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Research Paper

Antifungal Activity of Bacteriocins of *Lactobacillus plantarum* MTCC 9503 Purified using Diatomite Calcium Silicate

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Abstract

Bacteriocins as antimicrobial metabolites from lactic acid bacteria has potential to find application as a biopreservative agent both in processed as well as fresh food. The present investigation was performed to elucidate the antifungal activity of bacteriocins of *Lactobacillus plantarum* MTCC 9503 against two common food spoilage fungi, *Aspergillus awamori* and *Penicillium citrinum*. Bacteriocins were purified to contain 1,00,000 AU/g of food grade adsorbing agent Diatomite calcium silicate (Micro-Cel). Antifungal efficacy was determined by observing fungal growth, spore viability and spore germination in presence of 2000 AU/ml of bacteriocin preparation. A decrease of 46% (*A awamori*) and 34% (*P citrinum*) of fungal mat, on dry weight basis was observed over the control after 10 days of incubation. Spore viability of *P citrinum* decreased by 20.3% and that of *A awamori* by 24.2% after 30h of incubation. Control runs recorded 44% spore germination compared to 29.7% (*P citrinum*) and 27.6% (*A awamori*) in trial runs after 18h of incubation. Analysis using Sigma-Statistics (One way ANOVA) indicated statistical significance of differences in control and trial results at $p < 0.05$.

Keywords: *Lactobacillus*, bacteriocin, antifungal activity, Micro- Cel, purification

Microbes are the major agents of food spoilage especially in tropical and subtropical regions where conditions of high humidity and high average annual temperature favour their growth (Hall, 1970). One third of the food produced for human consumption is lost globally, which amounts to about 1.3 billion tons per year (Gustavsson, 2011). These losses can be attributed to inadequate infrastructure, lack of storage facilities, refrigerated transport etc. This issue is compounded by low level of processing in many countries like India, Bangladesh and some African countries. India

processes 2.2 % of its fruits and vegetables compared to 65% in USA, 23% in China, 83% in Malaysia, 78% in Philippines and 70% in Brazil (Anon, 2011). 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). *Aspergillus, Alternaria, Cladosporium* and *Penicillium* spp. are the important agents of microbial food spoilage. 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). They persist in food chain from harvest to transport and storage stage.

Various techniques of biological preservation can be used to prevent microbial food spoilage. Biopreservation refers to the use of natural microflora and their antimicrobial metabolites in food (Stiles, 1996). Bacteriocins are an important means of biopreservation. They are ribosomally synthesized, antimicrobial metabolites produced by lactic acid bacteria, which are being used as food additives to prevent food borne pathogenesis (Nissen- Meyer and Nes, 1997). *Lactobacillus plantarum*, a member of lactic acid bacteria, is an important bacteriocin producer given GRAS (generally regarded as safe) status (Schillinger *et. al.,* 1996). Bacteriocins produced by it have exhibited antimicrobial activity against *Escherichia coli* (Lade *et al*. 2006), *Acinetobacter baumanii* (Torodov and Dicks, 2005), *Listeria monocytogenes* (Ennahar *et al.,* 1996; Enan *et. al.,* 1996), *Bacillus cereus, Clostridium perfringens, C sporogenes* (Enan *et al.,* 1996), different species of *Enterococcus*, *Lactobacillus*, *Streptococcus, Staphylococcus*, *Bacillus* and *Clostridium* (Gonzalez *et. al.,* 1994), *Corynobacterium*, *Enterococcus*, *L.monocytogenes*, *L innocua* and *L ivanovii* (Eijsink et al.,1998).

Most of the work on bacteriocins is centered around isolation and identification of newer bacteriocin producers and elucidating their antimicrobial spectrum against bacterial agents of food borne diseases. There is limited information on their role as biocontrol agents of food spoilage fungi.

The aim of the present investigation was to examine antifungal potential of bacteriocins produced by *Lactobacillus plantarum* MTCC 9503 against two commonly encountered fungal food spoilage agents, *Penicillium citrinum* and *Aspergillus awamori.* The antifungal activity in terms of fungal mat growth (dry weight basis), spore viability and spore germination was studied using *in vitro* trials. Bacteriocins were purified by adsorption desorption method using inexpensive material, Diatomite calcium silicate as expensive three step downstream processing of bacteriocin limit their usage as biopreservative in food.

Materials and Methods

Microorganisms and Media

The microbial cultures used in present study namely, *Lactobacillus plantarum* MTCC 9503, *Lactobacillus brevis* MTCC 1750, *Penicillium citrinum* MTCC 2553 and *Aspergillus awamori* MTCC 2456 were obtained from the Institute of Microbial Technology, India. Source of these fungal cultures was decaying organic matter. Bacterial cultures were grown on de Man Ragosa Sharp (MRS) medium and incubated at 37°C/48h**.** Fungal cultures were cultivated on Czapek Yeast Extract (CYE) and incubated at 30°C/72h. *Lactobacillus plantarum* MTCC 9503 was used as bacteriocin producer and *Lactobacillus brevis* MTCC 1750 served as an indicator strain. All the microbial cultures were sub-cultured periodically and stained post-incubation to check for purity.

Purification of bacteriocin

It was performed by modified method of Coventry *et. al.,* (1996). Micro-Cel used as an adsorbent was obtained from M/s Gem Corporation, Mumbai, India. One litre of MRS broth was inoculated with metabolically active bacteriocin producer. After 21h of incubation, culture broth was centrifuged at 12,000×g for 30 min at 4°C (REMI CPR- 30 PLUS). To the culture supernatant, Micro-Cel (1% w/v) was added and mixed by vortexing at 10 min intervals for a total time period of 30 min at 20°C during which adsorption occurred. The supernatant was separated from Micro-Cel containing adsorbed bacteriocin by

centrifugation at 12,000×g for 10 min at 20°C. Micro-Cel with adsorbed bacteriocin was then, suspended in sterile water and centrifuged again at 12,000×g for 10 min at 20°C. Thereafter, the pellet was washed with sterile distilled water and resuspended in one litre of desorption solvent (SDS, 1% w/v, pH- 10.0). The suspension was vortexed three to four times for 30 min at 20°C. The Micro-Cel was recovered by centrifugation (12,000×g for 10 min at 20°C). The supernatant was collected and was again centrifuged at 12,000×g for 10 min at 20°C. SDS was removed by precipitation by cooling to 2°C for 2 days. It was pelleted by centrifugation (12,000×g for 1 min at 2°C). The pellet was dried at 100°C for 2h, and the weight of dried material was recorded to determine the extent of removal of SDS. The supernatant containing bacteriocin was lyophilized (Allied Frost, Mac Flow). Powdered form of bacteriocin was dissolved in 10 ml of 0.02M HCl and pH was neutralized with 0.1N NaOH to exclude the antimicrobial activity due to organic acids. Bacteriocin preparation was stored at -10°C during the time period of this study.

Assay and quantification of bacteriocin

It was done as per the method of Schillinger and Lucke (1989). Plates containing solidified MRS agar (2% agar) were overlaid with soft MRS agar (0.8% agar). Metabolically active culture of *Lactobacillus brevis* MTCC 1750 was spread plated onto solidified soft MRS agar. Wells of 0.7mm diameter were made in soft agar with a wide end of sterile microtip. 50 µl of bacteriocin preparation was transferred into each well. The plates were incubated at 37°C for 24 h and observed for presence/ absence of zone of inhibition. To quantify the bacteriocin**,** serial 10 fold dilutions of the bacteriocin preparation were used to perform the well diffusion assay.

Antifungal efficacy of bacteriocin

Commonly encountered food spoilage fungi i.e. *Penicillium citrinum* MTCC 2553 and *Aspergillus awamori* MTCC 2456 were used as agents of food spoilage in this investigation. Antifungal efficacy was determined by observing fungal growth, spore viability and spore germination in presence of bacteriocin.

One ml of spore suspension of fungal culture containing 10^6 spores/ml was added to 200 ml of CYE broth. 2000 AU/ml of bacteriocin was then, added to this broth and incubated at 30°C. A control was also run simultaneously. To determine the effect of bacteriocin on fungal growth, dry weight of fungal mat was recorded on 5^{th} and 10^{th} day of incubation. Effect of bacteriocin on spore viability was determined by withdrawing aliquots of 0.1 ml aseptically every 3h for a total time period of 30h and plating onto CYE for fungal colony count (Gourama and Bullerman, 1995a). Effect of bacteriocin on spore germination was studied by method of Coxon *et al.,* (1982). Aliquots of 0.15ml were withdrawn and pipetted onto sterile cavity slides. Microscopic slide culture was prepared using paraffin wax. The slides were incubated at 30°C for 18h. The slides were examined under microscope (Olympus- Magnus- MLX) every 3h for germination. Direct count of 300 germinated spores per slide was recorded and percentage spore germination was calculated.

Quantification and antifungal efficacy of commercially purchased Nisin

0.1g of Nisin purchased from National Chemicals, India was dissolved in 10 ml of 0.02M HCl and stored at -10°C. Antimicrobial component was quantified using Well Diffusion Assay. Nisin was also tested for antifungal efficacy using the methods mentioned above.

Statistical Analysis

Data acquired was subjected to statistical analysis using Sigma-Statistics. The significant difference between control and test values was compared using one way analysis of variance (ANOVA). For all analyses, differences were considered significant at $p< 0.05$.

Results and Discussion

Bacteriocin assay and quantification

Growth kinetics of the bacteriocin producer i.e. *Lactobacillus plantarum* MTCC 9503 was determined spectrophotometrically at 600 nm (data not shown). Growth

phases were related to bacteriocin production. *L plantarum* was found to produce maximum bacteriocin at 21h of incubation. Partially purified bacteriocins contained 1000 AU/ml. Commercially available Nisin was also subjected to antimicrobial assay and found to contain 1000 AU/ml. AU/ml is defined as the reciprocal of highest dilution which shows minimum zone of inhibition of 2mm (Graceila *et al*., 1995).

The supernatant obtained after separation of Micro-Cel containing adsorbed bacteriocin was subjected to antimicrobial assay against *Lactobacillus brevis* MTCC 1750. No zone of inhibition was observed indicating that whole of the bacteriocin was adsorbed on to the Micro- Cel.

By employing Micro-Cel as adsorbing agent, the amount of bacteriocins concentrated in present work was 1,00,000 AU/g of Micro-Cel. 25,600 IU of Nisin, 5,12,000 AU of Pediocin, 2,56,000 AU of Brevicin and 5,120,000 AU of Piscicolin adsorbed per gram of Micro-Cel have been reported earlier (Coventry *et. al.,* 1996).

Antifungal efficacy of bacteriocin preparation and Nisin

Effect on fungal growth

Aspergillus and *Penicillium* are important food spoilage agents. The effect of purified bacteriocin and Nisin on fungal growth was determined and the results are presented in Table 1. The extent of growth of fungal mat was evaluated (dry weight basis) on 5th and 10th day of incubation.

After 10 days of incubation, a dry weight of 2.025g of *A awamori* was observed in presence of bacteriocin, a significant ($p< 0.05$) decrease of 46% compared to control dry weight of 3.725g. *P citrinum* growing in presence of bacteriocin, recorded a dry weight of 1.94g compared to the a control of 2.935g, a significant decrease of 34% after 10th day of incubation. Dry weight of 1.86g of *A awamori* was recorded after 10 days in presence of Nisin.

In the present study, 2000 AU/ml of bacteriocins of *Lactobacillus plantarum* MTCC 9503 significantly inhibited the growth of both the investigated fungi as was statistically determined. Growth decreased by 46% (*A awamori*) and 34% (*P citrinum*) over the control after 10 days of incubation.

Table 1. Effect of bacteriocins of *Lactobacillus plantarum* MTCC 9503 and Nisin on growth of *Aspergillus awamori* MTCC 2456 and *Penicillium citrinum* MTCC 2553 (Dry weight basis)

Time period of incubation (days)	Fungal biomass in Control (g)	Fungal biomass (g) in presence of 2000 AU/ml of bacteriocin (% decrease)	Fungal biomass (g) in presence of 2000 AU/ml of Nisin (% decrease)
Aspergillus awamori MTCC 2456			
5	2.025±0.009	1.3±0.086 (36%)	1.10±0.063 (48%)
10	3.725±0.13	2.025±0.09 (46%)	1.86±0.107 (53%)
Penicillium citrinum MTCC 2553			
5	1.775±0.073	1.095±0.137 (38%)	0.84±0.141 (54%)
10	2.935±0.147	1.94±0.108 (34%)	0.96±0.09 (66%)

Effect on spore viability

Spore viability of *A awamori* was recorded in the presence of bacteriocin and Nisin (Figure 1). After 30h of incubation, spore viability was significantly ($p< 0.05$) reduced by 24.2% in presence of bacteriocin and 33.3% in presence of Nisin. The difference in spore viability of control and trial runs was observed after initial three hours of incubation and it increased with increase in time period of incubation.

Observations on spore viability of *P. citrinum* in presence of bacteriocin and Nisin were parallel to the ones arrived at in case of *A awamori*. Addition of bacteriocin significantly ($p< 0.05$) decreased spore viability of *P citrinum* by 20.3% and 31%, respectively after 30h of incubation. Nisin decreased spore viability by 31% (Figure 2).

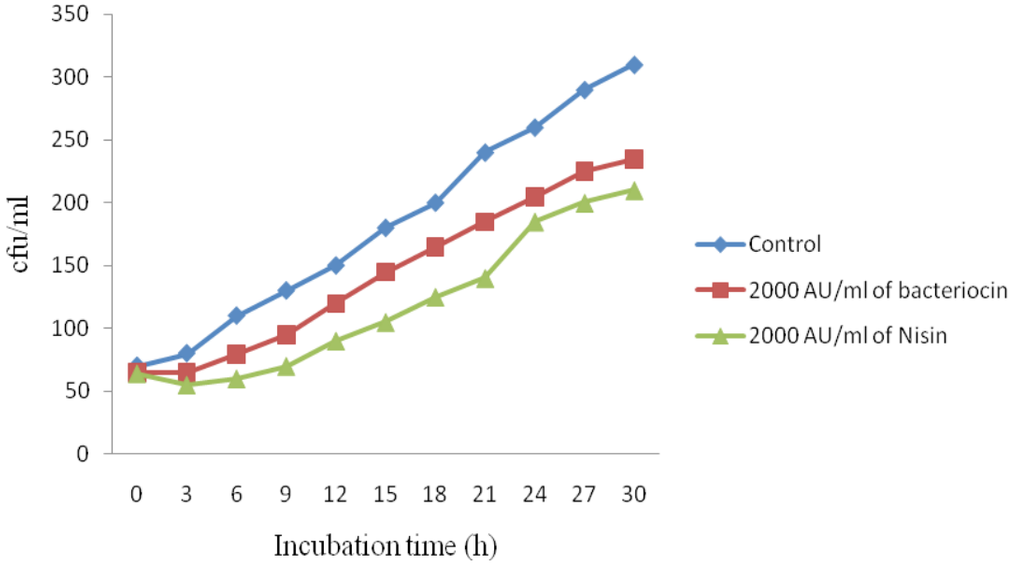

Fig 1: Spore viability of *Aspergillus awamori* MTCC 2456 in presence of bacteriocins of *Lactobacillus plantarum* MTCC 9503 and Nisin

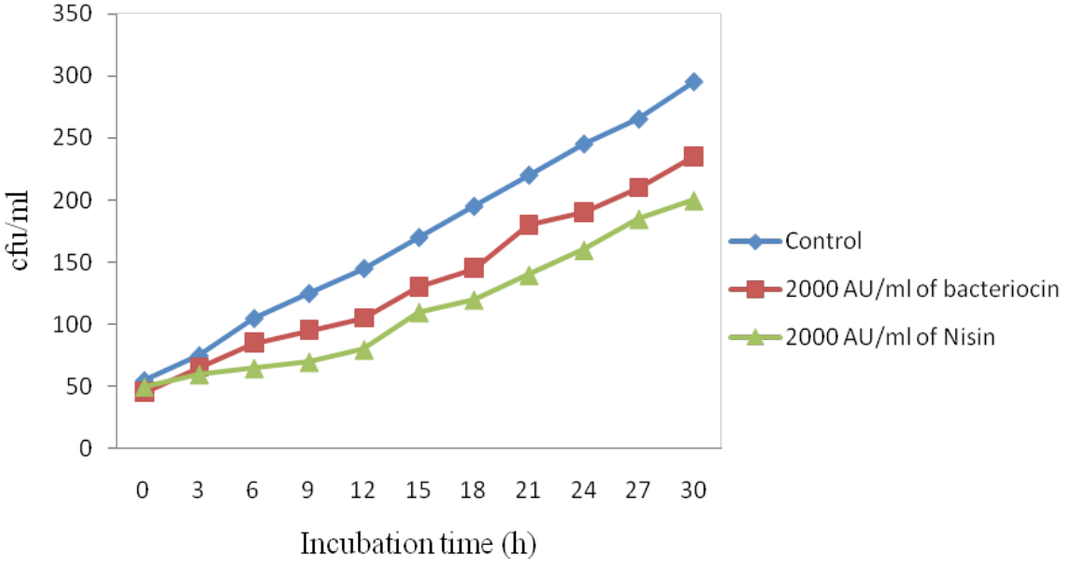

Fig 2: Spore viability of *Penicillium citrinum* MTCC 2553 in presence of bacteriocins of *Lactobacillus plantarum* MTCC 9503 and Nisin

Table 2: Effect of bacteriocins of *Lactobacillus plantarum* MTCC 9503 and Nisin on spore germination of *Aspergillus awamori* MTCC 2456 and *Penicillium citrinum* MTCC 2553

Time period of incubation (h)	Percentage spore germination-Control	Percentage spore germination in presence of 2000 AU/ml of bacteriocin	Percentage spore germination in presence of 2000 AU/ml of Nisin
Aspergillus awamori MTCC 2456			
0	0.0	0.0	0.0
3	0.0	0.0	0.0
6	13.3	0.0	0.0
9	22.3	8.0	5.7
12	29.6	10.3	6.7
15	37.6	30.0	18.7
18	43.0	29.7	22.3
Penicillium citrinum MTCC 2553			
0	0.0	0.0	0.0
3	0.0	0.0	0.0
6	15.6	0.0	0.0
9	21.0	6.3	3.7
12	25.3	9.3	6.7
15	38.0	29.0	22.3
18	44.3	27.6	24.7

Spore viability of *A. awamori* and *P. citrinum* decreased by 24.2% and 20.3%, respectively after 30h of incubation. Spore germination of both fungi was delayed and was also significantly reduced by 13.3% (*A. awamori*) and 16.7% (*P. citrinum*) after 18h of incubation.

Effect on spore germination

Spore germination by *A. awamori* and *P. citrinum* was exhibited at 6h of incubation in the control run compared to 9h in trial runs. 29.7% and 22.3% *A. awamori* spore germination was observed in presence of bacteriocin and Nisin, respectively, after 18h of incubation. 27.6% and 24.7% spore germination of *P citrinum* was observed in presence of bacteriocin and Nisin respectively, after 18h of incubation (Table 2). Bacteriocins are potent inhibitors of spore germination as delayed/ little spore germination occurred in their presence. Significant decrease in spore germination was observed in presence of bacteriocin as compared to the control ($p< 0.05$).

Liquid culture has been used in this study to facilitate diffusion of bacteriocins. Antifungal efficacy of lactic acid bacteria, *vis-à-vis* fungal growth only, is often evaluated by agar well diffusion assay by many workers. Batish *et al.,* (1989, 1990) reported the antifungal activity of *Lactobacillus acidophilus* R. Okkers *et al.,* (1999) reported that a bacteriocin like peptide pentocin TV35b, produced by *Lactobacillus pentosus* had a fungistatic effect. Prachyakij *et al.,* (2007) determined the antifungal activity of lactic acid bacteria isolated from Thai fermented foods. *Lactobacillus plantarum* strains DW1, DW3 and DW4 were effective against *Rhodotorula* sp., *Pichia* sp., *Hansenula* sp., *Saccharomyces* sp. and *Candida* sp. Adebayo and Aderiye (2010) demonstrated the antifungal activity of bacteriocins of lactic acid bacteria from some Nigerian fermented foods (Eko, Fufu, Iru and Ogi). Gourama and Bullerman (1995b)

showed that a commercially available silage inoculant with a combination of *Lactobacillus* species (*L plantarum, L bulgaricus* and *L acidophilus*) exerted antifungal and anti-aflatoxin activity against *Aspergillus flavus*.

Bacteriocins can find application in food preservation and can be used in various ways viz., inoculation of food with bacteriocin producer (Jeevaratnam *et al.,* 2005), addition of purified or semi- purified form of bacteriocin as food additive (Bower *et. al.,* 1995), use of product previously fermented with a bacteriocin producing strain as an ingredient in food processing, soaking food items directly into bacteriocin solution (Appendini and Hotchkiss, 2002).

Inoculation of bacteriocin producer into food systems has been reported by many workers (Lahti *et. al.,* 2001; Lisserre *et. al.,* 2002) but survival and bacteriocin production in that food system is imperative for successful inhibition of target bacteria. Efficacy of direct addition/ incorporation of bacteriocin as food additive in food depends on biochemical composition and physical condition of food (Galvez *et. al.,* 2007). Method of producing yoghurt with increased shelf-life containing bacteriocin has been developed by Vedamuthu (1992).

Bacteriocins play an important role in Hurdle Concept of food preservation. They have been found to exhibit synergy with other preservation methods like temperature, hydrostatic pressure, chemical preservatives and pulsed electric field (Szabo and Cahill, 1998; Kalchayanand *et. al.,* 1998; Schlyter *et. al.,* 1993; Pol *et. al.,* 2000). Bioactive packaging is also a 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).

Conclusion

Bacteriocins has proved potential in preventing fungal food spoilage. 1,00,000 AU/g of Micro-Cel of bacteriocin was concentrated in the present study. Bacteriocin of *Lactobacilus plantarum* inhibited fungal growth, which decreased by 46% (*A. awamori*) and 34% (*P. citrinum*) after 10 days of incubation. Bacteriocins also reduced spore viability by 24.2% (*A. awamori*) and 20.3% (*P citrinum*) after 30h of incubation. 13.3% (*A awamori*) and 16.7% (*P citrinum*) inhibition of spore germination was also recorded after observing for a time period of 18h. It is a potential tool in biological preservation of food. Delay in onset of microbial spoilage can enable phased marketing of fresh produce.

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