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RESEARCH PAPER

Immobilisation of Lactic Acid Bacteria and Application of Bacteriocin for Preservation of Fruit Juices

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ABSTRACT

Lactic acid bacteria are generally recognised as safe (GRAS) organisms producing bacteriocins that help in inhibiting food borne pathogens. However, its narrow spectrum activity is a major limitation for food applications. Thus, a combination of different methods were attempted to broaden the antimicrobial spectrum of bacteriocins produced using lactic acid bacteria. Initially, the antimicrobial activity of bacteriocin from various combinations of mixed lactic acid bacteria and single culture were determined after 56 h at 35 °C to select the desirable combination with maximum antimicrobial activity. Combination of bacteriocin produced by *Lactobacillus plantarum* DFR4, *Enterococcus faecalis* DFR4 and *E. faecalis* DFRP1showed maximum activity against *Listeria monocytogenes* with zone of 31.24±0.33 mm in agar disc diffusion assay. The specific activity and purification of bacteriocins produced by these isolates were found to be highest in gel permeation chromatography where as, the yield was highest in 80 percent acetone precipitation. The partially purified bacteriocins were further investigated for the effect of pH, enzymes and temperature stability and were found to be thermo stable and active at pH range of 3 to 5. Proteolytic enzymes such as protease and papain inactived the bacteriocins while amylase had no inhibitory effect. The antimicrobial activity of bacteriocin produced from bamboo (*Bambusa vulgaris*) fibre immobilised *Lactobacillus plantarum* DFR4 was found to be more (zone size=20.91±0.35 mm) in comparison to free cells (zone size=8.52±0.26 mm) after 24 h of incubation at 37 °C. Further, immobilisation of *Lactobacillus plantarum* DFR4, *Enterococcus faecalis* DFR4 and *E. Faecalis* DFRP1 using bamboo fibre aided in faster production of bacteriocins in 24 h. Mosambi juice (Citrus limetta) supplemented with bacteriocins (produced from mixed bacterial isolates) had a shelf life of 26 days at 5 °C.

Keywords: Bacteriocin; Immobilisation; Adsorption; Co-culture

1. INTRODUCTION

Food spoilage is a major challenge faced by the food industry during food storage. The incidence of food spoilage can be attributed to the action of foodborne pathogens and there are numerous techniques available to combat these harmful microbes. However, biopreservation is preferred by the consumers as they are natural, effective and safe for consumption. In recent times, bacteriocins have acquired wide acceptance in the food industry as a promising biopreservative. Bacteriocins are ribosomally synthesised, small antimicrobial peptides that are produced by the lactic acid bacteria. The mechanisms by which bacteriocins attack target microorganisms include; pore formation on the cell wall causing leakage of cell components[1], retarded peptidoglycan synthesis (arrests cell wall synthesis) and elicited cellular DNA damage, eventually causing cell death[2]. Earlier papers have extensively reported on achieving maximum bacteriocin activity by optimising the medium components, incubation period, pH and temperature but, adequate data involving their scale up process is rarely given[3, 4].Previous reports on immobilisation of microorganisms were carried out for the preparation of wine, large scale production of enzymes[5], organic acids[6] and bio-remediation of pollutants from industrial effluents[7].

Immobilisation of whole cells relates to the restricted movement or physical localisation of cells on a solid support for preserving their biological activity[8]. Techniques in cell immobilisation include covalent linkages, adsorption, entrapment, self-aggregation through flocculation and freeze thawing[9]. Solid supports that are generally used for microbial cell immobilisation are agar, carrageenan, calcium alginate, glass, polyacrylamide, cellulose and polyvinyl alcohol crosslinked with boric acid[10]. Adsorption is one of the immobilisation methods which operate on physical interactions between the cells and the surface of the water insoluble carrier. The immobilised cell aggregate comprise of mainly three components: the cells, the support material (carrier), and the interstitial solution (solution that fills the remaining space).

Bacteria generally compete with each other in a niche but, there are certain bacteria which coexist. Moreover, bacteria in consortia function in coordination for nutrition, protection and sometimes form biofilms. There are some reports available on culturing various lactic acid bacteria (referred as co-culture) to efficiently destroy food spoilage organisms, either acting as competitive microorganisms or by the production of bacteriocins[11-14]. In contrast, few papers have also stated that

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the co-culturing of lactic acid bacteria can suppress bacteriocin production[15,16]. Bacteria in consortium exert different relationships on one another. Synergistic interactions between various lactic acid bacteria enhanced bacteriocin production while, antagonistic interactions suppressed bacteriocin production[17,18]. However, selective combination of bacteriocins could widen the antimicrobial spectrum there by preventing the emergence of bacteriocin resistant strains.

This paper focuses on the immobilisation of lactic acid bacteria such as *Lactobacillus plantarum* DFR4*, Enterococcus faecalis* DFR4 and *Enterococcus faecalis* DFRP1 on a suitable carrier and their influence on the bacteriocin production in comparison to a free cell system. The difference in bacteriocin activity was demonstrated using antimicrobial susceptibility tests and cell density measurements. Synergistic and antagonistic interactions between various lactic acid bacteria were determined to select a desirable combination to improve bacteriocin production. The methods employed to inhibit food spoilage bacteria involved the combination of bacteriocins produced from selected mixed lactic acid bacteria *L. plantarum* DFR4*, E. faecalis* DFR4 and *E. faecalis* DFRP1, grown on an immobilisation matrix (bamboo fibre) using yeast extract dextrose medium. The bacteriocins thus produced were then incorporated into Mosambi juice and kept at 5 °C.

2. MATERIALS AND METHODS

2.1 Chemicals

Dextrose, yeast extract, tryptic soy agar, brain heart in fusion broth were procured from Himedia, Mumbai. Sodium chloride, hydrochloric acid and sodium hydroxide were procured from SD Fine chemicals (Mumbai, India).

2.2 Bacterial Cultures

The indicator organism *Listeria monocytogenes* 839 was procured from Microbial Type Culture Collection (MTCC), IMTECH, Chandigarh. *Lactobacillus acidophilus* 4356, *L. lactis* 19435 and *L. casei* 393 were procured from American Type Culture Collection (ATCC). *L. plantarum* DFR4, *E. faecalis* DFR4, *E. faecalis* DFRP1, *L. plantarum* DFR2 and *E. faecalis* DFRW1 were isolated from raw cabbage sample and decaying curry leaves.

2.3 Production, Partial Purification and Characterisation of Bacteriocin

A comparative study was conducted on the bacteriocin production obtained from single and various combinations of mixed lactic acid bacteria. The morphology of isolated lactic acid bacteria were studied by Gram staining and were characterised for biochemical nature. The genomic DNA was isolated to enable their identification via 16 S rRNA ribotyping. The 16 S rRNA ribotyping was carried out for the identification of the isolates using universal eubacterial forward primer 5'AGAGTTTGATCCTGGCTAG3' and eubacterial reverse primer 5'AAGGAGGTGATCCAGCC3'. Further, the 16S rRNA gene sequence analysis was performed using NCBI-BLAST homology search.

Lactic acid bacteria were grown in 100 ml Erlenmeyer flasks using yeast extract-dextrose medium, incubated at 35 °C for 56 h under static condition. The supernatant was obtained by centrifugation at 10,000 rpm for 15 min at room temperature. The supernatant was boiled at 75 °C for 15 min and then concentrated to $1/10^{th}$ of its quantity. Cold acetone precipitation (80 per cent) was performed to partially purify the crude bacteriocin samples[19]. The residual acetone present in the bacteriocin samples were removed using rotary flash evaporator (Heildolph, Rotaval, Germany) and dissolved in 50 mM acetate buffer (pH 4.5). Extraction of bacteriocin was also carried out using 70 per cent ammonium sulphate precipitation. Gel permeation chromatography was performed to further purify the bacteriocin samples. Crude bacteriocin preparation (3 ml) obtained via ammonium sulphate precipitation was passed through the G -25 column. Packed sephadex G -25 column (30 cm x 2 cm) was equilibrated with 50 mMacetate buffer (pH 4.5), and was used to elute the sample.

The pH for each bacteriocin sample was adjusted to 5.0 and agar well diffusion assay was performed to investigate the antimicrobial activity. The antimicrobial activity of the crude bacteriocin samples were checked after 24 h and their zone of inhibition were measured (Table1)The estimation of proteins from the bacteriocin samples was done using Lowry's method using Bovine serum albumin (BSA) as standard. The specific activity, yield and fold purification were determined for partially purified bacteriocins. Experiments were conducted to study the effect of pH 3.0-7.0, temperature at 80 ^{O}C, 100 ^{O}C, and 121 ^{O}C and enzymes such as amylase, papain and protease on the residual activity of bacteriocins produced by these isolates. The influence of pH on the bacteriocin production from these isolates was carried out by adjusting the pH of growth medium (pH 3.0-7.0), and inoculating with 2.0 % (v/v) of overnight cultures of *L. plantarum* DFR4, *E. faecalis* DFRP1 and *E. faecalis* DFR4 into different flasks, and incubated at 35 °C for 24 h under static condition. Thermal stability of the bacteriocins was evaluated by heating the bacteriocin preparations at different temperatures (80 °C and 100 °C for 30 min, followed by 121°C for 15 min). Further, the effect of enzymes (amylase, papain, and protease at 2.0 mg/ml) was determined by treating the bacteriocin samples with enzymes for 1 h at 4 °C.

2.4 Immobilisation of *L. plantarum* DFR4 on Bamboo Fibre

Immobilisation of *L. plantarum* DFR4 was carried out on bamboo fibre for enhancing bacteriocin production. Bamboo fibre was obtained from bamboo wood which was cut into small pieces, washed thoroughly, treated with 10 N NaOH, pasted using a blender (Cole Parmer, Model CB16TE, Made in USA) and dried at 55 °C in a hot air oven. Bamboo fibre (5 gm) were added to a 500 ml Erlenmeyer flasks containing 200 ml of yeast extract-dextrose medium (pH 7) and were auto claved at 121 psi for 15 min. The flasks were inoculated using 2.0 % (v/v) overnight culture of *L. plantarum* DFR4 and incubated at 35 °C for 24 h under agitation at 100 rpm. After 24 h, growth medium from the flasks were carefully collected in a sterile flask leaving behind the bamboo fibre. Thereafter, fresh medium was poured aseptically into the flask containing the leftover bamboo fibre, which constitute the first round/ first cycle of immobilisation. This process was repeated

for achieving 10 rounds of immobilisation (each cycle of immobilisation lasted 24 h). The bacteriocin production with respect to zone of growth inhibition and change in cell density was measured after each cycle of immobilisation. Bacteriocin production from free cells of *L. plantarum* DFR4 (culture) within 24 h served as control. Bamboo fibre was also used to immobilize selected mixed culture (*L. plantarum* DFR4, *E. faecalis* DFRP1 and *E. faecalis* DFR4) for achieving enhanced bacteriocin production with in 24 h.

2.5 Application of Bacteriocin Produced using Single and Mixed Culture of Lactic Acid Bacteria

2.5.1 Preparation of Mosambi Juice

Ripe mosambi fruits were procured from a local market in Mysuru. Mosambi juice was extracted from mosambis and made upto 250 ml using distilled water. The juice was centrifuged to remove seeds and fibre and then packed in retort pouches and treated using bacteriocins. Retort pouches containing Mosambi for Treatment (a) were sterilised by autoclaving at 121 psi whereas, pouches for Treatment (b) were not sterilised. Acetone precipitated (80%) bacteriocin samples were used for food application due to higher yield. Autoclaved retort pouches containing mosambi juice served as control.

2.5.2 Treatment (a)

In the laminar air flow, three sterile pouches containing 20 ml juice were spiked with *L. monocytogenes* (2.46×10^5 cfu/ml). After a time interval of 6 h, two pouches were supplemented with bacteriocin produced from single culture (*L. plantarum* DFR4) and selected co-cultured bacteria. The retort pouch without bacteriocin addition served as control. These pouches were stored at 35 °C.

2.5.3 Treatment (b)

Fresh mosambi juice without sterilisation was used for the study. Two retort pouches containing mosambi juice were supplemented with bacteriocins produced from *L. plantarum* DFR4 and from selected mixed bacteria, *L. plantarum* DFR4, *E. Faecalis* DFRP1 and *E. faecalis* DFR4. A control (mosambi juice without the addition of bacteriocins) was also kept. The retort pouch without bacteriocin addition served as control. These three pouches were stored at 5 °C for studying their shelf life.

3. RESULT AND DISCUSSION

Studies conducted on the bacteriocin production from monoculture and various combinations of co-cultured lactic acid bacteria lead to varied findings. Both, synergistic and antagonistic interactions between the bacteriocins and lactic acid bacteria were detected. Bacteriocin production from combinations of mixed lactic acid bacteria (combination 1, 2, 4 and 5) showed larger zone of inhibition than that of the single cultures (Table 1). Combination 1 comprised of individual cultures *Enterococcus faecalis* DFR4 (CL2C)23.50±0.32 mm, *Enterococcus faecalis* DFRP1 (P1) 23.50±0.30 mm, *Enterococcus faecalis* DFRW1 (W1) 24.20±0.16 mm and their combined activity was 30.02±0.24 mm, Combination 2 comprised of individual cultures *Lactobacillus plantarum* DFR4 (4dB) 23.34±0.14 mm, *Enterococcus faecalis* DFRP1 (P1) 23.50±0.26 mm, *Enterococcus faecalis* DFR4 (CL2C) 23.50±0.28 mm and their combined activity was 31.24±0.33 mm. Combination 4 comprised of individual cultures *Lactobacillus. acidophilus* ATCC 4356 23.25±0.20 mm, *Lactobacillus casei* ATCC 393 24.77±0.42 mm, *Lactococcus lactis* ATCC 19435 23.35±0.26 mm and their combined activity was 31.20±0.11. Combination 5 comprised of individual cultures *Lactobacillus plantarum* DFR4 (4dB)-23.30±0.10 mm, *Lactobacillus casei* ATCC 393 24.65±0.48

Table 1 . Bacteriocin production by single bacterial culture versus mixed culture of lactic acid bacteria

Bacteriocin producing strain	Zone of inhibition (mm) (Mean ±SD)
Enterococcus faecalis DFR4 (CL2C)	23.50±0.32
Enterococcus faecalis DFRP1 (P1)	23.50±0.30
Enterococcus faecalis DFRW1 (W1)	24.20±0.16
Mixed culture of *Enterococcus faecalis* DFR4, *Enterococcus faecalis* DFRP1 and *Enterococcus faecalis* DFRW1	30.02±0.24
Lactobacillus plantarum DFR4 (4dB)	23.34±0.14
Enterococcus faecalis DFRP1 (P1)	23.50±0.26
Enterococcus faecalis DFR4 (CL2C)	23.50±0.28
Mixed culture of *Lactobacillus plantarum* DFR4, *Enterococcus faecalis* DFRP1 and *Enterococcus faecalis* DFR4	31.24±0.33
Enterococcus faecalis DFRP1 (P1)	22.89±0.33
Lactococcus lactis ATCC 19435	23.02±0.35
Lactobacillus plantarum DFR2 (1d)	21.05±0.14
Mixed culture of *Enterococcus faecalis* DFRP1, *Lactococcus lactis* ATCC 19435 and *Lactobacillus plantarum* DFR2	23.50±0.45
Lactobacillus acidophilus ATCC 4356	23.25±0.20
Lactobacillus casei ATCC 393	24.77±0.42
Lactococcus lactis ATCC 19435	23.35±0.26
Mixed culture of *Lactobacillus acidophilus* ATCC 4356, *Lactobacilluscasei* ATCC 393 and *Lactococcus lactis* ATCC 19435	31.20±0.11
Lactobacillus plantarum DFR4 (4dB)	23.30±0.10
Lactobacilluscasei ATCC 393	24.65±0.48
Lactobacillus acidophilus ATCC 4356	23.34±0.11
Mixed culture of *Lactobacillus plantarum* DFR4, *Lactobacillus casei* ATCC 393 and *Lactobacillus acidophilus* ATCC 4356	28.92±0.55
Lactobacillus plantarum DFR2 (1d)	21.15±0.23
Enterococcus faecalis DFRP1 (P1)	23.52±0.26
Enterococcus faecalis DFRW1 (W1)	23.88±0.44
Mixed culture of *Lactobacillus plantarum* DFR2, *Enterococcus faecalis* DFRP1 and *Enterococcus faecalis* DFRW1	22.01±0.02

The values are means of three independent experiments carried out in duplicates. SD, standard deviation

mm, *Lactobacillus acidophilus* ATCC 4356 23.34±0.11 mm and their combined activity was 28.92±0.55 mm. Larger zone of inhibition from the combination of mixed lactic acid bacteria may be due to the additive effects of bacteriocins. In contrast, combination 6 (individual cultures *Lactobacillus plantarum* DFR2 (1d) 21.15±0.23, *Enterococcus faecalis* DFRP1 (P1) 23.52±0.26 mm, *Enterococcus faecalis* DFRW1 (W1) 23.88±0.44 mm and their combined activity was 22.01±0.02 mm) showed bacteriocin production from mixed culture to be lesser than that of monoculture, owing to antagonism. However, combination 3 (individual cultures *Enterococcus faecalis* DFRP1 (P1) 22.89±0.33 mm, *Lactococcus lactis* ATCC 19435 23.02±0.35 mm, *Lactobacillus plantarum* DFR2 (1d) 21.05±0.14 mm and their combined activity was 23.50±0.45 mm) did not show much difference in zone size from monoculture and mixed culture. Combination 2 (DFRL bacterial isolates DFR4, DFRP1 and DFRCL2C showed highest bacteriocin production among all the combinations tested (Fig. 1) and was selected for acquiring maximum bacteriocin production via immobilisation on bamboo fibre.

The 16S rRNA analysis confirmed the identity of the present isolates as *L. plantarum* DFR4 (4dB), *E. faecalis* DFRP1 (P1) and *E. faecalis* DFR4 (CL2C). The gene bank accession number assigned for the bacteriocin producing DFRL isolates were KT202829 for *L. plantarum* DFR4, KT 328588 for *E. faecalis* DFR4 and KT 328587 for *E. faecalis* DFRP1. *L. plantarum* DFR4 is a gram-positive, rod shaped, homo fermentative, non-spore forming and catalase negative bacterium whereas, *E. faecalis* DFRP1 and *E. faecalis* DFR4 are non-spore forming, catalase negative, hetero fermentative gram-positive cocci. The biochemical characterisation of these bacterial isolates is illustrated in Table 2. Amongst the three techniques used (cold acetone precipitation, ammonium sulphate precipitation and gel permeation chromatography), the specific activity and purity (fold purification of 85.25% for *L. plantarum* DFR4, 78.66% for *E. faecalis* DFRP1 and 75.07% for *E. faecalis* DFR4) for bacteriocin was found to be highest in gel permeation chromatography, whereas yield was highest in 80% acetone precipitated bacteriocin samples. The partial purification of the selected bacterial isolates is described in Table 3. pH ranging from 3.0-5.0 was found to be suitable for bacteriocin activity. pH above 5.0 affected the bacteriocin production drastically. Bacteriocins from selected bacterial isolates were found to be thermostable and could withstand higher temperatures such as 80 °C, 100 °C and 121 °C. with no loss of activity at 80 °C. However, loss of activity ranging from 20 % - 25 % was observed at higher temperatures 100 °C and 121 °C (22 % - 27 %).When a bacteriocin is thermostable, it can be applied to foods requiring cooking at higher temperature. Amylase (2.0 mg/ml) did not alter the bacteriocin activity. Bacteriocin was found to be inactive when treated with protease. Loss of bacteriocin activity (69 % -75 %) was noticed when for papain treatment. The effect of pH, heat and enzymes on the residual activity of bacteriocins produced from *L. plantarum* DFR4, *E. faecalis* DFRP1 and *E. faecalis* DFR4 is shown in Table 4.

The desirability of an immobilised system is determined by the kind of application and the physicochemical characteristics of the immobilizing matrix. Accordingly, the requirements will differ for a specific type but, characteristics such as non-toxicity, high surface to volume ratio, high cell mass loading capacity, ease of handling, being reusable and economical are considered necessary. Earlier, attempts were made to use solid supports such as agar, carrageenan and calcium alginate for immobilizing *L. plantarum* DFR4 but, none served as a good

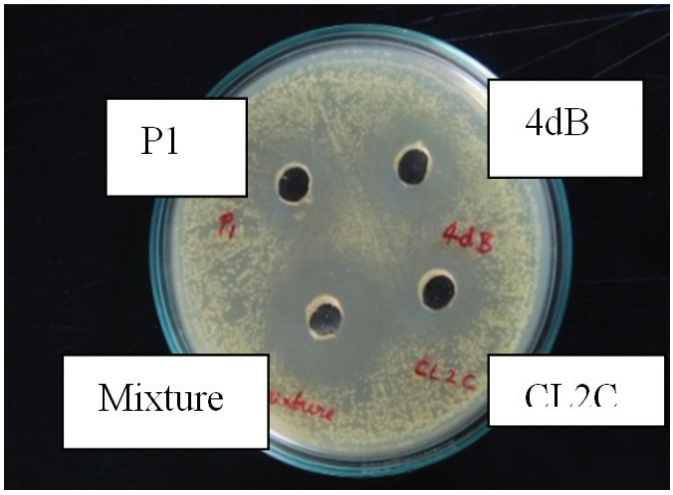

Figure 1. Antimicrobial activity of bacteriocins produced by4dB (*Lactobacillus plantarum* DFR4), P1 (*Enterococcus faecalis* DFRP1) and CL2C (*Enterococcus faecalis* DFR4) and their combined activity against *Listeria monocytogenes*.

Table 2. Biochemical characterisation of bacteriocin producing *Lactobacillus plantarum* DFR4, *Enterococcus faecalis* DFRP1 and *Enterococcus faecalis* DFR4.

Biochemical test	Bacteriocin producing isolates		
	Lactobacillus plantarum DFR4	*Enterococcus faecalis* DFRP1	*Enterococcus faecalis* DFR4
Catalase test	-	-	-
Production of gas from sugar	-	-	-
Production of ammonia	-	+	+
Citrate utilisation	-	-	-
Methyl red test	+	+	+
VogesProskauer test	-	-	-
Homo-heterofermentative test	homo	hetero	hetero

carrier. All these solid matrices were found to be unstable as they became weak and gradually dissolved along with the growth medium. However, these carriers may be suitable for dry conditions and are generally used for immobilizing enzymes and fungi (form spores). Therefore, the application of natural hydrophobic carriers was thought to be appropriate for such a set up. A simple and cost effective technique was developed for immobilisation. *L. plantarum* DFR4 was immobilised on bamboo fibre which is reusable, free of chemicals, non-toxic and easy to handle. Whole cell immobilisation through adsorption provides direct contact between nutrients from the growth medium and immobilised cells. Adsorption capacity and strength of binding were found to be essential factors for the selection of a suitable carrier. Bamboo fibre formed a spongy network, rendering enough hollow space for the attachment of bacteria. Scanning electron microscopic images displayed immobilised *L. plantarum* DFR4 cells on the surface of the bamboo fibre (Fig. 2). Bacteriocin production from immobilised cells (20.91±0.35 mm) were found to be higher than that of a free cell system (8.52±0.26 mm). Bacteriocin production within 24 h from *L. plantarum* DFR4 in form of free cells and immobilised cells are shown in Fig. 3.

Bacteriocin samples (obtained from selected immobilised single and cocultured lactic acid bacteria) were added to mosambi juice to investigate their preservative effect. Fruit juices are classified as easily perishable foods which are usually stored at low temperature. Higher level of humidity often relates to rapid food spoilage due to microbial contamination.

3.1 Treatment (a)

The pouch containing the control (Mosambi juice spiked with *L. monocytogenes* and without the addition of bacteriocin) showed bloating within 24 h (11.24 log), though microbial count declined in the pouches incorporated with bacteriocins. After 24 h, the initial viable count of *L. monocytogenes* in the pouch containing single bacteriocin was 5.56 log, which decreased to 3.04 log at 52 AU/ml and was 2.65 log at 104 AU/ml (Fig. 4.). Whereas the viable count decreased from 5.56 log to 2.57 log at 52 AU/ml and 1.77 log at 104 AU/ml for the pouch containing bacteriocins produced from selected mixed bacteria after 24 hours. Further reduction in the microbial count was noticed after 70 h as shown in Fig. 5. As the control did not contain any preservative, the inoculated *L. monocytogenes* overgrew in the juice and the bloating of retort pouches were observed.

Table 3. Partial purification and antimicrobial activity of bacteriocins obtained from *Lactobacillus plantarum* DFR4, *Enterococcus faecalis* DFRP1 and *Enterococcus faecalis* DFR4

Treatment	Total volume (ml)	Activity (AU/ml)	Protein concentration (mg/ml) mean ±SD	Specific activity (AU/mg) mean ±SD	Yield (%)	Purification (fold)
Bacteriocin CFSC from *L. plantarum* DFR4	100	385 (300-450)	130±4.0	2.96±0.44	100	1
40 % Acetone precipitation	10	2,312 (2,117-2,390)	172±2.05	13.44±0.42	60.05	4.54
80 % Acetone precipitation	5	5,508 (4,920-5,807)	167±5.24	32.98±0.6	71.53	11.14
70 % Ammonium sulphate precipitation	2	5,764 (5,300-5,788)	185±6.04	31.15±0.75	29.94	10.52
GPC purified active fractions	1	1,192 (947-1,307)	4.72±0.35	252.54±0.51	3.09	85.25
Bacteriocin CFSC from *E. faecalis* DFRP1	100	370 (290-422)	126±0.61	2.94±0.33	100	1
40 % Acetone precipitation	10	2,235 (2,016-2,460)	162±4.20	13.8±0.26	60.40	4.6
80 % Acetone precipitation	5	5,255 (4,881-5,608)	157±6.11	33.47±0.11	71.01	11.38
70 % Ammonium sulphate precipitation	2	5,425 (5,246-5,663)	176±3.43	30.82±0.15	29.32	10.48
GPC purified active fractions	1	1,050 (938-1,127)	4.54±0.22	231.28±0.28	2.72	78.66
Bacteriocin CFSC from *E. faecalis* DFR4	100	355 (287-460)	120±0.50	2.96±0.24	100	1
40 % Acetone precipitation	10	2,120 (2,005-2,401)	158±3.02	13.41±0.78	59.71	4.53
80 % Acetone precipitation	5	5,204 (4,990-5,502)	152±4.4	34.24±0.57	73.30	11.56
70 % Ammonium sulphate precipitation	2	5,318 (5,159-5,503)	173±2.71	30.74±0.13	29.96	10.38
GPC purified active fractions	1	1,000 (920-1120)	4.50±0.16	222.22±0.14	2.81	75.07

The values are means of three independent experiments carried out in duplicates, the range is given in parentheses. SD, standard deviation; CFSC, cell-free supernatant concentrate; GPC, gel permeation chromatography.

Table 4. Effect of pH, temperature and enzymes on the residual activity of bacteriocins produced from *Lactobacillus plantarum* DFR4, *Enterococcus faecalis* DFRP1 and *Enterococcus faecalis* DFR4

	Treatment residual activity (Mean ±SD)		
	***Lactobacillus plantarum* DFR4**	***Enterococcus faecalis* DFRP1**	***Enterococcus faecalis* DFR4**
pH			
3.0-5.0	100.0	100.0	100.0
6.0	84.35±3.20	82.04±0.26	79.66±1.35
7.0	68.87±2.70	65.56±0.78	63.88±0.53
Temperature			
80ºC (30 min)	100.0	100.0	100.0
100ºC (30 min)	80.12±2.40	77.5±1.26	75.12±0.50
121ºC (15 min)	78.48±5.73	75.22±1.76	73.17±1.44
Enzymes			
Amylase	100.0	100.0	100.0
Papain	28.25±0.36	27.77±0.42	25.26±0.25
Protease	0.0	0.0	0.0

The values are means of three independent experiments carried out in duplicates. SD, standard deviation

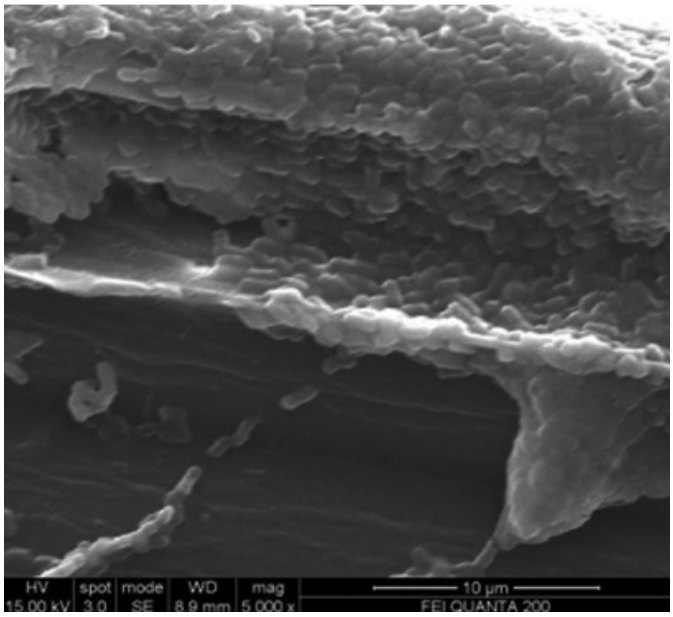

Figure 2. Scanning electron microscopy of immobilised *Lactobacillus plantarum* DFR4 cells on Bamboo (*Bambusa vulgaris*) fibre, x 5000.

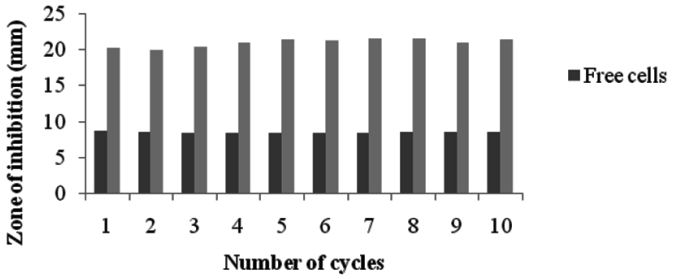

Figure 3. Bacteriocin production by free cells versus *Lactobacillus plantarum* DFR4 cells immobilised on bamboo (*Bambusa vulgaris*) fibre within 24 h.

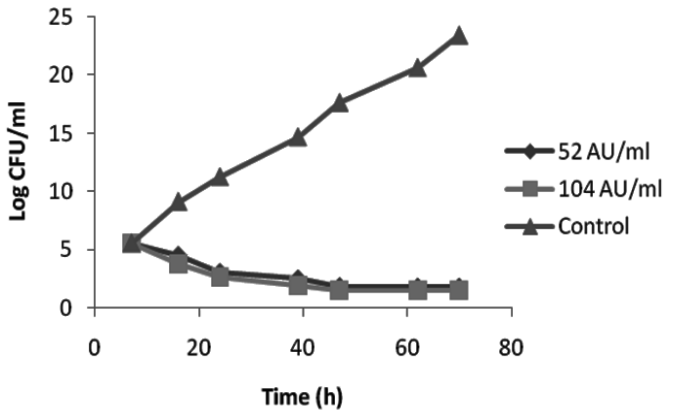

Figure 4. Retort pouches containing Mosambi (Citrus limetta) juice spiked with *L. monocytogenes* and addition of bacteriocins produced by single culture (*L. plantarum*).

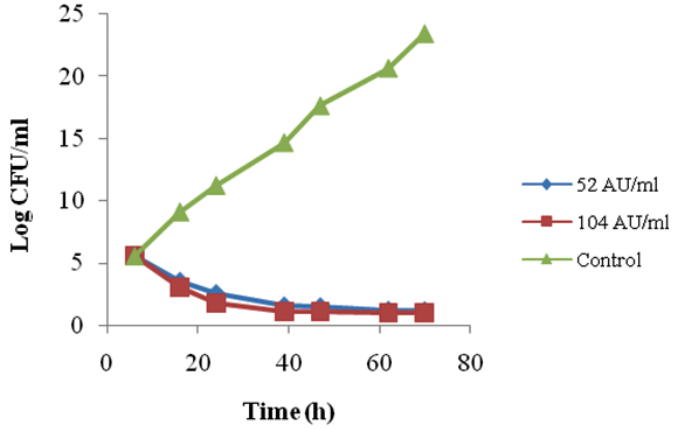

Figure 5. Retort pouches containing Mosambi (*Citrus limetta*) juice spiked with *L. monocytogenes* and addition of bacteriocins produced by mixed culture (*L. plantarum* DFR4, *Enterococcus faecalis* DFRP1and *Enterococcus faecalis* DFR4).

3.2 Treatment (b)

The pouch containing the second control (un autoclaved mosambi juice without bacteriocin) showed bloating after 72 h whereas, the pouch containing juice incorporated with bacteriocins (produced from immobilised selected cocultured bacteria) did not show bloating upto 20 days. The pouch containing juice supplemented with bacteriocins (produced from immobilised *L. plantarum* DFR4) did not show any signs of bloating until 17 days but, after 20 days slight bloating could be observed, owing to slow spoilage of the juice. Bacteriocin (obtained from immobilised *L. plantarum* DFR4) could preserve the juice up to 17 days while bacteriocins (produced from immobilised selected co-cultured bacteria) could preserve up to 26 days at low temperature (5 °C).

4. CONCLUSION

The suggested approaches for achieving large scale production of bacteriocins is simple, mild, fast and economical. The combination of selected co-cultured lactic acid bacteria showed a major improvement in the production of bacteriocins and its immobilisation facilitated faster production. Due to the presence of adsorbed bacterial cells on the bamboo fibre and their interaction with the growth medium, the production of bacteriocin became faster when compared to a free cell system. Low temperature is another physical parameter which restricts the growth of undesirable microorganisms. The combined effect of these approaches resulted in achieving maximum bacteriocin production. The advantages of using bacteriocins include extended shelf life, permit reduction in heat treatment, substitute chemical preservatives with bacteriocins and decrease food damage due to food spoilage bacteria.

REFERENCES

1. Todorov, S.D. Bacteriocin production by Lactobacillus plantarum AMA-K isolated from Amasi, a Zimbabwean fermented milk product and study of the adsorption of bacteriocin AMA-K to Listeria sp. *Braz. J. Microbiol.*, 2008, **39**, 178-187. doi: 10.1590/S1517-83822008000100035.
2. O'Sullivan, L.; Ross, R.P. & Hill, C. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality Biochimie., 2002, **84**, 593-604.
3. Pal, Ajay & Ramana, K.V. Purification and characterisation of bacteriocin from weissella paramesenteroides DFR-8, an isolate from cucumber (Cucumissativus). *J. Food Biochem.*, 2010, **34**, 932-948. doi: 10.1111/j.1745-4514.2010.00340.x
4. Neera; Patil, M.M.; Ramana, K.V. & Bawa, A.S. Statistical optimisation of bacteriocin production by pediococcusacidilactici in a simple food grade medium. *J. Food Process. Preserv.*, 2013, **37**, 179-187. doi: 10.1111/jfpp.12020
5. Chibata, I.; Tosa, T. & Sato, T. Immobilized aspartase-containing microbial cells: preparation and enzymatic properties. *Applied Microbiol.*, 1974, **27**, 878-885. http://aem.asm.org/content/27/5/878.short
6. Iqbal, M. & Saeed, A. Novel method for cell immobilisation and its application for production of organic acid. *Let. Appl. Microbiol.*, 2005, **40**(3), 178-182. doi: 10.1111/j.1472-765X.2004.01646.x
7. Zeynab, B.; Mehdi, H. & Simone, C. Immobilisation of microbes for bioremediation of crude oil polluted environments: A mini review. *Open Microbiol. J.*, 2015, **9**, 48-54. doi: 10.2174/1874285801509010048
8. Karel, S.F.; Libicki, S.B. & Robertson, C.R. Immobilisation of whole cells: engineering principles. *Chem. Eng. Sci.*, 1985, **40**, 1321-1354. doi: 10.1016/0009-2509(85)80074-9
9. Guy, A.J. & Thierry, J. Immobilized viable microbial cells: from the process to the proteome or the cart before the horse. *Biotechnology Advances.*, 2004, **22**(8), 633-658. doi: 10.1016/j.biotechadv.2004.06.003
10. Phadtare, S.; Vinod, V.P.; Wadgaokar, P.P.; Rao, M. & Sastry, M. Freestanding Nano gold membranes as scaffolds for enzyme immobilisation. *Langmuir.*, 2004, **20**, 3717-3723. doi: 10.1021/la035870j
11. Vignolo, G.; Palacios, J.; Farias, M.E.; Sesma, F.; Schillinger, U.; Holzapfel, W. & Oliver, G. Combined effect of bacteriocins on the survival of various Listeria species in broth and meat system. *Current Microbiology*, 2000, **41**(6), 410-416. doi:10.1007/s002840010159
12. Mathys, S.; Meile, L. & Lacroix, C. Co-cultivation of a bacteriocin-producing mixed culture of bifidobacteriumthermophilum RBL67 and pediococcus acidilactici UVA1 isolated from baby faeces. *J. Appl. Microbiol.*, 2009, **107**, 36-46. doi: 10.1111/j.1365-2672.2009.04186.x
13. Sobrino, A.; Lopez, O. & Martin, B. Use of nisin and other bacteriocins for preservation of dairy products. *Inter. Dairy J.*, 2008, **18**(4), 329-343. doi:10.1016/j.idairyj.2007.11.009
14. Chanos, P. & Mygind, T. Co-culture-inducible bacteriocin production in lactic acid bacteria. *Appl. Microbiol Biotechnol.*, 2016, **100**(10), 4297-4308. doi: 10.1007/s00253-016-7486-8
15. Garcha, S. & Sharma, N. Use of combinations of bacteriocins from Lactobacillus plantarum MTCC 1407 and Bacillus coagulans MTCC 492. *African J. Microbiol Res.*, 2013, **7**(47), 5338-5342. doi: 10.5897/AJMR12.2004
16. Jesus, D.; Manzano, R. & Jimenez, D. Suppression of bacteriocin production in mixed-species cultures of lactic acid bacteria. *Food Control*, **30**(2), 2013, 474-479. doi: 10.1016/j.foodcont.2012.09.014
17. Mulet, N.; Powell, A.M.; Lacoste, A.; Mignel, V. & Buochberg, M.S. Interactions between pairs of bacteriocins from lactic bacteria. *J. Food Protect.*,

1998, **61**(9), 1210-1212.
doi: 10.4315/0362-028X-61.9.1210
18. Hadas, H.; Farrah, B. & Curtis, M.L. Bacteriocin-mediated interactions within and between coexisting species, *Ecology and Evolution,* 2012, **2**(10), 2516-2521.
doi: 10.1002/ece3.354
19. Tagg, J.R. & McGiven, A.R. Assay systems for bacteriocins. *Applied Microbiology*, 1971, **21**, 943. PMCID: PMC377313

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