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Original Article

PURIFICATION AND CHARACTERIZATION OF PEDIOCIN PRODUCED BY PEDIOCOCCUS ACIDILACTICI NCIM 2292

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ABSTRACT

Objective: Pediocin, a bacteriocin produced by *Pediococcus acidilactici* NCIM 2292 showed broad inhibitory spectrum. The bacteriocin has sensitivity to proteolytic enzymes, NaCl, pH and temperature. The bacteriocin did not adhere to the surface of the producer cells. Its mode of action appears to be bactericidal, as determined against *Listeria monocytogenes* MTCC 839 and *Staphylococcus aureus* NCIM 2127.

Methods: Antimicrobial activity of pediocin has been tested against variety of microorganism. It was purified by ammonium sulfate precipitation followed by a Superose 12 fast performance liquid chromatography (FPLC). The purified bacteriocin was treated with proteases, NaCl, pH and temperature.

Results: Pediocin showed strong antimicrobial activity against *Listeria monocytogenes* and *Staphylococcus aureus* comparing to others orgamigms. Molecular weight of pediocin, estimated by SDS–PAGE was 5.0 kDa. The bacteriocin was inactivated by proteolytic enzymes such as trypsin, proteinase K, □-chymotrypsin, Pepsin, papain and pronase, but it was active when treated with α-amylase, catalase, lipase and NaCl. Pediocin activity was stable between pH 2.0-8.0 and heat resistant (15 min at 121°C). The bacteriocin was designated as pediocin 2292.

Conclusion: Experimental results showed that pediocin 2292 has special antagonism characteristics. The bacteriocin can be successfully utilized as potential bio-preservative in food industry.

Keywords: *Pediococcus acidilactici* NCIM 2292, FPLC, Pediocin 2292, *Listeria monocytogenes* MTCC 839, *Staphylococcus aureus* NCIM 2127, Bio-preservative.

INTRODUCTION

Listeria monocytogenes and *Staphylococcus aureus* have long been recognized as important food-borne pathogens. *Listeria monocytogenes*, a gram-positive bacterium is usually transmitted in human by ingestion of contaminated foods and causes a severe infectious disease in human known as listeriosis [1]. Listeriosis is characterized by meningoencephalitis, abortion, septicemia, and a high fatality rate (30%) and predominantly affects certain risk groups including pregnant women and their fetuses, newborns, elderly people and immunodeficient patients [1, 2]. *L. monocytogenes spp.* is commonly found in ready-to-eat food, mainly meat products without proper cooking [3], unpasteurized (raw) milk products [4], even in fresh salads [5]. *Staphylococcus* strains are facultative anaerobic gram-positive bacteria, produce staphylococcal enterotoxins which cause food poisoning. The symptoms of Staphylococcal food poisoning are nausea, violent vomiting, abdominal cramping, diarrhea and dehydration [6]. Foods that have been frequently contaminated in staphylococcal intoxication include meat and meat products, poultry and egg products, milk and dairy products, salads, bakery products, particularly cream-filled pastries and cakes, and sandwich fillings [7].

Bacteriocins produced by lactic acid bacteria showing antimicrobial activity against variety of gram-positive pathogens and food spoilers have been extensively studied to be used as an effective bio-preservative [8]. The use of nisin has been reported as biological food preservative to control *Listeria* and *Staphylococcus* in food [2]. However, pediocin produced by *peddiococcus* spp. has long been recognized as antilisterial bacteriocin [9] and has been shown as more effective against *L. monocytogenes* and *S. aureus* compared to nisin [10, 11].

Under the study, pediocin produced by *Pediococcus acidilactici* NCIM 2292 has been purified and has been characterized by different physicochemical properties including sensitivity to proteolytic enzymes and NaCl, stability in various ranges of temperature and pH, adsorption property to the producer cell. Mode of bacteriocin action has also been tested against *Listeria monocytogenes* and *Staphylococcus aureus.*

MATERIALS AND METHODS

Chemicals

MRS media, nutrient agar, brain heart infusion agar (BHIA) and trypticase soy broth (TSB) were purchased from Himedia, India. Low molecular weight marker, trypsin, proteinase K, □-chymotrypsin, pepsin, papain, pronase, catalase, lipase and α-amylase were purchased from Sigma-Aldrich India. Ammonium sulfate, sodium chloride, sodium phosphate and coomassie brilliant blue-R-250 were purchased from Sisco Research Laboratory Pvt. Ltd., India. All the fine chemicals used in the experiments were of analytical grade.

Microorganigms and media

Pediococcus acidilactici NCIM 2292 was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory (Pune, India) in freeze-dried form. MRS media was inoculated with 2 % (v/v) *Pediococcus acidilactici* NCIM 2292 culture and incubated for overnight at 30°C. The strain was propagated twice in media of goat meat processing wastes before use as inoculums. Preparation of goat meat processing wastes has been described in previous work [12].

Test organisms

The microorganisms employed as indicator strains to detect the antibacterial spectrum of pediocin produced by *Pediococcus acidilactici* NCIM 2292 were *Lactobacillus casei* NCIM 2360, *Lactobacillus acidophilus* NCIM 2909, *Lactobacillus plantarum* NCIM 2083, *Staphylococcus aureus* NCIM 2127, which were obtained from NCIM, Pune, India. *Enterococcus faecalis* MTCC 6845, *Bacillus cereus*

MTCC 430, *Bacillus subtilis* MTCC 441, *Listeria monocytogenes* MTCC 839, *Listeria monocytogenes* MTCC 657, *Staphylococcus aureus* MTCC 7443, *Escherichia coli* MTCC 41, *Proteus vulgaris* MTCC 742, *Pseudomonas aeruginosa* MTCC 647, *Salmonella typhimurium* MTCC 3224 were provided from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology, Chandigarh, India. Stock cultures of all strains were stored at−4 °C as slant culture and sub-cultured twice in their respective growth media before use in assays.

Analytical methods

The method of Lowry [13] was used for the determination of protein contents by comparing with standard curve. Bovine serum albumin (BSA) was used as marker. Pediocin activity was determined using well diffusion method as described in the previous article [12]. Instead of Staphylococcus aureus NCIM 2127, *Listeria monocytogenes* MTCC 657 was used as indicator strain.

Pediocin production

After 18 h of incubation at 30°C under optimized condition [12], *Pediococcus acidilactici NCIM* 2292 culture of 2 L volume was centrifuged (10,000× ,) for 20 min at 4°C. The supernatant was filtered through 0.22 µm membrane (Cellulose Nitrate Membrane Filters, Whatman) to remove all bacterial cells. The filtrate was known as cell free supernatant (CFS) or crude bacteriocin.

Purification of pediocin

Ammonium sulfate was gently added into CFS in 45% saturation for the precipitation of protein from the solution. Then the CFS was stirred continuously using a magnetic stirrer for 4 h and was left undisturbed at 4°C overnight till precipitation occurred. The mixture was centrifuged (20,000 rpm) for 1 h at 4°C. The precipitate was dissolved in 50 ml of 0.05 M sodium phosphate buffer (pH 7.0). The ammonium sulfate present in this solution was removed by subjecting the solution to dialysis using a spectra/por membrane with a molecular cutoff of 1000 Da against the same buffer overnight at 4°C. The dialyzed sample was again filtered through 0.22 µm membrane and purified using gel filtration technique. It was loaded to the gel filtration on Superose 12 fast performance liquid chromatography (FPLC, ÄKTAprime plus, Sweden) at room temperature, eluted using 0.02 M sodium phosphate buffer (pH 7.0) containing 0.15 M NaCl at flow rate 0.5 ml/min and monitored at 280 nm. The collected fractions were subjected for the assay of pediocin activity. The fractions exhibiting better titers of pediocin were dialyzed against distilled water overnight to remove sodium salt. The resulting sample was freeze-dried and stored at −4°C until for further use. At the end of each purification step, samples were collected and tested for pediocin activity and protein concentration.

Estimation of Molecular weight

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to estimate the molecular weight of pediocin in a slab gel system (Biotech, India). The freeze-dried pediocin of 10 µg was mixed up with the loading buffer of 5 ml, vortexed for 10 s followed by boiling in bath at 65°C for 5 min and cooled to room temperature. The wells prepared in stocking gel were loaded with samples of 10 µl volume and standard markers. The electrophoresis was conducted at constant current of 50 volts until the tracking dye reached at the bottom of the gel. Then the gel was divided in two parts. One half of the gel containing the sample and molecular weight markers was stained with 0.1% Coomassie billiant blue-R-250. Another half of the gel (not stained) was washed with sterile deionized water for 3 to 4 h and overlaid by 50 ml nutrient agar (1.0 % w/v) containing 500 µl overnight culture of test micro-organism (*Listeria monocytogenes* MTCC 839) as described by Powell *et al.,* (2007) [14]. The overlaid gel was incubated at 37°C for overnight and determined the positions of active pediocin. The molecular weight of pediocin was estimated by observing the possible band position with comparison to low molecular weight marker proteins and inhibited zone formed due to antimicrobial activity of pediocin on the gel.

Effect of proteases, NaCl, pH and temperature

The purified pediocin (freeze-dried) was dissolved in distilled water at a concentration of 457 AU/ml. It was treated with proteolytic enzymes, namely, trypsin, proteinase K, -chymotrypsin, pepsin, papain, pronase, catalase, lipase and α-amylase to test the sensitivity. All enzymes were used at a final concentration of 0.1 and 1 mg/ml individually. All samples were adjusted to pH 7.0 and then were sterilized by filtering through filter membrane of 0.22 µm. The filtrates were incubated at 30°C for 3 hr. Residual enzyme activities were finally stopped by heating with boiling water (65°C) for 5 min. After cooling at room temperature, the titers of pediocin were determined. Purified pediocin in buffers without enzyme, enzyme-buffer solutions and buffers were used as controls.

In a separate experiment, NaCl was added to purified pediocin (457 AU/ml) at the final concentration of 1% to test the effect of NaCl on its antimicrobial activity. NaCl in deionized water was used as control.

To determine the influence of pH on antibacterial activity of pediocin, the pH of the samples (457 AU/ml) were adjusted to various pH values ranging from 2.0-12.0 (at increments of two pH units), using 1N HCl or 1 N NaOH and incubated at room temperature (25°C) for 2 hr. Media contained meat processing wastes (pH range 2.0-12.0) was served as control. After that all samples were adjusted to pH 6.0 and sterilized by filtering through 0.22 µm filter membrane to determine the antimicrobial activity.

The effect of temperature on pediocin activity was tested by incubating the purified pediocin (457 AU/ml) in water bath at 40, 60, 80 and 100°C for 30 min, 60 min and 90 min, respectively. It was also separately treated with 121°C for 15 min and 20 min, respectively. Then antimicrobial activity of the samples was assessed against the indicator strain. The purified pediocin was stored for 6 weeks at−20, 4 and 30 °C and was assayed at 5 days intervals.

Mode of pediocin action

Purified pediocin (457 AU/ml) of 10 ml volume was added to 150 ml overnight culture of *L. monocytogenes* MTCC 839 and *Staphylococcus aureus* NCIM 2127 at different stages of growth (0, 3, 6 and 12 h) respectively and incubated at 37°C for 24 h. Optical density of collected samples were recorded at 600 nm. *L. monocytogenes* culture without bacteriocin was used as control.

Adsorption studies

To test the adsorption ability of pediocin to the producer cells, *Pediococcus acidilactici* NCIM 2292 was propagated at 30°C for 18 h. The pH of the culture was adjusted to 6.5 using 1 N NaOH to allow maximal adsorption of the bacteriocin to the producer cells, as described by Yang *et al.,* (1992) [15]. The cells were then harvested (10,000 ×g, 15 min, 4 °C) and washed with 0.1 M Sodium phosphate buffer (pH 7.0). The cells were re-suspended in 10 ml of 0.1 M NaCl. After adjusting the pH 2.0, the culture was stirred slowly for 1 h at 4 °C, allowing the release of pediocin from cells. Then the culture was then centrifuged (12,000 ×g, 15 min, 4 °C) and the CFS was re-adjusted to pH 7.0 with sterile 1 N NaOH to assay pediocin activity against indicator organism.

RESULTS AND DISCUSSION

Antimicrobial spectrum

Inhibitory spectrum of pediocin produced by *Pediococcus acidilactici* NCIM 2292 was tested against variety of gram-positive and gram-negative bacteria using CFS. As shown Table 1, pediocin was active against *Listeria monocytogenes, Staphylococcus aureus* and *Bacillus subtilis*. The bacteriocin was not able to inhibit the growth of *Bacillus cereus* and all of the lactic acid bacteria and gram-negetive bacteria. Similar observation have been previously reported for other pediocins such as pediocin PA-1 [16], Pediocin 05-10 [17] and Pediocin LB-B1 [18].

Purification of pediocin

The crude pediocin of 2285 AU/ml obtained from CFS was concentrated by ammonium sulfate precipitation followed by separation with suparose 12 FPLC. After precipitation with ammonium sulfate, the antimicrobial activity of pediocin against *L.*

monocytogenes was 18284 AU/ml. The purification and overall yield of pediocin at each step are shown in Table 2. Pediocin activity of 175552 AU/ml, 359-fold of purification and 130 % overall yield were achieved after separation by Superose 12 FPLC. SDS-PAGE was carried out to estimate the molecular mass of pediocin for the sample obtained from FPLC-active fraction as shown in Fig. 1. The analysis showed a single band with the size of 5.0 kDa (Fig. 1, lane 2). When the soft agar containing *Listeria monocytogenes* MTCC 839 was overlaid on gel for overnight, a clear inhibitory zone at approximately 5.0 kDa was detected (Fig. 1, lane 3).

Table 1: **Inhibitory activity of pediocin against indicator strains**

Indicator organism[a]	Fermentation condition			Inhibition[b]
	Medium	Incubation temperature/ time	Aeration	
Lactic acid bacteria				
Lactobacillus casei NCIM 2360	NA	30°C/24 h	anaerobic	–
Lactobacillus acidophilus NCIM 2909	NA	30°C/24 h	anaerobic	–
Lactobacillus plantarum NCIM 2083	NA	30°C/24 h	anaerobic	–
Enterococcus faecalis MTCC 6845	NA	30°C/ 24 h	aerobic	–
Other gram positive bacteria				
Bacillus cereus MTCC 430	NA	30°C/ 24 h	aerobic	–
Bacillus subtilis MTCC 441	NA	30°C/ 24 h	aerobic	+
Listeria monocytogenes MTCC 839	BHIA	37°C/ 24 h	aerobic	++
Listeria monocytogenes MTCC 657	BHIA	37°C/ 24 h	aerobic	++
Staphylococcus aureus NCIM 2127	NA	37°C/24h	aerobic	++
Staphylococcus aureus MTCC 7443	NA	37°C/24h	aerobic	++
Gram- negative				
Escherichia coli MTCC 41	NA	37°C/ 48 h	aerobic	–
Proteus vulgaris MTCC 742	NA	37°C/12 h	aerobic	–
Pseudomonas aeruginosa MTCC 647	NA	37°C/ 24 h	aerobic	–
Salmonella typhimurium MTCC 3224	TSB	37°C/24 h	aerobic	–

[b]–, no zone of inhibition, +, 1 mm< zone < 5 mm, + +: 5 mm < zone < 10 mm. NA: Nutrient Agar, BHIA: Brain Heart Infusion Agar, TSB: Trypticase Soy Broth.

Table 2: Purification of pediocin produced by *Pediococcus acidilactici* NCIM 2292

Purification step	Total volume (ml)	Total protein (mg)	Total units (×10³ AU)	Specific activity (×10³ AU/mg)	Overall yield (%)	Purification fold
CFS[a]	1800	4032	4113	1.02	100	1
ASP[b]	160	2023	2926	1.44	71	1.41
FPLC[c]	30.5	14.64	5354	365.71	130	359

[a]Cell Free Supernatant; [b]Ammonium Sulfate Precipitation; [c]Fast performance liquid chromato-graphy on a Superose 12 flow column.

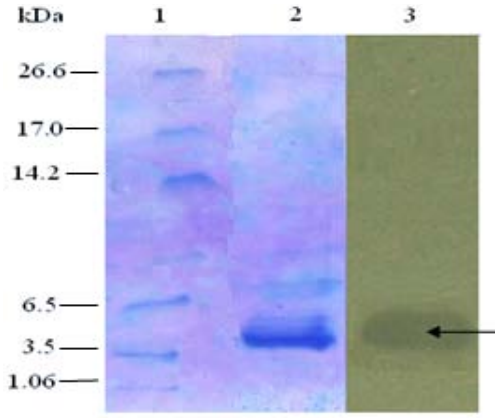

Fig. 1: Tricine-SDS-PAGE and detection of antimicrobial activity of the purified pediocin. (A) Gel stained with coomassie brilliant blue-R-250; lane 1, molecular weight standards; lane 2, purified bacteriocin by fast performance liquid chromatography. (B) Gel overlaid with indicator strain *L. mono*cytogenes MTCC 839. The arrow indicates the inhibition zone.

Sensivity of pediocin to enzymes, NaCl, pH and temperature

Antimicrobial activity of pediocin was completely inactivated after treatment of with trypsin, proteinase K, α-chymotrypsin, pepsin, papain and pronase as shown in Table 3. The sensitivity of pediocin to proteases indicated the proteinaceous character of the antimicrobial substance. However, treatment with catalase, lipase and α-amylase did not affect the antimicrobial activity (Table 3). Pediocin was stable after treating with 1% (w/v) of NaCl and was active at pH 2 to 8 after 2 h incubation. However, a decrease in pediocin activity was recorded at pH 10 and about 50% activity left at pH 12, which suggested that the bacteriocin was resistant at acidic condition. Significant reduction of antimicrobial activity was observed after treatment of purified pediocin (457 AU/ml) at 100°C for 90 min and 121°C for 15 min. But complete inactivation of its activity occurred at 121°C for 20 min. However, the activity was not reduced at 100°C for 60 min and others described temperature, which revealed that pediocin is high heat stable. Moreover, for necessary processing of food in different heating condition, heat stability is an important characteristic of food preservatives, because many food processing procedures need high temperature. In addition, storage of the purified pediocin for 6 weeks at -20, 4 and 30°C did not affect its antimicrobial activity. Similar observation has been previously reported for other pediocins [9, 10].

Mode of pediocin action

As shown in Fig. 2, the growth of both microorganisms namely *L. monocytogenes* MTCC 839 and *S. aureus* NCIM 2127 have been significantly decreased with the addition of pediocin (457 AU/ml) at four stages of growth (0, 3, 6 and 12 h) respectively. No inhibition of growth was observed in the sample of without pediocin. The experimental results suggested that the mode of pediocin action was bacteriocidal. So, the utilization of pediocin produced by *Pediococcus acidilactici* NCIM 2292 is a novel approach for inhibition or control *Listeria monocytogenes* and *Staphylococcus aureus* in food system.

Table 3: Factors affecting the antimicrobial activity of purified bacteriocin

Treatment	Pediocin
Enzymes (0.1 and 1 mg/ml)	
trypsin	–
proteinase K	–
□-chymotrypsin	–
pepsin	–
papain	–
pronase	–
catalase	+
lipase	+
α-amylase	+
1% (w/v) NaCl	+
pH (at 25°C for 2 hr)	+
2 – 8	+
8 –12	
(at increments of two pH units)	
Temperature (◦C)	
40 (30,60, 90 min)	+
60 (30,60, 90 min)	+
80 (30,60, 90 min)	+
100 (30,60 min)	+
100 (90 min)	–
121 (15 min)	+
121(20 min)	–
–20 (6 weeks)	+
4 (6 weeks)	+
30(6 weeks)	+

+: presence of inhibition zone (>2mm); –:no inhibition.

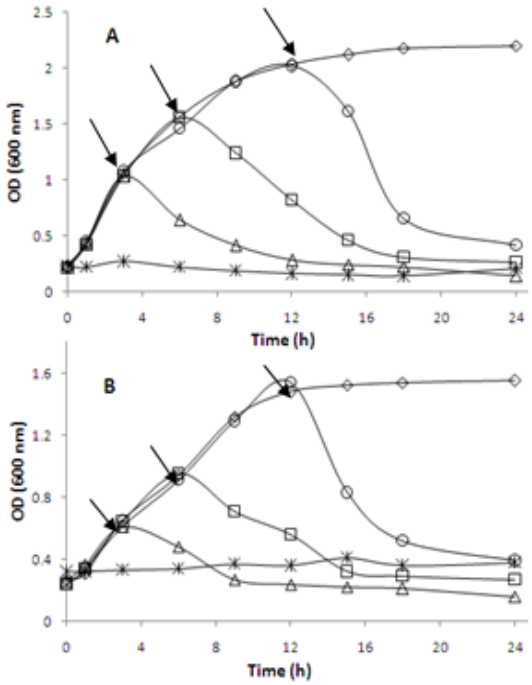

Fig. 2: Mode of pediocin action against (A) *Listeria monocytogenes* MTCC 839 and (B) *Staphylococcus aureus* NCIM 2127; Symbols (*, Δ, □ and ○ indicate the growth of test organisms with added pediocin (457 AU/ml) at 0 h, 3 h, 6 h and 12 h respectively; Optimal density of the cells measured at 600 nm; Arrows indicate addition of pediocin (457 AU/ml).

Adsorption studies of pediocin to producer cells

The results from the adsorption studies showed that pediocin did not adhere to the surface of the producer cell. Similar observations have been reported by other authors [9, 10, 19]. The purified pediocin was designated as pediocin 2292.

CONCLUSION

Pediocin produced by *Pediococcus acidilactici* NCIM 2292 was antagonistic against variety of food spoiler and food borne pathogen especially, *Listeria monocytogenes* MTCC 839 and *Staphylococcus aureus* NCIM 2127. It showed remarkable stability to heat (15 min at 121◦C) and cold treatments, as well as to a wide range of pH (2-8). It was sensitive to proteolytic enzymes, exception for catalase, lipase, α-amylase and NaCl. The mode of action of the purified pediocin appeared to be bactericidal. It did not adhere to the surface of the producer cell. It has high antilisterial activity. The purified pediocin designated as pediocin 2292 of 5.0 kDa molecular weight. Characteristics of pediocin 2292 indicated its potential application as a bio-preservativs in food products.

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