"*Isolation and characterization of bacteriocin and its producing micro-organism and further genetic modification and its characterization*."

Presented in partial fulfillment of the requirement for the Degree of Master of Technology (Biotechnology)

Submitted to

Department of Biotechnology
School of Biotechnology
Lovely Professional University
Jalandhar, Punjab

Submitted by

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Under the guidance of

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CERTIFICATE

This is to certify that **Sumit Sahu** bearing Registration no. **10905803** is doing project titled, **"Isolation and characterization of bacteriocin and its producing micro-organism and further genetic modification and its characterization"** under my guidance and supervision. To the best of my knowledge, the present work is the result of his original investigation and study. No part of the project has ever been submitted for any other degree at any University.

The project is fit for submission and the partial fulfillment of the conditions for the award of degree of M.Tech in Biotechnology.

Signature of Supervisor

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DECLARATION

I hereby declare that the project work entitled "**Isolation and characterization of bacteriocin and its producing micro-organism and further genetic modification and its characterization**" is an authentic record of my work carried out at Lovely Professional University as requirements of Project work for the award of degree of Master of Technology in Biotechnology, under the guidance of Dr. M.A. Khan, Professor, School of Biotechnology and Biosciences, Lovely Professional University, Phagwara, Punjab, India.

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I take this opportunity to present my votes of thanks to all those guidepost who really acted as lightening pillars to enlighten our way throughout this project that has led to successful and satisfactory completion of this study.

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Yours faithfully
Sumit Sahu

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Abstract

Two representative genera of rod shaped, gram-positive bacteria of class bacilli include few species of *Lactobacillus* and *Bacillus* can produce bacteriocin which is a polypeptide complex and shows antimicrobial activity. Bacteriocin is a class of antimicrobial and/or antibiotics which inhibit the biosynthesis of cell membrane of few of the gram negative bacteria including *E.coli*. But the strength of antimicrobial activity is very low and produced in very low concentration which can be improved by various genetic manipulation techniques such as mutation by UV radiations and different optimized conditions which were found favorable for increasing the bacteriocin concentration like pH 6-9, temperature 36^0C, selective MRS media, incubation time 0-96 hours and glucose concentration 1-5%. Bacteriocin producing organisms which were included in the present study were isolated from the curd (probiotic source of *Lactobacillus* sp.), which were also found inside human body like gastrointestinal tract (such as *L.acidophilus*), in vagina etc where they produce lactic acid and makes acidic environment to inhibit growth of harmful bacteria. Hence bacteriocin in non toxic to human body and could be a good antibiotic and biopreservative for industrial purpose to store food and other products for long time.

Key words: *Lactobacillus, Bacillus,* bacteriocin, UV treatment, antibiotic, biopreservative.

Chapter 1
INTRODUCTION

The word “antibiotic” is derived from Greek term antibiosis, which literally means “against life”. It can be purified from microbial fermentation and modified chemically or enzymatically for either chemical use or for fundamental studies (Robbers *et al*., 1996; De Mondena *et al*., 1993). The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage and compost. Of the several hundred naturally produced antibiotics that have been purified, only a few have been glass bead sufficiently non-toxic to be of use in medical practice. Those that are being currently of greatest use have been derived from a relatively small group of microorganisms belonging to the genera *Penicillium, Cephalosporium, Streptomyces, Micromonospora* and *Bacillus* (Zinsser *et al*., 1988). More than 5000 different antibiotics have been isolated from cultures of bacteria, fungi and plant cells, 60% of them are contributed by the genus *Streptomyces.*

Antibiotics are the chemical compounds which are actually obtained from microorganisms and they have the capacity of inhibiting the growth and proliferation and even destroying the microorganism in dilute solutions. First bacterial antibiotic tyrothricin was discovered by Dubos in 1939. It was obtained from a spore bearing soil *Bacillus, Bacillus brevis,* which was grown by surface culture in the medium containing salts, peptone or hydrolyzed casein or by the submerged culture in a medium containing glucose, salts and mixture of various amino acids. Influence and development of the culture medium (Ganchev and Kozhuharova, 1984), effect of different amino acids (Haavik, 1981), effect of inter-relationships between the primary and secondary metabolites (Supek *et al.,* 1985), development of solid-phase method (Lee *et al.,* 1996) and industrial aerobic parameters in real time (Iztok *et al.,* 2000) has been studied on the biosynthesis of bacitracin.

Bacitracin was named after a culture of *Bacillus* and the last name of a seven-year-old American girl, Margaret Tracey, that *Bacillus* was isolated from her wounds (Johnson *et al.,* 1945). The compound has bactericidal effect on gram positive but little activity against gram-negative organisms (Prescott and Baggot, 1993). It is one of the most important antibiotic used in human medicine, topical application and used after surgical operations (MacIver *et al.,* 2006; Katz and Fisher, 1987) and one most commonly used in animal and poultry feed which increases feed efficiency and reduce infectious diseases (Hampsun *et al.,* 2002). Despite its widespread use, bacitracin resistance is still scarce (Ming and Epperson, 2002).

Due to wide spread use of bacitracin, it is necessary to find out ways and measures to reduce the cost of this product. To achieve this, our focus is to utilize appropriate fermentation technology

and optimization of adequate control of fermentation processes that could minimize the product costs that allows microbial factories to yield higher titers of bacitracin.

The molecular formula is: $C_{66}H_{103}N_{17}O_{16}S$. Bacitracin is comprised of a polypeptide complex and Bacitracin A is the major component in this complex. The mol. wt. of Bacitracin A is 1422.71 Da. Bacitracin consists of one or more of the antimicrobial polypeptides produced by certain strains of *Bacillus licheniformis* and *Bacillus subtilis* var Tracy and yields the Amino acids L-cysteine, D-glutamic acid, D-phenylalanine, L-histidine, L-lysine, L-leucine, L-isoleucine, D-ornithine and DL-Aspartic acid on hydrolysis and functions as an inhibitor of cell wall biosynthesis (Azevedo, 1993). Bacteriocin from other micro-organism is an antibiotic as well as non-ribosomally produced by *Bacillus licheniformis*. Different types of Bacitracin like A, A1, B, C, D, E, F, F1, F2, F3 and G have been isolated. The most potent antibiotic is Bacitracin A, whereas Bacitracin B and C are less potent and the rest possess a very little antibacterial activity. This antibiotic is the undoubtedly effective against Gram-positive and a few Gram-negative species of bacteria. It is almost exclusively used as a topical preparation in the treatment of infections.

Chapter 2
AIMS AND OBJECTIVES

Isolation of Bacitracin producing microorganism from the probiotic source like curd, and characterization of the bacteria by performing various morphological and biochemical identifications tests. Further its genetic modification by the UV radiation exposure and the production of Bacteriocin, simultaneously. Followed by purification of this bacitracin and screening for the antimicrobial activity and also for change in the antimicrobial activity after UV exposure, and characterization.

Chapter 3
RATIONALE OF STUDY:

This study is based on *Lactobacillus* and *Bacillus* species and to study about their secondary metabolite secretion. As there are many products which are not available in bulk amount in nature but it is industrially important such as biopreservative in food industry. So there is need to exploit micro organisms for obtaining several products which are useless for bacteria but useful and beneficial for humans. Again a comparative study will give the clear picture which strain will give us maximum yield, which species and strain should be opted so that it would be industrially economical.

Bacteriocin is a different type of antibiotic which also act as a preservative at the same time. It was produced from *Lactobacillus species* isolated from curd, so it would have least or no toxic effects. Previously it had been used as antimicrobial agent for surgical process, but now a day's its antibiotic effect is also being researched. To obtain a different variety of bacteriocin, same isolates have been fermented under UV treatment and without UV treatment to study the new variety of peptide antibiotic. It could also affect the rate of bacteria multiplication and secondary metabolite production.

Chapter 4
HYPOTHESIS OF THE STUDY

The overall hypothesis is that to isolate the lactic acid bacteria form probiotic source and production of bacitracin, then further genetic manipulation/improvement by UV radiation either in the form of amount of production or the antimicrobial activity of bacitracin.

Bacteriocin producing organism: Null (H_o) Hypothesis, Alternate (H_1) Hypothesis.

H_o = Microorganisms which were isolated doesn't have the capability of producing bacteriocin

H_1= Microorganisms which were isolated have the capability of producing bacitracin like substances that shows antimicrobial activity or nothing.

Genetic manipulation: Null (H_o) Hypothesis, Alternate (H_1) Hypothesis.

H_o= Genetic manipulation by UV exposure in the genes of microorganism, might kill the bacteria or stops producing bacitrain and bacitracin like substances.

H_1=Genetic manipulation by UV exposure causes mutation which leads to initiate and/or enhance production of bacitracin. And could also produce bacteriocin with high/or low antimicrobial activity than wild strain or even inactive form of bacteriocin.

Chapter 5
REVIEW OF LITERATURE

5.1. Introduction

Awais et al (2007), Bacillus subtilis and *Bacillus pumilus* were isolated from soil and screened for the production of antibiotics by plate assay and then cultured in shake flask fermentation at 30^{o}C for further studies. Identification of antibiotics was done by paper chromatography. Bacteriocin was found to be produced by both the strains against *Micrococcus luteus* (ATCC 10240), whereas; *Staphylococcus aureus* (ATCC 6538) proved to be resistant to Bacteriocin produced by *Bacillus pumilus*. The maximum production of Bacteriocin from *Bacillus subtilis* and *Bacillus pumilus* against *Staphylococcus aureus* and *Micrococcus luteus* at different pH (6-9), incubation time (0-144 hours) and glucose concentration (1-5%) was checked by agar diffusion assay as detected by the size of zones of inhibition. Maximum zones of inhibition were observed at pH 8, 5% glucose and after 24 hours of incubation at 30^{o}C against *Staphylococcus aureus* and *Micrococcus luteus*.

5.2. Source of Bacteria

From Dairy Food

Yang et al (2012), the biopreservation of foods using bacteriocin producing lactic acid bacteria (LAB) was isolated directly from foods was an innovative approach. They isolate and identify bacteriocin producing LAB from various cheeses and yogurts and evaluate their antimicrobial effects on selected spoilage and pathogenic microorganisms in vitro as well as on a food commodity.

Bharadwaj et al (2012), Dahi (curd) is a fermented milk product, most commonly used by Indian population. Trials are in process to establish dahi as a source of health beneficial organisms (probiotics). Hence, their study was directed towards the study of prevalence of *Lactobacillus* species in dahi. They had collected a total of 40 samples of dahi for the isolation of *Lactobacilli* using *Lactobacillus* selective MRS agar media. Thirty-eight colonies were randomly picked based on colonial morphology. All the isolates were subjected to cell morphology, physiology and an array of biochemical characterization.

From Soil

Awais et al (2007), Isolated *Bacillus subtilis* and *Bacillus pumilus* from soil and screened for the production of antimicrobial agent by plate assay methods, and identified antibiotics by paper chromatography.

Joshi et al (2012), *Bacillus subtilis* isolated from soil sample. This bacterial strain identified as higher antibiotic producing species.

From Marine (Fishes)

Rajaram et al (2010), *Lactobacillus lactis* strain, which was isolated from marine sources produce bacteriocin .This bacteriocin showed a wide range of antimicrobial activity against pathogens found in our food.

From Fruits and Vegetables

Ravi et al (2011), Isolate, characterize, and identify the bacteriocin producing lactic acid bacteria (LAB) from mango pulp. Antimicrobial activity of the isolated strain was found useful against spoilage pathogens.

5.3. Isolation, Characterization and Biochemical Tests for Bacteria

For the isolation of bacteria specialized media provides a great help of the selective microbial growth like MRS, M17, TSB etc.

Yang et al (2012), LAB (lactic acid bacteria) was isolated using MRS and M17 media. The agar diffusion bioassay was specially used for screening of bacteriocin or bacteriocin-like substances (BLS) producing LAB using *Lactobacillus sakei* and *Listeria innocua* as test organisms. Out of 138 LAB isolates, 28 were found to inhibit the test organisms and were identified as strains of *Enterococcus faecium, Streptococcus thermophilus, Lactobacillus casei* and *Lactobacillus sakei subsp. sakei* using 16S rRNA gene sequencing.

Eight isolates of them were tested for antimicrobial activity at 5°C and 20°C against *L. innocua, Escherichia coli, Bacillus cereus, Pseudomonas fluorescens, Erwinia carotovora, and Leuconostoc mesenteroides subsp. Mesenteroides* using the agar well-diffusion bioassay, and also against *Penicillium expansum, Botrytis cinerea and Monilinia frucitcola* using the microdilution plate method. The effect of selected LAB strains on *L. innocua* inoculated onto fresh-cut onions was also investigated. Twenty percent of the isolates were producing Bacteriocin like Substance (BLS) inhibiting the growth of *Listeria innocua* and/or *Lactobacillus sakei*. Organic acids and/or H_2O_2 produced by LAB and not the BLS had strong antimicrobial effects on all microorganisms tested with the exception of *E. coli. Enterococcus faecium, Streptococcus thermophilus* and *Lactobacillus casei* effectively inhibited the growth of natural microflora and *L. innocua* inoculated onto fresh-cut onions. Bacteriocin producing LAB present in cheeses and yogurts may have potential to be used as biopreservatives in foods.

Bharadwaj et al (2012), 40 Samples of dahi were used for the isolation of *Lactobacilli* using *Lactobacillus* selective MRS agar media. Thirty-eight colonies were randomly picked based on colonial morphology. All the isolates were subjected to cell morphology, physiology and an array of biochemical characterization. The isolated bacteria showed different growth patterns at different temperatures (15°C and 45°C), oxygen and at different concentrations of Nacl (2.0, 4.0 and 6.5%). On the basis of various physiological tests and different sugar utilization pattern, all the seventy-eight isolates were confirmed to the different species of *Lactobacillus*: *Lactobacillus casei* (24.35%), *Lactobacillus brevis* (3.84%), *Lactobacillus fermentum* (6.41%), *Lactobacillus plantarum* (7.69%), *Lactobacillus helveticus* (5.12%), *Lactobacillus rhamnosus* (6.41%), *Lactobacillus viridiscence* (5.12%), *Lactobacillus lactis* (3.84%), *Lactobacillus acidophilus* (37.17%). Among isolates, *L. acidophilus* was found to be prevalent in dahi.

Bisht et al (2011). *Bacillus* species were isolated from their local habitats, which were capable of producing bacteriocin and the same was screened on nutrient media using test organism viz: *Micrococcus luteus*,(ATCC 9341) *& Staphylococcus aureus* (ATCC 13565). Culture conditions

and media composition was optimized for bacteriocin production by analyzing the effect of various nitrogen sources (like tryptone, meat extract, yeast extract), carbon sources (like maltose, glucose, fructose, sucrose, lactose), pH (3.6, 4.6, 5.6, 6.6), time (6hrs, 12hrs, 16hrs, 24hrs, 48hrs, 72hrs), temperature (4°C, 15°C, 25°C, 37°C, 48°C) on the growth of isolated species of *Bacillus*.

Rajaram et al (2010), Bacteriocin produced by *Lactobacillus lactis* strain , which was isolated from marine sources. This bacteriocin showed a wide range of antimicrobial activity against pathogens found in our food. According to this study, bacteriocin production is highest observed at 30^0C, pH 6.0 and 1.5 % Nacl solution. A range of that have positive effects on bacteriocin production are alpha-amylase, DNase, RNase and lipase, and that inhibit its production are Proteinase K and pepsin. Detergents have also been observed in increasing the production of bacteriocin, but Sodium dodecyl sulphate (SDS), Tween 80 and Tritone X-100, while EDTA and urea are strong inhibitors.

5.4. Production of Bacteriocin

Bacteriocin, a peptide antibiotic is the secondary metabolite secreted extracellularly by many bacteria which is can be optimized by different methods.

Bisht et al (2011). The isolated strains were introduced to solid state and submerged fermentation method with different production media having Soyabean meal & Wheat bran.

Joshi et al (2012), From the soil sample bacterial strain was screened for isolation of higher antibiotic producing species. That strain was identified as *Bacillus* sp. using Bergey's manual. Different parameters identified and optimized for synthetic media for the production of bacteriocin, a peptide antibiotic. For bacteriocin production, the nutrients were optimized as glucose (1%) and L-glutamic acid (0.5 %) as carbon and nitrogen source respectively. The identifiable production of bacteriocin was obtained after 48 hrs of incubation at 40^oC temperature and pH 7.

Haavik et al (1974), Bacteriocin is produced by some strains of *Bacillus* species. It is produced after growth and before sporulation. They proposed that bacteriocin is produced by the bacteria at this stage of their life cycle only. Other peptide antibiotics were also reported to be produced mainly after growth, e.g. penicillin, gramicidin S, cephalosporin C, tyrocidin, actinomycin, mycobacillin, edeine, polymyxins, triostin , etc. It is generally believed that the bacteriocin producing organisms initiate the synthesis of peptide antibiotics towards the end of the exponential phase of growth. It has also been suggested that peptide antibiotics, like other secondary metabolites, are also produced, only by organisms that have recently stopped dividing. During a study of bacteriocin production by *Bacillus Zicheniformis* in different synthetic media, both the kinetics of bacteriocin formation and the maximum titer of bacteriocin were greatly affected by the composition of the medium.

5.5. Genetic manipulations

Adrio et al (2005), Microorganisms have always been very amazing with a wide array of valuable products, whatever they produce are only in the limited quantity , according to their own

need and benefits, that's why they never overproduce their metabolites. By the genetic engineering method of strain improvements, could produce a strain of desired goal. By the mutagenesis we can tremendously increase the productivity and decrease the cost, by the application of recombinant technology.

Aftab et al (2010), The production of bacteriocin using synthetic media by *Bacillus licheniformis* UV-MN-HN-8 was achieved at 37ºC, pH 7 after 48 hrs. Effect of the addition of metal ions, phosphate sources, organic acids, and phosphate sources, inoculum age and size, pH, temperature, incubation time and aeration indicate that Ni^{+2}, KH_2PO_4, lactic acid, 20 hours old 6% inoculum, 37ºC, pH 7, 48 hours of incubation time and 25 ml medium gave higher yields of bacteriocin (51.3±1.29 IU/ml). But on the basis of kinetic variables, $Y_{p/s}$, $Y_{p/x}$, $Y_{x/s}$ (g/g), $Y_{p/s}$ mutant strain *Bacillus licheniformis* UV-MN-HN-8 was found 2.33 fold more bacteriocin production than wild type after optimization of different parameters.

5.6. Screening for Antimicrobial activity of Bacteriocin

Ravi et al (2011), The extracted antimicrobial compound was tested for bacteriocin assay and antimicrobial properties against to mango pulp spoilage bacteria. The antimicrobial compound was also tested for maintaining the decreased CFU during the mango pulp storage along with the different chemical preservative like Potassium Meta bisulphate (PMS). Total six bacterial isolates were isolated, out of all the six isolated strains one was selected for further work, which shows the probiotic characters like bacteriocin assay and antimicrobial properties. The extracted compound shows the decreased CFU used as preservative during the pulp storage when compared to a sample without any preservative and also to the chemical preservative. The proteinase K, pepsin shows the negative effect on the activity of extracted compound from Lactic acid bacteria. Enzymes like trypsin and α-chymotrypsin has no effect on bacteriocin, and there is no effect of surfactants on bactreiocin. The effect of pH on the culture supernatant was studied in range from 2 to 12. With increased pH after 9 decreases the activity of the compound.

Rajaram et al (2010), Strong cyotoxicity was showed by purified bacteriocin, in brine shrimp lethality assay and the LC50 value was 21.54µg/ml. And it is possible of using bacteriocin as a food preservative and *L. lactis* strain as probiotic.

5.7. Bacteriocin Extraction, Purification And Characterization

Bisht et al (2011). The bacteriocin that was produced during fermentation was extracted by Butanol-Ether solvent extraction system and further purified by Thin Layer Chromatography and characterized by SDS-PAGE for molecular weight of bacteriocin purified.

Rajaram et al (2010), In this study, bacteriocin was purified by ammonium sulphate precipitation method and ion exchange (DEAE cellulose) chromatography. Molecular weight was 94kDA by SDS-PAGE. Similar results were recorded by *Ivanova et al* (2000), *Karthikeyan and Santosh* (2009) and *Ogunshe et al*. (2007).

Haris et al (1999), With increased use of proteins in different industrial, domestic and therapeutic applications, ranging from pharmaceuticals to biosensors and biomaterials, and

various therapeutic applications, there has emerged a need for protein structural characterization in diverse environments/medium. Well it is not sufficient to just have the 3-dimensional structure of a protein in H_2O or in the crystalline state. More information on the structural properties of a protein is required in the presence of organic solvents, detergent micelles, phospholipid membranes and so on. Fourier transform infrared spectroscopy FTIR is one of the few techniques that can be applied for structural characterization of proteins in such environments. This technique is being frequently used to obtain information on protein structure and stability in both aqueous and non-aqueous media. It can help study of water soluble proteins and membrane proteins.

Chapter 6
SOURCE ORGANISM:

Bacillus sp.: In pharmaceutical industry, several peptide antibiotics of importance are produced by *Bacillus* species such as bacitracin, gramicidin, polymyxin, tyrocidine, bacilysin, subtilin etc. Bacilli are rod-shaped, Gram-positive, sporulating, aerobes or facultative anaerobes. Most bacilli species are saprophytes. Each bacterium creates only one spore, at a time, which is resistant to temperature(heat or cold), radiation, desiccation, and disinfectants. Bacilli exhibit an array of physiologic abilities that allow them to live in a wide range of habitats, including many extreme habitats such as desert sands, hot springs, and Arctic soils. Species in the genus *Bacillus* can be thermophilic, psychrophilic, acidophilic, alkaliphilic, halotolerant or halophilic and are capable at growing at temperatures, pH values, and salt concentrations where only a few other organisms can survive. Most of the peptide antibiotics produced by bacilli are active against gram-positive bacteria, however, compounds like polymyxin, circulin and colistin exhibit activity almost exclusively upon gram-negative bacteria, whereas bacillomycin, mycobacillin and fungi-statin are effective against molds and yeasts (Katz & Demain, 1977). Berdy (1974) reported the production of 167 peptide antibiotics from *Bacillus subtilis* and *Bacillus brevis.* Of this total, 66 different types of peptide antibiotics are elaborated by strains of *Bacillus subtilis* and 23 are products of *Bacillus brevis.*

Lactic acid bacteria: Lactic acid bacteria are traditionally used as starters for food fermentations. Since they have a capacity to inhibit spoilage and pathogenic bacteria, they are important in food preservation and intestinal prophylaxis. Lactic acid bacteria are the most important groups for industrial purposes, since their fermentative activity involves a notable preservative capacity as a result of the drop in the pH and the antimicrobial activity of their metabolites such as lactic and acetic acid, diacetyl or bacteriocins.

Bacteriocins are antimicrobial peptides produced by many lactic acid bacteria (LAB), which are directed mainly to inhibit the growth of related species or species with the same nutritive requirements. Some bacteriocins have been used to inhibit these pathogens in food.

Chapter 7
MATERIALS AND METHODS

Bacteria can be grown in different media according to their strains, like natural media, chemically defined synthetic media, serum, agar etc. also the effect of various physiological factors the rate of growth of bacteria, factors like pH, temperature, humidity, aeration, incubation period plays very important role, other factors like glucose conc., metal ions, amino acids, (protease) enzyme conc., organic solvent conc. etc also plays equally very important role. All these factors decide the rate of growth of bacteria and to reach to a maturation level at which bacteria starts the production of secondary metabolites (antibiotics: bacteriocin).

ISOLATION

7.1. Sample Collection

Two samples were collected from

1)The best source of *Lactoacillus* is curd also non-pathogenic at the same time, so the first sample was collected as the 5 different curd samples (Yang et al, 2012, Bhardwaj et al, 2012) from different dairies of Jalandhar.

2) Another sample was taken from pure stock culture of *Bacillus* species MTCC 6428.

7.2. Isolation of bacteria

Procedure for isolation of bacteria

1) 1ml of curd was dissolved in 9 ml of distilled water and then serial dilutions were done up to 10^{-6} and label it 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}.

2) Then spread plated on Petri-plate to obtain colonies of bacteria in 1% MRS media (for samples from curd) at 37^0C for 24 hrs at pH. MRS media is selective media for *Lactoacillus* species which is composed of and in 1% nutrient agar media (for *Bacillus* MTCC 6428).

3) After this, selected colonies had been inoculated in broth separately, to increase the cell count, which is incubated for 24 hours at 37 ^{0}C pH in the 1% nutrient broth. Now the next step is the calculation of viable titer:

Viable titer = (CFU/volume plated) x Dilution factor

MORPHOLOGICAL ANALYSIS

7.3. Gram staining

The inoculated colonies of bacteria over the Petri plates had been gram stained to study the morphological characteristics of the bacteria. (*Bhardwaj et al , 2012)*

The colony picked from different samples had been Gram stained and two were confirmed gram positive one from curd sample and another from stock culture of *Bacillus*.

Gram Positive: Purple to blue in color

Gram Negative: Pink to red in color

Procedure: To perform Gram Staining steps involved are:

1) First take the colonies and made smear of it, by heat fixing over burner flame.
2) After that add few drops of crystal violet, only enough to cover the smear and after 40-50 seconds, wash with water, gently.
3) Then add few drops on Gram's Iodine again enough to cover the smear only, wait for 20-30 sec.

4) And then wash with water, gently. Now add distain i.e. 70% ethanol drop wise and immediately add counter stain Saffaranin.

So the cell which are gram positive would appear blue/purple in color and those are gram negative appear pink/red in color.

7.4. Endospore Staining

In the inoculated broth to confirm that there had not been any spore formed by the bacteria it is confirmed by Endospore staining. (*Bhardwaj et al , 2012)*

Procedure: In this staining

1) the bacterial culture is heat fixed to form smear and then placed over steaming water bath,
2) now place a piece of blotting paper over area of smear only,
3) then saturate the blotting paper with 5 -6 % Malachite Green,
4) heat under water steam for 5 more minutes,
5) replenish stain don't let drying out the smear,
6) gently rinse with tap water,
7) then apply saffaranin for 1 min.,
8) rinse with water.
9) dry the slide and observe.

Endospore appears green while the vegetative cells appears red to pink in color

STOCK PREPARATION

7.5. Pure Culture

After staining, the sample of interest is inoculated by streaking over the nutrient agar plates and MRS agar media inside laminar air flow. The samples then incubated at 37 ^{0}C for 24 hours. And the pure isolated colonies were isolated and maintained in few eppendorf tubes and stored in refrigerator at 0 to 4 ^{0}C.

BIOCHEMICAL CHARACTERIZATION

7.6. Biochemical Test for Identification

7.6.1. Carbohydrate Fermentation Test:-

1) To perform this, trypticase (1g), Sodium chloride (.5g), and Phenol red (0.189g) were added to 100 ml distilled water and transferred into five conical flasks.
2) Then add 0.5% to 1% of desired carbon (carbohydrate) source into all flasks, here five carbohydrate sources have been used, and they are glucose, lactose, mannitol, maltose and sucrose. (Appendix A.a-A.e)
3) Then add 5 ml of broth to test tube and insert inverted Durham tubes into all flasks. Durham tube should be completely filled with broth without any bubble.
4) Now tubes are sterilized at 121 ^{0}C for 15 minutes at 15 p.s.i. by autoclave.
5) Phenol red in Carbohydrate should not be overheated otherwise carmelization of sugar may occur.

6) After cooling the test tubes are inoculated with different samples and one is kept as control without inoculation.
7) Now all these tubes are incubated at 37 ^{0}C for 24-48 hours and the results are observed, to find out among five major carbohydrate sources which source can be utilized by the cells of test culture.

7.6.2. Aerobic and Anaerobic Carbohydrate Breakdown Test:-

For this test,

1) Media is prepared with peptone (0.2g), sodium chloride (5.0g), Di potassium hydrogen phosphate (0.03g) , phenol red (0.03g) Agar (3g)into 100 ml of distilled water.(Table 2)
2) Media had been sterilized and three slants for each test sample were prepared, labeled as aerobic, anaerobic and control.
3) Two tubes out of three were inoculated by stabbing with the help of inoculation needle inside laminar air flow.
4) One tube is wrapped with a parafilm and labels it as anaerobic to provide anaerobic conditions.
5) Now these rubes are incubated at 37^{0}C for 48-96 hours.

If acid is produced only at the surface of the medium, where conditions are aerobic the breakdown of sugar is oxidative. If acid is found throughout the tube including the lower layers, where conditions are anaerobic, the breakdown is fermentative

***7.6.3. Casein Hydrolysis*:-** Casein is a milk protein which can be used as the carbon and energy source, if the isolated culture is able to utilize casein as the substrate material then clear zones of utilized casein might be visible. For this test,

1) 1% skim milk agar media (Appendix C) was prepared and autoclaved.
2) Now plates were made of this casein agar and inoculated with isolated culture as a single spot inside laminar air flow.
3) Incubate these plates 37^{0}C for 48-96 hours.

Results can be observed by observing the clearance zones around the spot where the isolated culture was inoculated; this shows the presence of casein hydrolyzing enzymes produced by isolated culture.

***7.6.4. Catalase Production Test*:-** As microorganism can produce hydrogen peroxide during photosynthesis. Accumulation of hydrogen peroxide leads to the death of organism until they are degraded enzymatically.

The test was performed in order to determine the ability of the isolated cultures to degrade the H_2O_2 by producing the enzyme catalase.

Anaerobes are unable to synthesize catalase this is the reason why oxygen is considered as toxic for whereas aerobes can synthesize catalase. To perform this test,

1) 50 ml nutrient agar was prepared and sterilized.
2) Then slants of this media was made and inoculated with isolated culture and one slant is kept as control without inoculation.
3) After incubation at 37^{0}C for 24-48 hours, 1ml of 3% hydrogen peroxide is trickled down the slant, and closely observed for the evolution of bubbles.

The production of bubbles indicates positive catalase reaction and was recorded accordingly for the presence or absence of enzyme.

***7.6.5. Citrate Utilization Test*:** Some organism can utilize citrate as the carbon source instead of glucose or lactose. So to perform this test:

1) Simmons citrate agar medium (Appendix D) was prepared and sterilized.
2) Two slants were prepared for each isolates, and one is inoculated while other one is kept as control and incubated at 37^0C for 48-96 hours

After incubation the appearance of blue color indicated the positive test for citrate utilization.

***7.6.6. Indole Production Test*: -** There are few organism which produce indole as tryptophan is decomposed into its metabolic products like indole, pyruvic acid and ammonia by enzyme tryptophanase. So to perform this test

1) Peptone broth (Appendix E) was prepared and 5ml was transferred to the test tubes and inoculated with isolated culture.
2) Incubation at 37^0C for 48-96 hours.
3) Following incubation, 0.2 ml of Kovac's reagent has been added.

A cherry red color in alcohol indicates a positive reaction.

***7.6.7. Methyl Red Test*: -** the significance of this test is to detect the ability of isolates to oxidize glucose, and the production and stabilization of high acid end products. To perform this experiment..

1) MR-VP broth (Appendix F) being prepared, sterilized and transferred into different test tubes.
2) One test tube was kept as control while other tubes were inoculated with isolates.
3) Then cultures were incubated at 37^0C for 48-96 hours.
4) Following incubation 5-6 drops of methyl red was added.

A bright red color after addition of methyl red indicates positive result and no color change indicates negative reaction.

***7.6.8. Starch Hydrolysis Test*:** - Starch is linear polymer of glucose molecule linked together by glycosidic bond. Because of its high molecular weight starch cannot be transported into the cell. So to assimilate starch for energy and catabolic reaction, it must be degraded into basic glucose units by starch hydrolyzing enzyme secreted extracellular, and allow starch to pass through cell wall. To perform this experiment..

1) Starch agar media (Appendix G) was prepared and sterilized by autoclaving and plates were made.
2) Dilution of isolates had been inoculated using glass spreader and one plate is kept as control.
3) All plates are incubated at 37^0C for 48-96 hours.

Hydrolysis of starch can be remarked by clear zones formed over the plate.

***7.6.9. Triple Sugar Iron Test*: -**

Procedure:

1) 6% TSI (triple sugar iron) agar was prepared and slants were made of this media.
2) Slants were streak inoculated with isolates using inoculation loop and incubated at 37^0C for 24-48 hours, including control slant.

Results can be of three types:

- **First** in which sugar fermentation observed where acid butt, alkaline slant (yellow butt, red slant) that means glucose has been fermented but not lactose nor sucrose,

- If acid butt, acid slant is observed (yellow butt, yellow slant) that means lactose or sucrose has been fermented,
- If alkaline butt, alkaline slant observed (red butt, red slant) that means neither glucose, lactose nor sucrose has been fermented.
- **Second** there may be gas production which is indicated by gas bubbling in butt it can be easily noted when agar may be broken or pushed upward.
- **Thirdly** hydrogen sulphide production which is indicated by the blackening of the butt. This is due to the reaction of hydrogen sulphide with the ferrous ammonium sulphate to form black ferrous sulphide.

***7.6.10) Acetonin Production (Voges Proskauer) Test*: -** This is performed to determine the capacity of some organism to ferment carbohydrates with production of non-acidic or neutral compound. Procedure:

1) MR-VP agar (Appendix F) slant were prepared after sterilization by autoclaving and inoculated with isolated cultures. One slant is kept as control, un-inoculated.
2) After that slants incubated at 37^0C for 48-72 hours.
3) Following incubation add 1 ml of 40% potassium hydroxide and 3 ml of 5% solution of alpha-naphthol in absolute ethanol.

A positive reaction is indicated by development of a pink color in 2.5 minute becoming crimson in 30 minutes. *(Bhardwaj et al, 2012)*

7.7. Identification of Bacteria by Bergey's Manual

Identification of bacteria by using the morphological and biochemical techniques using taxonomic scheme of Bergey's manual of Determinative Bacteriology.

GENETIC MANIPULATION AND CHARACTERIZATION OF BACTERIOCIN

7.8. Screening for the Production of Bacteriocin (Secondary Metabolite)

Almost all microorganisms secrete secondary metabolites extracellular or intracellular. In case of bacteriocin, it is secreted extracellular which is responsible for the antimicrobial activity of the secondary metabolite, produced by various species of *Lactoacillus* and *Bacillus*? Antimicrobial agents are produced in response to reduce the competition with other species or neighboring colonies. So to identify that, if any secondary metabolite is being produced by the isolates can be done by following procedure..,

1) After biochemical tests two isolates were inoculated in 4 different conical flasks containing nutrient broth under the laminar air flow.
2) Two flasks for each isolate had been inoculated and one of which was treated with UV light daily for 1 hour under laminar UV lamp.
3) And the flasks were placed in shaker incubator at 32^0C for 6 days.
4) Everyday 10 ml of culture sample were collected and after centrifugation at 5000 rpm for 20 minutes, supernatant stored in refrigerator while pellet was discarded

UV treatment is used to induce mutation in the type of metabolite production with varying activity of the metabolite (*Adrio et al,* 2005). UV rays cause thiamine dimer bond formation which could be lethal for the bacteria itself or it may affect only metabolite composition, but growth of bacteria observed even after the UV treatment this proves that bacteria is not dead.

After centrifugation pellet contains cells and heavy particles of media which was discarded but the pellet might contain bacteriocin, which is stored in refrigerator. Same procedure is repeated for the next 6 days and sample collected followed by UV treatment. (Haavik et al, 1974)

After the sample collection the protein content is quantitatively and qualitatively analyzed by the **Lowry's method on protein estimation**, by using BSA standard protein solution and Lowry's reagent 1 and reagent 2. After this spectrophotometer was used to estimate the quantity of protein in the metabolites.

The principle behind the Lowry method of determining protein concentrations lies in the reactivity of the peptide nitrogen[s] with the copper [II] ions under alkaline conditions and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Dunn, 1992). The Lowry method is sensitive to pH changes and therefore the pH of assay solution should be maintained at 10 - 10.5. The Lowry method is sensitive to even low concentrations of protein. Dunn (1992) suggests concentrations ranging from 0.10 - 2 mg of protein per ml while Price (1996) suggests concentrations of 0.005 - 0.10 mg of protein per ml. The major disadvantage of the Lowry method is the narrow pH range within which it is accurate. However, we used very small volumes of sample, which had very little or no effect on pH of the reaction mixture.

A variety of compounds might interfere with the Lowry procedure. These include some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids and sulphydryl reagents (Dunn, 1992). Price (1996) notes that ammonium ions, zwitter ionic buffers, nonionic buffers and thiol compounds may also interfere with the Lowry reaction. These substances should be removed or diluted before running Lowry's assays.

Procedure for Lowry's method of protein estimation:

1) 0, 0.1, 0.2, 0.3, 0.4, 0.5, ….., likewise up to 1ml of BSA (1mg/ml) working standard in 11 test tubes and make up to 1ml using distilled water.
2) The test tube with 1 ml distilled water serves as blank.
3) Add 4.5 ml of Reagent I and incubate for 10 minutes.
4) After incubation add 0.5 ml of reagent II and incubate for 30 minutes
5) Measure the absorbance at 660 nm and plot the standard graph .
6) Estimate the amount of protein present in the given sample from the standard graph.by repeating the steps 3, 4 and 5 for different samples collected as secondary metabolites.

7.9. Screening for Antimicrobial Activity of Protein

Different metabolite sample collected in 6 days were tested against the test organism E.Coli by agar well diffusion method.(Ravi et al, 2011) To check its antimicrobial activity following procedure was performed

1) Autoclaved nutrient agar was poured on petriplates and 3 wells per plate were made,
2) Plates inoculated with 5 µl sample of E.Coli and spread over media,
3) In each petriplate, one well is inoculated with 10µl distilled water and the other two were filled with 10 µl different collected metabolites of same isolates on same day
4) Incubate at 37^0C for 24 to 72 hours
5) Checked for zone of clearance around the wells and the diameter of the zones were measured every day.

If the zones are visible this means that antimicrobial agents are present in the collected sample and same is produced by isolated microbes. And different diameter of zone indicates the activity/strength of the antimicrobial agent

7.10. Protein Purification

To isolate a specific protein form a crude mixture it's physical and/or chemical need to be exploited. There is no single and simple way to purify all kinds of protein separately. Procedure for one protein could denature another one. So while choosing purification method it is very difficult and depends on the type of use, e.g., an enzyme which needed to be used in the washing powder could be a relatively in impure sample is sufficient: provided it does not contain any inhibitors. If the protein is to be utilized for therapeutic purpose it must be extremely pure and concentrated.

Main target behind the purification step is only to remove unwanted contaminants, but also the concentrated form of the desired protein and remain in stable conformation with its full activity.

Under different conditions proteins have the ability to be adsorbed on the surface to a variety of solid phases, and preferably in selective manner. The adsorption property is further applied in column chromatography. Due to their high resolving power, different chromatography techniques have become dominant for protein purification.

Column chromatography is the most common physical configuration, in which the stationary phase is packed into a tube, a column, through which the mobile phase, the eluent, is pumped. The degree to which the molecule adsorbs or interacts with the stationary phase will determine how fast it will be carried by the mobile phase. Chromatographic separation of protein mixtures has become one of the most effective and widely used means of purifying individual proteins. (Proath, et al, 1997: Rajaram et al, 2010)

In **SEC (size exclusion chromatography)**, the matrix consists of porous particles and separation is achieved according to size and shape of the molecules. The technique is sometimes also referred to as **gel filtration (GFC)**, molecular sieve chromatography or gel-permeation chromatography.

For separation in GFC, molecular sieve properties of a variety of porous materials are utilized. GFC matrices consist of a range of beads with slightly different pore sizes. The separation process depends on the different ability of various proteins to enter all, some or none of the channels in the porous beads. Molecules running through a GFC column have to solve a maze which becomes more complex the smaller the molecule is, as the small molecules have more potential channels that they can access. Larger molecules on the other hand, are for steric reasons excluded from the channels, and pass quickly between the beads. The detour through the channels will thus retard smaller molecules in comparison to larger proteins.

Although the separation in GFC is generally assumed to be according to molecular weight, it is more accurate to claim that it is achieved by the differential exclusion or inclusion within porous particles. The ease of diffusion is dependent on the hydrodynamic volume, which is the volume created by the movement of the molecule in water. The difference between hydrodynamic volume and molecular weight is shape. Proteins tend to be globular molecules while DNA or polysaccharides tend to be linear molecules. Linear molecules have much larger hydrodynamic volumes than globular molecules, so a 10,000 MW DNA molecule will elute much earlier than a 10,000 MW protein.

The stationary phase in GFC is composed of natural polymer such as agarose or dextran but may also be composed of synthetic polymers such as polyacrylamide. Gels may be formed from these polymers by cross-linking to form a three-dimensional network. Different pore sizes can be obtained by slightly differing amounts of cross-linking. The degree of cross-linking will define the pore size. The first commercial SEC media, Sephadex, composed of dextran that was crosslinked with Epichlorohydrin.

Here in this procedure we used DEAE Sephadex A-50 cross linked with Diethylaminoethy

And the mobile phase is a single buffer solution with a well defined pH and ionic composition to preserve the structure and biological activity of the protein of interest.

Here in this procedure we used phosphate buffer saline pH 7.2.

Procedure :

1. Column Preparation: Pack the column with glass wool of about 2-3 cm thickness and pour 2% sephadex porous stationary phase and allow it to settle for 1-2 days.
2. Column Equilibration: Choose a buffer for your specific application like PBS and use this same buffer for both equilibration and elution steps. To equilibrate the column, allow the equilibration buffer to enter the gel bed completely and continue elution until approximately 15 ml of buffer has been eluted. And set the flow rate at constant volume in per unit time.
3. Sample Application: Transfer up to 1 ml of your sample to Sephadex Column. Allow the sample to enter the gel bed completely. If the sample volume is less than 1 ml, add enough equilibration buffer so that the combined volume equals 1 ml before applying it to the column.
4. Elution: Place a tube/eppendorf for sample collection under the Sephadex Column and collect a fixed amount (say 1ml/tube). Transfer 1.5 ml of elution buffer to the column and elute the cleaned sample.

Now again by the Lowry's method of protein estimation we can find that which tube had maximum concentration of protein.

7.11. SDS PAGE

This is a technique for electrophoresis of protein molecules to separate protein molecules on the basis of their shape, size and molecular weight. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is the robust method to determine the molecular weight of unknown protein. In this technique protein of known molecular mass (called as SDS ladder / marker) are run along the on a track adjacent to protein of unknown weight. Each marker moves a specific distance through the gel , is measured and a calibration curve of log M_r versus distance moved is plotted, and the unknown protein distance is also measured and plotted against standard graph and hence M_r is calculated by comparision (Walker, 2005).

Procedure:

Stacking and resolving protein gels preparation according to Laemmli (1970)

1. Take two large glass plates (one large and one small), a comb, a casting block and the casting stand, and clean them using ethanol.
2. Arrange the sandwich, and take care that lower edges of the glass plates and spacers are well aligned.
3. Prepare fresh 10% (w/v) ammoniumpersulfate (APS) solution.
4. Prepare the separating gel mixture (at least 10 ml per gel when using the 1.5 mm spacers) (Appendix H):

	30% acrylamide	Buffer I	MilliQ
10 % gel	3.3 ml	2.5 ml	4.0 ml

5. Add 100 µl of 10 % SDS, 80 µl of 10 % APS and 10 µl of TEMED.
6. Pour gel between the glass plate sandwich with spacers, leaving 2 cm free from the top of the smaller glass plate. Carefully pipette water or water-saturated iso-butanol on top to create smooth top surface.
7. When the separating gel has polymerized, prepare the stacking gel mixture (3.75 % acrylamide):
   2.4 ml MilliQ
   1.0 ml Buffer II
   0.5 ml 30% acrylamide
   40 µl 10% SDS.
8. Pour the water from the top of the separating gel completely.
9. Add APS and TEMED to stacking gel then mix and pour it from the top and insert the comb. Be careful not to introduce air bubbles to the gel.
10. Let it polymerize.
11. Attach this gel sandwich to the electrode assembly. Grease the gasket to prevent leakage. If you are running only one gel, turn the gasket from the other side around and put only the acryl block on that side.
12. Pour running buffer inside the gel assembly. The buffer should be in good contact with the gel. Let stand for few minutes while preparing the samples to check for any leakage. Only if there is no leakage, fill the outer chamber (tank) with buffer to cover the bottom of the gels.
13. Prepare the samples by mixing one volume of sample and one volume of sample buffer. Heat at 95 ^{0}C for few minutes and load on the gel.
14. Run at 150V for about 1 h 20 min.
15. After when bands appear, stain the gel overnight and then destain.
16. Observe under UV lamp. And take images.
    (for solution see Appendix I)

FTIR Spectroscopy

This is the technique in the present arsenal of protein structural methods that are able to provide information on all aspects of protein structure. That's why a rational strategy has to be employing for a concerted approach in which the protein is being examined using several structural techniques. So that, information obtained from different techniques could be cross-correlated to provide a more complete picture of the chemical and physical state and/or bioactivity of the protein under different conditions and environment. One of the techniques which have recently become very popular for structural characterization of proteins is Fourier transform infrared spectroscopy FTIR

Infrared spectroscopy's working principle is based on molecular vibrations. Chemical bonds undergo various forms of vibrations such as stretching, twisting and rotating. The energy of most molecular vibrations corresponds to that of the infrared region of electromagnetic spectrum. Many of the vibrations can be localized to specific bonds or groupings, such as the C=O and O–H groups. This has led to the concept of characteristic group frequencies. Typical group frequencies of interest to biochemists include C=O, –COOH, COO^{---}, O–H and S–H. There are many other

vibration modes that do not represent a single type of bond oscillation but are strongly coupled to neighboring bonds. For example, the infrared spectrum of a protein is characterized by a set of absorption regions known as the amide modes.

With developments in FTIR instrumentation it is now possible to obtain high quality spectra from dilute protein solutions in H_2O. The overlapping H_2O absorption can be digitally subtracted from the spectrum of the protein solution. In addition to digital spectrum, the broad infrared bands in the spectra of proteins can be analyzed in detail using second-derivative and deconvolution procedures. These procedures can be utilized to reveal the overlapping components within the broad amide bands. Difference spectroscopy has the advantage in providing highly detailed information on conformational changes in proteins.

The most important advantage of FTIR spectroscopy for biological studies is that spectra of almost any biological material (synthetic or natural) can be obtained in a wide variety of environments. Spectra of a protein can be obtained in single crystals, in aqueous solution, organic solvents, detergents micelles, lipid membranes, etc. The chemical environment in which a peptide or protein exists influences its structure and stability, which has important implications for the formulation, storage and delivery mechanisms for protein therapeutics. There is increasing evidence indicating about the environment, which can be important in determining the secondary structure acquired by an amino acid sequence. Other advantages of the technique include:

- The amount of protein required is relatively small ~10 mg.;
- the size of the protein is not important;
- there is no light scattering or fluorescent effects;
- kinetic and time-resolved studies are possible;
- and inexpensive compared to the cost of X-ray diffraction, NMR, ESR and CD spectroscopic equipments.

CHAPTER 8
RESULTS

8.1. Isolation of the Microorganism Colony:

From the dilution sample of 10^{-5} separate colonies were identified and isolated with the help of loop

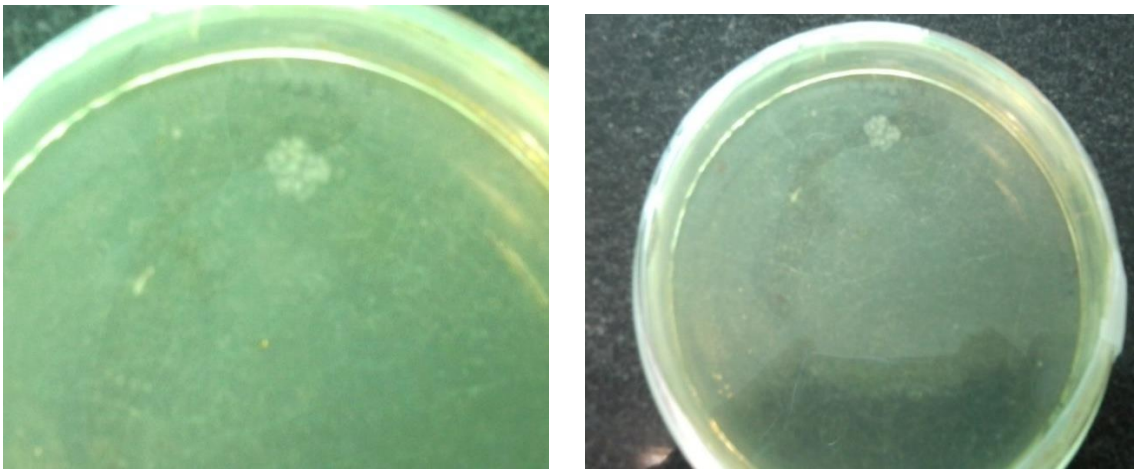


Fig. A)*Bacillus*

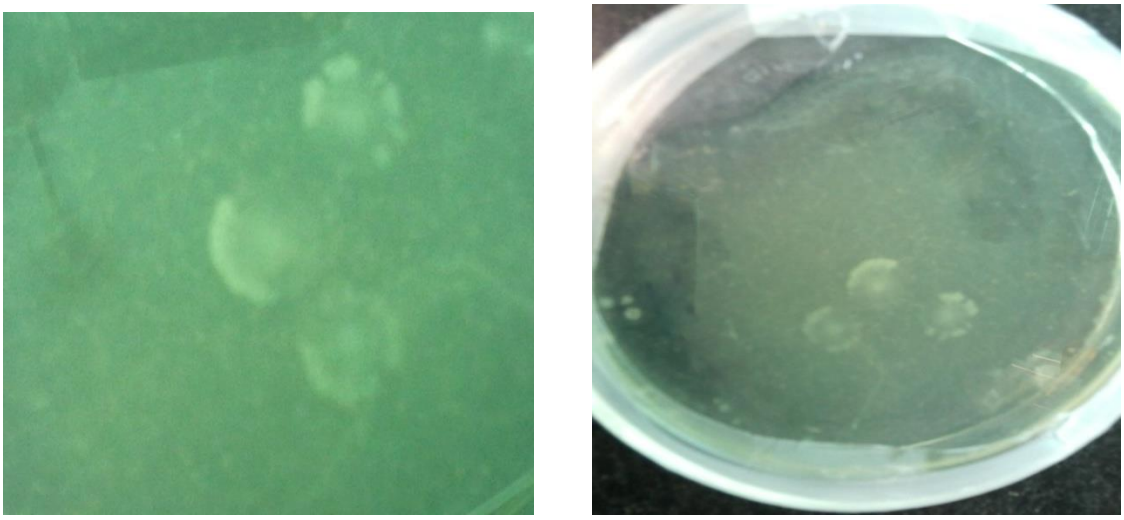
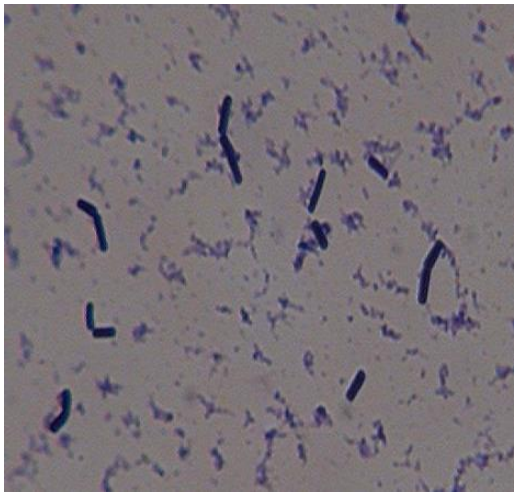
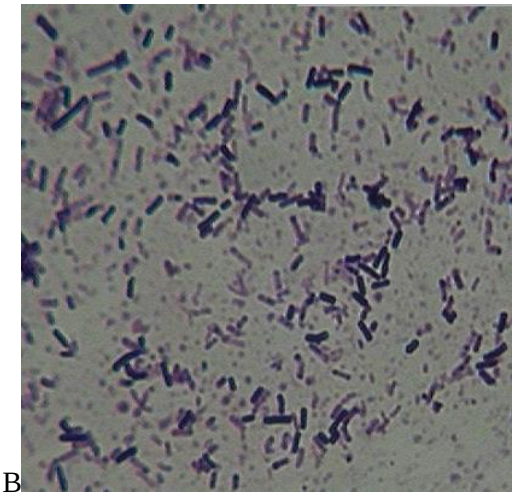


Fig. B) *Lactobacillus*

8.2. Morphological Identification of the Isolated Bacteria

8.2.1. Gram Staining

The single bacterial colony was selected and cultured in nutrient broth and after the gram staining in confirms the rod shaped (*Bacillus*) microorganism

A   B

A) *Bacillus* B) *Lactobacillus*

8.2.3. Endospore Staining

No endospore were present

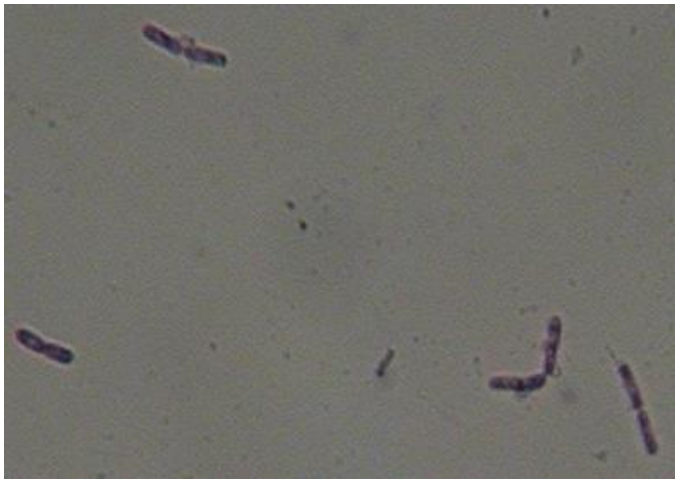


Fig. A. *Lactobacillus* sp.

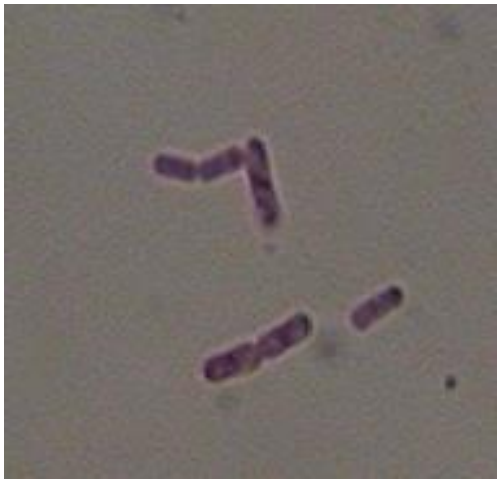


Fig. B. *Bacillus* sp.

8.3. Biochemical Test:

8.3.1.Carbohydrate Fermentation Test

8.3.1.1. Glucose: - Change in color from pink red to yellow indicates that here cells can utilize glucose as substrate and there is an Acid and Gas production which causes the change the medium color to yellow. Gas production can be detected by the presence of small bubbles in the inverted Durham tubes.

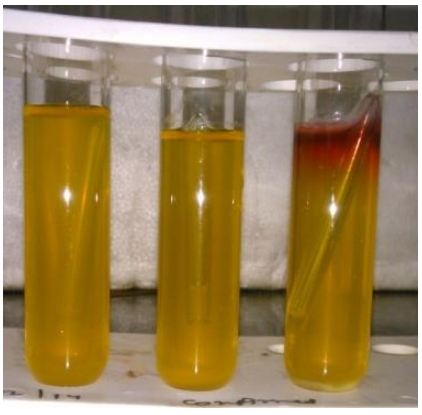


Fig. Left to right: control, *Bacillus*, *Lactobacillus*

8.3.1.2. Lactose:- No change in color indicates that present isolates were not utilizing lactose as its substrate and hence no growth observed.

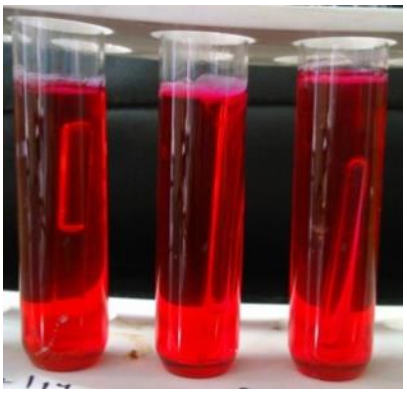


Fig. Left to right: control, *Bacillus*, *Lactobacillus*

8.3.1.3. Mannitol:- Growth was not observed in both of the inoculated tubes, this indicates both isolates cannot use mannitol.

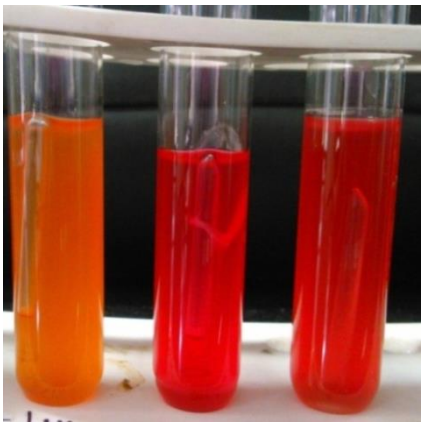


Fig. Left to right: control, *Bacillus*, *Lactobacillus*

8.3.1.4. Maltose:- Growth was observed in both isolates. Hence change in color confirms the result. Yellow color indicates change in pH because of acid production but no bubble formation indicates absence of gas production.

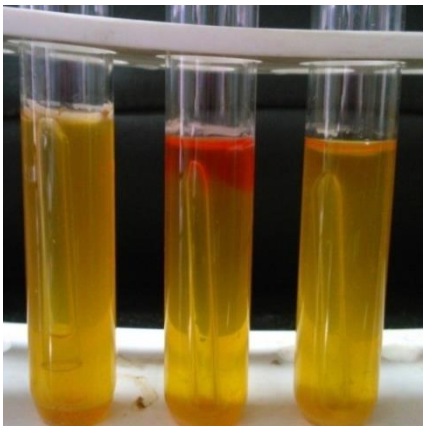


Fig. Left to right: control, *Bacillus*, *Lactobacillus*

8.3.1.5. Sucrose:- Acid as well as Gas production was observed in both isolates. Acidic breakdown of sucrose causes the shift in pH results in change in color to yellow while the bubbles formed in Durham tube indicates gas production.

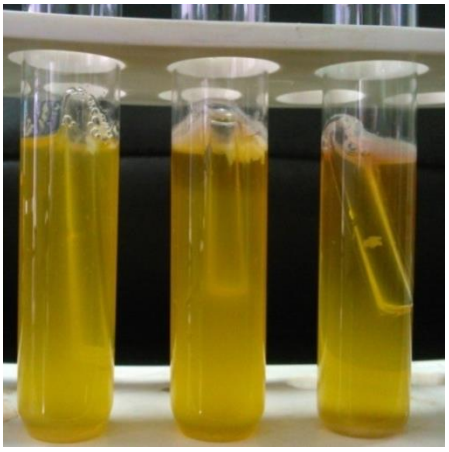
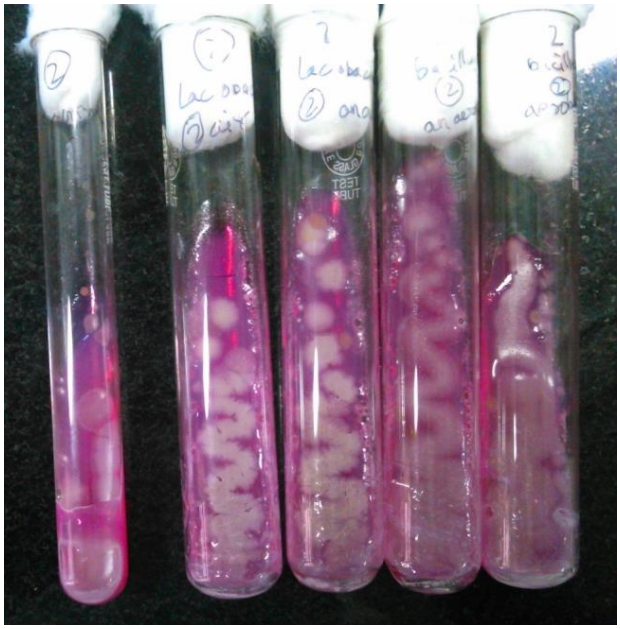


Fig. Left to right: control, *Bacillus*, *Lactobacillus*

8.3.2. Aerobic and Anaerobic Carbohydrate Breakdown Test:- For *Lactobacillus* isolates growth was observed in both conditions aerobic as well as anaerobic, which indicates isolates of *Lactobacillus* can break carbohydrate both oxidatively and fermentatively.

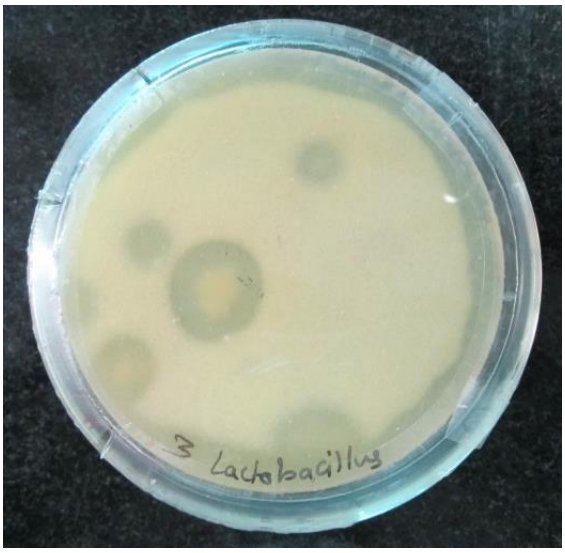


In case of isolates of *Bacillus*, growth is high in anaerobic condition while slight growth in aerobic condition, which indicates that isolates of *Bacillus* are more efficient in fermentative breakdown while weak in oxidative utilization of carbohydrate.

Fig. Left to right: control, *Lactobacillus*: aerobic, anaerobic; *Bacillus*: anaerobic, aerobic.

8.3.3. Casein Hydrolysis

Lactobacillus isolates were clearly indicate, visible zone of inhibition, hence it can digest the casein while the *Bacillus* species cannot, this means that *Lactobacillus* isolates could produce those casein hydrolysing proteolytic enzymes which can digest casein while the isolates of *Bacillus* cannot utilize casein as substrate.



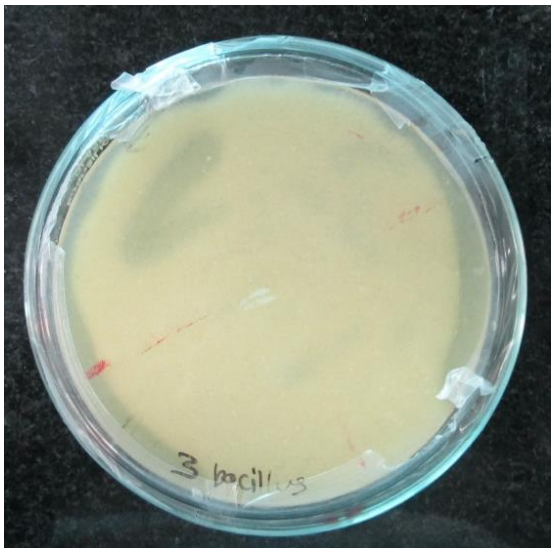
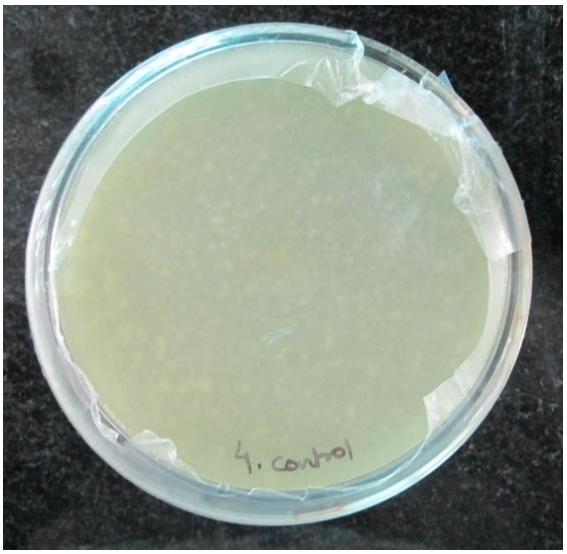


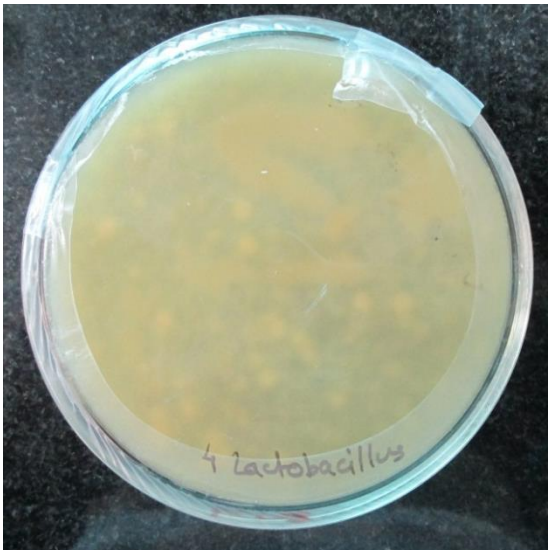
Fig. *Lactobacillus* *Bacillus*

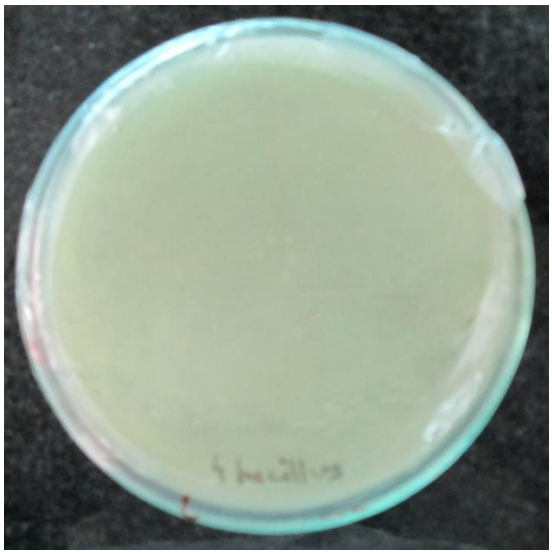
8.3.4. Catalase Test on nutrient agar

After the incubation of 48 hours at 37^0C on nutrient agar medium, only the *Lactobacillus* species were showing some of the catalase activity, but very poorly, while *Bacillus* species were not showing any sign on catalase activity even after 72 hours of incubation.

After that on the treatment with 1 ml of 3% hydrogen peroxide, *Lactobacillus* isolates responded by the formation of bubbles of free oxygen. This approves the ability of *Lactobacillus* species to produce catalase, while *Bacillus* species cannot.







8.3.5. Citrate Test:- Simmons citrate medium

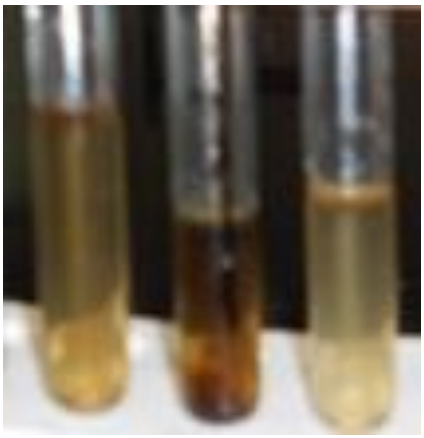
Result were negative for both the isolates

Fig. left to right: control, *Bacillus*, *Lactobacillus*

8.3.6. Indole Test:-Results were negative

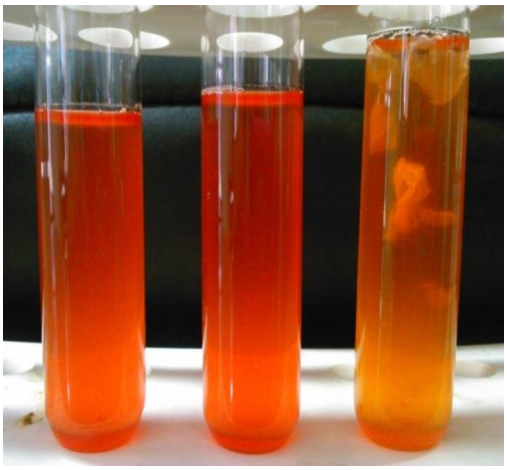
for control as well as both of the isolates.

Left to right: control, *Bacillus*, *Lactobacillus*



8.3.7. Methyl Red Test:- Positive results for both the isolates.

Left to right: control, *Bacillus*, *Lactobacillus*



8.3.8. Starch Hydrolysis. Starch agar slants were incubated at 37^0C for 24-48 hours, after the streaking. Results were positive for both the samples i.e. *Lactobacillus* and *Bacillus* species. Because the test organism can secrete starch hydrolysing enzymes that can degrade the starch present in the medium which degrade starch primarily into the glucose.

Left to right: control, *Bacillus*, *Lactobacillus*

8.3.9. Triple Sugar Iron Agar Test: Positive results for the *Bacillus* but negative for *Lactobacillus*

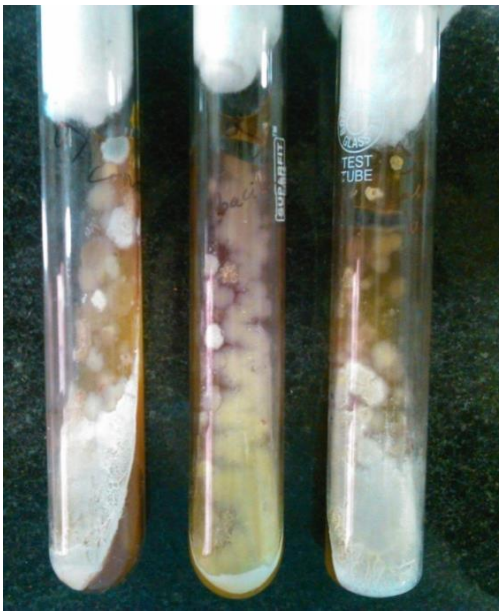


Fig. Front view: Left to right: control, *Bacillus*, *Lactobacillus*

Fig. Rear view: Left to right: control, *Bacillus*

Lactobacillus

8.3.10.Acetoin production:

Positive result for both the isolates

Left to right: control, *Bacillus*, *Lactobacillus*

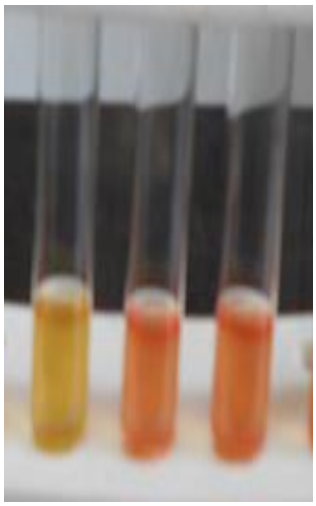


Table 1. Biochemical test for identification

Test	response of *Lactobacillus sp*	response of *Bacillus sp.*
Gram staining	Gram Positive	Gram Positive
Shape	Rod	Rod
Endospore staining	-	-

Carbohydrate fermentation Glucose Lactose Maltose Mannitol Sucrose	 + - + - +	 + - + - +
Aerobic and anaerobic carbohydrate breakdown	+ (both aerobic and anaerobic)	+anaerobic, weakly aerobic
Casein hydrolysis	+	-
Catalase production	+	-
Citrate utilization	-	-
Indole test	-	-
Methyl red test	+	+
Starch hydrolysis	+	+
Triple sugar iron test	-	+
Acetoin production	+	+

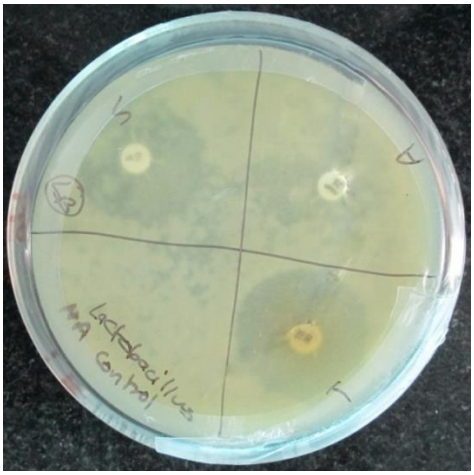
8.4. Identification of Bacteria by Bergey's Manual of Determinative Bacteriology

On the basis on Bergey's manual and results from Table 1, the isolate from curd is *Lactobacillus* sp. and the second isolate is *Bacillus sp.*

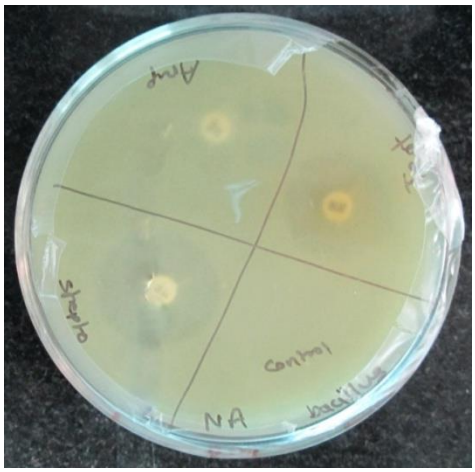
8.5. Antibiotic Susceptibility Test:

Lactobacillus species is showing zone of inhibition against streptomycin disc, tetracyclin disc, and little against ampicillin; and *Bacillus* species showing zone of inhibition of growth against streptomycin and tetracyclin (very clear) but little with ampicillin.

The results indicate both the isolates are susceptible to tetracycline and streptomycin but slightly resistant to ampicillin.



Lactobacillus spp.



***Bacillus* spp.**

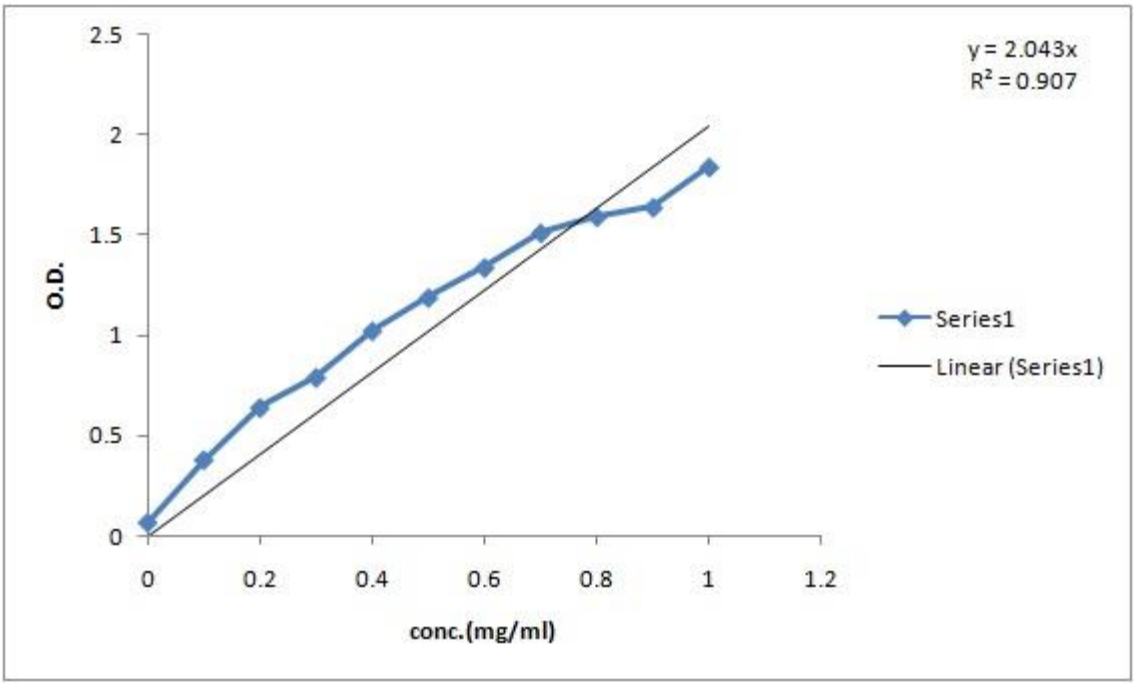
8.6. Screening for Bacteriocin Production

After the spectrophotometric analysis of the centrifuged supernatant at different time intervals, evidences the presence of protein by Lowry's method of protein estimation

Protein estimation by Lowry's method

Table 2. BSA standard estimation

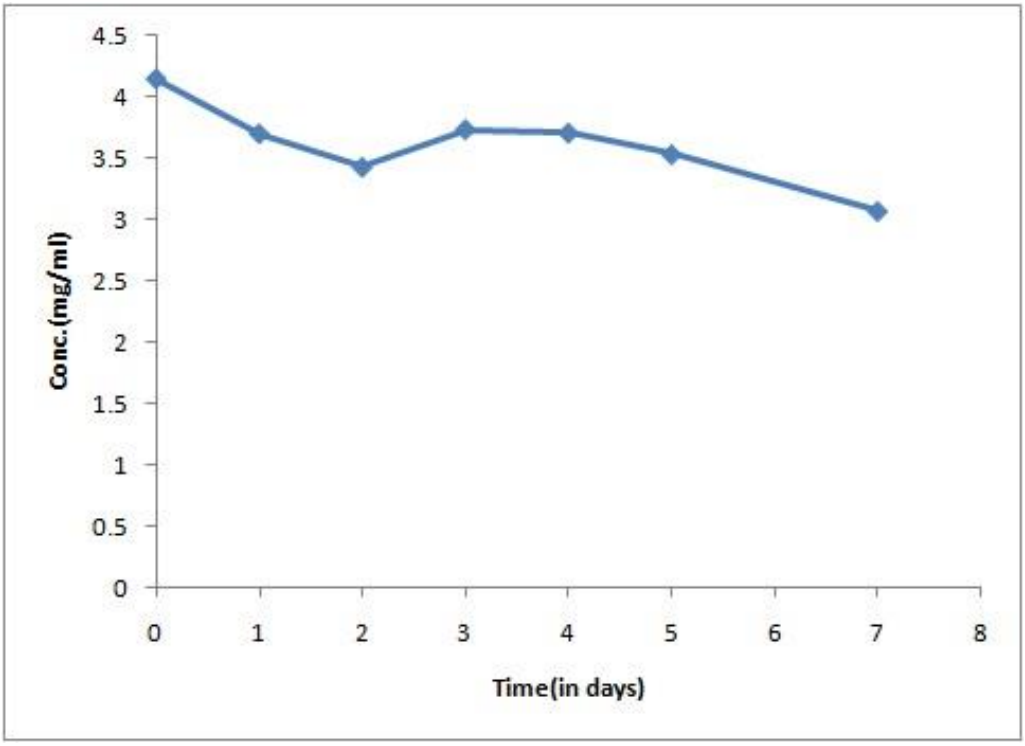
conc.	O.D.
0	0.07
0.1	0.383
0.2	0.643
0.3	0.793
0.4	1.025
0.5	1.191
0.6	1.341
0.7	1.514
0.8	1.594
0.9	1.641
1	1.842



Graph 1. BSA standard

Table 3. *Bacillus sp*. non UV treated protein estimation

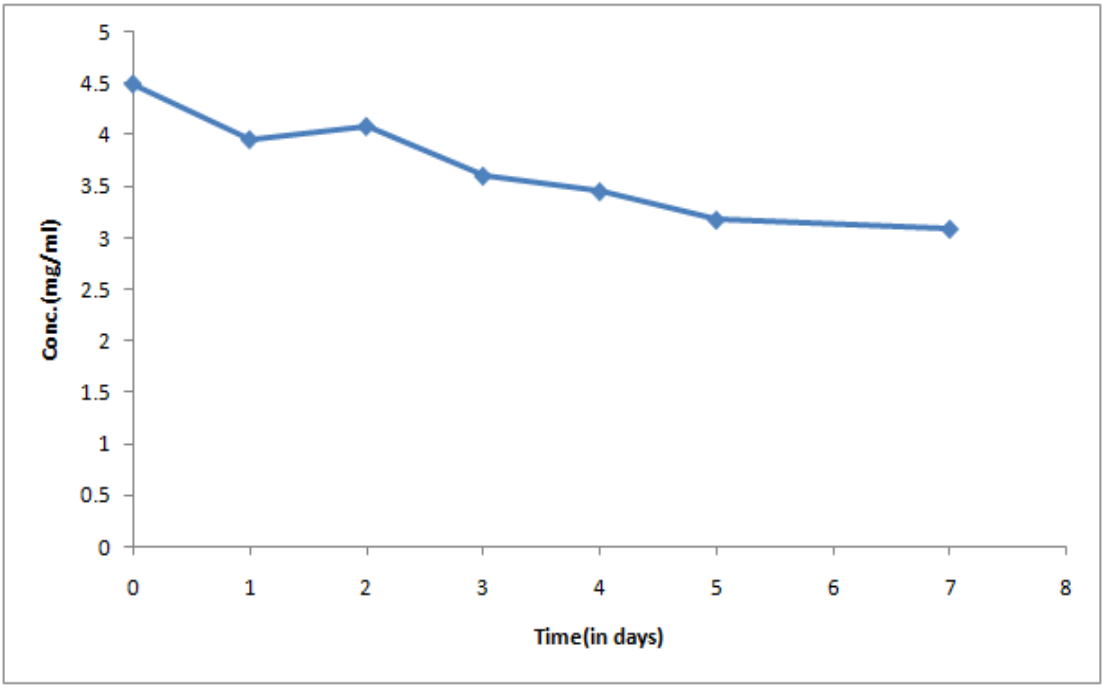
O.D.	**Day**	**conc.(mg/ml)**
0.848	0	4.150758688
0.756	1	3.700440529
0.7	2	3.426333823
0.763	3	3.734703867
0.757	4	3.705335291
0.721	5	3.529123837
0.626	7	3.06412139



Graph 2. *Bacillus sp*. non UV treated protein estimation graph

Table 4. *Lactobacillus sp.* non UV treated protein estimation

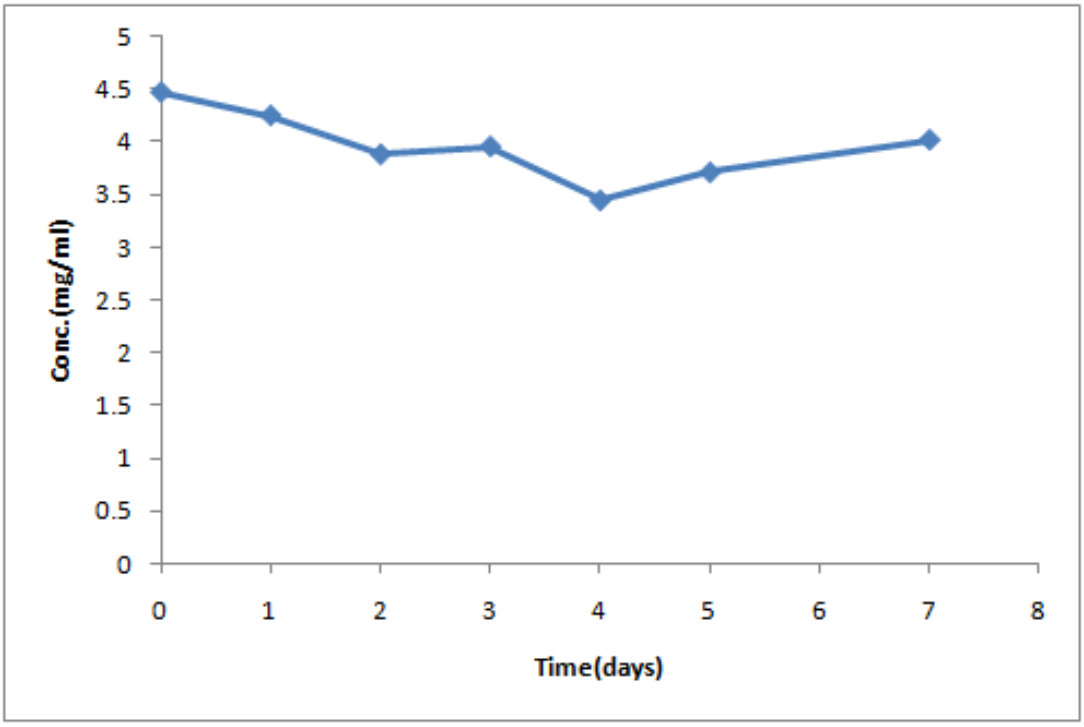
O.D.	**Day**	**conc.(mg/ml)**
0.919	0	4.498286833
0.809	1	3.959862947
0.835	2	4.087126774
0.737	3	3.607440039
0.706	4	3.455702398
0.65	5	3.181595693
0.632	7	3.093489966



Graph 3. *Lactobacillus sp.* non UV treated protein estimation graph

Table 5. *Bacillus* sp. UV treated protein estimation

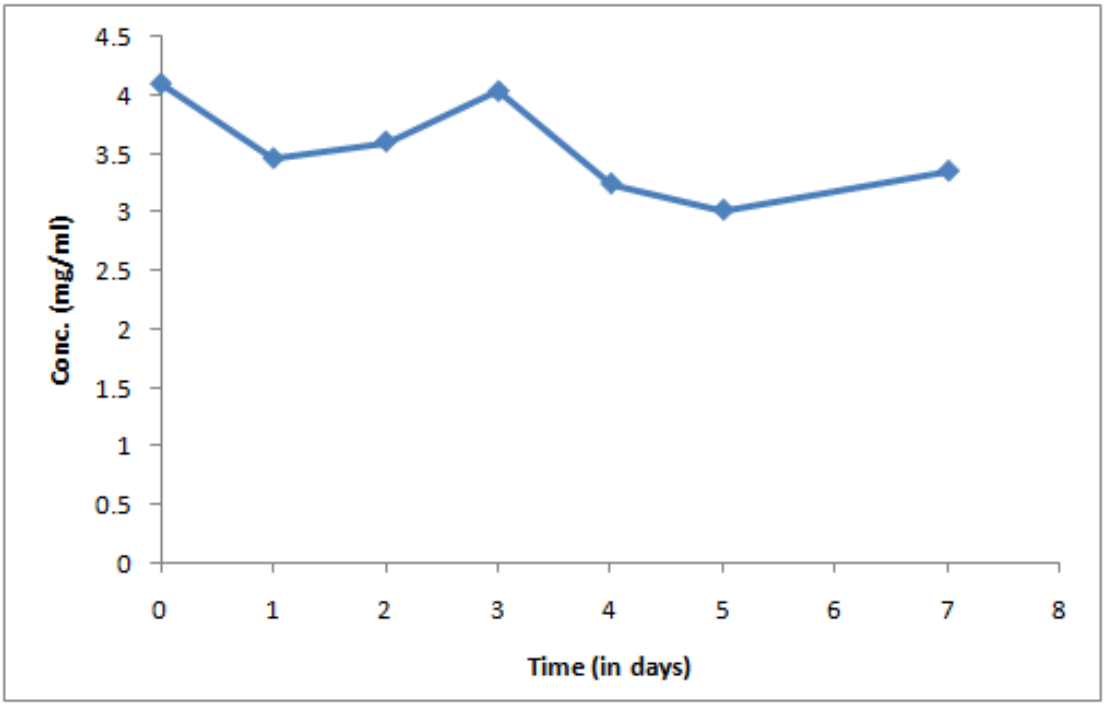
O.D.	Day	conc.(mg/ml)
0.913	0	4.468918257
0.868	1	4.24865394
0.793	2	3.881546745
0.808	3	3.954968184
0.704	4	3.445912873
0.758	5	3.710230054
0.821	7	4.018600098



Graph 4. *Bacillus sp*. UV treated protein estimation graph

Table 6. *Lactobacillus* sp. UV treated protein estimation

O.D.	Day	conc.(mg/ml)
0.837	0	4.0969163
0.707	1	3.460597161
0.736	2	3.602545277
0.824	3	4.033284386
0.663	4	3.245227606
0.617	5	3.020068527
0.685	7	3.352912384



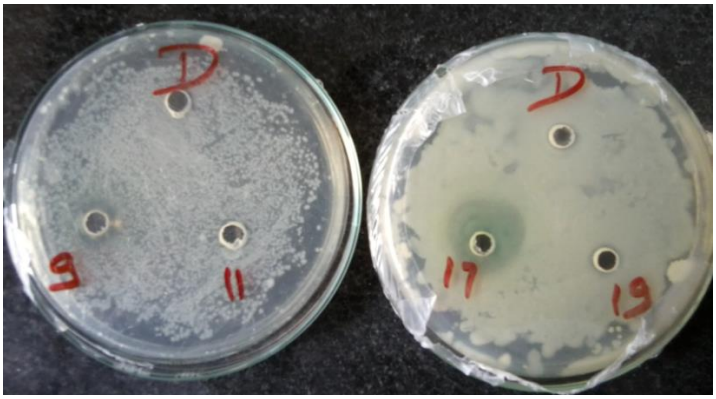
Graph 5. *Lactobacillus* sp. UV treated protein estimation

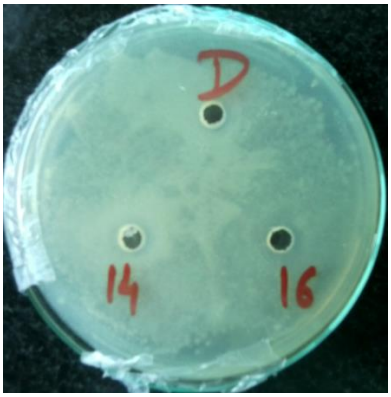
8.7. Screening for Antimicrobial Activity of the Bacteriocin

10 microlitre/well aliquot (cell free supernatant) was used to test antimicrobial activity against test organism (*E.coli*).

Table 7. Zone Of Inhibition (in milli-meter) of Antimicrobial activity of the purified protein

Zone Of Inhibition (in milli-meter) of Antimicrobial activity of the purified protein												
Secondary metabolite collection Time (in hrs)	Non UV treated Diameter of Zone of Inhibition						UV treated Diameter of Zone of Inhibition					
	Bacillus sp.			*Lactobacillus* sp.			*Bacillus* sp.			*Lactobacillus* sp.		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
0	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	0	11.5	12	-	-	-
48	10	13	14.5	-	-	-	0	13	13.5	-	-	-
72	18	20	21	0	9.5	9.5	-	-	-	0	11.5	11.5
96	18	20	21	-	-	-	-	-	-	-	-	-



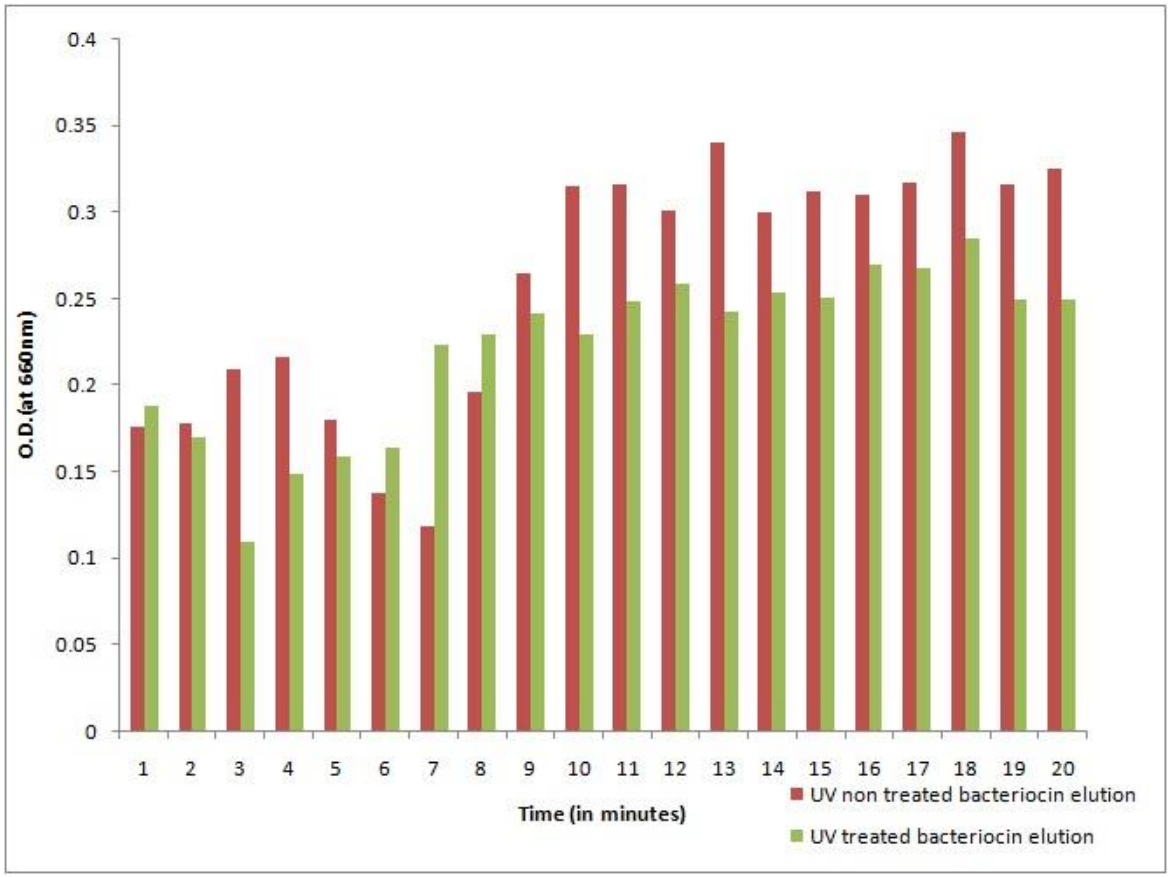


8.8. Bacteriocin Precipitation and Purification

After screening antimicrobial activity of bacteriocin, bacteriocin was purified by gel exclusion chromatograph. And purification of separated bacteriocin on after 72 hours of fermentation of *Lactobacillus* sp. of both UV treated and non treated had been carried out and the samples were eluted through the gel exclusion chromatography column of DEAE Sephadex A50, into 20 vials of 1ml volume each at the constant flow rate of 1ml/minute. And the protein concentration was estimated using spectrophotometer that was separated on the basis of the size.

Table 8. Purified bacteriocin spectrophotometric analysis

Time (in minutes)	O.D. (Bacteriocin from *Lactobacillus* sp. of UV non treated)	O.D. (Bacteriocin from *Lactobacillus* sp. of UV treated)
1	0.176	0.188
2	0.178	0.170
3	0.209	0.109
4	0.216	0.148
5	0.180	0.159
6	0.137	0.164
7	0.118	0.223
8	0.196	0.229
9	0.265	0.241
10	0.315	0.229
11	0.316	0.248
12	0.301	0.259
13	0.340	0.242
14	0.300	0.254
15	0.312	0.251
16	0.310	0.270
17	0.317	0.268
18	0.346	0.285
19	0.316	0.249
20	0.325	0.249

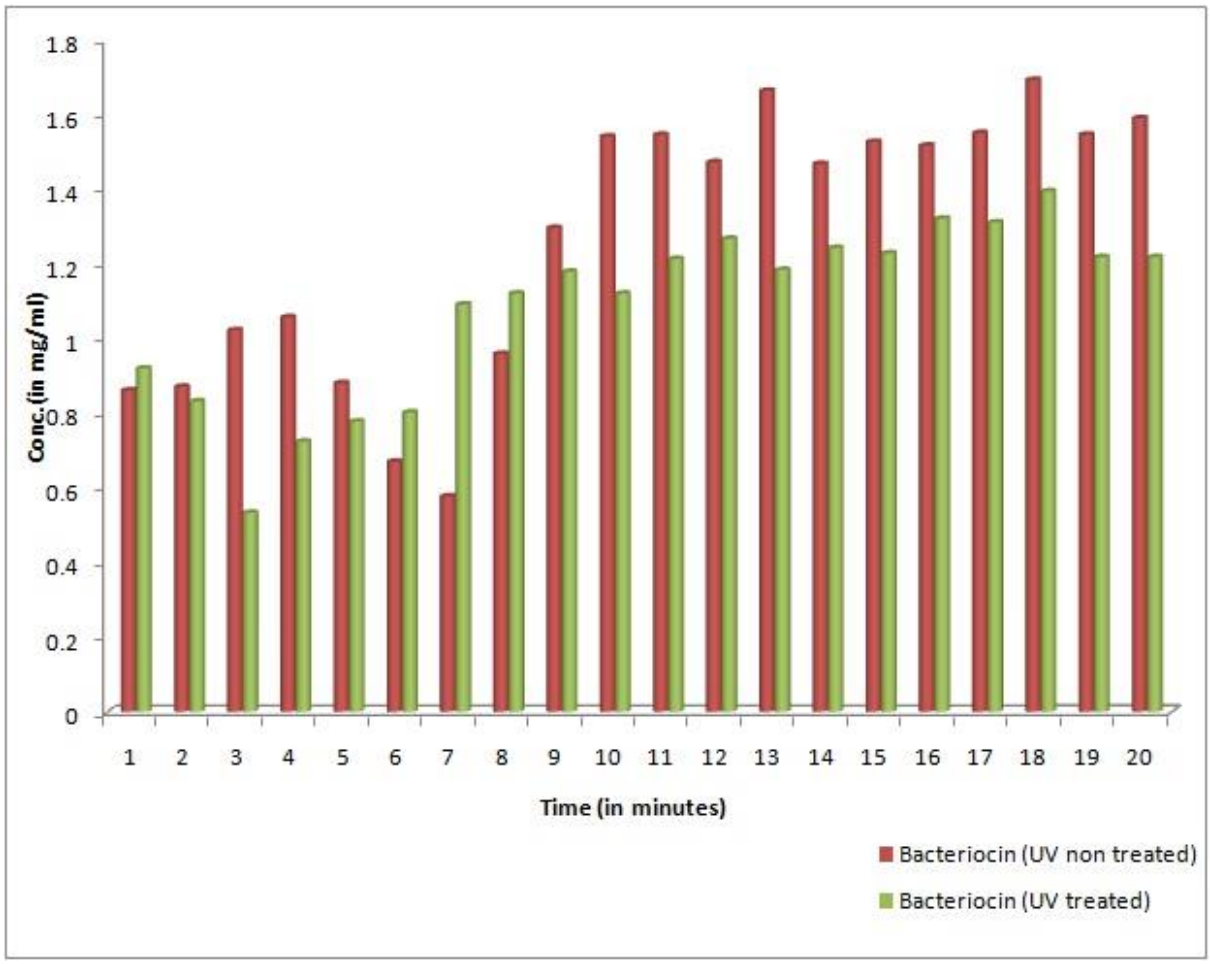


Graph 6. Purified bacteriocin O.D. graphical analysis

The elution obtained at 18 min in both the samples was containing high conc. of protein and with larger molecule size at the same time.

Table 9. Purified bacteriocin conc. estimation

Time (in minutes)	Conc.(mg/ml) (Bacteriocin from *Lactobacillus* sp. of UV non treated)	Conc. (mg/ml) (Bacteriocin from *Lactobacillus* sp. of UV treated)
1	0.8615	0.9202
2	0.8713	0.8321
3	1.023	0.5335
4	1.0573	0.7244
5	0.8811	0.7783
6	0.6706	0.8027
7	0.5776	1.0915
8	0.9594	1.1209
9	1.2971	1.1796
10	1.5419	1.1209
11	1.5467	1.2139
12	1.4733	1.2677
13	1.6642	1.1845
14	1.4684	1.2433
15	1.5272	1.2286
16	1.5174	1.3216
17	1.5516	1.3118
18	1.6936	1.395
19	1.5467	1.2188
20	1.5908	1.2188



Graph 7. Purified bacteriocin conc. graphical analysis

8.9. Molecular Weight Determination (by SDS PAGE)

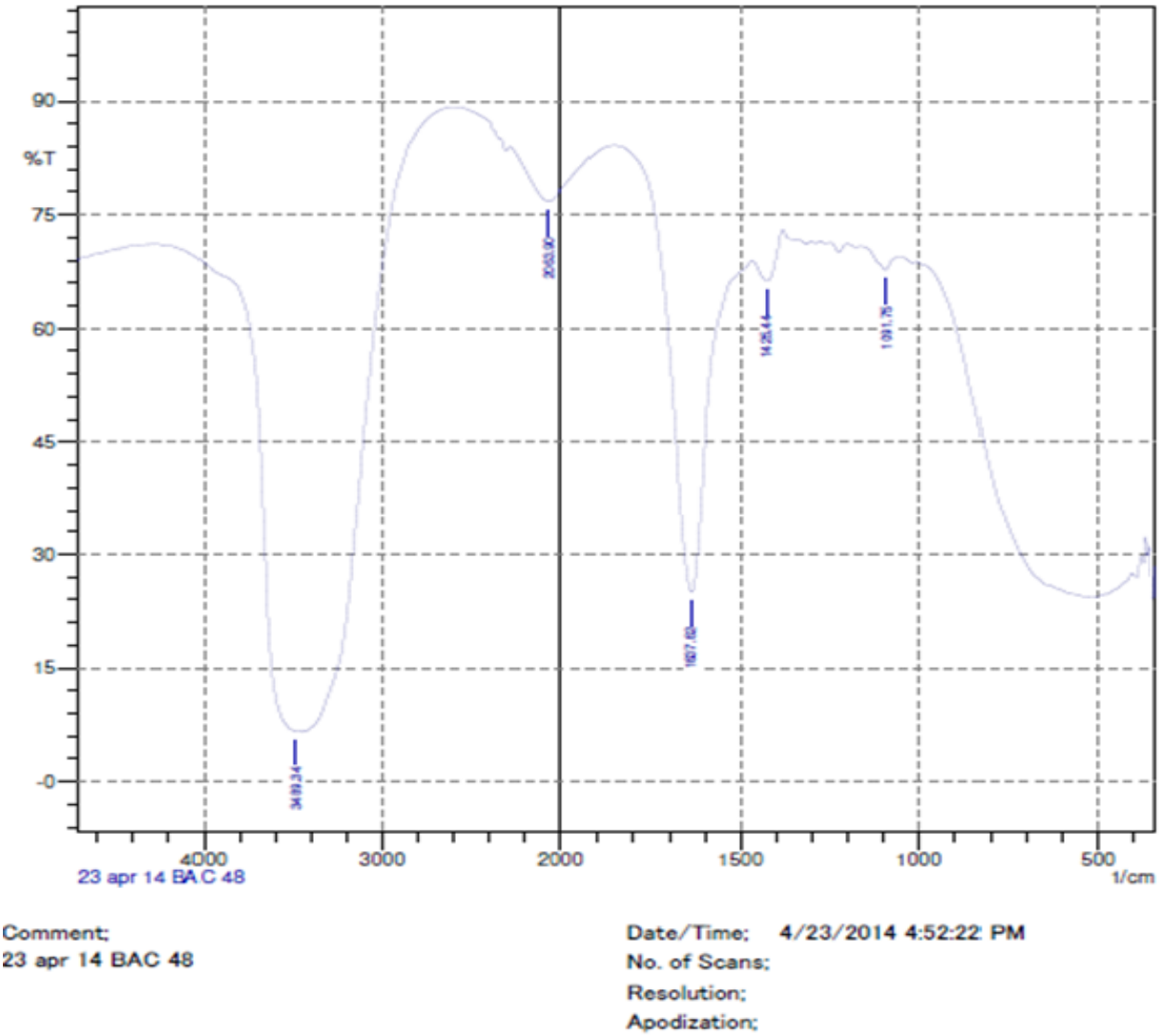
The molecular weight determined by SDS PAGE method for both samples was found to be same ~12kDa when compared with protein marker.

8.10. FTIR Spectroscopy

Bacteriocin eluted from UV non treated *Lactobacillus sp*. give following peaks of function group after FTIR spectroscopy analysis

Table 10.a. FTIR peak analysis for *Lactobacillus sp.* non UV treated

No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	1091.75	67.728	2.238	1163.11	1049.31	18.123	0.594
2	1425.44	66.293	4.619	1467.88	1381.08	14.374	1.425
3	1637.62	24.988	50.616	1855.58	1467.88	89.462	43.633
4	2063.9	76.755	7.326	2281.87	1855.58	40.826	8.72
5	3489.34	6.728	0.013	3495.13	3487.42	9.034	0.003

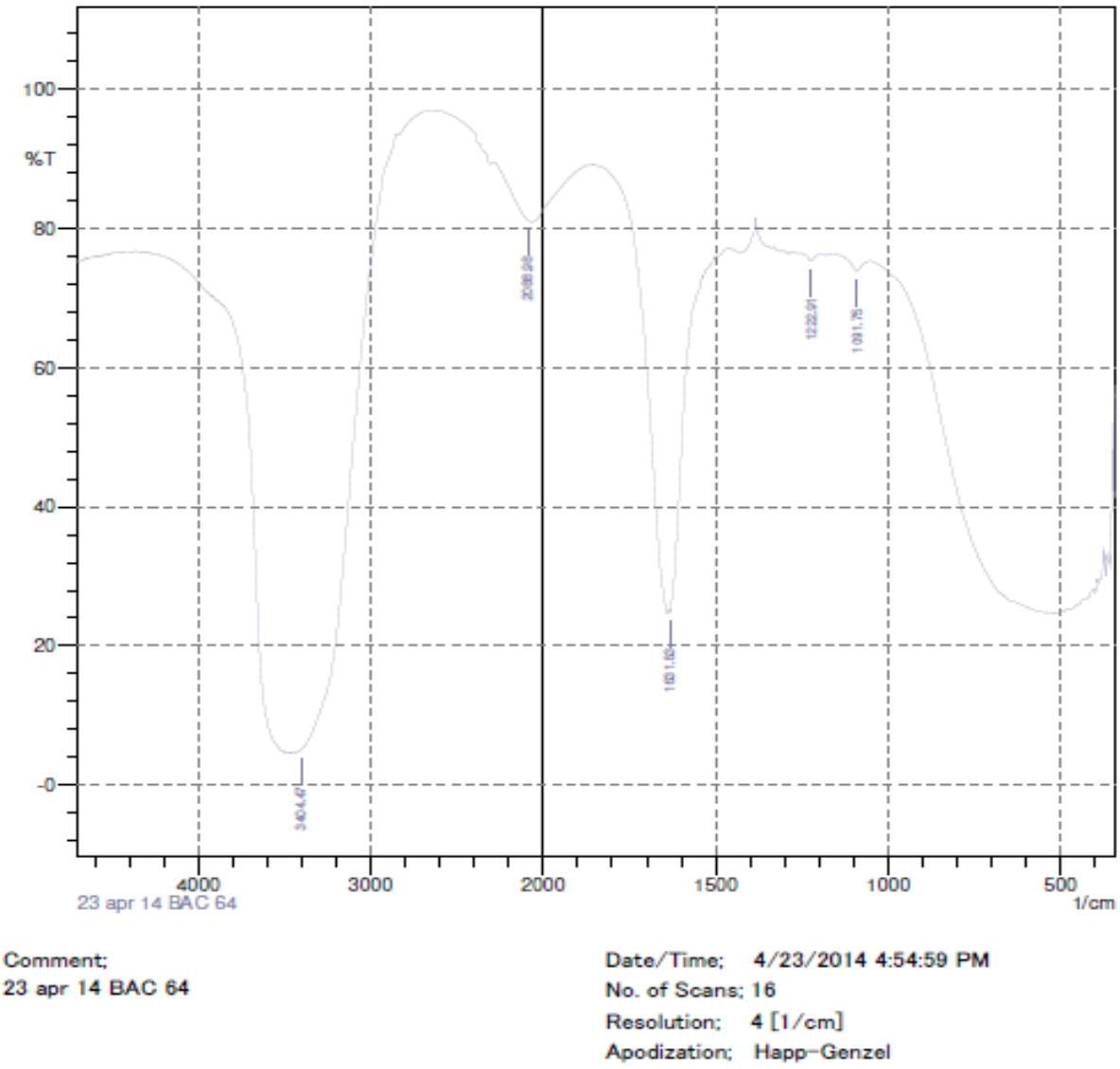


Graph 8. *Lactobacillus sp.* UV non treated FTIR characterization.

Bacteriocin eluted from UV treated *Lactobacillus sp*. give following peaks of function group after FTIR spectroscopy analysis.

Table 10.b. FTIR peak analysis for *Lactobacillus sp.* UV treated

No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	1091.75	73.836	1.846	1132.25	1049.31	10.397	0.356
2	1222.91	75.342	1.007	1251.84	1197.83	6.454	0.126
3	1631.83	24.804	1.543	1633.76	1548.89	26.574	-4.981
4	2088.98	81.149	0.101	2283.79	2087.05	13.635	0.018
5	3404.47	5.07	0.06	3406.4	3398.69	9.933	0.016



Graph 9. *Lactobacillus sp.* UV treated FTIR characterization.

From the graph it is clear that the peaks are different indicating different intensity for different functional groups present in FTIR sample (Appendix I).

Table 11. FTIR transmission frequency vs. function group prediction

Frequency (cm^{-1})	**Bond**	**Functional group**	
		Bacteriocin produced without UV treatment	**Bacteriocin produced after UV treatment**
1091.75	C–N stretch	aliphatic amines	aliphatic amines
1222.91	C–N stretch/ C–H wag (–CH_2X)/ C–O stretch	-	alkyl halides, alcohols, carboxylic acids, esters, ethers
1425.44	C–C stretch (in–ring)	aromatics	-
1631.83	N–H bend	-	1° amines
1637.62	N–H bend	1° amines	-
2063.9	–C≡C– stretch	alkynes	-
2088.98	–C≡C– stretch	-	alkynes
3404.47	O–H stretch, H–bonded	-	alcohols, phenols
3489.34	O–H stretch, H–bonded	alcohols, phenols	-

Chapter 9
DISCUSSION:

The objective of the present study was to isolate bacteriocin producing organism and mutation induced by UV radiation exposure which was lately recognized in the difference in antimicrobial activity of the two forms: one normal, and another UV treated. Bacteriocin was found to be antimicrobial (Prescott and Baggot, 1993), and in our present study it was tested against E.coli and remarkable zone of inhibition (9.5 mm to 10 mm in diameter) was observed with bacteriocin from *Lactobacillus sp.,* while bacteriocin from *Bacillus subtilis* showed a range of 9.5 mm to 21 mm in diameter of inhibition zones was reported.

This antimicrobial activity was observed from the aliquot (cell free supernatant) obtained from broth media fermentation at different time periods, and in case of *Lactobacillus sp.* it was found that after the incubation of 48 hours form normal non-UV treated sample and 72 hours for UV treated incubation at 34^{o}C, bacteriocin production was maximum, we also studied that the antimicrobial effect of bacteriocin from the aliquot separated after 72 hours from *Lactobacillus sp.* is highest.

In case of *Bacillus subtilis,* bacteriocin production was high after 72 hours of incubation under similar incubation conditions to *Lactobacillus sp* and the antimicrobial was effect highest from the aliquot separated after 96 hours for non-UV treated sample and 24 hours for UV treated sample.

Hence overall result, UV treatment delayed the production of bacteriocin in *Lactobacillus sp.* but increased the antimicrobial activity (increased zone of inhibition) after same time of exposure to test organism, and UV treatment was caused no effect on the time interval of bacteriocin production by *Bacillus subtilis,* but antimicrobial activity shown just after 24 hours of exposure to test organism by UV treated aliquot.

The antimicrobial activity was screened by the aliquot obtained from the broth, which was estimated to be contained 3-4 mg/ml bacteriocin but after purification from gel exclusion chromatography it was calculated to contain around 1-1.3 mg/ml bacteriocin was produced by *Lactobacillus sp.*

Lactobacillus and *Bacillus* sp. isolated both were morphologically identified as gram positive, rod shaped and non spore forming bacteria, and were later by various biochemical tests identified by using Bergey's manual.

The growth cycle of almost all bacteria follows the sigmoid curve behavior, and the secondary metabolite production is generally attained after log phase i.e. during stationary phase, when rate of bacterial call growth rate is equal to the death rate. Secondary metabolite include enzyme production, various antibiotics like penicillin, tetracycline, ampicillin etc, polymers like PHB, natural rubber etc, like wise few bacteria also secrete bacteriocin. Bacteriocin has various subclasses with different chemical structure. Bacteriocin is a polypeptide complex (Azevedo, 1993). And for the analysis of the structure of this protein various methods are present, but structural characterizations of proteins in diverse environment like in presence of organic solvents, detergent micelles etc these methods are not efficient. Hence FTIR was employed for structural characterization of protein in such environment (Haris et al, 1999). Because bacteriocin is produced in very less amount and in soluble form it could be analyzed by FTIR. FTIR results

shown that there is difference in the functional group in *Lactobacillus* samples because of UV treatment.

Scientifically curd is considered as the source of probiotics. (Curd is being used traditionally in India since very long ago. Curd is made from milk when its protein casein is coagulated due to some acid (lactic acid) secreted by LAB (lactic acid bacteria), hence LAB is found abundant in curd but not in milk and it is the easiest source to isolate LAB.) and LAB can produce bacteriocin which is non toxic to human beings and antimicrobial at the same time.

Exploiting the properties and studying the sources of production of bacteriocin can help to find a new potent antibiotic and preservative.

Chapter 10
CONCLUSION

Lactobacillus sp. and *Bacillus subtilis* were observed to produce antimicrobial agent, bacteriocin. Results obtained demonstrated the inhibitory effect of bacteriocin against test organism, and the genetic manipulation shows the positive effect in increasing the concentration of bacteriocin produced after different incubation time and hence the antimicrobial activity. Purification for bacteriocin characterization by FTIR showed the difference in types of function group and different frequency for same type of function group not only because of environment dependent changes in peptide but also of changes due mutation causes shifting in the position and synthesis of bacteriocin. And further study might show the actual three dimensional structural and chemical differences in bacteriocin produced by isolated organisms.

As a biopreservative, Ravi (2011) identified bacteriocin as a biological compound produced from lactic acid bacteria which showed a noticeable increase in acidity a, which results in decrease in pH during storage for 3-6 long months, and product remained acceptable even after three months of storage. And because of this antimicrobial property it can be used as a preservative in mango or fruit pulp industry.

In future bacteriocin could be major antibiotic because of no side effects at low concentration and no toxicity at all. Because of present development in Genetic Engineering and Molecular Medicine, it is possible to isolate the gene(s) that is/are responsible for the production of this polypeptide antibiotic and directly translate to produce bacteriocin, after transcription. And it is also possible to incorporate this gene into some other organism for different industrial applications e.g. in beverage industry, if the commercial strain of bacteria for beverage production contains this gene, so this strain could inhibit the growth of other harmful bacteria by producing bacteriocin, without inoculation of additional microorganism and no need to add preservative because it is already present and as it is non toxic to human so it is edible and no need to purify before packaging.

APPENDIX

Appendix

Appendix A.a

Media constituents	Amt. (in 100ml)
Trypticase	1g
Sodium chloride	0.5g
Phenol red	0.189g
Glucose	1g

Appendix A.b

Media constituents	Amt. (in 100ml)
Trypticase	1g
Sodium chloride	0.5g
Phenol red	0.189g
Lactose	1g

Appendix A.c

Media constituents	Amt. (in 100ml)
Trypticase	1g
Sodium chloride	0.5g
Phenol red	0.189g
Mannitol	1g

Appendix A.d

Media constituents	Amt. (in 100ml)
Trypticase	1g
Sodium chloride	0.5g
Phenol red	0.189g
Maltose	1g

Appendix A.e

Media constituents	Amt. (in 100ml)
Trypticase	1g
Sodium chloride	0.5g
Phenol red	0.189g
Sucrose	1g

Appendix B

Media constituents	Amt. (in 100ml)
Peptone	0.2g
Sodium chloride	5.0g
Di potassium hydrogen phosphate	0.03g
Phenol red	0.03g
Agar	3g

Appendix C

Media constituents	Amt. (in 100ml)
Sodium chloride	0.5g
Magnesium sulphate	0.02g
Ammonium dihydrogen phosphate	0.1g
potassium dihydrogen phosphate	0.01g
Sodium citrate	0.5g
Agar	2.5g
Bromoethanol blue	(0.2%)40 ml at pH6.8 in 100ml

Appendix D

Media constituents	Amt. (in 100ml)
Skim milk powder	10g
Peptone	5g
Agar	1.5g

Appendix E

Media constituents	Amt. (in 100ml)
Peptone	20g
Sodium chloride	5g

Appendix F

Media constituents	Amt. (in 100ml)
Peptone	5g
Dipotassium hydrogen phosphate	0.5g
Glucose (10%) sol	5ml

Appendix G

Media constituents	Amt. (in 100ml)
Peptone	0.5
Beef extract	0.3g
Starch	0.2g
Agar	1.5g (pH 7.0)

Appendix H. SDS PAGE solutions

Buffer I (4x)	1.5M Tris-HCl pH 8.8
Buffer II (4x)	0.5M Tris-HCl pH 6.8
30 % Acrylamide	29.2 % acrylamide
	0.8 % bis-acrylamide
Sample buffer	(2x) 0.125M Tris-HCl pH 6.8
	20 % glycerol
	4 % SDS
	2 % beta-mercaptoethanol (ME)
	0.02 % bromophenolblue
Running buffer	(1x) 25mM Tris
	192mM glycine
	0.1 % SDS

Appendix I: Characteristic IR absorption FTIR chart

frequency, cm–1	*bond*	*functional group*
3640–3610 (s, sh)	O–H stretch, free hydroxyl	alcohols, phenols
3500–3200 (s,b)	O–H stretch, H–bonded	alcohols, phenols
3400–3250 (m)	N–H stretch	primary, secondary amines, amides
3300–2500 (m)	O–H stretch	carboxylic acids
3330–3270 (n, s)	–C(triple bond)C–H: C–H stretch	alkynes (terminal)
3100–3000 (s)	C–H stretch	aromatics
3100–3000 (m)	=C–H stretch	alkenes
3000–2850 (m)	C–H stretch	alkanes
2830–2695 (m)	H–C=O: C–H stretch	aldehydes
2260–2210 (v)	C(triple bond)N stretch	nitriles
2260–2100 (w)	–C(triple bond)C– stretch	alkynes
1760–1665 (s	C=O stretch	carbonyls (general)
1760–1690 (s)	C=O stretch	carboxylic acids
1750–1735 (s)	C=O stretch	esters, saturated aliphatic
1740–1720 (s)	C=O stretch	aldehydes, saturated aliphatic
1730–1715 (s)	C=O stretch	alpha,beta–unsaturated esters
1715 (s)	C=O stretch	ketones, saturated aliphatic
1710–1665 (s)	C=O stretch	alpha,beta–unsaturated aldehydes, ketones
1680–1640 (m)	–C=C– stretch	alkenes
1650–1580 (m)	N–H bend	primary amines
1600–1585 (m)	C–C stretch (in–ring)	aromatics
1550–1475 (s)	N–O asymmetric stretch	nitro compounds
1500–1400 (m)	C–C stretch (in–ring)	aromatics

1470–1450 (m)	C–H bend	alkanes
1370–1350 (m)	C–H rock	alkanes
1360–1290 (m)	N–O symmetric stretch	nitro compounds
1335–1250 (s)	C–N stretch	aromatic amines
1320–1000 (s)	C–O stretch	alcohols, carboxylic acids, esters, ethers
1300–1150 (m)	C–H wag (–CH_2X)	alkyl halides
1300–1150 (m)	C–H wag (–CH2X)	alkyl halides
1250–1020 (m)	C–N stretch	aliphatic amines
1000–650 (s)	=C–H bend	alkenes
950–910 (m)	O–H bend	carboxylic acids
910–665 (s, b)	N–H wag	primary, secondary amines
900–675 (s)	C–H "oop"	aromatics
850–550 (m)	C–Cl stretch	alkyl halides
725–720 (m)	C–H rock	alkanes
700–610 (b, s)	–C(triple bond)C–H: C–H bend	alkynes
690–515 (m)	C–Br stretch	alkyl halides

m=medium, w=weak, s=strong, n=narrow, b=broad, sh=sharp

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