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Isolation of Lactobacillus Species from Sediments of Caspian Sea for Bacteriocin Production

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Abstract. Bacteriocins are heterogeneous groups of antibacterial peptides and proteins that vary in their spectrum of activity, mode of action, molecular mass, genetic origin and biochemical properties. In this study, we characterized the bacteriocins produced by *Lactobacillus* spp., which were isolated from the marine environment. Sediment samples were collected during 2011 from Caspian Sea. For isolation and identification of *Lactobacilli,* dilutions of one gram of sediments in sterile 50% aged seawater were prepared. Bacteriocin assay was determined using the well diffusion method. The sensitivity of the active substance to enzymes was tested on cell-free supernatant .The crude bacteriocin was precipitated with 80% ammonium sulphate saturation. The precipitate was dialysed against 20 mM potassium phosphate buffer for 12 h at 4ºC. Further purification was carried out in ion exchange chromatography. The molecular weight of the bacteriocin was determined by 15% Sodium dodecylsulfate polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Comassie Brilliant Blue R-250.The bacteriocin producing strains were identified as *L. acidophilus* and *L. plantarum* based on their physiological and biochemical characteristic. Maximum activity be served against *Enterococcus faecalis* and *salmonella typhi* and minimum activity observed against *Shigella boydii*.The maximum arbitrary unit as measured as 2647 AU/ml at 30ºC . Single protein band was observed when stained with Comassie blue and it clearly indicated the purity of the protein. Possession of bacteriocin by *L. acidophilus* and *L. plantarum* is an indication that the bacteria can be used as probiotic and as biopreservative.

Keywords: *L. acidophilus, L. plantarum,* bacteriocin, sediment

1. Introduction

Lactic acid bacteria (LAB) and physiologically related group of gram-positive bacteria produce a variety of compounds with antimicrobial activity, and they are termed bacteriocins. Bacteriocins are generally defined as extracellular released peptide or protein that shows a bactericidal activity against species closely related to the bacteriocin producing strain. Lactic acid bacteria and their metabolites have been shown to play an important role in improving microbiological quality and shelf life of many fermented food products and provide a good example of biopreservation [18]. Although bacteriocins may be found in many Gram positive and Gram-negative bacteria, those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives. Bacteriocins produced by LAB are small, ribosomally synthesized, antimicrobial peptides or proteins that possess activity towards closely related Gram-positive bacteria, whereas producer cells are immune to their own bacteriocin(s). Activity against Gram-negative bacteria such as *E. coli* and *Salmonella* has been shown, but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or chelating agent, or after pulsed electric field or high-pressure

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treatment. Among bacteriocins from LAB, distinction can be made between (i) lantibiotics or small, heat-stable, lanthionine- containing, single- and two-peptide bacteriocins (ii) peptide bacteriocins or small, heat-stable, non-lanthionine-containing bacteriocins (iii) bacteriolysins or large, heat-labile, lytic proteins, often murein hydrolases. Three major methods for the purification of bacteriocins by LAB to homogeneity can be distinguished [2]. In this paper, we characterized the bacteriocins produced by Lactobacillus spp. which were isolated from the marine environment, determined the antibacterial spectrum, the optimum condition for bacteriocin production, and estimate the molecular weight of the bacteriocin.

2. Materials and Methods

Sediment samples were collected during summer, 2011 from depths of 10 - 20 m at Caspian Sea in north of Iran. The sediment samples were stored in the laboratory at 4ºC in sterile specimen cups until they were used to presence of bacteriocin producing *Lactobacilli.* For isolation and identification of *Lactobacillus* spp. dilutions (10^{-1} - 10^{-6}) of one gram of sediments in sterile 50% aged seawater were prepared and plated on the Man Rogosa agar (MRS agar) medium. The strains were sub-cultured onto MRS agar slant (medium with 50% sea water), incubated at 30 ºC for 24 h and were preserved in 20% glycerol at -80 ºC. Two of the isolates were selected for further studies which exhibited strong inhibitory activity against indicator strains and identified on the basis of their morphological and biochemical characteristics according to Bergey's Manual of Determinative Bacteriology. Susceptible test organisms that were Bacillus *cereus*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes, Escherichia coli, Pseudomonas aeruginosa, Salmonella typhi, Shigella boydi, Shigella desenteriae* and *Yersinia enterocolitica* were isolated on nutrient agar plates. For production of crude bacteriocin the isolated strain s were grown in MRS broth (pH-6.0) seeded with 5% inoculums of overnight culture and maintained anaerobically at 30ºC for 48 h. After incubation, cells were removed from the growth medium by centrifugation (10,000×g for 15 min, 4ºC). The cell-free supernatants were adjusted to pH 6.0 using 1N NaOH and they were used as crude bacteriocin. Agar well diffusion method was used to check the cultures for the production of antimicrobial metabolites. Twenty - four hours fresh cultures of standard microorganisms were diluted with pre-sterilized normal saline and the turbidity of the cultures was adjusted with 0.5 McFarland, bacterial lawn and then wells were prepared over the Mueller hinton agar plates. The supernatant from a 48-h culture of *Lactobacillus acidophilus* and *Lactobacillus plantarum* were filter sterilized by passage through a 0.45 µm pore size membrane filter. About 80 µl cell free supernatants were added in the 4-mm-diameter wells and the plates were incubated at 37 ºC for 24 hours. After 24 hrs, the zones of inhibition were observed and measured. Antimicrobial activity was expressed in arbitrary units (AU/ml). One AU was defined as the reciprocal of the highest level of dilution resulting in a clear zone of growth inhibition (i.e. turbidity <50% of the turbidity of control culture grown without *Lactobacilli* supernatant).For optimization of culture conditions, the selected strains were subjected to different culture conditions to derive the optimum conditions for bacteriocin production. Growth and bacteriocin production were estimated at various temperatures (20, 25, 30, 35, 40 and 45ºC), pH (4.0, 5.0, 6.0, 7.0, 8.0, and 9.0), sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%) and incubation time (6, 12, 18, 24, 30, 36, 42 and 48 h). Samples were collected after 48 h and examined for bacteriocin production (AU/ml) as described earlier. The sensitivity of the active substance to enzymes was tested on cell-free supernatant (pH 6.0) of 24 h cultures incubated at 30 ºC and were treated for 2 h with 0.1 mg ml^{-1} and 1.0 mg ml^{-1} final concentration of the following enzymes: proteinase K, α amylase, DNase and lipase . The surfactants tested were sodium dodecyl sulphate (SDS), Tween 80, Tritone X-100, EDTA and urea at final concentration 0.1, 1, 2 or 5. Controls consisted of either active supernatant or detergents used. All samples and controls were incubated at 30ºC for 5 h and tested for activity. In due to purification of bacteriocin, the crude bacteriocin was precipitated with 80% ammonium sulphate saturation. The precipitate was dialysed against 20 mM potassium phosphate buffer (pH 7.0) for 12 h at 4ºC. Further purification was carried out in ion exchange chromatography. The dialyzed protein was applied to a DEAE- Cellulose A-50 column, pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). After washing the column with 3 vol. of equilibration buffer, bound proteins were eluted stepwise using phosphate buffers of increasing molarity and decreasing pH values at room temperature (approx. 25 ºC). The flow rate was adjusted to 24 ml h^{-1} and fractions (1 ml each) were collected. The fractions showing high bacteriocin activity were pooled and concentrated in lyophilizer.

Protein concentration of the bacteriocin in supernatant was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. The molecular weight of the bacteriocin was determined by 15% Sodium dodecylsulfate polyacrylamide gel Electrophoresis. After electrophoresis, the gel was stained with Comassie Brilliant Blue R-250. Range molecular markers (29-200 kDa) with five polypeptides were used as a marker.

3. Results

The bacteriocin producing strains were isolated from the marine environment of the Guilan province in north of Iran (Caspian Sea) and the selected strains were identified as *L. acidophilus* and *L. plantarum* based on its physiological and biochemical characteristics. Measurements of biomass and bacteriocin production by *L. acidophilus* and *L. plantarum* are shown in Fig 1, 2, 3 and 4. respectively. Results showed that *L. acidophilus* and *L. plantarum produced* bacteriocin in MRS broth. The strain *L. plantarum* exhibited a good bacteriocin activity of 2242 AU/ml, at pH 6.0, sodium chloride 1.5% and 30 ºC. The strain *L. acidophilus* exhibited a good bacteriocin activity of 2432 AU/ml, at pH 5.0, sodium chloride 1.5% and 25 ºC. The bacteriocin production was higher during the stationary phase of the growth of the organisms, whereas maximum biomass occurred at 18 h. The susceptibilities of various Gram-positive and Gram negative bacteria to growth inhibition by the supernatant of L. *acidophilus* and *L. plantarum are* presented in Table1.Among these, maximum activity observed against *Enterococcus faecalis, Salmonella typhi* and *Staphylococcus aureus* respectively by either *L. acidophilus* or *L. plantarum and* minimum activity observed against *Bacillus cereus*, *Shigella boydii* and *Shigella desenteriae* respectively by *L. plantarum and Shigella boydii*, *Bacillus cereus* and *Shigella desenteriae* by *L. acidophilus*.

Table 1: Inhibition of various tested microorganisms by bacteriocin produced by L. acidophilus and L. plantarum

Diameter zone of inhibition (mm)						
Standard microorganisms	Crude sample (80µl)		purified sample(80µl)		Ampicillin(30µl)	
Gram positive	*A*	*B*	A	B	A	B
Bacillus cereus	6	-	3	-	14	14
Staphylococcus aureus	25	20	17	14	20	20
Enterococcus faecalis	30	23	18	17	28	28
Listeria monocytogenes	25	20	19	14	5	5
Gram Negative						
Escherichia coli	18	14	9	10	19	19
Pseudomonas aeruginosa	19	17	12	11	17	17
Salmonella typhi	28	21	20	16	15	15
Shigella boydii	4	3	10	3	12	12
Shigella dysenteriae	7	4	2	-	10	10
Yersinia entrocolitica	8	5	5	2	3	3

A: *L. acidophilus, B: L. plantarum*

Temperature and pH played an important role in cell growth as well as bacteriocin production. The bacteriocin activity was tested with different temperatures (20, 25, 30, 35, 40 and 45ºC). Furthermore, the maximum arbitrary units by *L. acidophilus* and *L. plantarum* were measured at 25ºC and 30 ºC respectively. Minimum levels were recorded at 20ºC for both of them. Regarding pH the maximum arbitrary units were measured at pH 5 and pH 6 for *L. acidophilus* and *L. plantarum* respectively. Minimum of bacteriocin production was in pH 9 for both of them. Regarding various concentration of sodium chloride (NaCl %) tested from 0.5 to 3% NaCl, the high level of bacteriocin production was recorded at 1.5% and minimum was at 3%. The effect of enzymes used was: proteinase K, α - amylase, DNase, and lipase. In the presence of α - amylase, DNase and lipase were positive effect of Bacteriocin production. Proteinase K was strongly inhibited bacteriocin production. Effect of detergents used was: sodium dodecyl sulphate (SDS), Tween 80, Triton X-100, EDTA and urea. Sodium dodecyl sulphate (SDS), Twee 80 and Triton X-100 were could stimulate the bacteriocin production. In contrast, it was strongly inhibited by EDTA and urea. In the purification of filtrate culture, was removed by centrifugation, and the proteins were concentrated by 80% ammonium sulphate precipitation and dialysis. The recovered proteins were then fractionated by ion-

exchange chromatography, using DEAE- Cellulose. All procedures were done in cold room. Extracellular bacteriocin was purified up to 11.11 fold from.

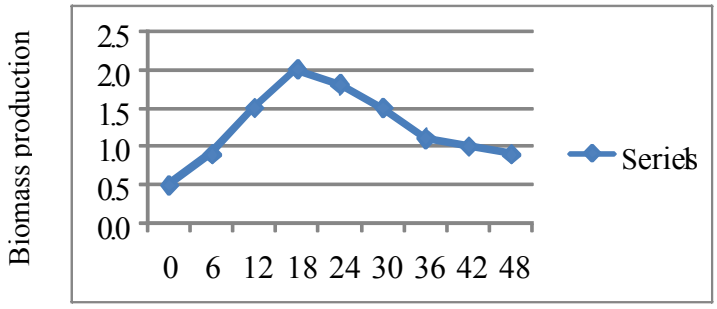

Figure 1: Biomass production by L.acidophilus

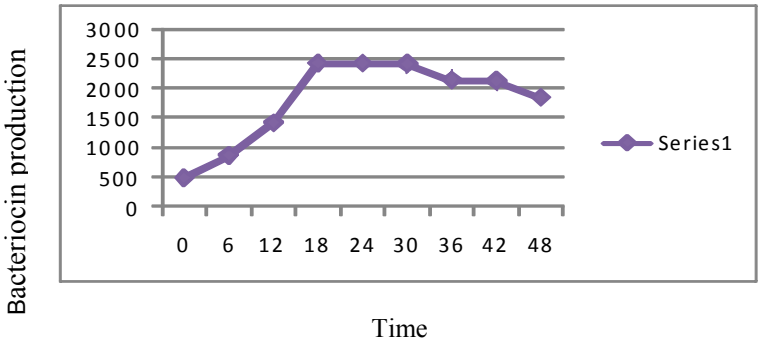

Figure 2: Bacteriocin production by L. acidophilus

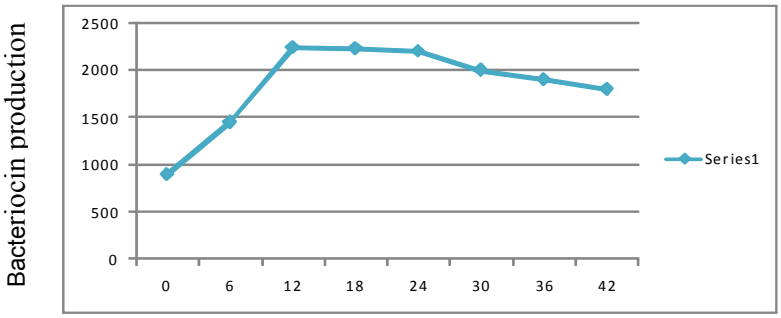

Figure 3: Bacteriocin production by L. plantarum

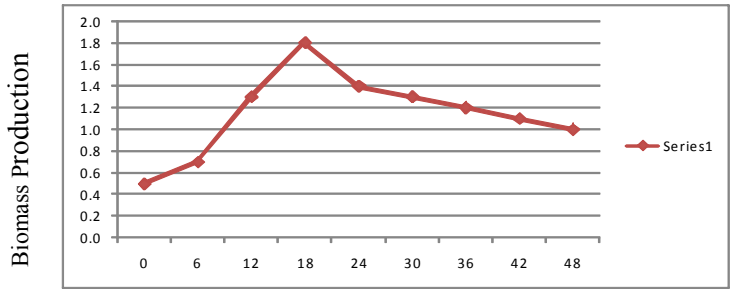

Figure 4: Biomass production by L. plantarum

4. Discussion

The present investigation highlights the isolation, characterization and activity of bacteriocin produced by *L. acidophilus* and *L. plantarum from* marine environment. Possession of bacteriocin by *L. acidophilus* and *L. plantarum* is an indication that the bacteria can be used as probiotic and as biopreservative. Todorov and Dicks showed that bacteriocin production was strongly dependent on pH, nutrients source and temperature [1 4]. Various physicochemical factors seemed to affect bacteriocin production as well as its activity. Maximum activities were noted at pH 5.0 and pH 6 .0, temperatures 25 ºC and 30 ºC and 1.5% NaCl. From the results proved that they could be used in acidic foods. MRS seemed to be more suitable medium for the bacteriocin production. Similar results were observed by Karthikeyan and Santosh [10] and Ogunshe *et al.* [1] . Bacteriocin production was influenced when incubated in different enzymes α -amylase; DNase, and lipase resulted in greater Bacteriocin production. Proteinase K was strongly inhibited bacteriocin production. This is in contrast to results obtained by Ivanova *et al*. [4] and Ogunbanwo *et al*. [13] . Among the detergents, Sodium dodecyl sulphate (SDS), Tween 80 and Triton X-100 stimulated bacteriocin production, which was strongly inhibited by EDTA and urea. But, stimulatory effect of Sodium dodecyl sulphate (SD S), Tween 80 and Tritone X-100 on bacteriocin in playing that the detergents act as co-factors, which are required to increase the bacteriocin production. During purification several different protocols were applied. Optimal recovery was achieved by including ammonium sulphate precipitation and ion-exchange chromatography. Purified bacteriocin from *L. acidophilus* and *L. plantarum revealed* homogeneity of a single protein band on 15% native PAGE. Similar results were recorded by Ivanovo *et al*. [4] and and Ogunshe *et al*. [1] .Numerous strains of bacteriocin producing *Lactobacillus plantarum* have been isolated in the last two decades and have also been reviewed by Olasupo [9]. Numerous small, heat-stable plantaricins have been described in the literature that has only been partially characterized. These include the following, of which the producing organisms have been isolated from various fermented food products: Meat: Several bacteriocin-producing *L. plantarum* strains have been isolated from fermented sausages obtained from different manufacturers at different times of ripening [8]. Schillinger and Lücke [16] isolated various bacteriocin producing lactobacilli including *Lactobacillus plantarum* from fresh meat and different meat products. Plantaricin UG1 [3] is produced by *L. plantarum* UG1 isolated from dry sausage. Plantacin 154 [6] is produced by *L. plantarum* LTF 154 isolated from fermented sausage. Bacteriocin-deficient mutants obtained after treatment of cells with acriflavin, coincided with the loss of a plasmid of 9.5 mDa, designated pLP1542. The bacteriocin-deficient mutants are immune to plantacin 154, suggesting that the genes coding for immunity are located on the chromosome. Plantaricin SA6, produced by *L. plantarum* SA6 was isolated from fermented sausage [17] . Plantaricin 1.25L is a thermo stable class two bacteriocin produced by *L. plantarum* TMW1.25 isolated from sausage fermentation [7]. Two bacteriocins, ST28MS and ST26MS, produced by two different strains of *L. plantarum* were isolated from molasses and partially characterized. Both bacteriocins showing unusual activity against Gram-negative bacteria, including *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumanii* Plantaricins S and T are produced by *L. plantarum* LPCO10, isolated from green olive fermentations [11, 12] . Plantaricin S is produced during the logarithmic phase of growth. These results were resembled with our findings. A second bacteriocin, plantaricin T, is secreted once the producing organism reaches the stationary phase of growth. Plantaricin T exhibits the same heat resistance as plantaricin S, but is not inactivated by αamylase or lipase A. Plantaricin T also exhibits a lower level of inhibition against the various organisms tested than plantaricin S. Amino acid sequence of plantaricin S was determined. Bacteriocin-producing strains of *L. plantarum* ST23LD and ST341LD were isolated from the brine of spoiled black olives [15] and there production optimized .Numerous reports have proved the ability of *L. acidophilus* strains to produce bacteriocins. *L. acidophilus* strains exhibiting antagonistic activity towards certain types of psychrotrophic microorganisms such as *Listeria monocytogenes*, *Bacillus cereus*, and *Clostridium* sp [5]. These finding had resemble with our results. The peculiar antimicrobial characteristics of *L. acidophilus* and *L. plantarum* can positively have impact on their use as starter cultures for traditional fermented foods, with a view to improving the hygiene and safety of the food products so produced. The bacteriocin produced by *Lactobacillus lactis* was assayed by agar well diffusion method and bacteriocin activity was measured in terms of AU ml^{-1}. The highest dilution that gave a define zone of growth inhibition was used to calculate AU ml^{-1}. Mode of action of bacteriocin produced by *L. acidophilus* and *L. plantarum* were tested and the behavior of the bacteriocin produced by isolated strain was considered as bactericidal.

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6. References

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