0.3214
2	8	5.17	5.64	4.96	5.2566	0.3481
3	12	5.35	6.14	5.98	5.8233	0.4176
4	16	6.39	6.29	6.24	6.3066	0.0763
5	20	6.26	6.32	6.83	6.47	0.3132
6	24	7	7.44	7.34	7.26	0.2306

7	28	8.46	7.99	7.91	8.12	0.2971
8	32	8.57	8.69	8.7	8.6533	0.0723
9	36	9.23	8.75	9.14	9.04	0.2551
10	40	9.17	8.98	9.34	9.1633	0.1800
11	44	8.97	9.67	9.71	9.45	0.4161
12	48	10.94	10	10.74	10.56	0.4951

- **Lactose standard curve:**



APPENDIX C:

➢ Antimicrobial activity of supernatant:

The zone of inhibition according to hours of incubation,

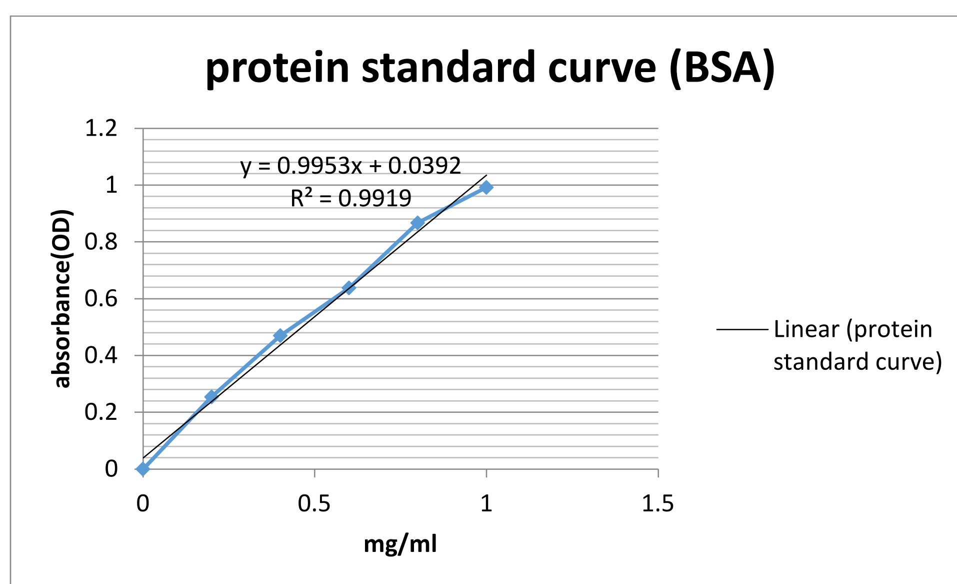
hours of incubation	zone of inhibition			Average	SD
	R1	R2	R3		
24	0	0	0	0	0
48	7	7	7.5	7.16666	0.28867
72	15	14.5	15	14.8333	0.28867
96	16.5	16	16	16.1666	0.28867
120	18	17.5	18	17.8333	0.28867
144	16	15.5	15	15.5	0.5
168	14	13.5	13	13.5	0.5

➢ Antimicrobial activity after ammonium sulphate precipitation:

% of ammonium sulphate precipitation	Zone of inhibition			Average	SD
	R1	R2	R3		
40	0	0	0	0	0
50	0	0	0	0	0
60	5.5	5.5	5.5	5.5	0

70	9	9.5	8.5	9	0.5
80	6	6	5.5	5.83333	0.28867
90	0	0	0	0	0

- **Protein standard curve:**



- **Effect of pH on bacteriocin:**

pH	Zone of inhibition(mm)			Average	SD
	R1	R2	R3		
4	7	7.5	7.5	7.33333	0.28867
5	7.5	7.5	7.5	7.5	0
6	8	8	8	8	0

7	8	8.5	9	8.5	0.5
8	8	8	8.5	8.1666	0.2886
9	7	7	6	6.6666	0.5773

- **Effect of temperature on bacteriocin:**

Temp ◦c	Zone of inhibition(mm)			Average	SD
	R1	R2	R3		
70	7.5	8	8	7.8333	0.2886
80	7.5	7	7.5	7.3333	0.2886
90	7	6.5	6.5	6.6666	0.2886
100	5	0	5	3.3333	2.8867
120	0	0	0	0	0

APPENDIX D:

Composition of MSA Agar:

Ingredients	**Gms / Litre**
Proteose peptone	10.000
Beef extract	1.000
Sodium chloride	75.000
D-Mannitol	10.000
Phenol red	0.025
Agar	15.000
Final pH (at 25°C)	7.4±0.2

Composition of MRS Agar:

Ingredients	**Gms / Litre**
Proteose peptone	10.000
Beef extract	10.000
Yeast extract	5.000
Dextrose	20.000
Polysorbate	80 1.000
Ammonium citrate	2.000
Sodium acetate	5.000
Magnesium sulphate	0.100
Manganese sulphate	0.050
Dipotassium phosphate	2.000
Agar	12.000
Final pH (at 25°C)	6.5±0.2

Composition of MHA Agar:

Ingredients	Gms / Litre
Beef, infusion	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

C omposition of Nutrient Broth:

Ingredients	Gms / Litre
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	5.000
Final pH (at 25°C)	7.0±0.2

APPENDIX E

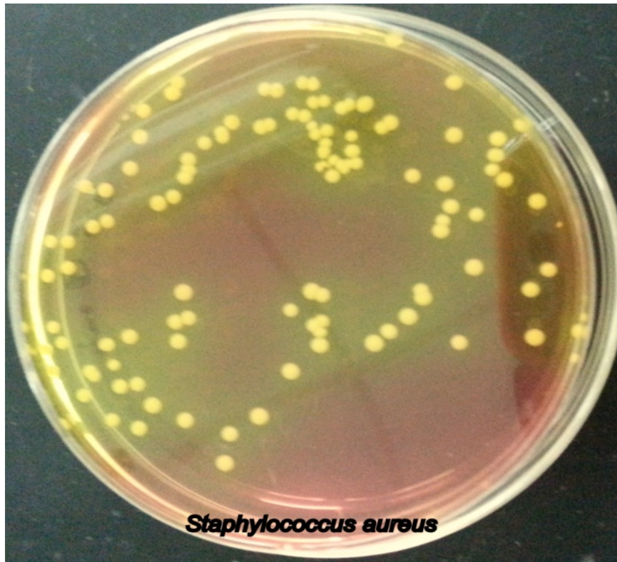


Figure: *Staphylococcus aureus*

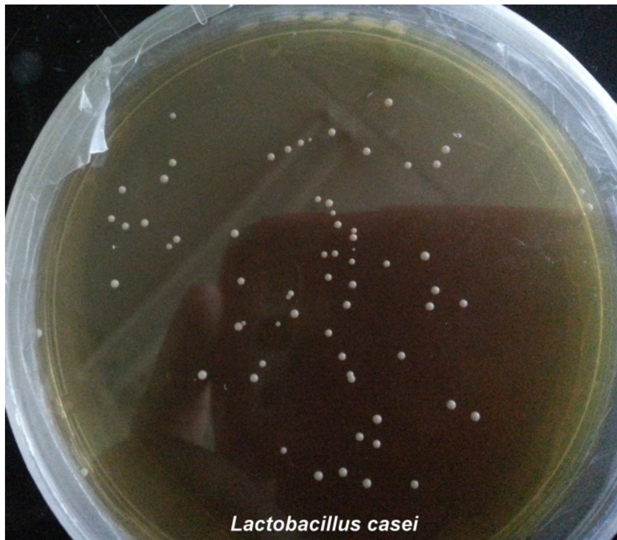


Figure: *Lactobacillus casei*