Original Article

Biopreservative Action of Bacteriocin from *Pediococcus pentosaceus* on the Microbial Load of Apple Juice

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ABSTRACT

Aim: The aim of the study is to explore the bio preservative role of *Pediococcus pentosaceus*. **Background:** Bacteriocins are proteinaceous or peptidic in nature produced by lactic acid bacteria and generally recognized as safe (GRAS). It ranges from 2 to 200kDa in molecular weight. It inhibits the growth of similar or closely related bacterial strains. **Materials and Methods:** In this experiment, "Appam batter" has been taken as our sample, collected from Vellore city. The sample was fermented for seven and fourteen days simultaneously from which 1mL of the samples were taken and inoculated in 100mL of production media consist of 3% skim milk. The bacteria were allowed to grow for 24 hrs and serially diluted and spread plate was done on MRS agar plate. Seventeen bacterial strains were isolated. The preliminary assays were carried out to identify the bacteria as *Lactobacillus* species, such as Gram staining and catalase. All the strains were screened for bacteriocin by agar well diffusion assay. The strains which showed zone of inhibition was further subjected to antibacterial activity. **Results:** The isolated strains were found to show a clear zone of inhibition against *Listeria monocytogenes* MTCC 5260 used as the indicator strain. Further the isolated strains were tested for antimicrobial activity against certain food borne pathogens which includes *Staphylococcus aureus* MTCC 5257, Salmonella typhi MTCC2501, *Escherichia coli* MTCC 2089 and *Pseudomonas aeruginosa* MTCC 2242. The isolated strain VITAB01 showed a broad spectrum activity against all the pathogens with a highest zone of inhibition of 13mm, 12800(AU/mg) and thus subjected to 16srRNA molecular level analysis and found out to be *Pediococcus pentosaceus* MH134499. The isolate upon characterization showed to be heat stable at 60°C and optimum pH 6. On purification it has a specific activity of 325.20(AU/mg) with a final yield of 3.12. The molecular weight of the bacteriocin was found to be 17kDa. **Conclusion:** The supernatant was mixed with apple juice and checked for its bio preservative effect. Decrease in colonies was found to be 26.82%.

Key words: Appam batter, Lactic Acid Bacteria, Purification, Bacteriocin, Apple juice, Preservative.

INTRODUCTION

The bacteriocins are considered to be or peptidic or proteinaceous toxins. They are obtained by bacteria to inhibit the growth of bacterial strains that are similar or closely related. They have similarity to paramecium and yeast killing factors and are functionally, structurally and ecologically diversified.[1] Lactic Acid Bacteria (LAB) of different strains are involved with systems related to food producing bacteriocins which exhibit bacteriocidal activity against closely related organisms[2] bacteriocins that are isolated by using gram positive bacteria have broad inhibitory spectrum and might be used in various practical applications as anti microbial agents.[3] This anti microbial property has extended the shelf life of many fermented food products.[4] Most

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bacteriocins are sensitive to certain proteolytic enzymes and are heat stable. LABs is playing a major role in production of aromatic compounds, food preservation and microbiological stability.[5] They have also been widely used as starter culture where LABs produced bacteriocins. In recent years, LAB have grabbed the attention for their potential use as biopreservatives in food which has resulted in the reduction of the use of chemical preservatives.[6] The preservation factors elaborated by LAB are known as "bacteriocins" and have been identified as "peptides". Raw food materials are generally preserved by lactic acid fermentation.[7] Fermentive activities are also performed by LAB in food products which may inhibit the growth of pathogenic bacteria growing on them.

In the preservation and processing of food materials, technologies help to maintain the nutrition values and ensures food safety. Many chemicals are being used to preserve foods for long durations in order to inactivate the food borne pathogens.[8]

MATERIALS AND METHODS

Mann Ragosa sharpe agar (MRS), Skim mik, Nutrient broth were purchased from HiMedia. The indicator strain such as pathogen was procured from MTCC. All the reagents used were analytical grade.

Isolation and Identification of Bacterial Strains

The research was performed from July to October 2019 to isolate and identify the naturally producing Lactic acid Bacteria from fermented appam batter sample. Appam batter were aseptically collected using sterilized sample bottles from Vellore district, Tamil Nadu, India. The sample was kept for fermentation at 37°C for 7-14days. The appam batter mixture was mixed with skimmed milk (10:3) incorporated in MRS broth media (Hi-media, India) and incubated at 37°C for 48 hr. Samples were serially diluted from 10^{-1} to 10^{-8} in sterile normal saline. A volume of 0.1ml of proper dilutions was spread plated on De Man Rogosa Sharpe (MRS) agar plates and incubated for 48 hr at 30°C.[9] Several streak were made to obtain pure isolate. Isolates with colony morphology similar to lactic acid bacteria were selected and sub cultured in MRS medium to obtain pure culture. Pure culture strains were numbered isolates from appam batter sample VITAB01 to VITAB13 and maintained at -20°C in MRS broth with 20% glycerol and enriched in MRS broth by incubating at 37°C for 24h for future study. These cultures were retrieved twice in MRS broth before the experiment.

Screening for bacteriocin activity from lactic acid bacteria

Isolated strains ware grown in MRS broth at 37°C for 48 hr. After incubation, centrifugation of the broth was performed at 10000 *g for 25 min at 4°C. The pellet were discarded and the supernatant were collected. Thus the supernatant that was free of cells were used as crude bacteriocin and were carried out for further assays. Screening for bacteriocin producing bacteria was carried out using extracellular protein as cell free supernatant. LAB was prepared in MRS broth and incubated for 48h at 37°C. After incubation the isolates were centrifuged at 8,000 rpm for 20 min at 4°C. Bacteriocin production from the isolated strains against *Listeria monocytogens* as an indicator strain were carried out, by performing agar well diffusion assay (AWDA) under aerobic condition. Mueller Hinton Agar plates were swabbed with 100µL of indicator microorganism *Listeria monocytogens*. 5mm deep wells were cut and 100µL of the cell free culture supernatant (crude bateriocin) of all the isolated strains were added into each well. Incubation of the plates were done for 3 hrs at 4°C which was followed by incubation at room temperature for 24 hr. The zone of inhibition were measured in order to determine the zone of inhibition.[10]

Determination of Antibacterial activity

Staphylococcus aureus (5257), *Salmonella typhi* (2501), *Escherichia coli* (2089) *and Pseudomonas fluoroscens* (2173) *Listeria monocytogenes* (MTCC 5260) used in the experiement were purchased from the Micobial Type Culture Collection (MTCC), Pune. The assay was carried out similarly using AWDA as described above.[11]

Biochemical characterization of the potent isolate

A potent isolate VITAB1 isolated from the appam batter sample, showed the most intense antibacterial activity and was selected for further work. Pure culture of strain was tested for Indole test, Methyl red, Voges proskeur and citrate utilization test. Characteristics of the isolate were compared with data from Bergey's Manual of Determinative Bacteriology (Bergey *et al.* 1923).[12]

Molecular characterization

The strain was further identified by 16S rRNA gene sequencing for species level identification. The nucleic acids of the isolate were extracted using a DNA purification kit (Amnion Biosciences Pvt Ltd, India), according to the manufacturer's instructions. Two primers namely Forward (5'AGAGTTTGATCCTGGCTCAG3') and Reverse

(5'AAGGAGGTGATCCAGCCGCA3') were used for the DNA amplification isolated from VITAB1 isolate for 16S rRNA sequencing. The obtained sequences were subjected to BLAST in the NCBI database. Phylogenetic tree was constructed in MEGA 4.0.2 using neighbor-joining (NJ) method.[13]

Characterization

Heat stability and effect of pH

Various temperature like 37°C, 60°C, 100°C and 120°C were applied to the sample for 15 min. The samples were cooled down and were checked for its activity on by using agar well diffusion assay. Partially purified bacteria of about 5ml were collected in several test tubes. The pH values were adjusted of pH was done at 2, 4, 7, 9 and 12. The MRS broth was prepared and adjusted to pH 2 to 4 using Acetic acid buffer followed by 7 using Sodium phosphate buffer and pH 9 to 12 was maintained with Glycine buffer. Also, the sentence was reframed in the revised manuscript. It was kept at room temperature for 2 hr and checked for it activity on well diffusion agar.[14]

Effect of enzyme on bacteriocin activity (proteolytic assay)

Partially purified protein sample of 1 mL was collected in a test tube and mixed with proteinaseK (1mg/ml) and kept at 4°C for overnight. Further the untreated and treated samples were boiled for 2 min at 100°C, in order to inactivate the enzyme. Agar well diffusion assay were performed to check the activity.[15]

Purification of bacteriocin

The 300ml culture of *Pediococcus pentosaceus* was incubated for 48 hr at 30°C. the purification technique has been carried out with modification of the process as described by Nieto Lozano JC *et al*.[16]

The sample was subjected to 80% ammonium sulfate precipitation. The precipitate obtained was further dissolved in sodium acetate buffer 1mM (pH 5). The sample was centrifuged (4°C, 4000 rpm, 30min) using ultrafiltration membrane (Amicon Ultra-15, Millipore, India) with a 10kDa molecular cutoff. The permeate and retentate were both collected and and the active fraction was subjected to gel filtration chromatography using self-packed Sephadex G-50 column (1.5 in diameter, 15 cm gel bed height, 1 mL sample volume) equilibrated with sterile column buffer, 1mM Sodium acetate buffer (pH 5). All the twenty five filtrate fractions obtained were pulled off and in each step of the purification, the protein concentration and activity using Lowry's method and AWDA were checked consequetively. The molecular weight was determined using 12% tricine SDS–PAGE and compared with standard protein marker (4.6- 42kDa) bought from Genei.

Application

Efficacy of bacteriocin as biopreservative

Apple juice was refrigerated after adding 5% bacteriocin to it.[17] Serial dilution from 10^1 - 10^6 was performed. The plates were incubated at 37°C for 24 hr. The number of colony was calculated and compared with the control (without bacteriocin) the juice were further kept for a week and every day the microbial load was checked.[18]

RESULTS AND DISCUSSION

Isolation and identification of bacterial strains

Based on the colony morphology 13 different strains were isolated from appam batter samples. All 13 bacterial strains were characterized to be lactic acid bacteria based on the Grams and Catalase reaction. Out of 13 strains 3 are shown to be gram positive cocci and 10 were shown to be gram positive bacilli. All the isolates were shown to be negative for catalase reaction. From the results it was evident that bacilli were high in number compared to cocci. All the strain were subjected to antimicrobial activity against different food borne pathogens purchased from MTCC. The strain that produced bacteriocin was isolated from the source of appam batter and the selected strain *Lactobacillus* VITAB01 was identified as on its physiological and biochemical characteristic. The isolate was gram positive, rod shaped, oxidase positive and catalase negative displaying smooth round colonies on the MRS agar media.The morphological characterization of the isolates are shown in Table 1.

Screening for bacteriocin activity from lactic acid bacteria

The isolates VITAB01 VITAB02, VITAB03 and VITAB04 showed to produce bacteriocin with a clear zone of inhibition against the indicator organism.

Determination of Antibacterial activity

Among 13 isolates which were obtained from appam batter sample, one potent isolate VITAB01 were very effective against all 5 pathogens with high zone of inhibition. Among the rest VITAB02, VITAB03 and VITAB04 were resistant to *E. coli* and *Pseudomonas fluoroscens*. Other isolates from VITAB05 to VITAB13 were completely resistant to all pathogens. Isolates VITAB01 were considered for further studies such as biochemical, molecular and application. The bacteriocin

isolated from the selected strains was treated against 4 different types of major food borne gram positive and gram-negative pathogens for antibacterial activity. The isolated bacteriocin showed inhibitory activity against *Staphylococcus aureus* MTCC 5257*, Salmonella typhi* MTCC2501*, Escherichia coli MTCC* 2089 and *Pseudomonas fluoroscens* MTCC 2173.

Among all the strain VITAB01 showed strong zone of inbition of 13mm as shown (Table 2).

Biochemical characterization of potent isolate VITAB01

The isolate VITAB01 showed Methyl red positive, Indole, voges prauskauer and citrate utilization test negative (Table 3).

Molecular characterization

16S rRNA was amplified using universal primers (forward and reverse). The amplified 16s rRNA was identified as *Pediococcus pentosaceus* (NCBI accession no. MH134499) as Figure 1.

Morphology

Table 1: The Gram's Reaction, Catalase test and morphology of the 13 isolates were shown in the table.

Isolate No.	Gram's Reaction	Catalase Test	Shape
VITAB01	Gram Positive	Negative	Cocci in chains and tetrads
VITAB02	Gram Positive	Negative	Cocci in tetrads
VITAB03	Gram Positive	Negative	Bacilli in long rods
VITAB04	Gram Positive	Negative	Bacilli in long rods
VITAB05	Gram Positive	Negative	Bacilli in chains
VITAB06	Gram Positive	Negative	Bacilli in chains
VITAB07	Gram Positive	Negative	Cocci in chains
VITAB08	Gram Positive	Negative	Bacilli in long rods
VITAB09	Gram Positive	Negative	Bacilli in short rods arranged singly
VITAB10	Gram Positive	Negative	Bacilli in long rods
VITAB11	Gram Positive	Negative	Bacilli in long rods
VITAB12	Gram Positive	Negative	Bacilli in long rods
VITAB13	Gram Positive	Negative	Bacilli in chains

Characterization

Heat stability and effect of pH

At different temperatures (37, 60, 100 and 121C), the protein activity was checked. The inhibition zones were observed at 37 and 60°C. The maximum inhibitory zone was measured at pH 6 as shown in Figure 2. Result obtained by Saad MA *et al.*[19] showed the activity was stable even after heated for 30min at 100°C, which showed a better performance than this study.

Effect of enzyme on bacteriocin activity (proteolytic assay)

No zone of inhibition was found when the protein of interest was treated with proteinase K as shown in Figure 3. So it can be concluded that the partially purified protein sample got degraded and confirmed to be proteinaceous in nature. Similar result by Saad MA *et al.*[19] on the activity of Lactobacillus Acidophilus bacteriocin supported the present study.

Purification of Bacteriocin

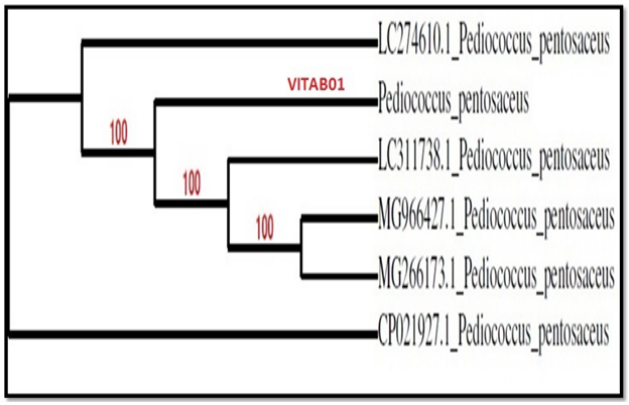

Figure 1: The phylogenetic position of the *Pediococcus pentosaceus* strain among neighbouring species.

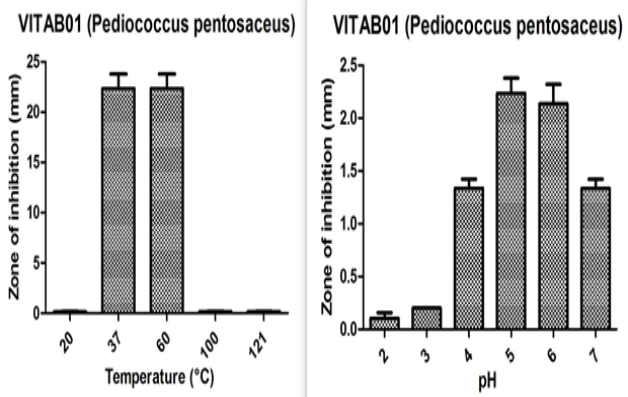

Figure 2: (a) Heat stability (b)Effect of pH on bacteriocin.

The cell free supernatant protein concentration was 100mg with specific activity of 128 (AU/mg). The purification data as obtained showed, the protein of interest got precipitated at 80% ammonium sulfate saturation. The total protein concentration of the precipitate was found to be 40mg, with 160.00 as the specific activity. The precipitate of 19.20mg on being subjected to ultrafiltration, was found in the retentate of the 10 kDa ultrafiltration membrane with specific activity of 166.66(AU/mg). In the gel filtration chromatography, the protein was loaded and all the fractions were pulled off and checked at 220/280nm using UV Visible spectrophotometer. The protein concentration of the active fraction was found to be 1.23mg, with 325.20(AU/mg) specific activity, 2.54 purification fold and a final yield of 3.12 as shown in Table 4. The molecular weight of the purified was determined using 12% Tricine SDS-PAGE and observed to be 17kDa as shown in Figure 4. Similarly, Marianne *et al*.[20] reported the specific activity of pediocin was found to be 300 AU/mg using the new procedure for purification. There are reports which showed very less yield of 0.98% for Enterocin LR/6.[21] Our results shows better specific activity when compared with the report

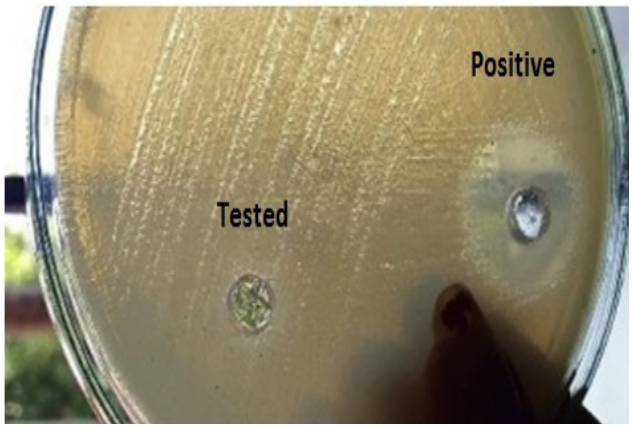

Figure 3: Effect of enzyme on the bacteriocin. The positive control without the enzyme showed a clear zone, with Proteinase K treatment, no zone of inhibition was obtained.

Antibacterial activity

Table 2: The table shows the antibacterial activity of the four major potent lactic acid isolates.

Isolates	*E.coli*	*Pseudomonas fluoroscens*	*Salmonella typhi*	*Staphylococcus aureus*
VITAB01	++	+	++	+
VITAB02	++	+	-	-
VITAB03	++	+	-	-
VITAB04	++	++	-	-

Plus(+) indicates zone of inhibition. (+) <1cm , (++) >1cm, (+++) >1.5cm

Biochemical characterization

Table 3: The table shows the biochemical characteristic of the potent isolate.

Isolate	Indole	Methyl red	Voges proskeur	Citrate utilization
VITAB01	(-)ve	(+)ve	(-)ve	(-)ve

Purification table

Table 4: The purification table shows the increase in specific activity with each purification step.

Purification fraction	Total Volume (ml)	Total bacteriocin activity (AU/mL)	Total Protein (mg)	Specific activity (AU/mg)	Fold Purification	Yield (%)
Cell free supernatant (CFS)	300	12800	100.00	128.00	1	100
Ammonium sulphate precipitation (80%)	150	6400	40.00	160.00	1.25	50
Ultra-filtration (10kDa)	10	3200	19.20	166.66	1.30	25
Gel filtration chromatography	5	400	1.23	325.20	2.54	3.12

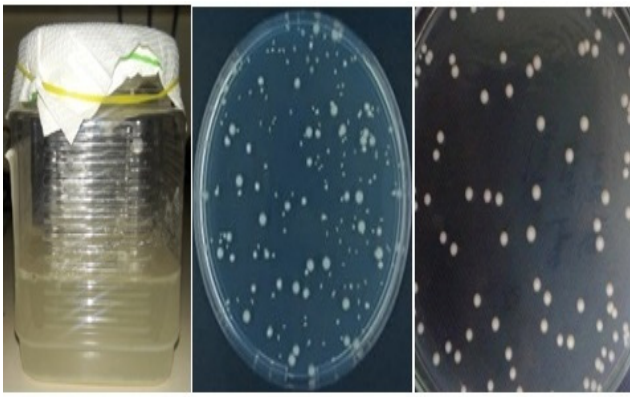

Figure 5: (a) The apple juice (b)Colonies before and (c) after the application of bacteriocin like substance.

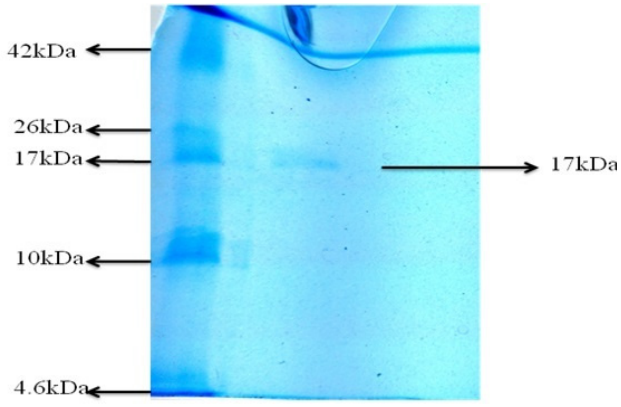

Figure 4: The 12% Tricine SDS-PAGE showed a single band at 17kDa with comparion to the protein marker (4.6- 42kDa).

of Tichaczek *et al.*[22] for bacteriocin purified by the earlier standard procedure shows a specific activity of about 45 AU/mg for 400ml.

Application

Efficacy of bacteriocin as biopreservative

A hand on preservative assay was done to check the activity of the partially purified protein sample on apple juice. The result showed that there was a drastical decrease in the microbial count on action with the protein sample ashown in Figure 5.

Number of colonies observed on 10^{-6} dilution of apple juice =82

CFU /mL = (No. of colonies * dilution factor) / volume of culture

= $(82 * 10^{-6})/0.1$

= $8.2 * 10^{-4}$ Cfu

On action of bacteriocin the colony count reduced to 60.

Cfu per mL = (no. of colonies * dilution factor) / volume of culture

= $(62 * 10^{-6})/0.1$

= $62 * 10^{-4}$ Cfu

Percentage decrease in colonies = 100 - [(60/82) * 100]

= 26.82 %

Our result suggested that bacteriocin producing *Pediococcus pentosaceus* naturally occurs and survives in appam batter.

In the present study used phenotypic characteristics for grouping the isolates and employed 16s rRNA sequence analysis to identify the representative isolate. The representative isolate has high similarity with the reference strain *Pediococcus pentosaceus.* The antibacterial activity exhibited by *Pediococcus pentosaceus* was proteinaceous in nature and stable at 60°C and in pH 6. Thus the study performed revealed that the protein from *Pediococcus pentosaceus* VITAB01 isolated from appam batter contains a wide range of inhibitory activity against pathogens like *Staphylococcus aureus* MTCC 5257, *Salmonella typhi* MTCC 2501, *Escherichia coli* MTCC 2089 *and Pseudomonas fluoroscens* MTCC 2173. It also showed a considerable decrease in microbial count when applied to apple juice. Similar study has also carried out by Mohammad Shaokat Ali *et al.*[23] which support this study. In their case the microbial count was reported to reduce drastically on incorporation of 5%bacteriocin, but in this study it has been clearly stated the reduction found to be 26.82 %.

For biopreservative effect of refrigerated food product for the longevity the organism producing bacteriocin *Pediococcus pentosaceus* will probably be useful in food products as it showed to maintain the microbial load as low as the 24th hr till 4th after incorporation. Hence this property will be useful to extend the shelf life of the refrigerated food material approximately for 3-4 days as can be concluded.

CONCLUSION

The present study revealed the bacteriocin from *Pediococcus pentosaceus* isolated from natural lactic acid fermentation of appam batter posess a wide variety of inhibitory activity against *Staphylococcus aureus* MTCC 5257, *Salmonella typhi* MTCC 2501, *Escherichia coli* MTCC 2089 and *Pseudomonas fluoroscens* MTCC 2173. Since lactic acid fermentation is employed mostly for the development of flavor and taste of fermented products, the production of bacteriocin in such products assumes more significance as biopreservative.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

LGRAS: Generally Recognized As Safe; **MTCC:** Microbial Type Culture Collection; **LAB:** Lactic Acid Bacteria; **MRS:** De Mann Rogosa and Sharpe; **AWDA:** Agar Well Diffusion Assay; **BLAST:** Basic Local Alignment Search Tool; **NCBI:** National Centre for Biotechnology Information; **MEGA:** Molecular Evolutionary Genetics Analysis; **SDS-PAGE:** Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis; **CFU:** Cell-Free Unit.

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SUMMARY

Lactic acid bacteria are considered to be a distinguished bacterial group because of their ability to produce lactic acid, natural antimicrobial peptide i.e., bacteriocins. Bacteriocins are antimicrobial peptides synthesized ribosomally which can be used as bio-preservative reducing the risk of chemical preservatives and also replacing the thermal treatments. The main aim of the study is to produce bacteriocin from *Pediococcus pentosaceus* isolated from appam batter to assess the bio-preservative aspect of apple juice.

- Among different isolates, one promising isolate VITAB01 *Pediococcus pentosaceus* was found positive for bacteriocin screening on Agar Well Diffusion Assay for foodborne pathogens such as *E.coli*, *Pseudomonas aeruginosa*, *Salmonella typhi*, and *Staphylococcus aureus*.
- The potent strain VITAB01 was further subjected to biochemical characterization and found to be *Pedioccoccus* by comparing with Bergey's manual and further identified as *Pediocccoccus pentosaceus* in 16S rRNA sequencing.
- Preliminary characterization confirms the zone of inhibition on heat stability at a maximum temperature of 37°C and 60°C. The effect of bacteriocin is stable at pH 5 and 6.
- Bacteriocin purified by using 80% ammonium sulfate precipitation method followed by 10kDa ultrafiltration membrane and fractionated in gel filtration chromatography (Sephadex G-50 column).
- The molecular weight was determined via a 10% SDS PAGE and found to be of 17kDa with a yield of 3.12% and increased with 325.20 (AU/mg) in a specific activity.
- Purified bacteriocin analysed for efficacy on bio preservative on the microbial load apple juice. In this case, the microbial count was reported to reduce drastically on the incorporation of 5% bacteriocin, but in this study, it has been clearly stated the reduction found to be 26.82 %.
- Hence this property will be useful to extend the shelf life of the refrigerated food material approximately for 3-4 days.

About Authors

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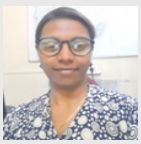
Jannatul Firdous Siddqiue: Pursuing Ph.D. (full-time- Scholar) in Industrial Microbiology at VIT,Vellore. Research interest is to work with protein and peptide based studies. Current focus is on the role of peptides from Lactic Acid Bacteria (LAB) as an anti-cancerous agent.

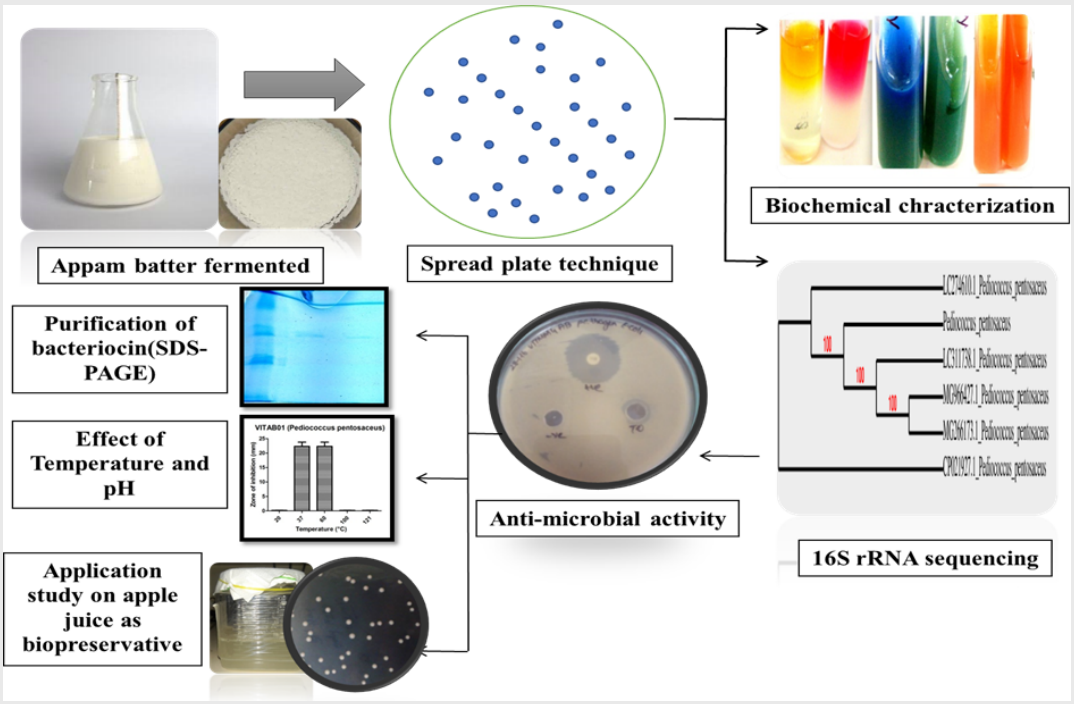


Nivetha A: Senior Research Fellow (SRF) – ICMR: She is pursuing a full-time Ph.D. in Industrial Microbiology at VIT, Vellore. Research focus is on the the production of beta-galactosidase for lactose free product development.

Dr. C. Subathra Devi is currently an Associate Professor at Vellore Institute of Technology , Vellore, Tamil Nadu, India. Her main research focus is on actinobacteria. Her lab is currently working on clot buster enzymes, actinokinases, nattokinase and recombinant streptokinase. Dr. Subathra"s lab focuses on a diverse array of actinobacteria related to anti-cancer, anti-bacterial and other bioactive metabolites. From her research lab two marine actinobacterial strains were deposited in National collection of industrial microorganisms (NCIM). She has published over 130 research papers, 3 books and 5 book chapters in the past 10 years in the field of microbial biotechnology.

Dr. Mohanasrinivasan is a Microbiologist working as an Associate Professor at Vellore Institute of Technology, Vellore ,Tamil Nadu, India. He has vast experience in teaching and research for about 15 years. He has published 107 papers in reputed journals and filed two research patents. Currently his research is focused on industrial enzymes and proteins to combat myocardial infarction and bacterial proteins for biopreservation with the application of nanobiotechnology.

PICTORIAL ABSTRACT



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