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STUDY ON FRUIT JUICE PRESERVATION TECHNIQUES INCLUDE IMMOBILIZING LACTIC ACID BACTERIA AND USING BACTERIOCIN

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ABSTRACT

Bacteriocins produced by lactic acid bacteria, which are generally recognized as safe (GRAS) species, aid in the inhibition of food-borne infections. For culinary applications, its narrow spectrum activity is a significant drawback. In order to increase the antibacterial range of bacteriocins made with lactic acid bacteria, a variety of techniques were combined. In order to choose the optimal combination with the highest antibacterial activity, the antimicrobial activity of bacteriocin from various combinations of mixed lactic acid bacteria and single culture was first tested after 56 hours at 35 °C. In an agar disc diffusion testing, a mixture of the bacteriocins generated by Lactobacillus plantarum DFR4, Enterococcus faecalis DFR4, and E. faecalis DFRP1 had the greatest effectiveness against Listeria monocytogenes with a zone of 31.24 0.33 mm. The yield was highest in 80 percent acetone precipitation, whereas the specific activity and purity of the bacteriocins produced by these isolates were shown to be highest in gel permeation chromatography. The influence of pH, enzymes, and temperature stability on the partially purified bacteriocins was further explored, and it was discovered that they were thermally stable and active at pH ranges of 3 to 5. Proteolytic enzymes like papain and protease rendered the bacteriocins inactive, whereas amylase had no such impact. After 24 hours of incubation at 37 °C, it was discovered that bacteriocin made from bamboo (Bambusa vulgaris) fiber immobilized Lactobacillus plantarum DFR4 had higher antibacterial activity (zone size=20.910.35 mm) than free cells (zone size=8.520.26 mm). Additionally, bamboo fiber was used to immobilize Lactobacillus plantarum DFR4, Enterococcus faecalis DFR4, and E. faecalis DFRP1 to speed up the generation of bacteriocins in 24 hours. At 5 °C, Mosambi juice (Citrus limetta) treated with bacteriocins, which are made from a variety of bacterial isolates, had a 26-day shelf life.

Keywords: *Bacteriocin; Immobilisation; Adsorption; Co-culture*

INTRODUCTION

The food sector faces a lot of difficulties when storing food because of food deterioration. Foodborne pathogens are responsible for a significant portion of food spoilage, and there are several methods available to fight these dangerous microorganisms. However, customers choose biopreservation since it is healthy, efficient, and safe to ingest. Bacteriocins, a promising biopreservative, have recently gained widespread recognition in the food sector. The lactic acid bacteria create bacteriocins, which are tiny antimicrobial peptides that are ribosomally synthesized. Bacteriocins kill their target microorganisms by inducing cellular DNA damage, which finally results in cell death1, hole development on the cell wall, which allows cell components to leak out, delayed peptidoglycan production (which halts cell wall synthesis), and cell leakage. Prior studies have thoroughly discussed how to maximize bacteriocin activity by adjusting the temperature, pH, incubation time, and medium components, but adequate information regarding their scale-up procedure is rarely provided.3, 4.In the past, studies on the immobilization of microorganisms were conducted for the manufacture of enzymes5, organic acids6, and bio-remediation of contaminants from industrial effluents7, as well as for the making of wine.

Immobilization of entire cells refers to the limited motion or physical localization of cells on a solid platform in order to maintain their biological activity8. Covalent connections, adsorption, entrapment, self-aggregation through flocculation, and freeze thawing are methods for immobilizing cells9. Agar, carrageenan, calcium alginate, glass, polyacrylamide, cellulose, and polyvinyl alcohol crosslinked with boric acid are common solid supports used for microbial cell immobilization10. One immobilization technique that relies on physical interactions between cells and the surface of the water-insoluble carrier is adsorption. The interstitial solution (solution that fills the empty space) and the support material (carrier) are the three primary components of the immobilized cell aggregate.

In a niche, bacteria often compete with one another, however certain bacteria can cohabit. Additionally, bacteria work together in groups to coordinate functions for nourishment, defense, and occasionally the formation of biofilms. There are few studies on using co-cultures of different lactic acid bacteria to effectively eliminate food spoilage organisms, either by acting as rival microorganisms or by producing bacteriocins11–14. Contrarily, a small number of articles have also claimed that the co-culturing of lactic acid bacteria can inhibit the synthesis of bacteriocin15,16. The associations that bacteria in a consortium have with one another vary. While antagonistic interactions reduced bacteriocin production17,18, synergistic interactions between different lactic acid bacteria

increased bacteriocin production. The formation of bacteriocin resistance organisms could be avoided, though, by carefully combining several bacteriocins to broaden the antibacterial range.

The immobilization of lactic acid bacteria such Lactobacillus plantarum DFR4, Enterococcus faecalis DFR4, and Enterococcus faecalis DFRP1 on an appropriate carrier and their impact on the generation of bacteriocin in comparison to a free cell system are the main topics of this research. Cell density measurements and antimicrobial susceptibility tests were used to show the difference in bacteriocin activity. The selection of a favorable combination to increase bacteriocin production was done by analyzing the synergistic and antagonistic interactions between different lactic acid bacteria. The mixture of bacteriocins produced from selected mixed lactic acid bacteria L. plantarum DFR4, E. faecalis DFR4, and E. faecalis DFRP1 cultured on an immobilization matrix (bamboo fiber) using yeast extract dextrose medium was used to suppress the growth of bacteria that cause food rotting. The resulting bacteriocins were then added to Mosambi juice and maintained at 5 °C.

MATERIALS AND METHODS

1.1 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).

1.2 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.

1.3 Production, Partial Purification and Characterisation of Bacteriocin

A comparative study was conducted on the bacteriocin production obtained from single and various combinationsof mixed lactic acid bacteria. The morphology of isolatedlactic 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 1/10th 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 50mM acetate buffer (pH 4.5). Extraction of bacteriocin was alsocarried out using 70 per cent ammonium sulphate precipitation. Gel permeation chromatography was performed to furtherpurify the bacteriocin samples. Crude bacteriocin preparation (3 ml) obtained via ammonium sulphate precipitation waspassed 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 theantimicrobial activity. The antimicrobial activity of the crude bacteriocin samples were checked after 24 h and their zone ofinhibition were measured (Table1)The estimation of proteins from the bacteriocin samples was done using Lowry's methodusing Bovine serum albumin (BSA) as standard. The specific activity, yield and fold purification were determined for partially purified bacteriocins. Experiments were conducted to study theeffect 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 for24 h under static condition. Thermal stability of the bacteriocinswas evaluated by heating the bacteriocin preparations at different temperatures (80 °C and 100 °C for 30 min, followedby 121°C for 15 min). Further, the effect of enzymes (amylase,papain, and protease at 2.0 mg/ml) was determined by treatingthe bacteriocin samples with enzymes for 1 h at 4 °C.

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

Immobilisation of *L. plantarum* DFR4 was carried out onbamboo 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 wereauto claved at 121 psi for 15 min. The flasks were inoculated using 2.0 % (v/v) overnight culture of *L. plantarum* DFR4and 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.

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

1.4.1 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.

1.4.2 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.

1.4.3 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.

2. 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, 4and 5) showed larger zone of inhibition than that of the singlecultures (Table 1). Combination 1 comprised of individual cultures *Enterococcus faecalis* DFR4 (CL2C)23.50±0.32mm, *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 was31.24±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 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 bacteriamay 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 cultureto 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 highestbacteriocin production among all the combinations tested (Fig. 1) and was selected for acquiring maximum bacteriocin production via immobilisation on bamboo fibre.

Table 1 . Bacteriocin production by single bacterial cultureversus 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

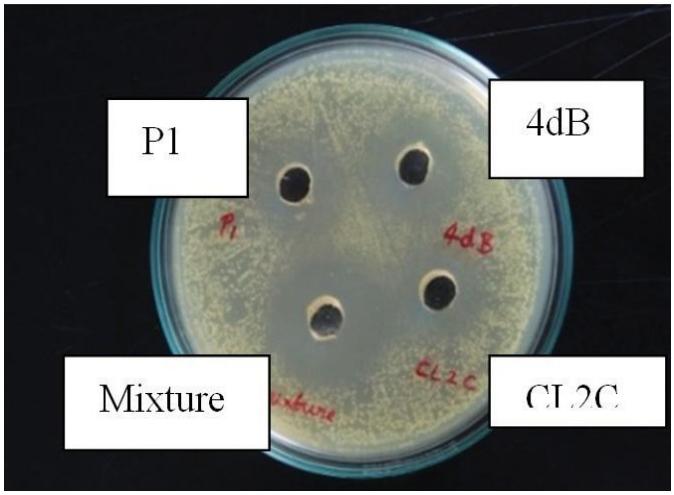

Figure 1. Antimicrobial activity of bacteriocins produced by4dB(*Lactobacillus plantarum* DFR4), P1 (*Enterococcus faecalis* DFRP1) and CL2C (*Enterococcus faecalis* DFR4) and their combined activity against *Listeria monocytogenes*.

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 tobe 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 activityranging from 20 % - 25 % was observed at higher temperatures100 °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 whentreated with protease. Loss of bacteriocin activity (69 % -75 %)was noticed when for papain treatment. The effect of pH, heatand enzymes on the residual activity of bacteriocins producedfrom *L. plantarum* DFR4, *E. faecalis* DFRP1 and *E. faecalis* DFR4 is shown in Table 4.

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

CONCLUSION

The suggested methods are easy, mild, quick, and affordable for producing bacteriocins on a big scale. The synthesis of bacteriocins significantly improved with the combination of chosen co-cultured lactic acid

bacteria, and its immobilization permitted quicker manufacturing. When compared to a system using free cells, the generation of bacteriocin became faster because of the adsorbed bacterial cells on the bamboo fiber and how they interacted with the growing media. Another physical factor that prevents the growth of harmful germs is low temperature. The sum of these strategies allowed for the highest possible bacteriocin output. The benefits of employing bacteriocins include increased shelf life, the ability to reduce heat treatment, the ability to swap out chemical preservatives for bacteriocins, and a reduction in food damage caused by bacteria that cause food rotting.

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