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Research Article

PURIFICATION, CHARACTERIZATION AND IN VITRO CYTOTOXICITY OF PLANTARICIN 9496: A BACTERIOCIN PRODUCED BY LACTOBACILLUS PLANTARUM MTCC 9496

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ABSTRACT

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by lactic acid bacteria. The objective of this study is to investigate antibacterial and antifungal spectrum of bacteriocin that was purified and characterized from *Lactobacillus plantarum* MTCC 9496 and to determine toxicity of this peptide, named plantaricin 9496 on eukaryotic cells. Plantaricin 9496 was purified by adsorption desorption method followed by gel permeation chromatography. Molecular weight of plantaricin 9496 was found to be of 5.8 kDa. It exhibited broad antimicrobial spectrum against fruit and vegetable spoiling organisms, especially *Staphylococcus aureus*, *Listeria monocytogenes*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Aspergillus niger*, *Fusarium oxysporum*, *Botrytis cinera*, *Penicillium expansum*, *Rhizopus stolonifer* and *Candida albicans*. Plantaricin 9496 retained activity after heating upto 100°C and at pH range of 2.0-7.0. Cytotoxicity of plantaricin 9496 on spleenocytes showed that it was non toxic upto concentration of 40- 2600 µg/ml and CC_{50} value is above 20800 µg/ml, indicating that cytotoxicity of plantaricin 9496 is very low. Hence, this bacteriocin can be used as safe biopreservative to extend shelf life of perishable food products.

INTRODUCTION

Agriculture continues to be backbone of Indian economy. India is one of the key food producing countries in the world next to China. The wastage of agricultural produce in India is massive (Bhatia *et al.* 2016). Perishable foods such as fruits and vegetables undergo the greatest proportion of post harvest losses as they represent ideal conditions for the survival and growth of microorganisms (Moss, 2008). The principle of food preservation is to prevent the microbial spoilage of food so as to provide a reasonable shelf life to the product (Parish *et al.*, 2003). Consumer awareness about the ill effects of chemical preservatives raised the demand for research on biological origin, safe and environment friendly food preservatives (Linda *et al.*, 2009). In this regard, bacteriocins play an important role and act as strong candidate of biopreservation. These are antimicrobial peptides produced by lactic acid bacteria (LAB) that kill or inhibit the growth of other bacteria and fungi. *Lactobacillus* is an important genera of lactic acid bacteria. Many *Lactobacillus* sp. like *Lactobacillus plantarum* (Powell *et al.*, 2007; Xie *et al.*, 2011; Hu *et al.*, 2016; Wen *et al.*, 2016), *L. acidophilus* (Muriana and Klaenhammer, 1991; Rani *et al.*, 2016), *L. pentosus* (Torodov and Dicks, 2004), *L. paracase*i subsp. *paracasei* (Caridi, 2002), *L. delbrueckii* (Miteva *et al.*, 1998); L. curvatus (Massani *et al.*, 2016; Marques *et al.*, 2017) have been exploited as bacteriocin producers. Lactobacillus plantarum is an industrially important organism with probiotic properties and given GRAS status (Parente *et al.*, 2010). In the past, many bacteriocins have been purified and characterized from Lactobacillus plantarum with broad antibacterial spectrum (Rekhif *et al.*, 1995; Messi *et al.*, 2001; Todorov and Dicks, 2004; Noonpakdee *et al.*, 2009; Xie *et al.*, 2011; Hu *et al.*, 2013). Most of the work in field of biopreservation is centered around purification and characterization of bacteriocins and elucidating their antimicrobial spectrum against bacterial agents of food borne diseases. There is limited information on their role as biocontrol agents of food spoiling fungi. Based on the applications of bacteriocins as a food component, evaluation of their safety is an important aspect. This study reports the purification and characterization of bacteriocin from Lactobacillus plantarum MTCC 9496. Antagonistic effect of purified bacteriocin against major food spoiling organisms have been elucidated and toxicity of this peptide has been investigated on eukaryotic cells.

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MATERIALS AND METHODS

Microbial strains

Bacteriocin producer, *Lactobacillus plantarum* MTCC 9496 was used in present study. It was procured from Microbial type culture collection (MTCC), Chandigarh, India and

cultivated on MRS broth/37ºC. *Staphylococcus aureus* NCDC 109 was employed as indicator organism and cultivated on nutrient agar/37ºC.

Growth kinetics of bacteriocin production

Lactobacillus plantarum was inoculated in MRS broth for 48h. After every 3 h, 1 ml of sample was taken and optical density was recorded at 600 nm and pH was checked by digital pH meter. To determine bacteriocin activity at different time periods during growth of organism, cell free supernatant (CFS) was obtained and diluted ten folds. Well diffusion assay was performed using *Staphylococcus aureus* as indicator organism (Marques *et al*., 2017). The antimicrobial activity is defined as the reciprocal of the highest dilution showing inhibition of the indicator lawn and it is expressed in arbitrary units per ml (AU/ml).

Purification of bacteriocin

One litre of MRS broth was inoculated with overnight grown metabolically active culture of *Lactobacillus plantarum.* Bacteriocin was purified using two step procedure involving cell adsorption desorption method followed by gel permeation chromatography.

Cell Adsorption- Desorption: Cell adsorption desorption method of Yang *et al*. (1992) with some modifications was employed for bacteriocin purification from culture supernatant. The cell culture after 16 hours of incubation was heated in boiling water bath for 20 minutes and was cooled down. pH of the culture was adjusted to 6.5 with 4M NaOH and kept for overnight stirring at 4ºC. It facilitates bacteriocin adsorption on surface of producer cells. Cells were harvested by centrifugation at 9000 rpm/20 minutes. Cells were washed with sterile 5mM sodium phosphate buffer (pH 6.5) and suspended in 100ml of NaCl (pH 1.5, adjusted with 5% phosphoric acid) and was stirred overnight at 4ºC. After stirring, the cell suspension was centrifuged. Pellet was discarded and antimicrobial activity of supernatant was determined.

Gel Permeation Chromatography: A column was packed witrh Sephadex G-50 (Sigma- Aldrich) and equilibrated with 0.05mM ammonium acetate buffer, pH 4.8. After equilibration, 1 ml of bacteriocin preparation obtained from cell adsorption desorption technique was loaded on to the column. Fractions were collected at flow rate of 0.5 ml/min and evaluated for protein concentration by Lowry method and antimicrobial activity was also determined against *Staphylococcus aureus*. Subsequently, active fractions were pooled, concentrated and stored at -20 ºC for further studies.

Molecular mass determination of purified bacteriocin

The molecular mass of the bacteriocin was determined by 15% SDS- PAGE (Laemmli, 1970) employing a vertical slab gel system (Banglore Genei, India). After electrophoresis at 100V, the gel was stained with Coomassie Brilliant blue G-250 and destained using methanol and glacial acetic acid (20:10) mixture. Low range protein ladder (Thermo scientific) ranging from 1.7- 40 kDa was run simultaneously.

Sensitivity to enzymes, temperature and pH

First, the sensitivity of the purified bacteriocin towards different enzymes, i.e. α-amylase, catalase, pepsin, proteinase K, lipase and trypsin was evaluated. Enzymes were added at 5mg/ml of bacteriocin and incubated at 37ºC for 2 hours. pH stability of bacteriocin was assessed by adjusting pH value from 2.0 to 9.0 with 0.1N HCl and NaOH. To determine thermal stability of purified bacteriocin, it was heated to different temperatures (4, 25, 30, 37, 45, 60, 80 and 100ºC for 30 minutes and in autoclave at 121ºC for 15 minutes). After treatment of enzymes, temperature and different pH, antibacterial activity of bacteriocin was determined against *Staphylococcus aureus* by well diffusion assay. Untreated bacteriocin sample was used as a control.

Antimicrobial spectrum and MIC values of Plantaricin 9496

Antimicrobial spectrum of plantaricin 9496 was determined against fruit and vegetable spoiling bacteria, fungi and yeast given in Table 3. Minimum inhibitory concentration (MIC) was determined against bacteria by broth dilution method (Zhao *et al*., 2016). Overnight grown different Indicator bacteria (10^6 cfu/ml) were added to each well of microtiter plate. Stock solution of bacteriocin (2048 µg/ml) was 2- fold serially diluted in normal saline in microtiter plate. After incubation at 37°C for 24 hours, 10 µl of 0.5% triphenyl tetrazolium chloride (TTC) was added. Live microorganisms reduce colorless TTC by enzymatic action, originating red coloured formazan.

MIC against fungi was determined by method of Garcha and Rani (2014) by observing the fungal growth. Spore suspension of fungal culture containing 10^6 spores/ml was added to 200 ml of broth. Bacteriocin at different concentrations was added to this broth and incubated. A control flask with indicator strain and without bacteriocin was also run simultaneously. Dry weight of fungal mat was recorded after 5 days of incubation. MIC was considered as maximum dilution that completely inhibited growth of indicator strain.

Storage stability of Plantaricin 9496

Storage stability of plantaricin 9496 was checked at 4ºC and -20ºC for a period of 60 and 90 days respectively. Samples were taken to determine residual antimicrobial activity after every 15 days (Ogunbanwo *et al*., 2003).

In vitro cytotoxicity assessment

Effect of plantaricin 9496 on the viability of eukaryotic cells was determined using MTT assay as described by Mosmann (1983). Spleenocytes at density of 2×10^9 /ml were added to RPMI 1640 (supplemented with 10% FBS) and then cultured in a CO_2 chamber at 37ºC/24h. After which, plantaricin 9496 was added at different concentrations ranging from 40 to 20800 µg and further incubated for 24h. After incubation, 150 µl of MTT (0.5mg/ml) was added and incubated for 4h at 37ºC. Consequently, 1.5ml of DMSO was added and mixture was thoroughly mixed to dissolve blue formazan crystals. Absorbance was determined at 590nm using DMSO as a blank. Percentage inhibition of cells was determined using formula:

$$(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{test}}) / (\text{Absorbance}_{\text{control}}) \times 100$$

Statistical analysis

All the experiments were carried out in triplicates and results are expressed as mean± standard deviation (SD). Comparison of various groups and trials was performed using analysis of

variance (ANOVA) followed by Tukey's test. Results are considered statistically different at p value of <0.05.

RESULTS AND DISCUSSION

Growth kinetics of bacteriocin production

The growth kinetics of *Lactobacillus plantarum* MTCC 9496 was studied in order to identify its various growth phases. The lag phase continued upto initial 6 hours. Exponential phase of the culture was observed from 8 to14 hour. Thereafter, a stationary growth rate was observed. A lowering in pH was observed in the culture supernatant during the growth cycle from initial 6.55 to a final pH of 1.6 after 48 hours of incubation (Fig 1). Lactic acid bacteria produce lactic acid as one of their major metabolic products. This acid production causes a lowering of pH in the growth medium (Leroy and Vuyst, 2004).

As observed by Avaiyarasi *et al*. (2016) there was maximum bacteriocin production after 18h of incubation by *Lactobacillus sakei* GM3 at early logarithmic growth phase.

Purification of bacteriocin

Bacteriocin produced by *Lactobacillus plantarum* MTCC 9496 was purified from the culture supernatant by cell adsorption desorption method followed by gel permeation chromatography (GPC). Results are shown in Table 1. Adsorption desorption method for bacteriocin purification is based on the property of bacteriocin to adsorb on the surface of producer cells at high pH values and then desorption in solution of low pH values. In this study, at pH- 6.5, adsorption of bacteriocin onto producer cells was 100% and no antimicrobial activity left in the supernatant. After bacteriocin desorption at pH 1.5, recovery of 6400 Arbitrary units/ml was reported and there is increase in specific activity

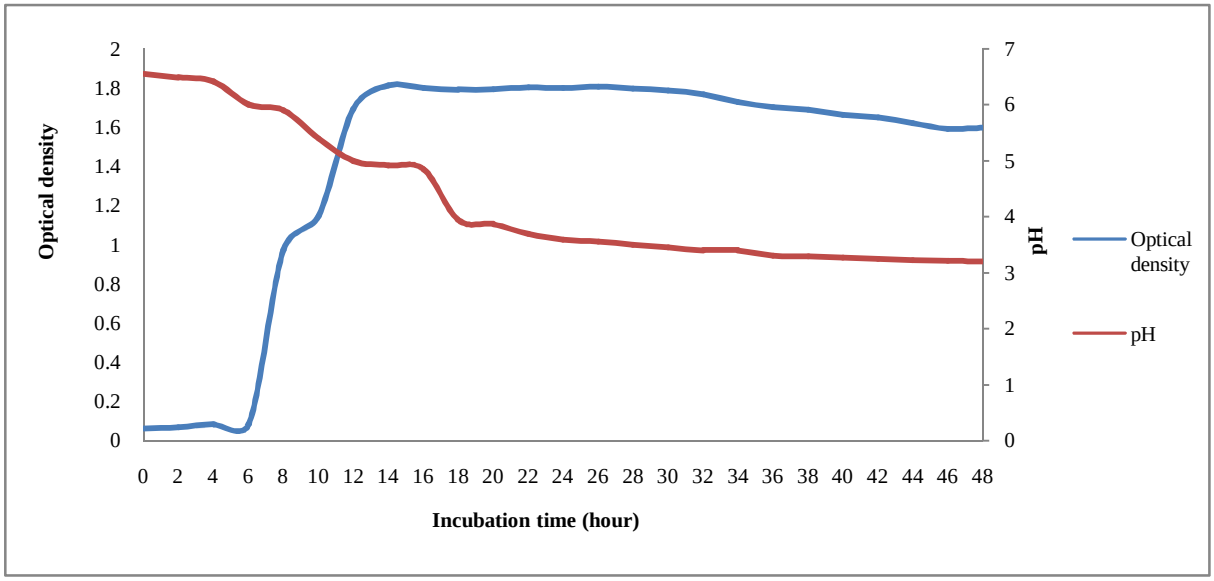

Fig 1 (a) Growth profile of *Lactobacillus plantarum* MTCC 9496
(b) Variation in pH with incubation time

Synthesis of bacteriocin by *Lactobacillus plantarum* was observed after 4h of incubation. Maximum bacteriocin activity (3200AU/ml) was found after 16h of incubation during stationary phase. Bacteriocin production remains constant from 16- 32h of incubation and decrease in bacteriocin activity was observed upto 48h. Optimum incubation time for production of plantaricin 9496 by *Lactobacillus plantarum* was found to be 16h.

Decrease in activity of plantaricin 9496 with incubation time could be due to production of protease enzyme (Ondaa *et al*., 2003).

Bacteriocin preparation obtained from cell adsorption desorption technique was subjected to GPC for further purification. Bacteriocin of *Lactobacillus plantarum* was eluted in six fractions (fraction No. 22-27) during chromatography and the specific activity of the combined fractions was 7013.7 AU/mg. The elution profile of bacteriocin on sephadex G-50 coloumn is depicted in Fig 2. Further, GPC using sephadex G-50 as matrix results into 43.8 times increase in purification folds and 8% recovery of the bacteriocin from culture supernatant.

Table 1 Purification of bacteriocin from *Lactobacillus plantarum* MTCC 9496

	Volume (ml)	AU/ml	Total activity	Protein (mg)	Specific activity* (AU/mg)	Purification fold**	Percentage recovery***
CFS	200	3200	64×10^4	4000	160	1	100
CAD	10	6400	64×10^3	26.8	2388	14.9	10
GPC	2	25600	512×10^2	7.3	7013.7	43.8	8

CFS: Cell free supernatant; CAD: Cell adsorption desorption; GPC: Gel permeation chromatography
*Specific activity: Total activity divided by protein concentration
**Purification fold: Increase in specific activity
***Percentage recovery: Total activity divided by initial total activity ×100

Molecular weight of purified plantaricin 9496 by SDS PAGE and coomassie brilliant blue staining was estimated to be around 5.8kDa (Fig 3). In past few years, many bacteriocins produced by *Lactobacillus plantarum* have been purified and characterized. Plantaricin SA6, a bacteriocin produced by *L. plantarum* SA6 with molecular weight of 3.4 kDa (Rekhif *et al*., 1995); Plantaricin 35d produced by *L. plantarum 35d* has molecular weight of 4.5kDa (Messi *et al*., 2001) and plantaricin ZJ008 was reported with molecular weight of 1.3 kDa (Zhu *et al*., 2014). Molecular weight of the bacteriocin purified in this study is different from previously reported bacteriocins and it indicates its novel nature.

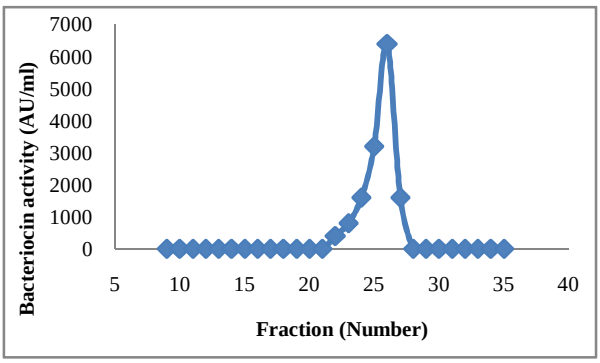

Fig 2 Gel permeation chromatography of bacteriocins of *Lactobacillus plantarum* MTCC 9496. Fractions were collected and analysed for bacteriocin activity

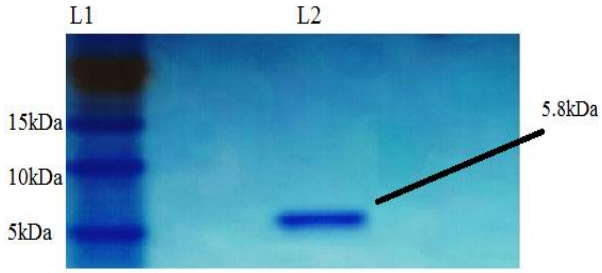

Fig 3 Molecular weight determination of purified bacteriocin by 15% SDS PAGE

L1- Molecular wight marker (1.7- 40 kDa), L2- Purified bacteriocin from gel permeation chromatography

Sensitivity to enzymes, temperature and pH

Stability of bacteriocin to different treatments is necessary to determine before its application as food preservative. Antimicrobial activity of plantaricin 9496 remains unaffected after treatment with lipase and amylase. It indicates that no lipid and sugar moiety is responsible for its antimicrobial activity. Treatment of plantaricin 9496 with catalase did not result in loss of antibacterial activity showing that activity was not due to hydrogen peroxide. Antimicrobial activity was completely lost after treatment with pepsin and proteinase K. These results implied that plantaricin 9496 is proteinaceous in nature.

Stability of plantaricin 9496 was evaluated at different temperatures and it was found to be thermostable upto 100°C. However, its activity decreased by 21% after heating at 121°C for 15 min. Temperature stability is crucial for the application of bacteriocins as a component of hurdle concept in food preservation. Plantaricin 9496 also remained stable at pH values of 2.0- 7.0 and antimicrobial activity decreased by 8% in alkaline conditions (Table 2). Apple, cherry, citrus, peach, plum, yoghurt and vinegar are high acid foods (pH 1.0-3.0). Banana, melon, papaya, pineapple and tomato are medium acid foods (pH 4.6). Most vegetables like bean, beet, carrot, cucumber, onion, potato are low acid foods with pH 6.0- 7.0 (Anon, 1992). Bacteriocins of *Lactobacillus plantarum* MTCC 9496 showed an advantage for its application in above mentioned foods as preservative, as it can remain stable at pH of 2-7. It can lower the use of chemical preservatives which have adverse effects on human health. Overall characterization of bacteriocin of *Lactobacillus plantarum* MTCC 9496 suggests that it is heat and acid stable proteinaceous compound.

Table 2 Sensitivity of bacteriocin to temperature, pH and enzymes and its storage stability

Treatment	Residual ativity (%)
Temperature	
4, 25, 30& 37/30 minutes	100
45, 60, 80 &100°C/30min	100
121°C/15 min	79
pH	
2.0-6.0	100
7.0	97.5
8.0-9.0	92
Enzymes (5mg/ml)	
Pepsin	0
Proteinase K	0
Trypsin	0
α-Amylase	100
Lipase	100
Storage stability	
4°C/45 days	100
4°C /60 days	88
-20°C /75 days	100
-20°C /90 days	84

Antimicrobial spectrum and MIC value of Plantaricin 9496

Bacteriocin produced by *Lactobacillus plantarum* MTCC 9496 exhibited broad antimicrobial spectrum against fruit and vegetable spoiling organisms. It inhibited Gram positive (*Bacillus subtilis, Staphylococcus aureus, Listeria monocytogenes, Leuconostoc mesenteroids, Lactobacillus brevis, L. bulgaricus, L. casei* and *L. delbrueckii)* and Gram negative *bacteria* (*Pseudomonas fluorescens, P. aeruginosa, Erwinia sp.* and *Escherichia coli*). Minimum inhibitory concentration (MIC) values of bacteriocin against *Listeria monocytogenes* and *Erwinia sp* was 32µg/ml whereas MIC value is low for bacteria belonging to related genera like *Lactobacillus* and *Leuconostoc*. Inhibition of *Pseudomonas aeruginosa* and *Escherichia coli* was reported at high MIC values of 64µg/ml (Table 3). Plantaricin 163 exhibited wide antibacterial spectrum against *Staphylococcus aureus, Listeria monocytogenes, Bacillus pumilus, Pseudomonas aeruginosa* and *Escherichia coli* (Hu *et al*., 2013). Plantaricin UG1 was also reported to be inhibit some food borne pathogens such as *B. cereus, Clostridium perfringens* (Enan *et al*., 1996).

Bacteriocin of *Lactobacillus plantarum* MTCC 9496 also showed broad antifungal spectrum against *Aspergillus awamori, Aspergillus niger, Fusarium oxysporum, Botrytis cinera, Penicillium expansum, Rhizopus stolonifer* and against yeast such as *Candida albicans, C. parapsilosis* and *Saccharomyces cerevisiae*. MIC values of bacteriocin against fungi and yeast was also determined and given in Table 3.

Antibacterial efficacy of bacteriocins of *Lactobacillus plantarum* have been determined by many co workers but there is limited information on their antifungal spectrum. Poor postharvest practices damage the integrity of fresh produce

and make them vulnerable to bacterial and fungal spoilage which compromise food quality and safety. Out of total world food production, 5-10% is spoiled by fungi (Gwiazdowska *et al.*, 2008). Mycotoxins produced by them are both acutely and chronically toxic to humans, effecting normal hepatic functioning, causing immunosuppression and cancers of colon and kidney (Williams *et. al.*, 2004). Bacteriocins purified in the present investigation had broad antibacterial and antifungal spectrum and hence can be employed as safe biopreservative to minimize post harvest losses.

Table 3 Antimicrobial activity and MIC of bacteriocin of *Lactobacillus plantarum* MTCC 9496

Microorganism	Source	Growth medium/ Temperature(°C)	MIC (µg/ml)
Bacteria			
Bacillus subtilis	MTCC[a] 2451	Nutrient broth/ 37	16
Staphylococcus aureus	NCDC[b] 109	Nutrient broth/ 37	8
Listeria monocytogenes	MTCC 657	BHI[c]/ 37	32
Leuconostoc mesenteroids	NCDC 29	MRS broth/ 37	16
Lactobacillus brevis	MTCC 1750	MRS broth/ 37	16
Lactobacillus bulgaricus	NCDC 253	MRS broth/ 37	16
Lactobacillus casei	NCDC 17	MRS broth/ 37	16
Lactobacillus delbrueckii	NCDC 290	MRS broth/ 37	16
Pseudomonas fluorescens	MTCC 103	Nutrient broth/ 37	32
Pseudomonas aeruginosa	NCDC 105	Nutrient broth/ 37	64
Erwinia sp	MTCC 2760	Nutrient broth/ 37	32
Escherichia coli	NCDC 135	Nutrient broth/ 37	64
Fungi			
Aspergillus awamori	MTCC 2879	CYE[d] broth/ 25	128
Aspergillus niger	MTCC 281	CYE broth/ 25	256
Fusarium oxysporum	NCDC 284	Potato sucrose broth/ 30	512
Botrytis cinera	MTCC 359	Potato dextrose broth/ 25	256
Penicillium expansum	MTCC 8241	CYE broth/ 25	512
Rhizopus stolonifer	MTCC 2591	CYE broth/ 30	256
Yeast			
Candida albicans	MTCC 7253	Malt yeast broth/ 30	128
Candida parapsilosis	NCDC 279	Nutrient broth+ 1% glucose/ 30	128
Saccharomyces cerevisiae	NCDC 42	Potato dextrose broth/ 30	256

[a] MTCC: Microbial type culture collection, [b] NCDC: National Collection of Dairy Culture, [c] BHI: Brain heart infusion broth, [d]CYE: Czapek yeast extract broth

Storage stability of bacteriocin

Bacteriocin of *Lactobacillus plantarum* was found to be stable during stoarage at 4°C and -20°C. Bacteriocin retained full antimicrobial activity for 45 days at 4°C whereas on further storage, activity decreased by 12% after 60 days. At -20°C, bacteriocin activity remains stable for 75 days of storage (Table 2).

In vitro toxicity assessment of the bacteriocin

The effect of purified bacteriocin was determined on avian spleenocytes by MTT assay. Bacteriocin at concentration of 40- 2600 µg/ml, results into no cell death and 100% cell viability is reported. Further increase in bacteriocin concentration, decreased the cell viability. At concentration of 10400 and 20800 µg/ml, 11.4 and 20% inhibition of cells was reported respectively (Table 4.). CC_{50} (bacteriocin concentration (µg/ml) required to lower the cell viability by 50%), value is above 20800 µg/ml, indicating that cytotoxicity of bacteriocin is very low. Hence, this bacteriocin can be used as safe biopreservative without harmful effects for the consumers.

(The experiment was performed in triplicates. The absorbance was represented as mean ± standard deviation. (P-value < 0.05). The absorbance values were statistically significant as compared to control as depicted by Tukey test)

Table 4 Evaluation of cytotoxicity of bacteriocin of *Lactobacillus plantarum* MTCC 9496 on splenocytes *in vitro*

Group	Absorbance (Mean± S.D.)	Percentage Viability of cells
Control	0.352 ± 0.017	100
40	0.352 ± 0.017	100
160	0.352 ± 0.017	100
650	0.352 ± 0.017	100
1300	0.352 ± 0.017	100
2600	0.352 ± 0.017	100
5200	0.334± 0.015	94.8
10400	0.311± 0.025	89.6
20800	0.280± 0.021	79.8

(The experiment was performed in triplicates. The absorbance was represented as mean + standard deviation. (P-value < 0.05). The absorbance values were statistically significant as compared to control as depicted by Tukey test).

Bacteriocin safety is must to be evaluated before their use as food component or as an alternative to antibiotics in medical applications. Unfortunately, only few bacteriocins have been previously characterized regarding their cytotoxicity. Bacteriocin ST202Ch and ST216Ch of *L. plantarum* at concentration of 50 µg/ml showed 44% and 34% inhibition of human hepatocytes respectively (Carneiro *et al*., 2014). As reported by Martinez *et al*. (2013), CC50 of bacteriocin produced by *L. plantarum* ST71KS is above 1200 µg/ml. Vaucher *et al.* (2010) reported cytotoxicity of antimicrobial peptide P34 and nisin on Vero cells. EC50 values of the peptide P34 and nisin in MTT assays were 0.60 and 0.50µg/ml respectively. In the present study, purified bacteriocin of *L. plantarum* MTCC 9496 has shown mild toxicity to eukaryotic cells at much high concentrations than its MIC values for food spoiling bacteria and fungi. Nisin, a commercial bacteriocin, also reported to be mild toxic to human cells (Reddy *et al*., 2004).

Fresh produce being highly perishable should be marketed as early as possible after harvest for economic benefits. However, inadequate transportation facility and lack of cold storage facility at the farm level leads to post harvest losses. A more practical and easy to use by the modest farmer is a wash water solution containing bacteriocin. Bacteriocins can be used synergistically with other preservation methods and can be an important tool in Hurdle concept of preservation without compromising on food safety.

Bioactive packaging is a further potential application of bacteriocins which can prevent microbial growth on food surface by direct contact. They can be incorporated into packaging films in two main ways- By direct incorporation into polymers (Padgett *et al.*, 1998) i.e. they can be made a component of film formation or bacteriocins can also be used to coat polymeric substances (Appendini and Hotchkiss, 2002). Moreover, they do not have any therapeutic application and are not known to cause allergies. Being of LAB origin they are probiotic in nature also and help in restoring the normal gut microflora (Thomas *et al.*, 2000).

CONCLUSIONS

Plantaricin 9496 produced by *Lactobacillus plantarum* MTCC 9496 remained active after different treatments of temperature, pH and during storage. Plantaricin 9496 showed an advantage for its application as a strong candidate of

hurdle concept of food preservation. Plantaricin 9496 showed antagonistic activity against food borne pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Penicillium expansum* and *Botrytis cinera*. MTT assay on avian spleenocytes proved that this antimicrobial peptide is safe to use at high concentrations as food component without adverse effects on human health.

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