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Isolation and Characterisation of Bacteriocin from *Lactobacillus* both in Native as well as UV treated state.

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ABSTRACT

Two representative genera of rod shaped, gram-positive bacteria of class *Bacilli* includs 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 varying from 0-96 hours and varying glucose concentration ranging from 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

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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

INTRODUCTION

Term “antibiotic” (grk term: antibiosis) that means “against life” and could be purified through fermentation of microbes and can have chemical as well as enzymatic modification for the purpose of fundamental studies [21, 28]. Antibiotics play major part in regulating the population of microbes of present in agriculture and industrial effluents. One which is of greatest use are derived from comparitively that belongs to genera of *Penicillium, Cephalosporium, Streptomyces, Micromonospora* and *Bacillus* [33]. Approximately 4500 multiple antibiotics have been extracted from cultures of microbes such as fungi,bacteria and plant cells, and 70% of them are mainly contributed from genus *Streptomyces*

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Antibiotics are the chemical compounds which are actually obtained from microorganisms and they have the ability of restricting the growth and proliferation and also 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 hydrolysed 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 [7], development of solid-phase method [18] and industrial aerobic parameters in real time [12] has been asessed on the synthesis 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* isolated from her wounds [14]. Compound has bactericidal effect on gram positive but very little activity against gram-negative organisms [25]. It is most commonly utilised in veterinary and poultry feed that helps in increasing efficiency of feed and also to reduction in infectious diseases incidences. Resistance against bacitracin is still very less despite its widespread use, [20].

Molecular formula is of Bacitracin is: $C_{66}H_{103}N_{17}O_{16}S$. Bacitracin consist of a complex which is chemically a polypeptide with Bacitracin A as major component in this. Mol. wt. of Bacitracin A is 1422.71 Da. Bacteriocin from micro-organisms are used as antibiotic and are also produced non-ribosomally by *Bacillus licheniformis*. Multiple varities of Bacitracin isolated such as A, A1, B, C, D, E, F, F1, F2, F3 and G so far and amongst them the most potent one is Bacitracin A, while Bacitracin B and C are less

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effective and others possess lower activity against bacterial cells. Such antibiotics are undoubtedly more effective against both Gram +ve and few Gram-ve sp. of bacteria.

MATERIAL AND METHODS

I. Sample Collection

Lactoacillus sample was collected from 5 different curd samples [5,32] from different dairies of **Jalandhar**,Punjab,India.Standard used is *Bacillus* species with MTCC number 6428.

II. Isolation of bacteria

Serial dilution method utilized to isolate bacteria in MRS media followed by incubation at 37^0C for 24 hrs. MRS media is selective media for *Lactoacillus* species and 1% nutrient agar media used for *Bacillus* (MTCC 6428). 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. viable titer calculated as:

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

III. Morphological Analysis

III.(i) Gram staining

The inoculated colonies of bacteria over the Petri plates had been gram stained to study the morphological characteristics of the bacteria. [5].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*.

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IV.Biochemical characterization

- Carbohydrate fermentation test was achieved by utilizing different sources of carbohydrates such as Glucose,Lactose,Mannitol,Maltose,Sucrose etc.
- Aerobic and Anaerobic Carbohydrate Breakdown Test.
- Casein Hydrolysis
- Catalase Test
- Citrate Test
- Indole Test
- Methyl Red Test
- Starch Hydrolysis
- Triple Sugar Iron Agar Test
- Acetoin Production

V. Identification of Bacteria using Bergey's Manual

VI. Antibiotic Susceptibility Test

VII. Screening for Bacteriocin Production :

Protein production assay done using Lowry estimation using BSA as standard.

RESULTS

I. Isolation of the Microorganism Colony:

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

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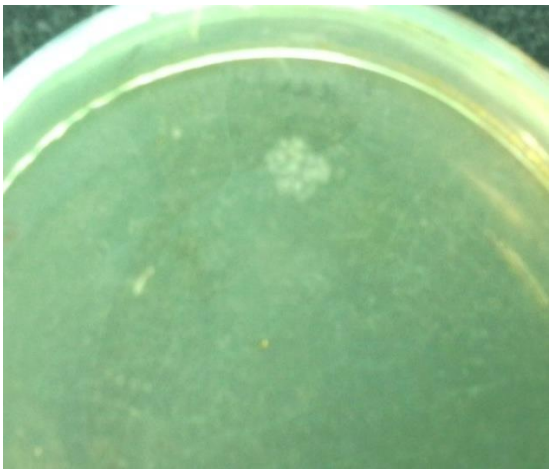
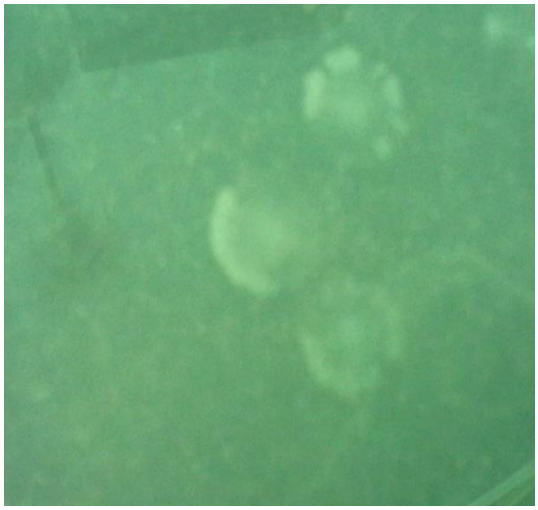


Bacillus



Lactobacillus

II. 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

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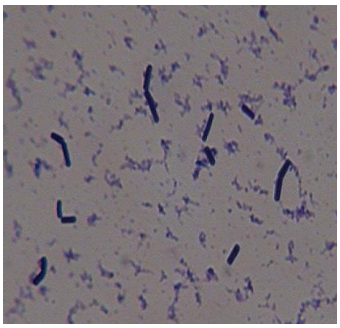
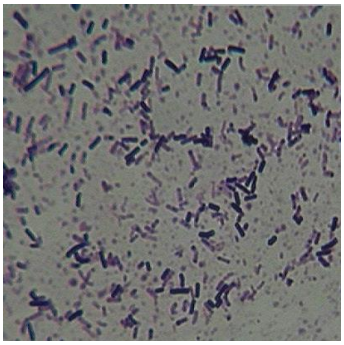


Bacillus



Lactobacillus

Biochemical Test:

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	+	+

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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	+	+

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 indicates both the isolates are susceptible to tetracycline and streptomycin but slightly resistant to ampicillin.

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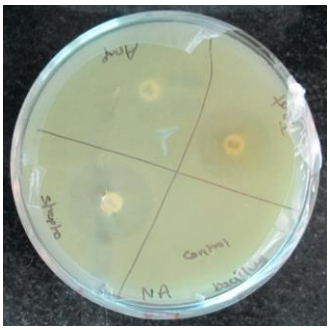
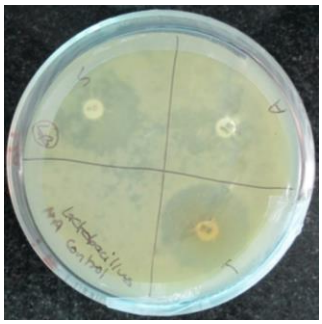


Bacillus

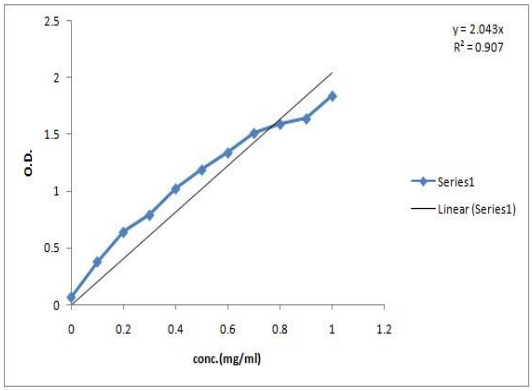


Lactobacillus

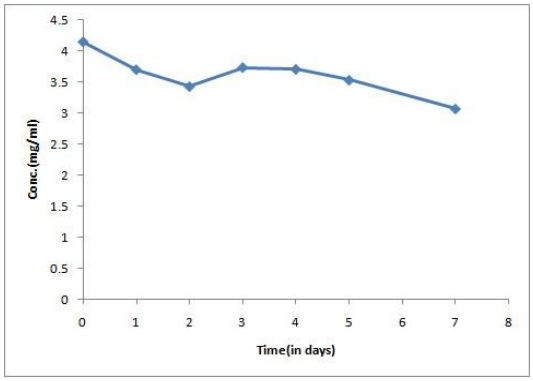
Screening for Bacteriocin Production

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

I. Protein estimation by lowry's method (Native State)



A



B

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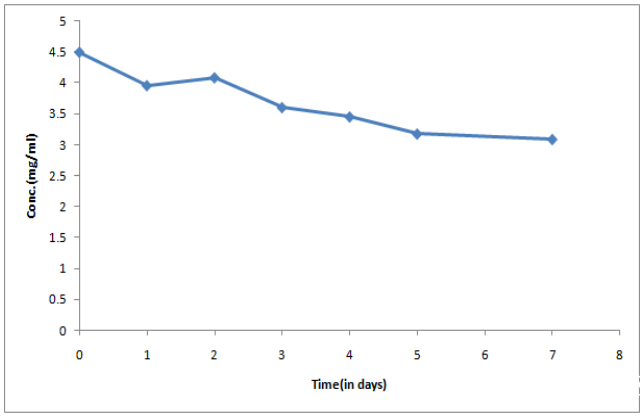
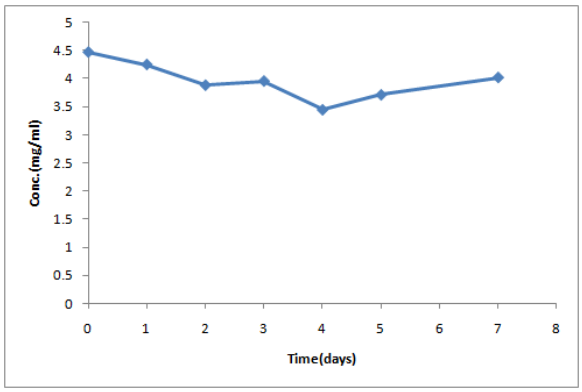


C

Fig: Representing Protein Estimation in A) Standard BSA;B) *Bacillus Sp* and C) *Lactobacillus Sp*

UV Treated



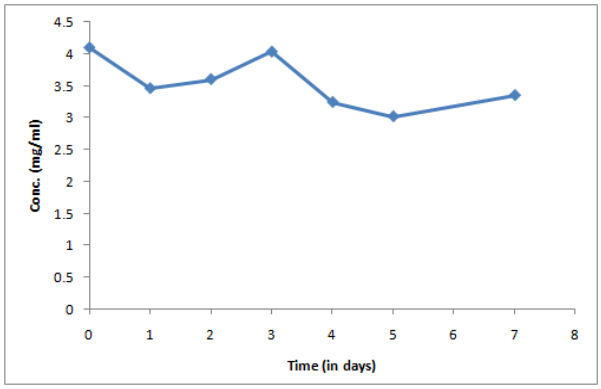


Fig representing Bacteriocin production when Bacillus and Lactobacillus treated with UV light

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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*).

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	48	72	24	48	72	24	48	72	24	48	72
0	-	-	-	-	-	-	-	-	-	-	-	-
24	-	-	-	-	-	-	0	11.5	12	-	-	-
48	10	13	14.5	-	-	-	0	13	13.5	-	-	-
72	-	-	-	0	9.5	9.5	-	-	-	0	11.5	11.5
96	18	20	21	-	-	-	-	-	-	-	-	-
120	0	11	11	-	-	-	0	10	10	-	-	-
168	-	-	-	-	-	-	0	9.5	11	0	10	10

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

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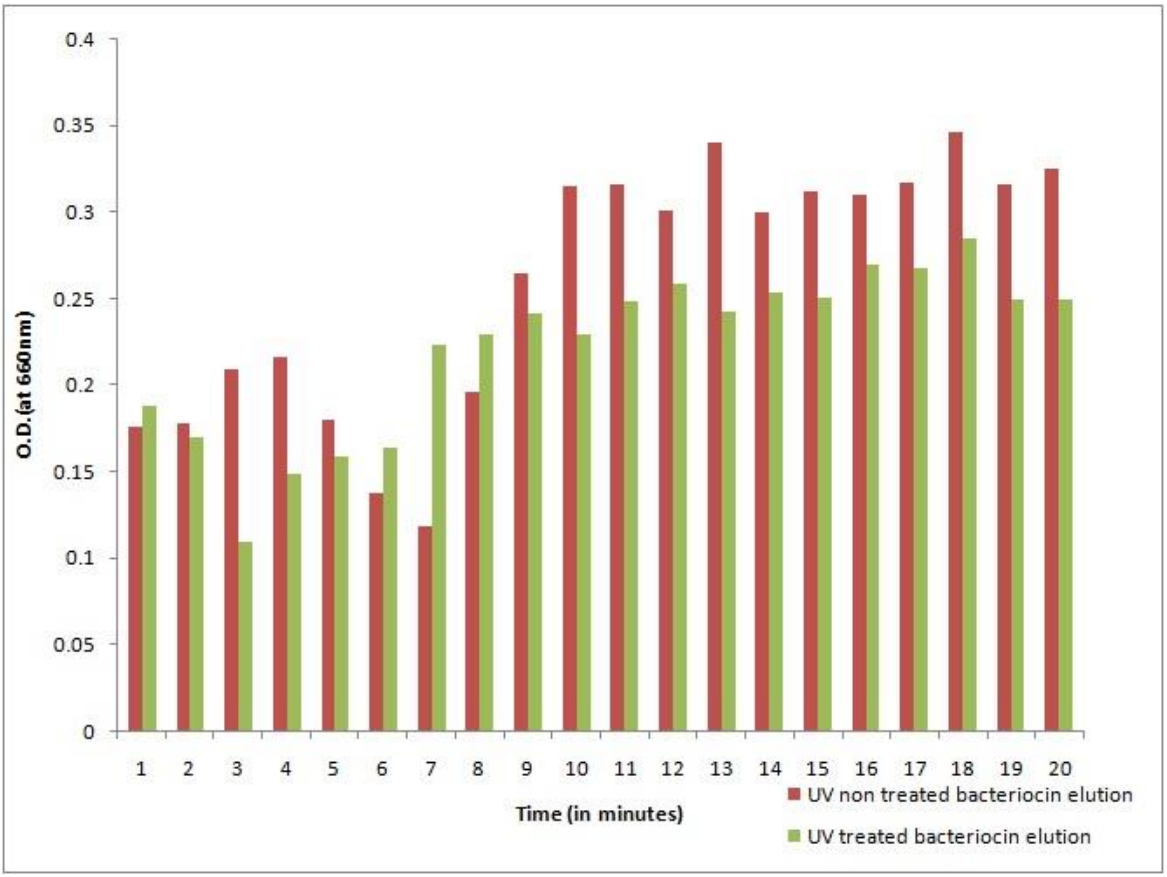
lactobacillus sp. of both UV treated and non treated had been carried out and the samples were eluted through the gel exclusion chromatography column of DEAE Sephadex A50, into 20 vials of 1ml volume each at the constant flow rate of 1ml/minute. And the protein concentration was estimated using spectrophotometer that was separated on the basis of the size.



FTIR Spectroscopy

I. For Non UV Treated

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

Table 1: FTIR peak analysis for *Lactobacillus sp.* non UV treated

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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

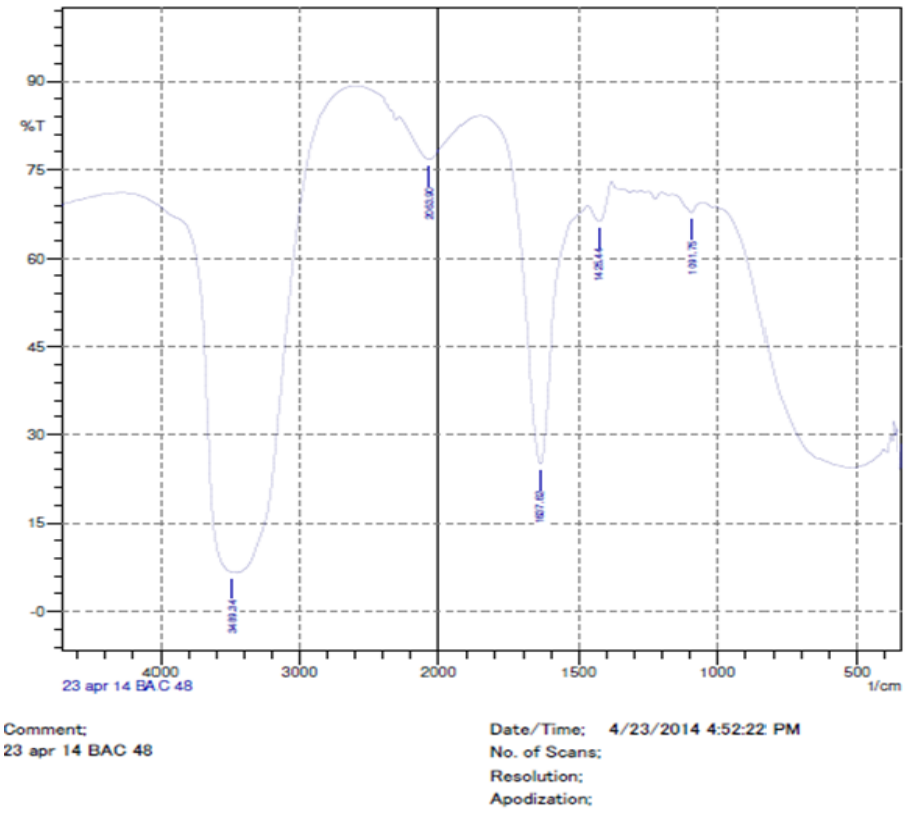

Fig: *Lactobacillus sp.* UV non treated FTIR characterization.

II. For UV Treated

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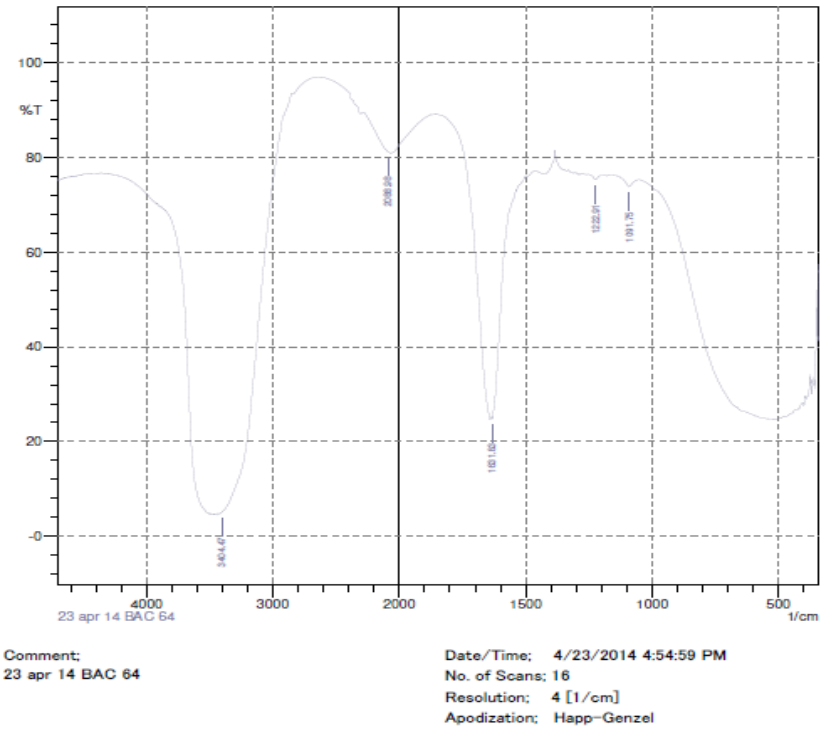
No.	Peak	Intensity	Corr. Inte	Base (H)	Base (L)	Area	Corr. Are
1	1091.75	73.836	1.846	1132.25	1049.31	10.397	0.356
2	1222.91	75.342	1.007	1251.84	1197.83	6.454	0.126
3	1631.83	24.804	1.543	1633.76	1548.89	26.574	-4.981
4	2088.98	81.149	0.101	2283.79	2087.05	13.635	0.018
5	3404.47	5.07	0.06	3406.4	3398.69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



From the graph it is clear that the peaks are different indicating different intensity for different functional groups present in FTIR sample (as mentioned in Table 2 below)

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Table 2:

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

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3489.34	O–H stretch, H–bonded	alcohols, phenols	-

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

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producing bacteriocin, without inoculation of additional microorganism and no need to add preservative because it is already present and as it is nontoxic to human so it is edible and no need to purify before packaging.

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