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RESEARCH ARTICLE

DETECTION OF ANTIMICROBIAL EFFICASY OF NOVEL BACTERIOCIN PRODUCED FROM *Lactobacillus similis RL7*

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Abstract

Production of antimicrobial substances is often important trait in the context of bacterial fitness but also in terms of probiotic efficacy. Several probiotic bacteria produce a variety of antimicrobial compounds *viz.* short chain fatty acids, Hydrogen peroxide, Nitric oxide, Bacteriocins etc. Bacteriocins are peptide or protein complexes showing antibacterial activity against closely related species. These antimicrobial agents are gaining more and more attention as an alternative therapeutics not only in pharmaceutical but also as a preservative in food industries. In present study, bacteriocin production was carried out by *Lactobacillus similis RL 7* isolated from rose inner petals and identified by 16s rRNA sequence analysis. This newly isolated strain showed antibacterial activity against several Gram positive and Gram negative bacteria. To obtain maximum bacteriocin yield, Production study was carried out in three different production media *viz.* MRS broth, BSM broth and M17 medium. An isolate RL 7 showed the maximum bacteriocin production in MRS medium. The produced bacteriocin was purified by Ammonium Sulphate and RP – HPLC method. Molecular mass determination study was done by SDS – PAGE and it was found that, the new bacteriocin having molecular weight less than 6.4 KDa was observed. Bacteriocin from *Lactobacillus similis RL 7* is capable of capable of showing antimicrobial activity at wide range of temperatures and pH but lost activity in presence of proteolytic enzymes. Hence *Lactobacillus similis RL 7* it is an ideal strain for bacteriocin production for food and pharmaceutical industries.

Introduction

Resistance of bacteria to antibiotics has become one of the main problems in human health. In addition, in agronomy, microbial diseases are largely responsible for the decrease in agricultural production. The discovery of new antibiotic families is a way to circumvent such problems and antimicrobial peptides may represent a new type of such antibiotics. A recent review lists the antimicrobial peptides / polypeptide isolated from bacteria and shows the rapid increase in the number of molecules that have been characterized (Shafer*et al*., 1997).

Lactic acid bacteria (LAB) have been widely used as probiotics in various fermented foods since antiquity. The preservative and health benefit of such traditional foods has been recognized for thousands of years and accordingly, lactic acid fermentation played an important role in the early years (Naidu and Clemens, 2000). Metchnikoff (1907) proposed the health benefits related to the regular consumption of fermented milks based on his research findings with " Bulgarian bacillus" an organisms closely related to *Lactobacillus delbreukii spp. bulgaricus*, a common LAB starter of yogurt. Lactobacilli continue to remain the most commonly used probiotic microorganisms. Currently available probiotic preparations contain *Lactobacillus delbreuckii spp. bulgaricus, L. acidophilus, L.casei, L. fermentum, L.plantarum, L. brevis, L. lactis and L. Reuteri* (Naidu and Clemens, 2000).

Several investigations have demonstrated that various species of LAB exert antagonistic action against intestinal and food – borne pathogens (Gibson *et al*., 1997). LAB are capable of preventing the adherence,

establishment, replication and / or pathogenic action of specific enteropathogens (Saavedra, 1995). These antagonist properties may be manifested by (a) decreasing the luminal pH through the production of volatile short – chain fatty acids (SCFA) such as acetic, lactic or propionic acids. (b). rendering specific nutrients unavailable to pathogens. (c). decreasing the redox potential of the luminal environment. (d). producing hydrogen peroxide under anaerobic conditions. (e). producing specific inhibitory compounds such as bacteriocins (Havenaar et al., 1992). Bacteriocins are allelopathic, proteinaceous compounds produced by bacteria, which acts as anticompetitor toxins against the same or closely related species (Reeves 1972, Chao *et al*., 1981, Rice 1984, Jack *et al*., 1995, Nes *et al*., 1996). The term bacteriocin encompasses an array of structurally different molecules produced by a number of phylogenetically distinct Gram positive and Gram negative bacterial groups (Reeves 1972).

Historically, much of the work on these substances has focused on the colicins, which are a group of related bacteriocins produced by *Escherichia coli*and some other Gram negative bacteria (Jack *et al*., 1995). Several types of bacteriocins from food associated lactic acid bacteria have been identified and characterized, of which nisin, Diplococcin, Acidophilin, Bulgarican, Helveticins, Lactacins, Lactolin and Plantaracins are the important bacteriocins (Deshmukh and Thorat, 2013).More recently, however there has been a vast increase in research on bacteriocins from other bacterial groups, including the Gram positive lactic acid bacteria. The increased interest in these compounds has been fuelled by the potential industrial applications of bacteriocins for use as safe and novel food preservatives (De Vuyst and Vandamme 1994). However, studies relating to the antibacterial properties of these organisms are limited and not fully exploited for use (Deshmukh and Thorat, 2013).

The present work focuses on isolation, production, purification, characterization and antimicrobial spectrum of bacteriocin produced from *Lactobacillus similis* isolated from inner rose petals.

Materials and Methods

Chemicals and Media

Analytical grade chemicals were obtained from Qualigenes, Thomas Baker, Sigma and SD Fine India, while media were obtained from Hi-media, India.

Test Microorganisms

The test microorganisms *viz. Alcaligenes fecalis, Escherichia coli, Klebsiella pneumoniae, Proteus vulgaris, Pseudomonas aeuroginosa, Staphylococcus epidermidis, Staphylococcus aureus, Bacillus coagulans, Enterobactoraerogenes, Staphylococcus aureus, Aspergillus bombycis, Saccharomyces cerevisieae and Saccharomyces bayanus* were procured from local pathological laboratory and P.G. dept. of Microbiology and Research Center, Barshi, Solapur, Maharashtra.

Isolation and identification of LAB

Rose flowers were collected in aseptic condition from local agricultural farm in Barshi and processed within five hours for further studies. MRS broth and agar were used for enrichment and isolation of culture of Lactic acid Bacteria respectively (De Mann *et al*., 1960). The outer flower whorls were removed and inner petals were aseptically transferred into the sterile saline (0.85% Nacl). 1 ml sample was added into the 10 ml MRS broth and incubated at 37^0C for 24 hrs. under microaerophilic condition. After incubation, loopful of culture was spreaded on sterile MRS agar plate and all plates were incubated microaerobically at 37^0C for 24 hrs. well isolated colonies were selected randomly and transferred in MRS broth. They were streaked on MRS agar to check the purity of the isolates and then stored in MRS soft agar (0.5%) overlaid with glycerol at -20^0C.

Screening of bacteriocin producer

The selected lactic acid bacterial isolates were cultured in MRS broth and incubated at same above mentioned conditions for 20 hrs. Aliquots of cultures were spotted on the sterile MRS agar plates and incubated micro aerobically at 37°C or 24 hrs. After incubation plates were overlaid with soft nutrient agar inoculated with culture of *Bacillus coagulans* and incubated at 37°C for 24 hrs. under aerobic condition. Isolate showing maximum zone of growth inhibition of *Bacillus coagulans* was selected and designated as RL7 and used for further studies.

Bacteriocin production from selected isolate

Bacteriocin production from selected isolate were carried out in three different medium *viz.* MRS broth, BSM (Bacteriocin Screening Medium) and M17 Medium.14The selected culture was inoculated into above mentioned medium and incubated micro aerobically at 37°C for 24 –48 hrs. During fermentation, aliquots of samples were removed for Growth curve and protein estimation analysis and results were recorded. The medium showing maximum optical density in growth curve analysis and protein concentration was selectedand used for further Production studies.

Production of bacteriocin using optimum conditions

Bacteriocin production was performed in above optimized medium. 5ml of inoculum of RL 7 isolate was inoculated in optimized medium and incubated micro aerobically at 37°C for 24 – 48 hrs. After fermentation broth was centrifuged at 12000rpm for 15 min. and supernatant was collected. pH of the supernatant was adjusted to 7.0 with 1N NaOH. Precipitation was done with Ammonium Sulphate at 40% and 70% saturation level at 4°C. After precipitation, broth was centrifuged at 15000 rpm for 20 min., precipitate was collected and stored in 0.2M Sodium Phosphate buffer (pH 6.9) and labeled as Crude Bacteriocin Preparation (CBP) (Navarro *et al*., 2000, Muriana *et al*., 1991).

Extraction of Bacteriocin

Chloroform – Methanol (2:1 v/v), was used for crude bacteriocin extraction. However produced precipitate at solvent – aqueous interphase was collected aseptically. Solvent was evaporated and precipitate was kept in buffer which was used for antimicrobial study (Deshmukh and Thorat, 2013).

Antimicrobial activity of extracted bacteriocin

Agar well diffusion and paper disc methods were used to study antimicrobial activity of extracted bacteriocin. Agar well diffusion technique was performed as 0.1ml culture of test microorganisms is spreaded on sterile nutrient agar and wells were prepared. 100µl of extracted bacteriocin preparation (CBP) was added wells and plates were aerobically incubated at 37°C for 24 hrs.

Purification of Crude Bacteriocin by RP – HPLC

Final purification step was performed by Reverse Phase Chromatography on a Agilent TC – 18 column (Analytical 4.6 X 250 mm) integrated in a High Performance Liquid Chromatography system (Agilent 1100 series). The gradient portion was generated by 0.1% TFS and acetonitrile containing 0.1% TFA. The column was equilibrated with 40ml of 0.1% TFA with flow rate 1 ml / min., then CBP sample that contain bacteriocin activity was applied to a column by pass through sucker line with flow rate of 0.5ml / min. The column was washed with 0.1 % TFA until the baseline became stable. Before elution, 200µl of starting elution buffer was injected to the system and elution was performed by using linear gradient from 0 – 100% acetonitrile or 20 – 80 % acetonitrile in 55 min. and fractions were collected and antimicrobial activity was checked. Polypeptides were detected spectrophotometrically by measuring the optical density at 280nm (Ennahar *et al*., 2001).

Purified bacteriocin sample were characterized with respect to thermal and pH stability and biochemical nature was determined by treating sample with different enzymes.

Thermal stability

Purified bacteriocin (Fraction showing antimicrobial activity) was exposed to various heat treatments: 40^0, 60^0, 80^0, 100^0, and 121^0C. Aliquot volume of each fraction was then removed after 30 and 60 min. and assayed for antimicrobial activity (Ten Brink *et al*., 1994).

pH stability

Purified bacteriocin was treated with buffer having different pH *viz*. 2, 4, 6, 8, and 10 with Hydrochloric acid (HCL) and Sodium hydroxide (NaOH) and incubated for 4 hrs. at room temperature and assayed similarly (Ten Brink *et al*., 1994).

Treatment with enzymes

Bacteriocin was assessed for its sensitivity to various enzymes *viz*. Lipase (8.6 U / mg) in 0.05M Tris hydrochloride (pH 8.0), Pepsin (3200 U / ml) in 0.2M Citrate (pH 6.0), Trypsin (15000 U / mg) in 0.05M Tris hydrochloride (pH 8.0), α- amylase (1000 U / mg) in 1N NaOH (pH 6.5) and Proteinase K (11.5 U / mg) in 1 N NaOH (pH 6.5). Samples of bacteriocin were incubated with 500mg of each enzyme per ml of bacteriocin for 60 min. at 37^0C, except for samples containing trypsin and pepsin which was incubated at 25^0C prior to being assayed for antimicrobial action (Wanda and Bonita 1991).

Molecular size determination

The molecular size of purified bacteriocin was analyzed by Sodium dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS - PAGE). After electrophoresis, the gel was stained with Silver nitrate staining.

Results

Isolation, Identification and Phylogenetic Analysis

Screening of bacteriocin producers were carried out by agar overlay method. 10 strains of Lactic Acid Bacteria were isolated from rose petal sample. Antibacterial activities of 4 isolates were detected and strain RL 7 showed maximum antibacterial activity to the indicator strain *Bacillus coagulans* (Fig. 1) .Hence, Isolate RL 7 was used for production studies.

Lactic acid Bacterial isolate designated as RL7 was selected as good candidate for bacteriocin production, based on its ability to form clear and large zone of growth inhibition of *Bacillus coagulans*. Molecular identification

was carried out of this isolate based on 16S rRNA gene sequencing. The phylogenetic tree was constructed by using Neighbor Joining method by Kimura 2 parameter with 1000 replicas in MEGA 4.0 (Fig. 2).According to sequence similarities and multiple alignments, the isolate RL 7 was found to be in close relation to *Lactobacillus similis*.

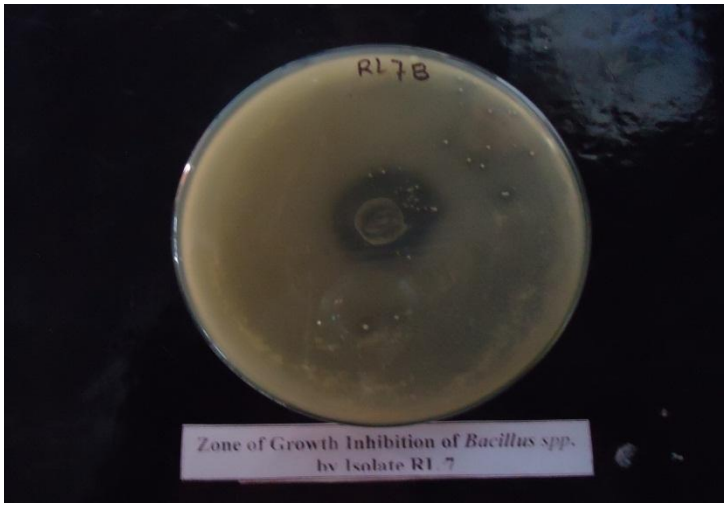

Figure 1: Zone of growth inhibition of *Bacillus coagulans* by RL7 isolate

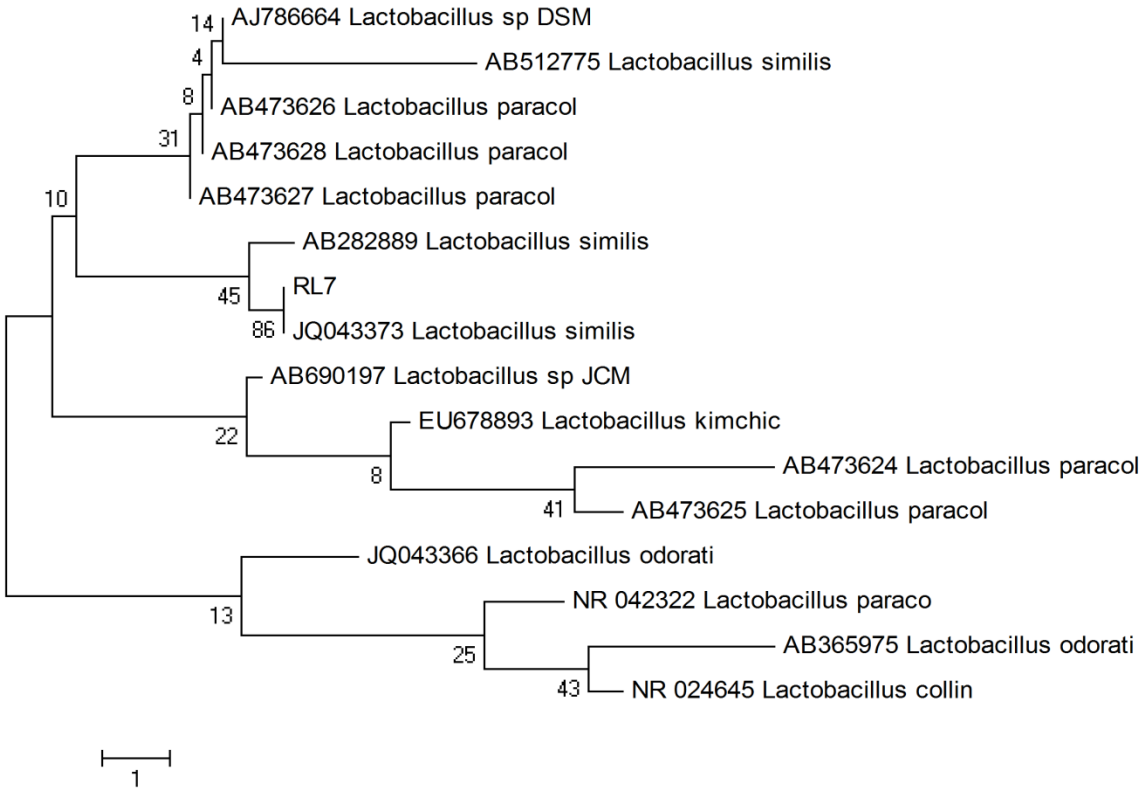

Figure 2: Phylogenetic tree of RL 7 isolate

Production of Bacteriocin

Bacteriocin production was carried out in three different medium *viz*. MRS broth, BSM and M17 broth. The inoculum of selected RL7 isolate was added and incubated microaerobically for 24 – 48 hrs. during incubation, protein estimation and growth curve was studies at time intervals (Fig. 3 & 4). Among them MRS is best medium for growth and Bacteriocin production by *Lactobacillus similis RL7*, as it shows maximum protein concentration.

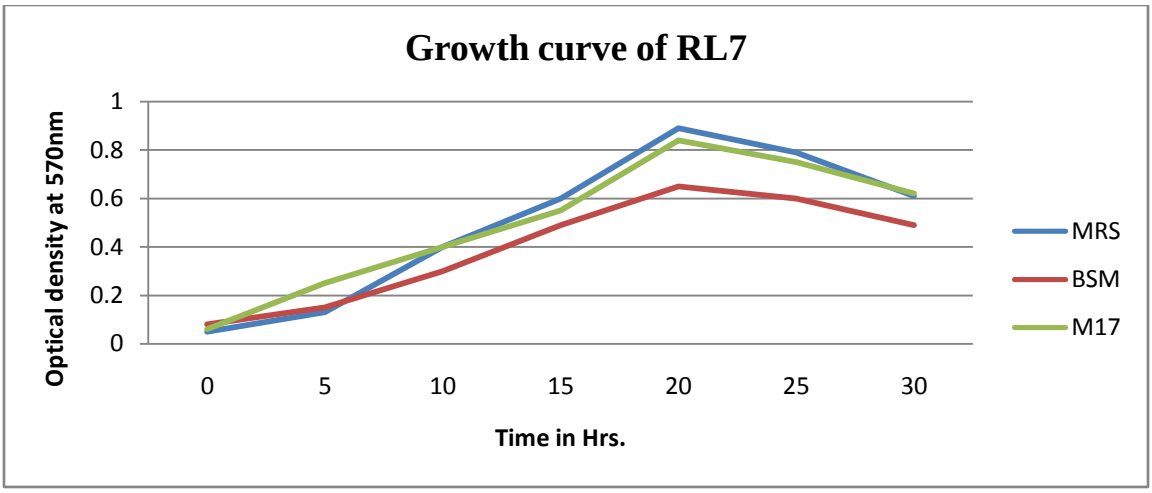

Figure 3: Growth curve of RL 7 isolate in three different media

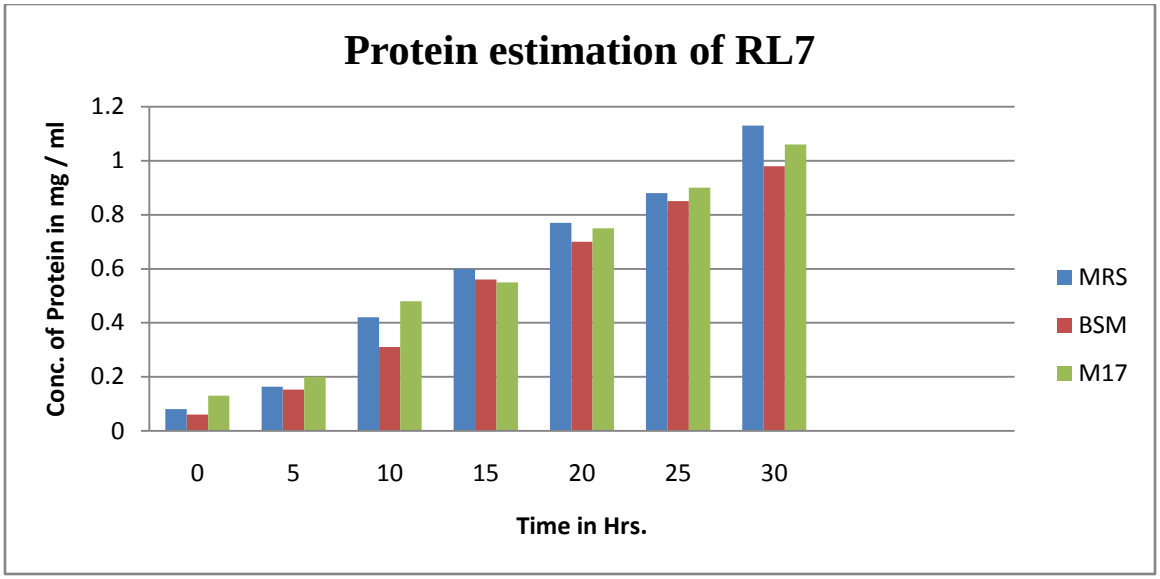

Figure 4: Estimation of protein in three different medium during fermentation by RL 7 isolate

Purification and characterization of Bacteriocin

The purification of bacteriocin produced by RL7 was performed by Ammonium Sulfate precipitation and RP-HPLC as described in methods. During RP – HPLC, a fraction collected at 6.339 time shows maximum protein concentration (Fig. 5) and antimicrobial studies of same fraction was performed with above listed test microorganisms (Table. 1).

The effect of heat, pH and enzymes on bacteriocin was determined using *Bacillus coagulans* as indicator organism. Bacteriocin produced by *Lactobacillus similis RL7* was considered to be the heat stable i.e. showing activity at 80^0C (Table 2) and showing activity at high pH i.e. 8 & 10 (Table 3), as it shows considerable zone of growth inhibition of test microorganism. Bacteriocin produced by RL7 was tested for their sensitivity to various enzymes. From that it was observed that, antimicrobial activity was lost after treatment with all proteolytic enzymes

whereas treatment with lipase and catalase did not affect the antimicrobial activity (Table 4).Bacteriocin sample used in above studies was used for molecular weight determination using SDS – PAGE, from that it was observed that, bacteriocin sample contain single peptide chain having molecular weight less than 6.4KDa (Fig.6)

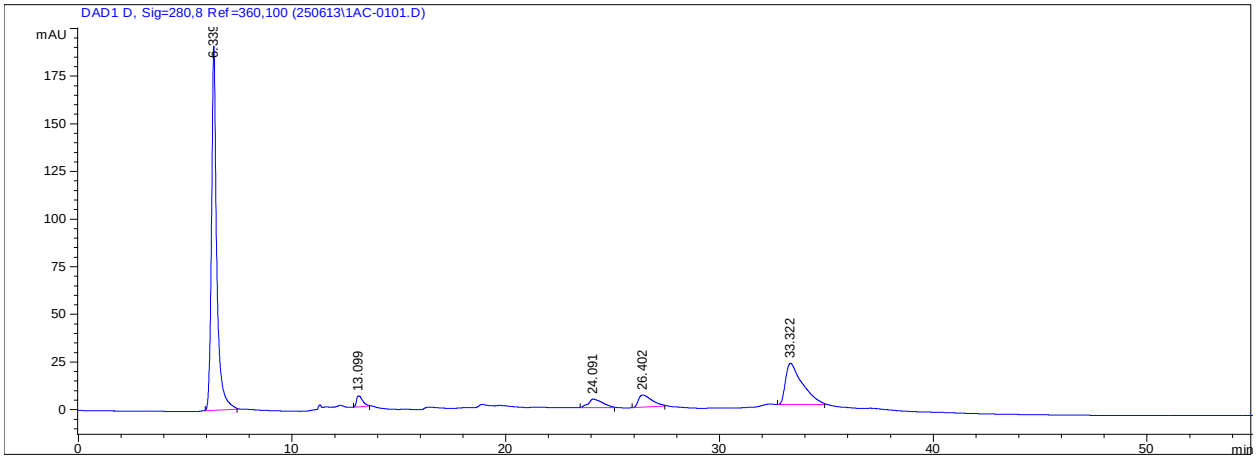

Figure 5: Reverse – Phase HPLC 280nm profile of the Bacteriocin produced from *Lactobacillus similis RL7.*

Figure 6: Molecular weight determination of Bacteriocin from *Lactobacillus similis RL 7* by SDS - PAGE

Table 1: Antibiogram of *Lactobacillus similis RL 7* against test microorganisms

Name of test Microorganisms	**Zone diameter of growth inhibition of test microorganisms in mm**
Alcaligenes fecalis	22
Escherichia coli	27
Klebsiella pneumoniae	23
Proteus vulgaris	25
Pseudomonas aeuroginosa	25
Staphylococcus epidermidis	28

Staphylococcus aureus	30
Bacillus coagulans	25
Enterobactoraerogenes	24
Aspergillus bombycis	24
Saccharomyces cerevisieae	21
Saccharomyces bayanus	26

Table 2: Temperature stability of Bacteriocin from *Lactobacillus similis RL7*

Temperature in ^{0}C	**Time in min.**	**Zone diameter of growth inhibition of test microorganism *Bacillus coagulans* in mm.**
40	30	28
	60	25
60	30	25
	60	22
80	30	25
	60	20
100	30	23
	60	20
121	30	00
	60	00

Table 3: pH stability of Bacteriocin from *Lactobacillus similis RL7*

pH	**Zone diameter of growth inhibition of test microorganism *Bacillus coagulans* in mm.**
2	12
4	18
6	26
8	29
10	29

Table 4: Enzyme sensitivity of Bacteriocin from *Lactobacillus similis RL7*

Enzymes used	**Zone diameter of growth inhibition of test microorganism *Bacillus coagulans* in mm.**
Lipase	30
Pepsin	03
Trypsin	05
α- amylase	29
Proteinase K	02

Discussion

A large number of bacteriocins of lactic acid bacteria have been described over the last few years. Frequent reports have been made of bacteriocin of lactic acid bacteria in milk product, fermented food and feed, but studies on bacteriocin from rose petals isolated lactic acid bacteria remain scarce. Antagonistic microorganisms and their antimicrobial metabolites may have some potential as natural preservative to control the growth of pathogenic or spoilage microbiota in foods. Most bacteriocin produced by LAB appear to have relatively narrow inhibitory spectra (Klaenhammer 1988), while some bacteriocins, such as pediocin and plantaricin, are active against a wide range of bacteria comprising Gram positive and Gram negative food borne pathogenic and spoilage bacteria (Cintas *et al*., 1995). Thus, those antimicrobials that have relatively broad inhibitory spectra with high biological activity, improved stability and improved solubility are expected to possess great bio preservative potential.

It was seen in our investigation that, there are large number of lactic acid bacteria habituated on inner petals having antimicrobial activity. So this is may be the first report on bacteriocin from lactobacilli isolated from inner rose petals and we report first time, the isolate *Lactobacillus similis RL7* as a novel bacteriocin producers as it shows antimicrobial activity. Species identification was authenticated by partial 16s r RNA gene sequencing (Schleifer and Ludwig, 1995).

From antimicrobial spectrum it was observed that, bacteriocin from *Lactobacillus similis RL7* inhibit growth of many Gram positive and Gram negative bacteria, fungus and yeast (Showing considerable zone diameter). This result correlates with the Tagg*et al*., (1976) and Sanni*et al*., (1999)in that they have shown that, some bacteriocin produced by Gram positive bacteria have a broad spectrum of activity.

Bacteriocin can be denatured by proteolytic enzymes results into loss of antimicrobial activity. In our investigation, we used different enzymes to check biochemical nature of purified bacteriocin. And we observed that, loss of activity in proteolytic enzyme treated sample. Similar behaviors were observed by Khalil *et al*., (2009), they found that Pepsin and trypsin treatment inhibited the bacteriocin activity. Cherif et al., (2001), also found similar results; they found that inhibitory activity was only susceptible to proteinase K.

The thermal stability of bacteriocin produced by isolate RL7 may constitute an advantage for potential use as bio preservative in combination with thermal processing in order to preservefood products. In present work, the antimicrobial activity of bacteriocin was completely lost at 121^0C for 15 min. our results are similar with results of Joshi *et al*., (2006), in that they reported that bacteriocin from *Lactobacillus CA 44* remained stable at 25, 30, 45, 60, and 100^0C, but activity was reduced when heated with 121^0C for 20 min.

The molecular weight of the obtained purified bacteriocin RL7 was less than 6.4 KDa. These result shows similarity with results of Todorov and Dicks, (2004) in that bacteriocin from *Lactobacillus plantarum ST 13 BR* having molecular mass approximately 10KDa. This result also similar to bacteriocin from *Lactobacillus sakei R 1333* produced 3800 Da bacteriocin. Todorov *et al*., (2010).

Conclusion

Depending on the proven stability and safety of the herein obtained bacteriocin from the non – pathogenic bacterium *Lactobacillus similis RL7*, the purified bacteriocin would be investigated as food bio preservative and as therapeutic agent.

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