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FULL PAPER

Antimicrobial activity of *Lactobacillus pentosus* against the *Bacillus cereus* and *Klebsiella pneumoniae* strains

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SUMMARY

Food safety is the primary concern of the food industry. The aim of the current research is to study the antimicrobial effects of cell-free supernatant of *Lactobacillus pentosus* against *Bacillus cereus* and *Klebsiella pneumoniae*. *B. cereus* and *K. pneumoniae* were isolated from infant formula milk product and meat sample, respectively. Their identification was performed through morphological characterization and biochemical testing. Molecular identification of *K. pneumoniae* was based on 16s ribotyping. A previously isolated and reported strain of *L. pentosus* was used for the isolation of CFS (Cell-free supernatants). Antimicrobial activity was studied through agar well diffusion assay. Inhibitory activity was recorded by measuring the zone of inhibition. CFS activity was evaluated for temperature and pH. The antimicrobial activity of CFS of *L. pentosus* produced at different temperatures and pH was investigated against *B. cereus* and *K. pneumoniae*. A clear zone of inhibition was observed against *B. cereus* while no ZOI was formed against *K. pneumoniae*. *K. pneumoniae* was found resistant to the CFS. Crude bacteriocin exhibited heat stability for a temperature of 121°C for 30 minutes and pH range of 3-7. The current study concluded that bacteriocin produced from *L. pentosus* can be used for the control of *B. cereus*. Its heat and pH stability allows its potential therapeutic use in the food industry as a food preservative and to control food poisoning cases due to *B. cereus*. *K. pneumoniae* was found resistant to the isolated bacteriocin, and therefore *L. pentosus* cannot be used for control against *K. pneumoniae*.

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INTRODUCTION

Probiotics, used to control foodborne bacteria, are live microbes with beneficiary effects on host bodies (Simons *et al.,* 2020). The isolation of new promising probiotics from fermented food is now an emerging field in the food processing and health sector because of the above-mentioned features (Rajoka *et al.,* 2018). Lactic acid bacteria are considered safe sources of probiotics. Bacteriocins produced from lactic acid bacteria are getting more attention (Bhattacharya *et al.,* 2022). Bacteriocins have proved to be good substitutes for chemical preservatives. These are ribosomally-formed peptides or proteins with antimicrobial potential to inhibit or kill closely-related bacteria, and are degraded by enzymes present in the gut with no residual generation. These are heat-tolerant, non-toxic, safe substances with high efficacy against pathogens and food spoilage bacteria at nanomolar concentration (Meade *et al.,* 2020). Only nisin is a widely-used natural diet preservative (Des *et al.,* 2018). Some bacteria have developed resistance against nisin (Draper *et al.,* 2021). Still, the number of applicable bacteriocins is very low because of less heat tolerance, narrow inhibitory spectrum, and ecological instability. So, there is a need to identify more ecologically stable bacteriocins produced from different strains due to potential benefits, particularly in food processing as biopreservation.

Foodborne microbes not only cause pathogenicity but also mortality of consumers. This a public hygiene concern because it can approach endemic level due to contamination of food at any stage from processing to consumption. It has been reported that milk and its products can be associated with foodborne ailments due to *S. aureus, L. monocytogen, Campylobacter spp., Salmonella spp., E. coli, and Bacillus cereus* (Shabbir, 2021). *B. cereus* is a Gram positive, facultative anaerobic, spore-forming rod (Abdeen *et al.,* 2020). It is an opportunistic foodborne pathogen found in various foods and causes food poi-

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soning. *B. cereus* causes illness because of enterotoxins and emetic toxins (Berthold-Pluta *et al.,* 2019). Storage of raw milk without pasteurization can change milk quality because of proteolytic and psychrophilic and lipolytic microbial enzymes affecting the quality of food (Hamid and Fuzi, 2021).

Klebsiella pneumoniae causes numerous contagious diseases in immunocompromised persons (Effah *et al.,* 2020). It is the most common causative agent of nosocomial and community-acquired infection in immunocompromised individuals. It causes pneumonia, urinary tract infection, mastitis and endometritis (Jiang *et al.,* 2020). Food products may also be carriers for *K. pneumoniae*, which has been isolated from different food sources such as fruit juice, uncooked meat, fresh vegetables and ready-to-eat food. Several antibiotics are used to combat infections from *K. pneumoniae*, most are futile in treatment of these infections. *K. pneumoniae* is considered a multidrug-resistant bacteria. Consequently, an alternative strategy is needed to combat infections caused by resistant *K. pneumoniae*. The situation is even more alarming because it has been reported that *K. pneumoniae* is resistant to more than three classes of antibiotics (Junaid *et al.,* 2022).

The goal of the current research is the isolation and characterization of foodborne pathogenic bacteria from different locally-available food samples. Antimicrobial activity of a bacteriocin produced from *Lactobacillus pentosus* was evaluated to determine its ability to control the isolated foodborne pathogenic strains.

MATERIAL AND METHODS

Isolation and identification of Lactobacillus and test strains

Identified *Lactobacillus pentosus* was used for the present study (Akhtar *et al.,* 2020). Infant milk formula product and raw meat samples were used to isolate and identify two test strains. Characterization was based on morphology of colonies and biochemical test. Gram staining, catalase test, growth at various temperature and pH, growth on selective media and sugar fermentation tests, Indole, o-Nitrophenyl-D-galactoside (ONPG), oxidase, arginine dihydrolase (ADH), gelatinase, VogesProskauer (VP), tryptophan deaminase (TDA), citrate, urease, hydrogen sulfide (H2S) and ornithine decarboxylase (ODC) test (Merck, Germany), and tests according to Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1872; Orskov, 1984; Patel *et al.,* 2017; Rasool *et al.,* 2017) were performed. The test strain isolated from infant milk formula product was cultured on MRS agar. The other test strain isolated from raw meat was cultured on SS agar. Molecular characterization of the test strain cultured on SS agar was performed on the basis of 16S ribotyping. The genetic homology of the resulting sequences was found by comparison with other sequences present on the NCBI database through BLAST. The phylogenetic location of the strain was studied with the same bioinformatics tool.

Characterization of bacteriocin and antimicrobial assay

Cell-free supernatant (CFS) was isolated from *Lactobacillus pentosus*. The presence of bacteriocin in the supernatant was noted in the same manner as described earlier (Akhtar *et al.,* 2020). An agar well diffusion assay was performed to evaluate the inhibitory effects of the bacteriocin produced from *Lactobacillus pentosus*. The antimicrobial activity of bacteriocin was observed by measuring the zones of inhibition against test strains (Teixeira de Carvalho *et al.,* 2006). The CFS was treated with proteinase enzyme to evaluate that the antimicrobial activity was due to the bacteriocin (Pato *et al.,* 2022). Growth parameters such as pH, temperature, and incubation period were set and investigated for growth of *Lactobacillus*, which was linked with the production of bacteriocin (Akhtar *et al.,* 2020). Bacteriocin production was noted at different pH and temperatures. *Lactobacillus pentosus* was cultured in MRS broth at 30°C, 35°C, 40°C, 45°C, and 50°C (in triplicate). The antimicrobial efficacy of bacteriocin was evaluated by means of agar well diffusion assay. The effect of pH (3, 5, 7, 9, and 11) on bacteriocin activity was also noted by the same method. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was performed following the standard protocol (EZTM Pre stained protein ladder marker; cat # PM001; size ~12kDa to ~160kDa; bright reference bands: ~27kda to ~100kDa) to detect the molecular weight of the bacteriocin. The gel was then stained with coomassie brilliant blue R-250. The bacteriocin was characterized for heat stability and pH range. Isolated CFS of the *L. pentosus* culture in MRS broth (Lab M Ltd, UK) was heated at 50°C, 60°C, 70°C, 80°C, 90°C, 100°C and at autoclaving temperature (121°C). Thermally treated CFS samples were used to study the antimicrobial efficacy by means of agar well diffusion assay. Furthermore, the pH of the CFS supernatant was changed to 3, 5, 7, 9, and 11 and evaluated by means of antimicrobial well assay.

Statistical analysis

One way ANOVA followed by post hoc Duncan's test was used to compare the means of ZOI recorded at different pH and temperatures to ascertain the difference in significance.

RESULTS

The objective of the present work was to assess the antimicrobial efficacy of bacteriocin produced from *L. pentosus* against test strains, one belonging to the genus *Klebsiella*, the other to *B. cereus*.

Table 1 - *Colony morphology of test strains.*

Colony Parameters	*Strain I (N= 10)*	*Strain II (N= 10)*
Size (Mean ± S.E.M)	6.67 ± 0.882 mm	2-3 mm
Shape	Spherical	Round
Texture	Smooth	Smooth
Color	Creamy	Pinkish
Elevation	Low Convex	Convex
Margin	Undulate	Entire
Opacity	Opaque	Opaque

Table 2 - *Biochemical characterization of tests strains.*

Biochemical Test	*Strain I (N= 3)*	*Strain II (N= 3)*
Gram staining	+	–
Catalase	+	+
Oxidase	–	–
H2S	–	–
M.R	–	–
Citrate	+	+
Indole	–	–
Urease	+	+
Gelatinase	–	–
VP	+	+
ONPG	–	+
LDC	–	+
ADH	+	–
ODC	–	–
TDA	–	–
Glucose	+	+
Sucrose	+	+
Fructose	+	+
Maltose	+	+
Arabinose	–	+
Lactose	–	+
Rhamnose	–	+
Ribose	+	+
Melibiose	–	+
Mannitol	–	+
Sorbitol	–	+
Inositol	–	+
Amygdalin	–	+
Identification as	*Bacillus cereus*	*Klebsiella*

Screening and Identification of test strains

Colony morphology and biochemical testing results of test strains are shown in *Table 1* and *2*. Gram staining results specify that the strain cultured on SS agar was Gram negative rods. The strain grown on MRS broth was Gram positive rods. Biochemical test results indicated that the strain cultured on SS agar (Strain II) belongs to genus *Klebsiella* while the test strain cultured on MRS (Strain I) belongs to *Bacillus cereus* (*Table 2*). Molecular identification of the *Klebsiella* strain was performed through 16S ribotyping to get species-level identification. The obtained sequences were aligned using BLAST at NCBI to check genetic homology with other sequences in the database in order to identify the strain. Sequences confirmed homology to accession numbers NR_117683.1, CP010523.2, NR_117686.1 and NR_036794.1. Isolated *Klebsiella* strain got a 99% similarity index with *Klebsiella pneumoniae*, showing the highest score of 2706 bits. The result of sequencing for 16S ribotyping is shown in *Table 3*. Resulting sequencing peaks are shown in *Figures 1* and *2*.

Characterization of bacteriocin and antimicrobial assay

Treatment of CFS of *L. pentosus* with proteinase K enzyme showed loss of antimicrobial activity, as no zone of inhibition was observed against the test strain (*B. cereus*). Clear zones of inhibition were observed against the *B. cereus* strain by the bacteriocin in a temperature range of 30- 45°C. The largest zone of inhibition was observed at 40°C. *Figure 3A* depicts the ZOI of CFS against *Bacillus cereus*. Clear ZOI formed at 30°C, 35°C, 40°C, and 45°C, which indicates the antibacterial activity of the bacteriocin at these temperatures. No ZOI was seen at 50°C. *Table 4* shows values for the ZOI formed due to the antibacterial activity of CFS yielded by *Lactobacillus pentosus* against *B. cereus* at different temperatures. In the same way, clear zones of inhibition formed in the range of 3 to 9pH. The largest ZOI formed at pH 5. Bacteriocin produced from *L. pentosus* showed no inhibitory effects against *K. pneumoniae*, as no ZOI were observed at any temperature or pH (*Figure 3B*).

Table 3 - *Results of 16S ribotyping for the molecular characterization of strain Klebsiella pneumoniae.*

Subject	*Identification*	*Score*			*Identities*			*Strand*
Accession numbers	*Gene*	*Bit*	*Raw*	*E-value*	*Match*	*Total*	*Pct(%)*	
NR_117683.1	*Klebsiella pneumoniae* strain DSM 30104 16S ribosomal RNA gene, partial sequence	2706	1465	0	1469	1471	99	Plus/Plus
CP010523.2	*Klebsiella variicola* strain DSM 15968, complete genome	2700	1462	0	1468	1471	99	Plus/Plus
NR_117686.1	*Klebsiella pneumoniae* strain DSM 30104 16S ribosomal RNA gene, partial sequence	2684	1453	0	1465	1471	99	Plus/Plus
NR_036794.1	*Klebsiella pneumoniae* strain DSM 30104 16S ribosomal RNA gene, complete sequence	2684	1453	0	1466	1472	99	Plus/Plus

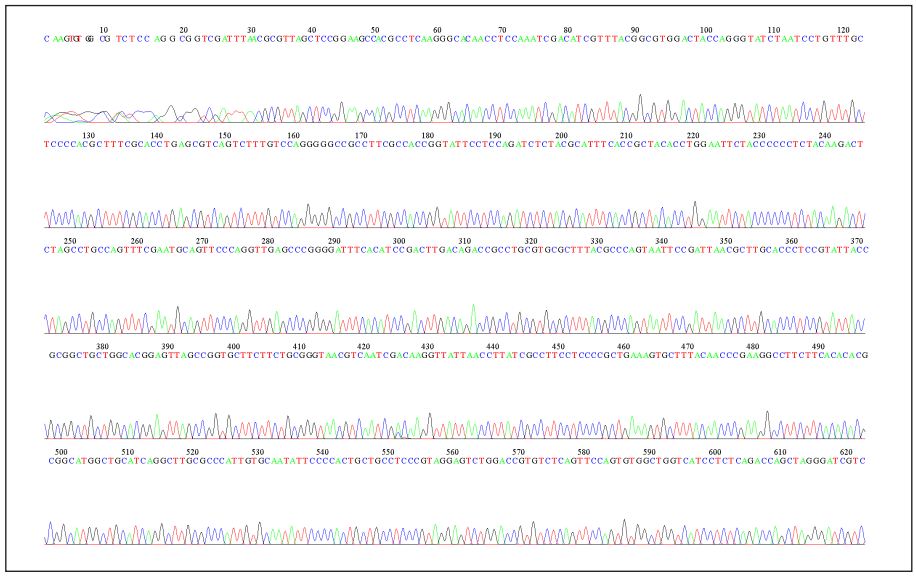

Figure 1 - *Obtained nucleotide sequencing peaks for 785F 5' (GGA TTA GAT ACC CTG GTA) 3' primer.*

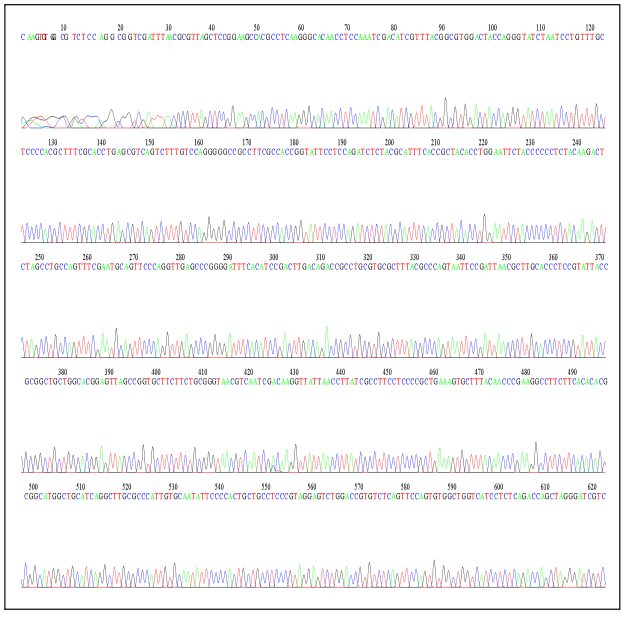

Figure 2 - *Obtained nucleotide sequencing peaks for 907R 5' (CCG TCA ATT CMT TTR AGT TT) 3' primer.*

SDS PAGE revealed that the molecular weight of the bacteriocin is almost 3 KDa. A heat stability experiment revealed that CFS heated to 50°C, 60°C, 70°C, 80°C, 90°C, and autoclaved produces clear ZOI and that the bacteriocin is heat stable. pH stability experiments revealed that from acidic to neutral pH range 3-7 antimicrobial activity was visible, although it decreased at alkaline pH.

DISCUSSION

B. cereus presence was observed by Meena *et al.* (2019) in milk and its products. Aman *et al.* (2021) studied the prevalence, antibacterial resistance, physiological features and enterotoxin genes (ces and hblA) of *B. cereus* in Egyptian dairy products. In a study, *B. cereus* was detected in 41.7% of formula milk samples and in 30% of UHT milk samples (Lesley *et al.,* 2017). In the present study, *B. cereus* was also isolated from formula milk product. Kurittu *et al.* (2021) also isolated *E. coli* and *K. pneumoniae* from different food samples to study their antibiotic-resistant genes.

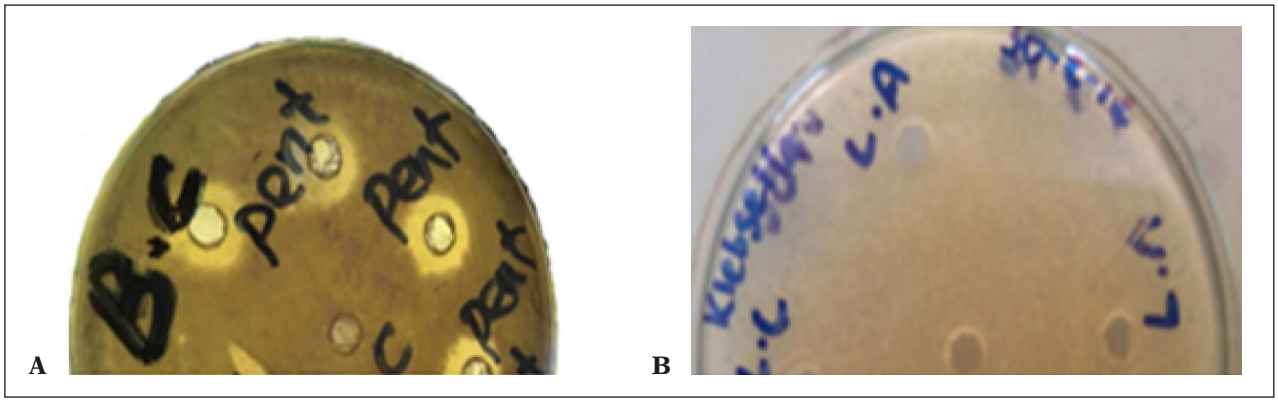

Figure 3 - *Antimicrobial activity of bacteriocin against B. cereus (A), and K. pneumoniae (B).*

Table 4 - *Effect of variation of pH and temperature on antimicrobial activity of bacteriocin produced by Lactobacillus pentosus against Bacillus cereus.*

No	*pH*	*ZOI (mm) Mean±S.E.M (N=3)*	*Temperature (°C)*	*ZOI (mm) Mean± S.E.M (N=3)*
1	3	3.62±0.130[c]	30	2.32±0.246[c]
2	5	5.32±0.101[a]	35	4.25±0.375[b]
3	7	4.48±0.101[b]	40	7.35±0.592[a]
4	9	1.53±0.117[d]	45	4.80±0.541[b]
5	11	0.00±0.00[e]	50	0.00±0.00[d]

One way ANOVA following post hoc Duncan's test performed to compare strain wise means. p≤0.0001. Means with same alphabets are not statistically significantly different. Distilled water was used as negative control.

A wide range of antagonistic action is a benchmark for choosing bacteriocin as a biopreservative. The current study reported a narrow range of antimicrobial activity of bacteriocin, since no inhibition was observed against *K. pneumoniae* but inhibitory effects were observed against *B. cereus*. It was reported that bacteriocin K2N7 had a narrow range of antimicrobial action with no inhibition against *E. faecium*, *E. coli*, *L. monocytogen, S. aureus,* and *B. cereus* (Watthanasakphuban *et al.,* 2016). The present study agrees with Rushdi and Rushdi (2022), whose study reported that CFS of *L. plantarum* and *L. acidophillus* has no inhibition against *Klebsiella pneumoniae*. Pentocin MQ1 was found to have strong inhibition against *B. cereus* ATCC14579, *M. luteus* ATCC, and *L. monocytogen* NCTC 10890. The broad antagonistic spectrum of pentocin MQ1 resulted in its wide application in medicine and in the food industry. It was demonstrated that *in situ* application of pentocin MQ1 was useful to extend the shelf life of bananas. The wide antimicrobial range of pentocin MQ1 makes it good for food preservation. Pentocin MQ1 reduces the number of spoilage bacteria. Reduction in the cell count of spoiling microbes on bananas favors the growth of LAB, which ultimately increases the shelf life of bananas (Wayah and Philip, 2018). Ren *et al.* (2022) reported antimicrobial activity of new *Lacticaseibacillus rhamnosus* strain A5 against *B. subtilis* and *E. coli*.

The heat, pH, and chemical stability of bacteriocin makes it suitable for use with food exposed to extreme conditions (Wayah and Philip, 2018; Hemu *et al.,* 2016). Bacteriocin activity was recorded between values 2-5 pH. Temperature stability was recorded up to 121°C for 30 minutes (Ren *et al.,* 2022). Wayah and Philip (2018) reported that Pentocin MQ1 produced from *Lactobacillus pentosus* CS2 was found heat and pH stable. Antimicrobial activity was recorded at acidic pH range of 2-5. Alkaline pH 8 causes an extreme decline in activity. Complete inactivity is seen at pH 10. Bacteriocin K2N7 lost activity at 121°C, and a pH stability range of 2-12 was recorded (Watthanasakphuban *et al.,* 2016). The current study reported that bacteriocin is heat resistant up to 121°C for 30 minutes. The pH stability range was 2-7 with complete loss of activity at alkaline pH.

The current study reported that the molecular weight of bacteriocin is 3 KDa. Our findings agree with Jiang *et al.* (2017). Pentocin JL-1 isolated from *L. pentosus* had a molecular weight of 2.987 KDa. It was also found sensitive to proteinase K enzyme, heat stable, and with a pH tolerance range of 5-7. Pentocin JL-1 exhibited antimicrobial activity against multi-drug resistant *S. aureus*. Wayah and Philip (2018) reported 2.1 KDa of molecular weight of pentocin MQ1. Dai *et al.* (2021) reported that pentocin ZFM94 had a molecular weight of 3,547.74 Da with a wide range of heat and pH stability. Pentocin ZFM94 also revealed a broad range of antimicrobial efficacy against Gram positive and Gram negative bacteria.

In the present study, bacteriocin produced from *L. pentosus* was susceptible to the proteinase enzyme. Similar findings were reported by Wayah and Philip (2018) for pentocin MQ1. Pentocin MQ1 was found susceptible to the proteinase enzyme, which caused the loss of antimicrobial activity. Treatment with catalase, lyticase and hyaluronidase retain activity, which proves the proteinaceous nature of pentocin MQ1. Bacteriocin activity was lost when treated with pepsin, trypsin and papain (Ren *et al.,* 2022). No loss of antimicrobial activity occurred when CFS of *L. pentosus* was treated with the catalase enzyme, revealing that antimicrobial activity derived from proteinaceous substances.

CONCLUSION

The present work described the therapeutic role of *L. pentosus* against *B. cereus*. *K. pneumoniae* was found resistant to CFS of *L. pentosus*.

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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