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Production, optimization and characterization of broad spectrum bacteriocins from *Lactobacillus plantarum* DP2 and *Lactobacillus casai* DD1.

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ABSTRACT

Two broad spectrum bacteriocins producing lactobacilli isolated from dairy samples were identified as *L. plantarum* DP2 and *L. casai* DD1. Bacteriocin production from *L. plantarum* DP2 was recorded maximum in the presence of maltose as sole source of carbon, whereas glucose was found to be best carbon source in case of *L. casai* DD1. Both bacteriocin showed maximum production when three nitrogen sources tryptone, yeast extract and meat extract were present together in the medium at pH 6 and 37°C. Bacteriocins from *L. plantarum* DP2 and *L. casai* DD1 were purified up to 4 fold and 6.3 fold with a recovery of 146.8% and 130.2% respectively. Molecular weights of purified bacteriocins were 4.8 kDa (*L. plantarum* DP2) and 9.2 kDa (*L. casai* DD1). Bacteriocin from *L. plantarum* DP2 was thermal stable, active at pH 4 to 8 and also showed stability at high salt concentrations (2-10%). Increased activities of this bacteriocin were also recorded with 2% NaCl, 1% EDTA and 1% tween 80. Whereas bacteriocin produced by *L. casai* DD1 was heat unstable and also unstable at high salt concentrations. However, both bacteriocins were completely inactivated by proteolytic enzymes.

Keywords: Bactetiocin, *Lactobacillus plantarum, Lactobacillus casai*, optimization, purification, characterization

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INTRODUCTION

Lactobacilli are recognized for their fermentative ability including their health and nutritional benefits. The antimicrobial properties of lactobacilli are of special interest in developing strongly competitive starter cultures for food fermentation. Lactobacilli exhibit strong antagonistic activity against many microorganisms including food spoilage organisms and pathogens by producing various compounds such as organic acids, diacetyl, inhibitory enzymes, hydrogen peroxide and other inhibitory substances, such as bacteriocins or bactericidal proteins during lactic fermentations [1,2]. Bacteriocins are ribosomally-synthesized peptides or proteins having antimicrobial property. The bacteriocins produced by LAB are suitable for food preservation as they are recognized as safe, nontoxic on eukaryotic cells, become inactivated by digestive proteases and usually pH and heat- tolerant.

They have a relatively broad antimicrobial spectrum against many food-borne pathogens and spoilage bacteria but having little influence on the gut microbiota. Bacteriocins show a bactericidal mode of action, usually by affecting the bacterial cytoplasm membrane. Nisin produced by *Lactobacillus lactis* is well characterized bacteriocin and approved in more than 60 countries to be used in food industries. Many lactobacilli such as *L.*

amylovorus, L. plantarum, L. fermentum, L. brevis reported to produce bacteriocin. Several LAB bacteriocins offer potential applications as biopreservative, more over its use in the food industries can help to reduce or completely replace the addition of chemical preservatives as well as the intensity of heat treatments, as a result naturally preserved and organoleptic richer and nutritional properties. This can be an alternative to satisfy the increasing consumers demands for safe, fresh-tasting, ready-to-eat, minimally-processed foods and also to develop “novel” food products (e.g. less acidic, or with a lower salt content). The present study has been aimed to the detection, purification and characterization of bacteriocins to exploit their use in food preservation strategies.

MATERIALS AND METHODS

Isolation and screening of bacteriocin producing lactobacilli: Homemade cheese, raw milk, dosa paste and curd were serially diluted (10^{-1} to 10^{-6}) and 0.1 ml of each sample was spreaded onto deMan Rogosa Sharpe agar medium. Plates were incubated anaerobically for 24-48 hrs at 30^{o}C [3]. Colonies initially observed were subjected for screening of bacteriocin production by well diffusion method against indicator bacteria i.e. *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes, Pseudomonas aeruginosa*, *Salmonella thyphimurium*, *Staphylococcus aureus*. Screening of bacteriocin producing lactobacilli was carried out by well diffusion assay on Muller Hinton agar. *Lactobacillus* sp. showing broader range of antagonistic activity against indicator bacteria were selected and identified on the basis of morphological and biochemical characteristics.

The selected lactobacilli were subjected for bacteriocin production, purification and further characterization. The isolates were maintained as frozen stock culture at -20°C in MRS broth with 5% glycerol and propagated twice before use in experiments.

Determination of bacteriocin activity

The isolated strains were propagated in MRS broth (100 ml) seeded with 10% inoculum (108 CFU/ml) of overnight culture and incubated for 48 hrs at 150 rpm and 30°C. After incubation, the whole broth was centrifuged at 10,000 rpm for 20 min at 4^{o}C. The cell-free supernatant was neutralized by 1 M NaOH and treated with catalase and then used as crude bacteriocin [4]. The antimicrobial activity of the bacteriocin was defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and was expressed in activity units per ml (AU/ml) [5].

Optimization of carbon and nitrogen source

MRS production media were prepared by taking glucose, sucrose, maltose, lactose and fructose (2% each) separately and then inoculated with *Lactobacillus* sp. and incubated at 30^{o}C for 24 hrs. After incubation the broth cultures were subjected for the extraction and bacteriocin activity. Five Nitrogen sources were

added in different combinations in a total of 2% (w/v) concentration in the MRS medium. The combinations taken were: tryptone (10g/l) plus yeast extract (10g/l); tryptone (10g/l) plus peptone (10g/l); Yeast extract (10g/l) plus peptone (10g/l); tryptone (10g/l), yeast extract (5g/l) and meat extract (5g/l) and they were designated as N1 (T+Y), N2 (T+P), N3 (Y+P) and N4 (T+Y+M) respectively. Each medium was inoculated with isolated *Lactobacillus* sp. and after 24 hrs bacteriocin activity was determined.

Optimization of pH and incubation temperature

pH of MRS broths were adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0. Each of the medium was inoculated with isolated *Lactobacillus* sp. and after 24 hrs bacteriocin activity was determined. For optimization of incubation temperature MRS broth was adjusted at above optimized culture conditions. Then inoculated and incubated at different temperature i.e. 20^{o}C, 27^{o}C, 30^{o}C, 37^{o}C and 40^{o}C. After 24 hrs bacteriocin activity was determined.

Purification of Bacteriocin

Bacteriocin was purified by three steps purification procedure involving ammonium salt precipitation and ion-exchange chromatography [6]. Cell free supernatant was precipitated with 80% ammonium sulphate and stored overnight at 4^{o}C and then the pallet was collected by centrifugation at 10000 g at 4^{o}C for 30 min. The pallet was dissolved in phosphate buffer (pH 7.0, 0.05 M) and dialyzed against two liters of same buffer overnight at 4^{o}C. The dialyzed protein was applied to DEAE column (anion exchanger) and elution was performed by taking 0.1 M NaCl in the same buffer. Each fraction was checked for the concentration of protein by Bradford method.

Molecular weight determination

The partially purified fractions were analyzed by SDS-PAGE [7]. The gel was run at constant current 50 V until the tracking dye (bromophenol blue) had migrated to the end. Molecular weight marker was used as standard marker (Genei, India).

Effect of heat treatment

Purified bacteriocin was exposed to various heat treatments i.e. 60 ºC for 180 min, 80ºC for 120 min, 100ºC for 40 min and 121ºC for 20 min. Aliquot volumes of each Fraction was then removed and assayed for bacteriocin activity [2].

Effect of pH

Purified bacteriocin was adjusted to pH 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 with hydrochloric acid (1M HCl) and sodium hydroxide (1M NaOH) and incubated for 4 h at 37ºC and than similarly assayed [2].

Salt Tolerance

The purified bacteriocin (1ml) was incubated at 37ºC with varying concentrations of NaCl (2, 4, 6, 8 and 10%) (Merck, Germany). The bacteriocin activity was assessed.

Treatment with Enzymes

The nature of the antagonistic agent was evaluated by treating the purified bacteriocin samples to various enzymes such as lipase, protease, trypsin, proteinase K and α amylase (all obtained from Genei). Purified bacteriocin was incubated with enzyme (1:1 w/v) at 37ºC. The remaining activity was determined after 2 h against indicator organism by well diffusion assay [8].

Effect of surfactants

The surfactants (such as tween 20, tween 80, EDTA, sodium dodecyl sulphate) were added to purified bacteriocin at a concentration of 0.01 ml or 0.01 g of surfactant per ml of bacteriocin solutions.

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These preparations was incubated at 37ºC for 60 min [9] and assayed for bacteriocin activity against indicator organism by using well diffusion method.

RESULTS AND DISCUSSION

Lactobacilli synthesize bactericidal agents that vary in their spectra of activity. Out of fifteen, two *Lactobacillus* sp. showed broad spectrum of antibacterial activity against seven selected pathogenic and food spoilage bacteria i.e. *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes, Pseudomonas aeruginosa*, *Salmonella thyphimurium*, *Staphylococcus aureus*. They were identified as *Lactobacillus plantarum* DP2 and *Lactobacillus casai* DD1 on the basis of morphological, physiological and biochemical characteristics.

Broad-spectrum bacteriocins present potential wider uses as food biopreservatives and these was reported earlier [10-15].

Effect of pH and temperature on production of bacteriocin: Bacteriocin production was recorded maximum at pH 6 and 37°C by both *L. plantarum* DP2 (1800 AU/ml) and *L. casai* DD1 (1200 AU/ml). Meera and Devi [16] also reported best production at pH 6 but at 30°C. Similarly, optimum temperature for production of some other bacteriocins such as lactocin A, enterocin 1146, nisin Z, lactocin S [17] and bacteriocin from *L. plantarum* F12 [15] was also recorded at 37°C. In contrast, maximum bacteriocin production by *L. plantarum* AA135 was also recorded at 30°C [37]. Bacteriocin production was comparatively low in other studied pH and temperatures (Fig.1 and Fig.2).

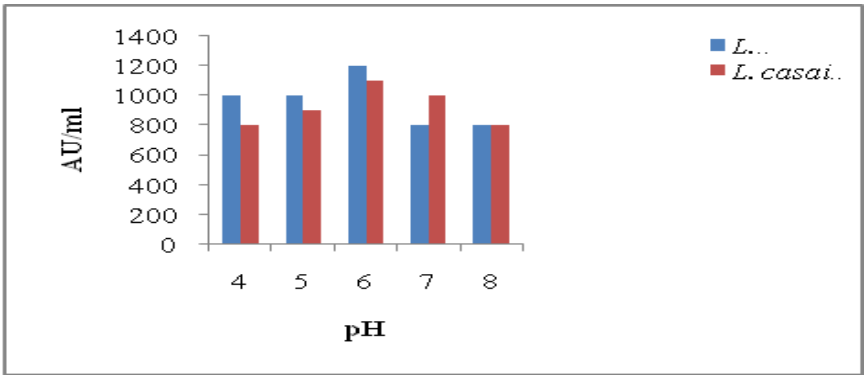

Figure 1: Effect of pH on bacteriocin production

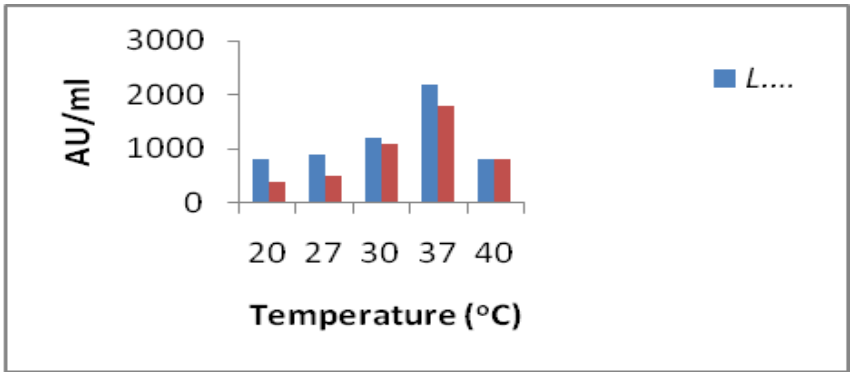

Figure 2: Effect of temperature on bacteriocin production

Effect of carbon and nitrogen on production of bacteriocin

Maltose was found to be best carbon source for the production of bacteriocin by *L. plantarum* DP2 (3200 AU/ml). Similar observation was reported for bacteriocin, produced by *Lactobacillus plantarum* [17]. In contrast, *L. casai* DD1 showed highest activity (1800 AU/ml) in the presence of glucose and no significant effect was observed by addition of other carbon sources (Fig. 3).

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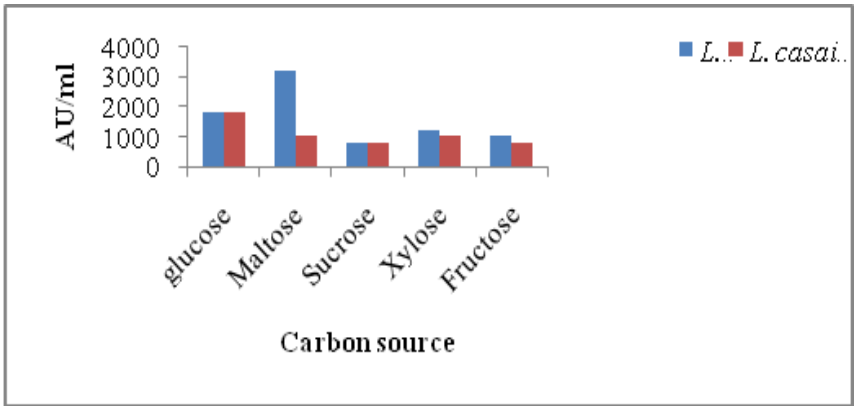

Figure 3: Effect of different carbon sources on bacteriocin production

Previously probiotic lactic acid bacteria also showed maximum production by adding glucose as carbon source [16]. N4 (T+Y+M), in which Tryptone, yeast extract and meat extract were present together in the medium, was found to stimulate bacteriocin production by both lactobacilli (Fig.4). Similar findings were reported for *L. plantarum* ST13BR and *L. plantarum* AA135 respectively [17,1].

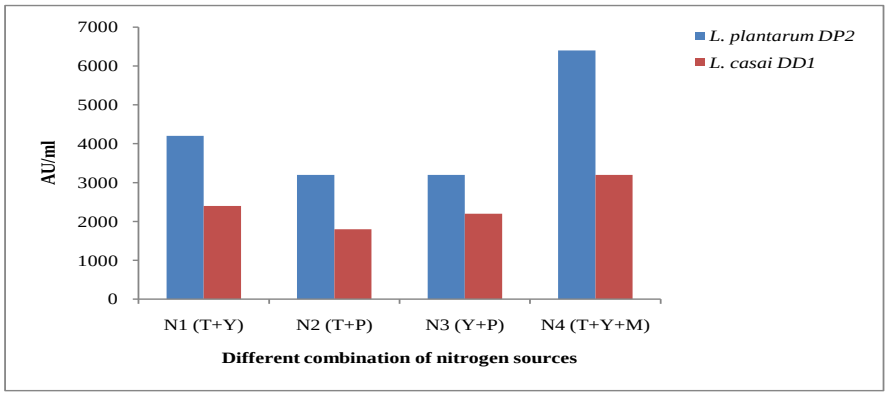

Figure 4: Effect of different nitrogen sources on bacteriocin production

Purification of bacteriocin:

Table 1: Screening of bacteriocin production by isolated *Lactobacillus* spp.

Isolated Lactobacilli	1 ZI (mm)	2 ZI (mm)	3 ZI (mm)	4 ZI (mm)	5 ZI (mm)	6 ZI (mm)	7 ZI (mm)
DD1	7mm	6mm	5mm	8mm	6mm	5mm	5mm
DD2	-	-	3mm	4mm	-	-	4mm
DD3	-	-	-	6mm	5mm	-	-
DD4	-	-	-	-	-	-	-
DP1	5mm	-	6mm	-	-	-	-
DP2	8mm	7mm	6mm	8mm	8mm	5mm	6mm
DP3	-	6mm	-	-	-	-	-
DP4	-	-	5mm	-	6mm	-	-
DP5	-	-	-	-	-	-	4mm
DA1	-	-	-	-	-	-	-
DA2	5mm	6mm	6mm	-	-	5mm	-
DC1	-	6mm	-	7mm	-	4mm	4mm
DC2	-	7mm	6mm	-	6mm	-	-
DC3	-	-	7mm	-	5mm	-	-
DC4	6mm	6mm	-	7mm	-	4mm	-

ZI- Zone of inhibition in mm; Indicator bacteria- 1. *Bacillus cereus* 2. *Bacillus subtilis* 3. *Escherichia coli* 4. *Listeria monocytogenes* 5. *Pseudomonas aeruginosa* 6. *Salmonella thyphimurium* 7. *Staphylococcus aureus*.

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Optimized culture conditions (summarized in table 2) were applied for bulk production and purification of bacteriocin. The results of purification procedure were summarized in table 3. Highest bacteriocin activity was achieved at 80% saturation with ammonium sulphate for *L. plantarum* DP2 and *L. casai* DD1 both. After final purification step the bacteriocins from *L. plantarum* DP2 and *L. casai* DD1 were purified up to 4 fold and 6.3 fold with a recovery of 146.8% and 130.2% respectively. During the purification procedures, each step resulted in considerable loss of protein concentration while specific activity was found to increase. Caseicin 80 from *L. casai* was also purified by cation exchange chromatography [18]. The Increase in activity could be due to release of active monomers from bacteriocin complexes. The above fractions were subjected to DEAE cellulose column and production of active fraction of bacteriocin was achieved.

Table 2: Optimized culture conditions

Optimized Parameters	*L. plantarum* DP2	*L. casai* DD1
Carbon source	Maltose (2%)	Glucose (2%)
Nitrogen source	Tryptone(10g/l)+Yeast extract (5g/l) + Meat extract (5g/l)	Tryptone(10g/l)+Yeast extract (5g/l) + Meat extract (5g/l)
pH	6	6
Temperature	37°C	37°C

Table 3: Summary of purification steps of bacteriocin produced by *L. plantarum* DP2 and *L. casai* DD1

Organisms	Purification Stages	Volume (ml)	Activity (AU/ml)[a]	Total activity (AV)[b]	Total Protein (mg/ml)[c]	Specific activity (AU/mg)[d]	Purification fold[e]	Recovery (%)[f]
L. plantarum DP2	Culture supernatant	500	6400	3200000	58.7	109	1	100
	Ammonium sulphate	50	10400	520000	6.2	1677.4	16.3	15.38
	Dialysis	40	10000	400000	5.2	1923	12.5	17.64
	DEAE cellulose	10	12800	128000	0.8	16000	4	146.8
L. casai DD1	Culture supernatant	500	3200	1600000	32.7	97.9	1	100
	Ammonium sulphate	50	5600	280000	4.7	1191.5	17.5	12.17
	Dialysis	40	5200	208000	4.2	1238.1	13.0	12.64
	DEAE cellulose	10	10200	102000	0.8	2266.7	6.3	130.2

[a]Activity (AU/ml): Reciprocal of highest dilutionX1000/volume of bacteriocin added
[b]Total activity (AV): Determined by multiplying volume by activity
[c]Protein concentration(mg/ml): Determined by the Bradford method
[d]Specific activity (AU/mg): Activity of the subsequent purification step/Protein concentration of the same step
[e]Purification fold: Specific activity of subsequent step/Specific activity of crude preparation
[f]Recovery (%): Total activity of subsequent step X100/Total activity of crude preparation

Determination of Molecular weight

SDS PAGE of purified bacteriocins showed the molecular weight of bacteriocin from *L. plantarum* DP2 and *L. casai* DD1 were 4.8 kDa and 9.2 kDa respectively. Bacteriocin from *L. plantarum* DP2 was considered as class I bacteriocin as its molecular weight was less than 5 kDa and bacteriocin from *L. casai* DD1 was considered as class II bacteriocin as its molecular weight was between 5-10 kDa. In contrast, caceicin 80 was reported to have molecular weight between 42 kDa. [18,19]. Some previously reported bacteriocins from *L. plantarum* ST13BR [20] and *L. plantarum* F1 [21] having 10 kDa and 9.5 kDa molecular weight respectively, were reported as class II bacteriocins.

Effect of heat treatment

The bacteriocin produced by *L. plantarum* DP2 was considered to be heat stable as it retained its activity (10,200 AU/ml) after heating at 121°C for 20 min. The phenomenon of heat stability of LAB bacteriocins have been reported earlier for different bacteriocin including plantaricin A, plantaricin C19, plantaricin S, plantaricin 149, plantaricin SA6, plantaricin 423, lactocin RN 78, bacteriocin GP1 [22,23,10,24,25]. The thermostability of bacteriocin from *L. plantarum* DP2, place it within heat stable low

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molecular weight group of bacteriocins. This quality of the bacteriocin makes it superior in processed food stuffs where high heat is applied and also avoids their storage at low temperature. However, bacteriocin from *L. casai* DD1 was heat unstable and completely lost its activity after heating at 121°C (Fig. 5).Similar finding was recorded for lacticin NK 24 [26].

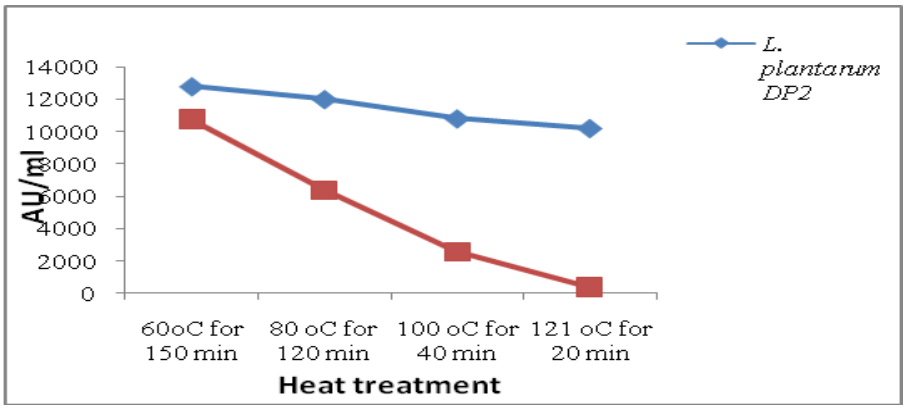

Figure 5: Effect of Heat treatment

Effect of pH

The activity of bacteriocin elaborated by the test isolates was also pH dependent. The bacteriocins produced from both lactobacilli showed stability between pH 4 to 8 and significantly loss of bacteriocin activity was observed at extreme acidic (from pH 2 to 3) and alkaline pH (pH 9 to 10). Similar observation was reported by Ogunbanwo and co-workers for *Lactobacillus plantarum* F1 [12]. Maximum activity of bacteriocin from *L. plantarum* DP2 was recorded at pH 6 to 7 (12800 AU/ml) whereas for *L. casai* DD1, it was found in pH 5 and 6 (Fig. 6). This was also shown by different bacteriocins, namely bulgarican, lactobulgarican and a bacteriocin from *L. plantarum* F12 [28,27,15].

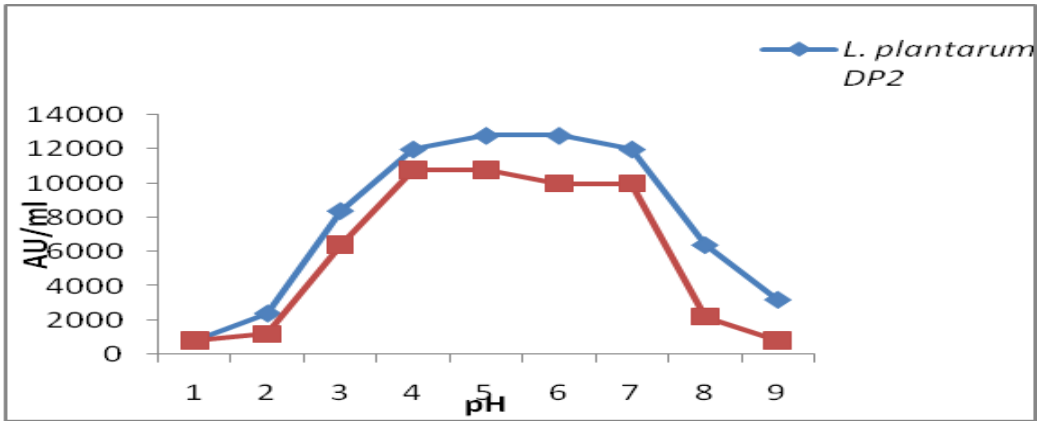

Figure 6: Effect of pH

Effect of salt concentrations

Bacteriocins produced by *L. plantarum* DP2 showed increased antimicrobial activity (14,800 AU/ml) in 2% salt concentration. The increased bacteriocin activity in low salt concentration, agreed with previous studies which have shown that the presence of NaCl enhanced the antimicrobial action of bacteriocins such as nisin, leucocin F10, enterocin AS-48 [29,30,31,32]. Bacteriocin from *L. plantarum* DP2 was found to be stable in high salt concentrations (6-8%) whereas bacteriocin from *L. casai* DD1 lost its activity (Fig. 7). Negative effect of high salt concentration in bacteriocin activity was also observed in lactocin 705 at 5–7% NaCl [33], pediocin at 6.5% NaCl [33] and curvacin [34]. The protective effect of sodium chloride may be due to interference with ionic interactions between bacteriocin molecules and charged groups involved in bacteriocin binding to target cells [35]. It is suggested that sodium chloride may induce conformational changes of bacteriocins or may

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cause changes in the cell envelope of the target organisms [33,36]. The bacteriocin produced by *L. plantarum* DP2 were stable at different pH as well as in high salt concentrations, make it an attractive applicant in food supplies i.e. they can be used in acidic foods like pickle, yogurt etc. Bacteriocin produced by *L. casai* DD1 was not stable at high temperature and high salt concentrations, limits its use as biopreservative.

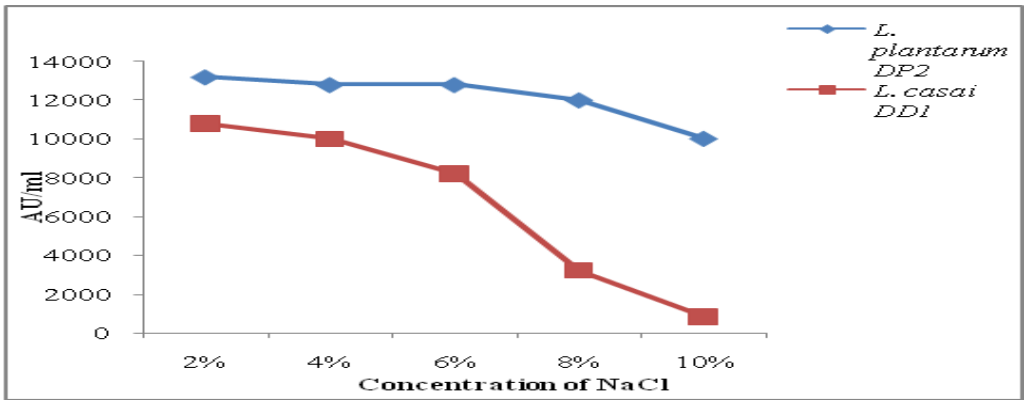

Figure 7: Effect of salt concentration

Effect of surfactants:

Bacteriocin activity of *L. plantarum* DP2 was significantly increased with EDTA (16,800 AU/ml) and tween 80 (16,200 AU/ml). Similarly bacteriocin from *L. casai* DD1 showed enhanced activity (14,200 AU/ml) only with EDTA (Fig. 8). These results agreed with the findings observed for made for *Lactobacillus plantarum* G2 and *Lactobacillus plantarum* F1 [12, 14]. This increase might be due to the effect of surfactant on the permeability of the cell membrane [5]. Whereas tween 20 had adverse effect on both bacteriocins as their activity was completely demolished after subjection to this surfactant. However there were no significant effects of other surfactants on bacteriocin.

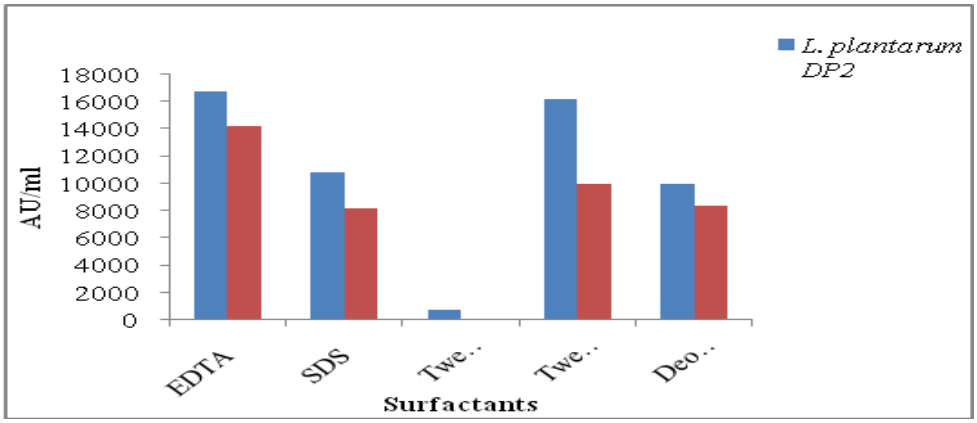

Figure 8: Effect of surfactants

Effect of enzymes

Proteinase K, pepsin and trypsin completely inactivate both bacteriocins (Fig. 9). These proteinaceous bacteriocins can be broken down easily by gastric enzymes, thus making them completely safe for human consumption. Bacteriocin from *L. casai* DD1 showed some loss of activity in the presence of lipase and amylase. This observation showed that protein contributes major part in the total molecular weight of bacteriocins with some contribution of sugar and lipid moieties. Whereas, bacteriocin from *L. plantarum* DP2 was not affected by the action of lipase and α amylase. Similar results were observed by bacteriocin produced from *L-plantarum* F1 and *L. brevis* OG1 [12] and for bacteriocin from *L. plantarum* G2 [14]. Bacteriocin activities were not affected by catalase, proved that the antibacterial activity was not due to H_2O_2.

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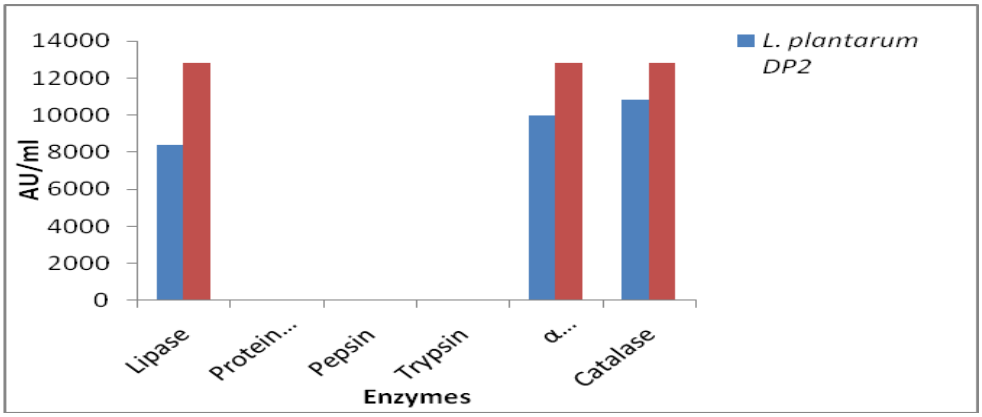

Figure 9: Effect of enzymes

CONCLUSIONS

The peculiar broad spectrum antibacterial characteristic, technological properties and especially heat and pH stability and salt tolerance capacity of *L. plantarum* DP2, can positively has impact on their use as biopreservative or this could be directly used as starter culture with a view to improving the hygiene and safety of the food products. This can be an alternative to satisfy the increasing consumers demands for safe, fresh-tasting, ready-to-eat, minimally-processed foods and also to develop "novel" food products (e.g. less acidic, or with a lower salt content).

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