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Research Article

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Inhibition of Food- Borne Pathogens by Pediococcus Pentosaceus DS1 and in Silico Analysis of the Pediocin Gene

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Abstract: Global food safety is a huge concern, costing food industries billions of dollars every year. A healthy eating habit has become a myth due to an increase in food borne diseases. It is therefore necessary to avoid economic losses due to microbial spoilage and to preserve foods naturally in order to solve many of the current issues with food. Antimicrobial peptides isolated from bacteria have garnered considerable attention because of their potential benefits in extending the shelf-life of food products. *Listeria monocytogenes* and *Staphylococcus aureus* are two opportunistic pathogens which cause various food borne diseases. The aim of the study was to evaluate the production of antimicrobial compounds by the strain *Pediococcus pentosaceus* DS1 isolated from *ekung*, a fermented bamboo shoot product of North- East India. The main objectives of the study were to perform agar well diffusion assay for antimicrobial activity followed by characterization of the antimicrobial compound present in the cell free supernatant of the bacteria. It was observed that the antimicrobial peptide containing cell surface supernatant extracted from *P. pentosaceus* DS1 was able to inhibit *Listeria monocytogenes* (MTCC 839) and *Staphylococcus aureus* (MTCC 3160). PCR amplification led to the detection of a gene sequence in the genome of the strain *P. pentosaceus* DS1 which showed maximum similarity to pediocin, a 406 bp sequence. Pediocin belongs to a group of antimicrobial proteins known as bacteriocins which possess antimicrobial activities against food borne pathogens and spoilage bacteria. *In- silico* analysis indicated the presence of class IIa bacteriocin superfamily motif in the sequence. Class IIa bacteriocin producing bacteria isolated from fermented foods have a proven history of being used safely as antimicrobial agents in the food industry. Thus, this study reveals that *P. pentosaceus* DS1 has the potential to produce bacteriocinogenic agents that can be used safely to inhibit food pathogens.

Keywords: Fermented Food; Antimicrobial Agent; Food Pathogens; Bacteriocin; PCR; Gene Sequencing

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1. INTRODUCTION

Food- borne diseases are caused due to ingestion of contaminated food that triggers mortality and morbidity worldwide. The progress in industry encompassed by scientific inventions led to the improvement of human health and well-being. However, in order to cope up with modern lifestyle, food habits dramatically changed and preference of ready to eat food over home –made food are exposing people to food-borne pathogens that have the capacity to withstand food-processing conditions and cold storage. For instance, the link of the fast-food hamburger to E. coli O157:H7 and Listeria and Yersinia to cold storages can be taken into account [1]. As a consequence of direct contact of food- poisoning bacteria with antimicrobial residues present in food, resistant traits have emerged that pose a greater threat to human health [2]. In the light of such findings, it has become a necessity to adopt alternative strategies such as the use of probiotics for the control of food pathogens as well as their entry into the gut.
Probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host [3]. They comprise mostly of lactic acid bacteria (LAB) and Bifidobacteria that are added to foods as health beneficial adjuncts [4]. Many probiotics are formulated as adjuncts for different health benefits [5,6]. Antimicrobial compounds produced by LABs are known as bacteriocins which are ribosomally synthesized peptides that show antagonistic activity. Recently, the focus is on class II bacteriocins since they show activity against many food- borne pathogens including LIsteria monocytogenes and Staphylococcus aureus [7.8.] Pediocin is a class IIa bacteriocin that contains a conserved N-terminal region characterized by an amino acid motif known as pediocin box (YGNGVXCXXXXC XV) along with two cysteines joined by a disulphide bond 9. Pediocin PA-1/AcH contains an extra C- terminal disulphide bond that renders heat- stability of the bacteriocin and antimicrobial spectrum [10.] PCR based detection of YGNGV- motif containing pediocin is reported only by a few authors in some dairy starters of Pediococcus [11], although pediococcus is a common LAB present in wide varieties of fermented foods [12,13.] Different fermented bamboo shoot products are consumed by the ethnic people living in the biodiversity- rich northeast region of India including Arunachal Pradesh which provide optimum climate for the growth of many edible bamboo species. Ekung is such a product prepared by the Nyshi community of Arunachal Pradesh that employs their unique methods of fermentation [13.] In this study, the cell free supernatant was extracted from the strain Pediococcus pentosaceus DS1 and its antimicrobial activity was evaluated against different food-borne pathogens. Moreover, the sequence obtained after PCR amplification was screened for potential antimicrobial motifs and it was found to contain the YGNGV motif.

2. MATERIALS AND METHODS

2.1. Isolation of Lactic Acid Bacteria and Growth Conditions

Fermented bamboo shoot products were collected aseptically in sterile containers and stored at 4 °C. 1g of sample was blended with sterile 0.85% (w/v) saline solution using mortar and pestle under aseptic conditions and 10- fold serial dilution was performed. Different dilutions were spread on de Man, Rogosa and Sharpe (MRS) agar (Himedia Labs, Mumbai) and incubated at 37 °C under anaerobic conditions for 24h. Isolates were evaluated for their biochemical characteristics and Gram positive and catalase negative strains were selected for further evaluation. The indicator strains Listeria monocytogenes (MTCC 839), Bacillus cereus (MTCC 430), Staphylococcus aureus (MTCC 3160), Mycobacterium smegmatis (MTCC 14468) and Salmonella enterica typhimurium (MTCC 1252) were purchased from MTCC, IMTECH, India and were grown in tryptic soy broth (TSB) at 37 °C.

2.2. Determination of Bacteriocin Production

For the determination of bacteriocin production, a previously described method was adopted [14.] Fresh overnight culture was centrifuged at 10,000 rpm for 15 min min at 4°C to obtain the cell free supernatant (CFS) which was then filter-sterilized (0.22 µm, Millipore, Massachusetts, USA) and the pH was adjusted to 7.0 with NaOH. Fresh cultures of indicator strains (about 107 cfu/ml)) were spread on tryptic soy agar (TSA) plates and 6 mm wells were punctured onto the agar surface. 100 µl of CFS was placed on each well and incubated for 24 h at 37°C. Clear zones formed around the wells indicated pathogen inhibition and diameters of those zones were measured.

2.3. Template DNA Preparation

A colony was picked using a sterile tooth- pick and added to a PCR tube containing 90µl MilliQ water and 10µl 0.5M NaOH and heated for 5 min at 95°C. After heat treatment was over, the tube was centrifuged for 10 min at 10,000 rpm. 1µl of supernatant was used as a PCR template for the subsequent PCR reactions.

2.4. Molecular Identification of Bacteriocin- Producing Strain

The identification of bacteriocins- producing strains was done by 16S rDNA gene sequencing followed by phylogenetic tree construction. Universal primers analysis followed by phylogenetic studies. Universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGT TACGACTT-3′) were used for the amplification of 16S rRNA gene sequence. The PCR amplifications were performed in Eppendorf thermocycler in a total volume of 25 µl reaction mixture containing 5 µl of 10X Taq Buffer containing MgCl2, 14.8 µl nuclease free water, 2 µl dNTPs,1 µl of each primer, 0.2 µl of Taq DNA polymerase, and 1 µl of DNA template for each strain. Amplification parameters consisted of an initial denaturation step at 95 °C for 5 min, followed by 30 cycles of primer annealing for 30 sec at 53 °C, elongation for 1 min at 72 °C, and finally 10 min extension at 72 °C. PCR products were separated by electrophoresis in 1% (w/v) agarose gel. The amplified PCR product was purified and subjected to automated DNA sequencing using 3130 Genetic Analyzer (Applied Biosystem, Rotkreuz, Switzerland). The sequence was analyzed using the BLAST algorithm (http:// www.ncbi.nlm.nih.gov/blast) for homology searching. The phylogenetic tree was constructed by the neighbor- joining (NJ) method using MEGA 5.05 software. Data consistency was tested by bootstrapping the alignments 1000 times.

2.5. PCR Amplification of Pediocin Gene

Gene encoding for pediocin was amplified using the primers and conditions adopted by a previously published report 9.A forward primer (PFP2): 5´-TGGCCAATATCATTGGTGGT-3´ and reverse primer (PRP1): 5´-CTACTAACGCTTGGCTGGCA-3´ targeting a 406 bp sequence encoding pediocin gene that contains a signal peptide, mature pediocin and an immunity protein were designed and synthesized by Imperial Life Sciences (P) Limited, India. The product obtained after PCR amplification was resolved in 2% agarose gel and sequencing was done.

2.6. Nucleotide Sequence Translation And Structural Analysis

The nucleotide sequence of the bacteriocin gene obtained after sequencing was subjected to translation using the Translate tool from ExPASy server 15. The translated ORF which showed maximum similarity to bacteriocin II superfamily after BLAST analysis was taken for further analysis. The secondary structure prediction was done using the online server PSIPRED 16. The physicochemical properties of the translated protein such as molecular weight, theoretical pI, amino acid composition, atomic formula, extinction coefficients, instability index and Grand average of hydropathicity (GRAVY) were calculated using ProtParam 17.

2.7. Sequence Submission

The sequenced nucleotide sequences were submitted to the NCBI GenBank (http:// www.ncbi.nlm.nih.gov/genbank).

3. RESULTS

3.1. Isolation And Biochemical Characterization

From Gram staining and catalase tests the strain DS1 was found to be Gram positive cocci and catalase negative. Hydrolysis of starch, gelatine and citrate utilization was absent. Utilization of L-arabinose, ribose, D-xylose, galactose, glucose, fructose, mannose, rhamnose, n-acetyl-glucosamine, amygdalin, arbutin, esculin, salicin, cellobiose,lactose, melibiose, trehalose, raffinose, gentiobiose, and tagatose was observed.

3.2. Determination Of Bacteriocin Production

Neutralized cell free supernatants of DS1 showed activity against different Gram positive and negative bacteria as shown in the Table 1. The activity was lost in presence of proteinase K indicating proteinaceous nature of the antimicrobial substance. In presence of catalase the activity was not lost which indicates that the activity is not due to the production of hydrogen peroxide. In our study CFS of DS1 showed antimicrobial activity against Listeria monocytogenes, S. aureus and B. cereus but failed to inhibit Gram negative bacteria such as Salmonella and Yersinia and also Candida albicans. This implies the possibility of production of class IIa type bacteriocin by DS1.

Table 1: Antimicrobial activity of cell free supernatant of *Pediococcus pentosaceus* DS1

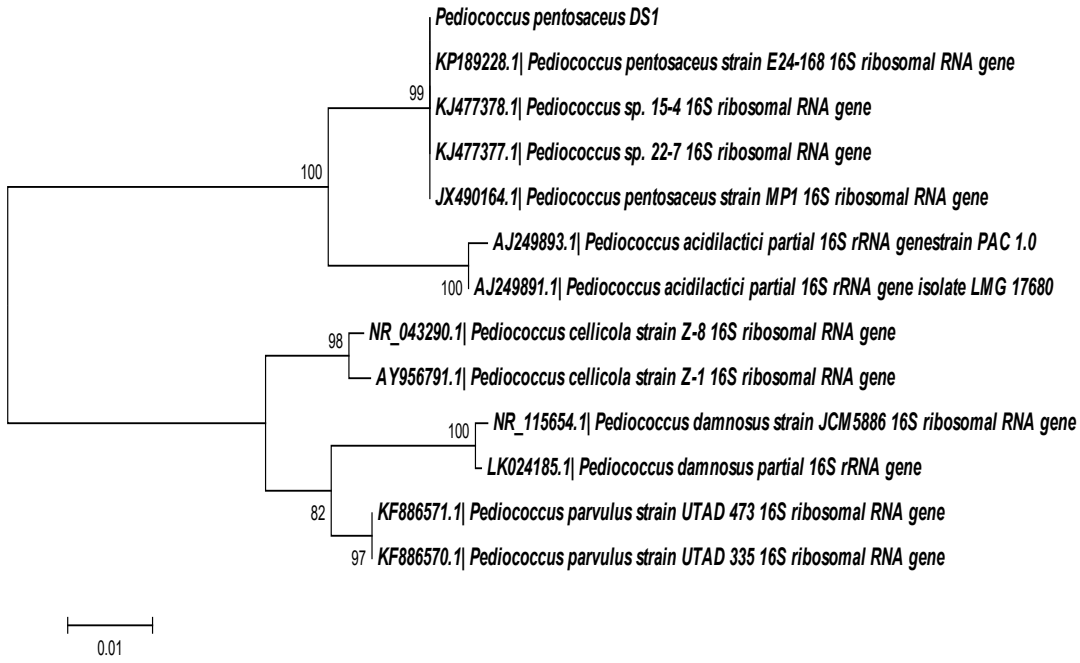
Strains	CFS	CFS (neutralized)	CFS (Proteinase- K treated)	CFS (Catalase treated)
Bacillus cereus (MTCC 430)	+	+	–	+
S. enterica typhimurium (MTCC 1252)	–	–	–	–
Y .enterocolitica (MTCC 859)	–	–	–	–
P. duminita (MTCC 3361)	–	–	–	–
Listeria monocytogenes (MTCC 839)	+	+	–	+
Staphylococcus aureus (MTCC 3160)	+	+	–	+
Mycobacterium smegmatis (MTCC 14468)	–	–	–	–
C. albicans (MTCC 183)	–	–	–	–

Table 1 shows the antimicrobial activity against different pathogen indicator strains. '+' indicates positive and '-' indicates negative result.

3.3. Molecular Identification Of Bacteriocin- Producing Strain

PCR amplification of 16S rDNA using the universal primers resulted in the generation of products with amplicon sizes ~1500 bp. Homology searching of the sequence which was obtained after sequencing resulted in sequence similarity of 99% with *Pediococcus pentosaceus* strain E24- 168. Fig. 1 depicts the phylogenetic tree constructed for the strain DS1 with closely- related strains using the neighbor- joining method. It was observed that the strain DS1 formed a monophyletic group with the strains having GenBank Accession Numbers KP189228.1, KJ477378.1, KJ477377.1 and JX490164.1.

Phylogenetic analysis of the strain DS1



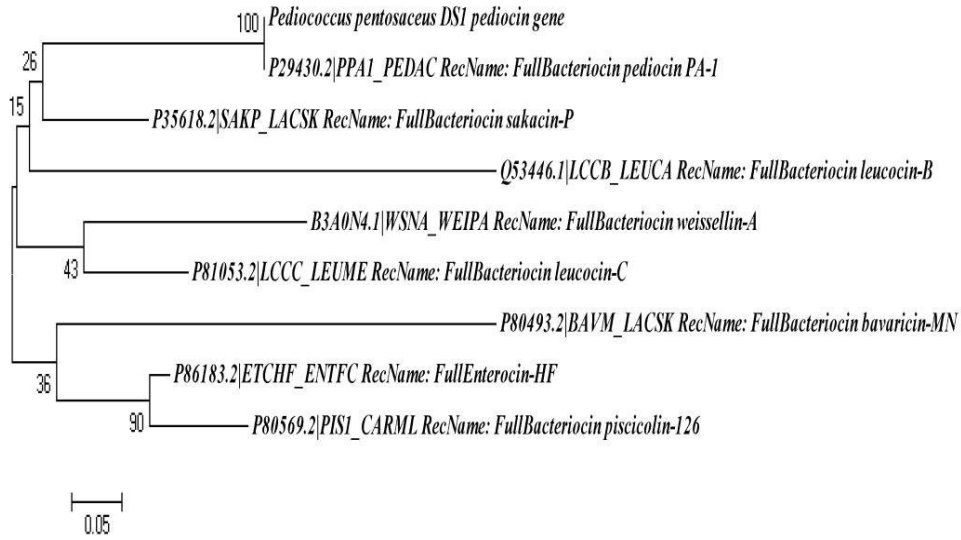
The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

FIG 1. Phylogenetic tree showing the relationship of strain DS1 with closely related species.

3.4 PCR Amplification of Pediocin Gene And Structural Analysis

PCR amplification using the primers PFP2 and PRP1 resulted in a 406 bp band after gel electrophoresis. Phylogenetic tree was constructed from the translated nucleotide sequence which was obtained after sequencing showed 100% similarity with bacteriocin pediocin PA-1 (Fig 2).

Phylogenetic analysis of the Pediocin gene



The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Multiple sequence alignment of data (Fig 3) clearly indicates the presence of the YGNGVXCXXXXCXV sequence motif at the N terminal terminus.

Fig 2. Phylogenetic tree showing the relationship *of P. pentosaceus* DS1 pediocin gene sequence with closely related species.

Multiple sequence alignment of pedicin gene

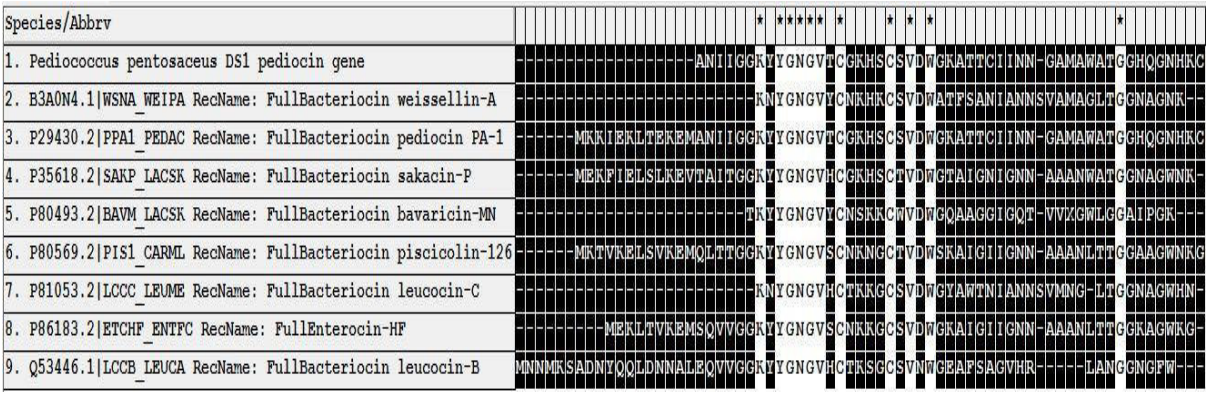


Fig 3. Multiple sequence alignment of *P. pentosaceus* DS1 pediocin gene

The secondary structure analysis reveals the presence of multiple strand- loop structures (Fig 4). Starting from the YGNGV motif there are three short β strands connected by a short loop or turn followed by a helix at the C terminus. The DISULFIND server which was used to predict disulphide bridges confirmed the presence of two disulphide bridges.

Secondary structure of pediocin gene

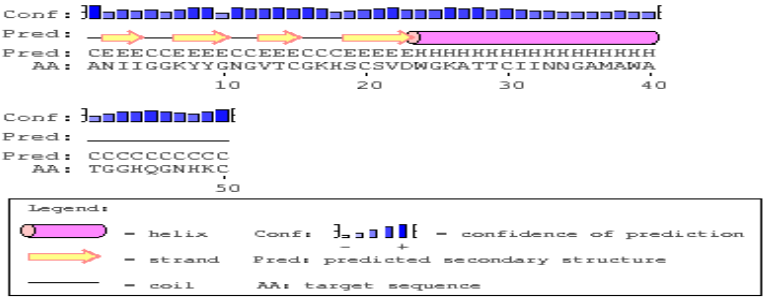


Fig 4. Secondary structure of *P. pentosaceus* DS1 pediocin gene as predicted by the PSIPRED server.

From the physicochemical properties as calculated by ProtParam analysis tool it was found that the protein has a molecular weight approximately 5.15 KDa with isoelectric pH of 8.85. The aliphatic index (AI) of a protein is the relative volume occupied by aliphatic side chains (alanine, valine, leucine and isoleucine). Higher values of AI, which in this case is 52.80 indicates thermal stability which is a property of class IIa bacteriocin. Moreover, the instability index was calculated as 10.34 that is lower than 40% indicates that the protein is stable under *in- vitro* conditions. Grand average hydropathicity (GRAVY) -0.304 indicates the protein is hydrophilic in nature.

Table 2. Physicochemical properties of *P. pentosaceus* DS1 pediocin gene

Amino acid	Molecular Weight (KDa)	Theoretical pI	Formula
50	5.1537	8.85	$C_{219}H_{336}N_{68}O_{67}S_5$
Aliphatic index	**Ext. coefficient (M^{-1} cm^{-1})**	**Instability index**	**GRAVY**
52.80	14230	10.34	-0.304

Table-2 shows different physiochemical properties of P. pentosaceus DS1 pedicin gene as calculated using the ProtParam analysis tool.

3.6 Sequence Submission

After submission to the NCBI GenBank 16S rDNA of P. pentosaceus DS1 obtained an Accession No KP723364 and the pediocin gene obtained an Accession No. KT345707

4. DISCUSSION

Diverse studies have been conducted for the isolation of probiotics from different sources. However, isolation of probiotics from fermented foods has been given preference because of their functionality and safety attributes [18, 19]. In this study, we isolated lactic acid bacterium DS1 from fermented food product and screened for antimicrobial activities using both microbiological as well as molecular tools. Based on the morphology and biochemical characteristics, the strain was presumptively identified as Pediococcus pentosaceus DS1 [20, 21]. Furthermore, the pattern of utilization of different sugars was found to be corroborating with previous observations [22]. P. pentosaceus DS1 showed activity against a number of gram positive and gram negative bacteria which includes food borne pathogens. Previously published reports give an idea about the antimicrobial spectrum of bacteriocins produced by P. pentosaceus which has activity against gram positive bacteria [23, 24]. In bacteria 16s rRNA is a component of the 30S subunit of ribosome which is also known as the small subunit of prokaryotic ribosome. 16s rDNA, or the gene coding for the 16s rRNA is used for the determination of phylogenetic

relationship among different bacterial species which remains highly conserved in different species of bacteria and archaea [25].Bacteriocins have the potential to play an important role in food biopreservation by acting as antimicrobial agents. The 406 bp of the pediocin gene which was sequenced showed 100% homology with pediocin gene can confirm the presence of the particular gene [26]. The structural analysis revealed the presence of YGNGV motif. This supports the findings of Sood et al [9]. Previous works on the secondary structure of class IIa bacteriocins yielded similar results [27]. Among different servers that predict the secondary structure of protein, PSIPRED is the most accurate one that employs two forward neural networks to analyze the outputs obtained from PSI- BLAST [16]. Presence of disulphide bridge is a characteristic of pediocin PA-1/AcH type bacteriocin and our finding also support the previously published works [28,29]. For different types of in vitro experiments, the bacteriocin has to be stable. Understanding a protein's physicochemical properties also supports the development of drugs and quality control [30]. Protparam analysis revealed that the protein is stable under in vitro conditions.

5. CONCLUSIONS

Bacillus cereus, L. monocytogenes and S. aureus are documented as food poisoning bacteria which possess the risk of becoming antibiotic resistant upon frequent contact with antibiotics. Because of the diverse epidemiology of infections and the development of antibiotic resistance of most of the pathogens, it is now necessary to substitute standard antimicrobial agents with alternative treatment agents. The use of inexpensive and "natural" food preservatives like lactic acid bacteria that produce antagonistic agents like bacteriocins can be regarded as a safe alternative for the treatment of food related diseases. This study mainly explores the effectiveness of bacteriocin producing strain Pediococcus pentosaceus DS1 against selected food borne pathogens. The molecular characterization results in the identification of pediocin which is a bacteriocin that is active against many food- borne pathogens. The study gives an insight into a strategy for the eradication of food poisoning and other related conditions without the creation of any antibiotic resistant traits.

6. AUTHOR CONTRIBUTION STATEMENT

All the authors have equal contribution in preparing the manuscript and revising. Surjya Loying performed the experimental part. All authors read and approved the final version of the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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