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PARTIAL CHARACTERIZATION AND PURIFICATION OF A NOVEL CLASS II A PEDIOCIN PRODUCED BY *PEDIOCOCCUS PARVULUS* STRAIN MF233 ISOLATED FROM BATTER OF IDLI A TRADITIONAL FERMENTED FOOD OF SOUTH INDIA FOR MANAGEMENT OF FOOD BORNE PATHOGENS

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ABSTRACT

Pediococcus parvulus MF 233 isolated from batter of idli a traditional fermented food of south India, identified based on its 16S rRNA gene sequence, produced pediocin that had broad spectrum of inhibition against Gram positive and Gram negative antibiotic resistant pathogenic, food spoilage organisms, Salmonella typhi, Escherichia coli., Pseudomonas aeruginosa and Staphylococcus aureus. Pediocin (designated as pediocin MF233) was purified by ammonium sulphate precipitation, dialysis (MWCO 1000D) and ion exchange chromatography (DEAE Cellulose) showed increased specific activity from 3.66 to 9.52, 27.50 and 216, with 2.6, 2.88 and 7.87-fold increase in purification of protein respectively. The molecular weight of pediocin MF233 was 3000 Da. Pediocin MF233 found to be most heat stable and showed full activity at 121 ^{0}C for 10 minutes and maintained full stability after storage for 60 days at –20 ^{0}C and for 40 days at 4^{0}C, partial stability at 37^{0}C for 20 and 40 days while stability decreased slowly thereafter and lossed completely by 60 days. Pediocin MF233 showed stability at pH 2 to 6. Active principle of Pediocin MF233 was proteinaceous in nature since it was inactivated by proteolytic enzymes, but not by non–proteolytic enzymes. UV radiation did not affect the activity of Pediocin MF233. The studies concluded that the ability of Pediocin MF233 produced by Pediococcus parvulus MF233 in inhibiting a wide-range food pathogenic and spoilage bacterium, is of potential interest for food safety and may have future applications as food preservative.

KEY WORDS

Pediocin MF233, partial purification, characterization, foodborne pathogens, inhibitory activity.

INTRODUCTION:

The empirical use of microorganisms and their natural products for the preservation of foods (biopreservation) have been a common practice in the history of mankind.[1] The lactic acid bacteria generally considered as food grade organisms since they are involved in numerous food fermentations, known to man for millennia, do not pose any health risk to man and are designated as generally regarded as safe (GRAS) status.[2] The inhibition of food spoilage microbes could be attributed to the production of antimicrobial compounds including organic acids, hydrogen peroxide, antibiotics and bacteriocins.[3] Many species of *Lactobacillus*, used in the manufacture of fermented dairy products, inhibit the growth of other bacteria

including the intestinal pathogens and spoilage organisms by producing anti-bacterial compounds or bacteriocins. Bacteriocins are polypeptides, with bactericidal or bacteriostatic activity against those bacteria which are closely related to the producer strain.[4] The bacteriocins produced by Gram positive bacteria, in particular, the lactic acid bacteria display fairly broad inhibitory spectra with food preservative and therapeutic potentials.[5-6] Considering this quality, there has been an increased concern in recent years on usage of bacteriocins due to the wide spread over-prescribing use of antibiotics and consequent increased development of antibiotic resistance. In order to use bacteriocin as food biopreservative and as a therapeutic agent, large-scale production is required with high level of activity. Bacteriocin of *Pediococcus* sp. have antilisterial activity, are thermostable and fall within the size range from 2867 to 4685 Da.[7]

Nisin and Pediocin are the well-studied bacteriocin products of lactic acid bacteria and are widely used in the food industry for over the past 40 years.[8-9] Several strains of *Pediococcus acidilactici* and *P. pentosaceus* are known to produce pediocins.[10] Most of pediocins are plasmid encoded, post translationally modified, small, heat-stable, non-lanthionine-containing peptides, belonging to the class II of pediocins.[4] So far, only nisin, the lantibiotic produced by *Lactococcus lactis*, is a commercial product and an approved food additive in most major food producing countries. However, increased interest has been developed in bacteriocins produced by *Pediococcus* spp., which are very likely to be used by the food industry in near future.[11-12] Most class IIa bacteriocins kill the targeted cells by membrane permeabilization and successive leakage of intracellular metabolites.[13]

Only a few bacteriocins have been purified and characterized, including SRCAM 602 from *Paenibacillus polymyxa*[14,] OR-7 from *Lactobacillus salivarius* [15], and E-760 and E50-52 from *Enterococcus.*[16-17] Purified pediocin AcH from a strain of *P. acidilactici* isolated from fermented sausage have been shown to possess inhibitory activity against a range of Gram-positive bacteria including *Listeria monocytogenes.* [18-19]

Many LAB pediocins have potential applications in the food industry for inhibiting the growth of foodborne bacterial pathogens. In this report, new pediocin producing *Pediococcus parvulus* strain MF233 isolated from idli batter. The pediocin MF233 with antimicrobial activity against Gram negative and Gram positive antibiotic resistant organisms were partially purified and characterized.

MATERIALS AND METHODS:

Bacterial strains and culture conditions

The pediocin producing *Pediococcus* isolated from idli batter and grown aerobically at 37^0 C in de Man Rogosa Sharpe (MRS) broth (HI media, India). *Escherichia coli.* MTCC 443, *Bacillus cereus* MTCC 430, *Staphylococcus aureus* MTCC 096, *Pseudomonas aeruginasa* MTCC 424, *Salmonella typhi* MTCC 734 and *Proteus vulgaris* MTCC 426 obtained from Microbial Type Culture Collection, Chandigarh, India. Food borne pathogens were isolated from meat. All the cultures were maintained in brain heart infusion broth (BHI broth, HI Media, India, 37°C). and stored as a 10% inoculum in the above media at - 80°C until required.

Strain identification

The *Pediococcus* was identified by sequencing the 16S rRNA (Euroffins Labs, Banglore, India) followed by a BLAST homology search with the database of NCBI Gene bank aligned using multiple alignment software program Clustal W and the phylogenetic tree was constructed using MEGA 4.

Isolation and identification of food borne pathogens (FBP)

FBP were isolated from perishable food meat. Ten meat samples were examined for initial pH, total viable count, proteolytic organisms and FBP. Sampling was done according to, the International commission on Microbiological Specification for Foods (ICMSF, 1986) and the International Standard Organization (ISO 948).[20] 10 gms of meat sample was homogenized with blander and 1:10 dilutions were prepared in 90 ml of 0.85% sterile physiological saline. Serial dilutions were made upto 10^{-7} to obtain different number of bacteria. The diluted samples were inoculated on Eosine methylene blue agar (EMB agar), Cystine Lactose Electrolyte Deficient medium (CLED agar), Mannitol salt agar (MS agar), Pseudomonas isolation agar (PI agar), Salmonella Shigella agar (SS agar) and Mannitol Egg Yolk Polymixin agar (MYP agar) and incubated at 37^0C for 24 h. The colonies of FBP obtained were identified on the basis of Gram staining , sugar fermentation (glucose,lactose and mannitol), hydrogen sulphide production, indole production, methyl red test, vogues proskauer test,

citrate utilization test, production of enzyme coagulase, urease and gelatinase.

Antibiotic sensitivity of FBP

The susceptibility of FBP studied against different antibiotics using the method of Brashears and Durre[21]. Antibiotic discs OCTA (HI Media) Imipenem10 mcg, Meropenum 10 mcg, Ciproflaxacin 05 mcg, Tobramycin10 mcg, Moxifloxacin 05 mcg, Ofloxacin 05 mcg, Sparfloxacin 05mcg, Levofloxacin 05mcg, Cephalothin 30 mcg, Clindamycin 02mcg, Chloramphenicol 30 mcg, Erythromycin 15 mcg, Gentamycin 10 mcg, Oxacillin 01mcg, Vancomycin 30 mcg and Ampicillin 10 mcg were used. Inhibition zone produced after incubation were recorded.

Antibacterial activity of Pediocin

Pediococcus inoculated in MRS broth and incubated at 37 ^{0} C for 24 h. Cell free supernatant (CFS) obtained by centrifugation at 12000 rpm, 4^{0}C for 13 min. To eliminate the possible inhibitory effect of hydrogen peroxide or lactic acid, pH was neutralized, and catalase treated supernatant of overnight culture was used. [22] Supernatant was sterilized by filtration through 0.22 µm millipore membrane filter and used as crude pediocin. 500 µl of 24 h broth culture *E. coli.* MTCC 443, *B. cereus* MTCC 430, *S. aureus* MTCC 096, *P.aeruginasa* MTCC 424, *S. typhi* MTCC 734 and *P.vulgaris* MTCC 426 and FBP was seeded on Muller- Hinton agar. Pediocin activity was, evaluated by measuring zone of inhibition.[23]

Effect of proteolytic enzyme, heat, pH, UV radiation and stability on storage

500 µL of pediocin was treated with 500 µL (1mg/ml) pepsin, lipase and catalase (Sigma) and incubated at 37^0 C for 2 h for testing the effect of proteolytic enzyme**.** To study the effect of heat and pH, 5 ml of pediocin was heated at 37, 50, 75, and 100^{0}C for 15 min and 121^{0}C for 10 and 15 min. pH was adjusted to 2, 4, 6, 8 and 10, using either NaOH or HCl (1M), allowed the samples to stand at room temperature for 4 h. For studing the effect of radiation, pediocin exposed to UV (254 nm.) from a distance of 30 cm for 1, 2 and 3 min. To study the effect of storage, pediocin was stored at -20, 4 and 37 ^{0} C for 20, 40 and 60 days. The antimicrobial activity of all the treated samples was determined by the agar well diffusion method using test organism *S. aureus.* [22]

FTIR of crude pediocin

The Infra-red spectrum of CFS was done by using FTIR (Make- Bruker Alpha model) to determine chemical nature of the compound.

Electrophoresis

SDS–PAGE electrophoresis was carried out by electrophoresing the gel at 100 V, until dye front reached 0.5 cm above the sealing gel at 5 °C for determining molecular weight of pediocin. Molecular weight standards (Bio Era, India) were employed as a protein marker.

Purification of pediocin

Crude pediocin purification was carried out by 80% ammonium sulphate saturation to get semi-purified pediocin.[24] This semipurified pediocin was subjected to dialysis using dialysis membrane MWCO 1000 D (Spectra) against 20 mM potassium phosphate buffer (pH 7.0) for 12 h at 4 ^{0} C. The precooled buffer after every 4 to 6 h was changed for three times (1 lit each time). Dialysed sample was purified by using ion exchange coloum Sephadex G 100 (Hi media, India). Column was pre equlibrited with 20 mM potassium phosphate buffer (pH 7.0) and bound proteins were eluted. The flow rate was adjusted to 24 ml / h and fractions of 1 ml each were collected. The concentrated extract was stored at -80 °C until use. After each step concentration, purification and activity/ml of protein was determined.[25]

RESULTS:

Strain identification

A blast search homology of 16s rRNA sequences identified the producer strain as *Pediococcus parvulus* MF233. Phylogenetic analysis of closely related sequence of *Pediococcus parvulus* MF233, revealed that *Leuconostoc mesenteroides* and *Lactococcus lactis* were clustered in one lineage whereas other three species *Lactobacillus plantarum*, *Pediococcus parvulus* and *Streptococcus agalactiae* were shown to diverge from each other (Figure 1).

Phylogenetic tree of *Pediococcus parvulus* strain MF233 (among on five species / genus of Lactobacillales).

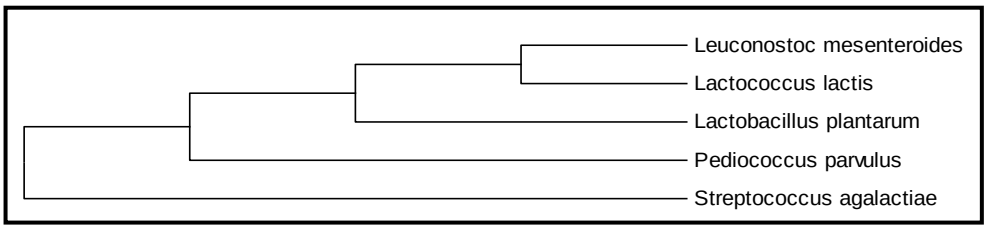

Fig 1

Assay for antimicrobial activity

The results of antimicrobial activity of pediocin MF233 are shown in table 1. Pediocin MF233 significantly inhibited the growth of both food borne pathogens isolated from meat and control strains, commonly associated with food spoilage. Inhibitory zones for isolated pathogens (13.4 to 14.6 mm) were slightly higher than control strains (12.2 to 14.0 mm).

Table 1: *Antibacterial spectrum of crude Pediocin MF233*

FBP / Control strain	ZOI in (mm)
E. coli	13 .8
S. aureus	14 .6
P. vulgaris	13 .4
S. typhi	13 .5
P. aeruginosa	13 .7
B. cereus	14 .5
E. coli. MTCC 443	13.0
B. cereus MTCC 430	13.9
S. aureus MTCC 096	14.00
P.aeruginosa MTCC 424	12 .8
S. typhi MTCC 734	12 .5
P.vulgaris MTCC 426	12.2

Antibiotic sensitivity of FBP

The data for antibiotic sensitivity of FBP is presented in Table 2. With few exceptions all the antibiotics exhibited good inhibitory action towards FBP. Of the sixteen different antibiotics used, no resistance was observed in *Proteus vulgaris*, however *S. typhi* was resistant to four different antibiotics. Sensitivity of food borne pathogens varies with the antibiotics.

Table 2: *Antibiotic sensitivity of FBP*

Antibiotics	Conc. in mcg	*E. coli.*	*S. aureus*	*P. vulgaris*	*S. typhi*	*P. aeruginosa*
Imipenem	10 mcg	ND	ND	10 mm	ND	ND
Meropenum	10 mcg	25 mm	ND	18 mm	ND	18 mm
Ciproflaxacin	05 mcg	22 mm	ND	25 mm	17 mm	26 mm
Tobramycin	10 mcg	10 mm	R	11 mm	11 mm	19 mm
Moxifloxacin	05 mcg	21 mm	ND	16 mm	18 m	24 mm
Ofloxacin	05 mcg	20 mm	11 mm	15 mm	14 mm	26 mm
Sparfloxacin	05 mcg	21 mm	ND	16 mm	ND	25 mm
Levofloxacin	05 mcg	23 mm	ND	17 mm	16 mm	28 mm
Cephalothin	30 mcg	ND	11 mm	18 mm	12 mm	ND
Clindamycin	02 mcg	ND	14 mm	18 mm	ND	R
Chloramphenicol	30 mcg	ND	10 mm	16 mm	R	R
Erythromycin	15 mcg	R	15 mm	12 mm	R	12 mm
Gentamycin	10 mcg	R	17 mm	13 mm	14 mm	15 mm
Oxacillin	01mcg	ND	R	12 mm	R	ND
Vancomycin	30 mcg	ND	18 mm	10 mm	11 mm	R
Ampicillin	10mcg	ND	R	ND	R	ND

ND- Not determined, R- Resistant

Effect of proteolytic enzymes, heat, pH, UV radiations and stability of pediocin on storage

A total loss of activity was observed with proteolytic enzyme, pepsin exposed samples indicating the protein nature of the compound while the catalase and lipase exposed samples did not show any change in the activity. Pediocin exposed to 37 ^{0}C, 50 ^{0}C, 75 ^{0}C, 100^{0}C for 15 min and at 121^{0}C for 10 min, retained full stability, with 1100 AU/ml. At 121^{0}C for 15 min, activity was lowered to 700 AU/ml, indicating partial stability to 121^{0}C for 15 min. pH 2, 4 and 6 retained full activity, while activity was reduced to 700 AU/ml at pH 8 and further reduced to 300 AU/ml pH to 10. Pediocin remained stable when exposed to UV 254 nm. (Table 3).

Table 3: ***Effect of heat, pH, enzyme and UV radiation on activity of Pediocin MF233***

Treatment		Pediocin activity (AU/ml)
Control		1100
Heat treatment	37^{0}C 15 min	1100
	50^{0}C 15 min	1100
	75^{0}C 15 min	1100
	100^{0}C 15 min	1100
	121^{0}C 10 min	1100
	121^{0}C 15 min	700
Enzyme	Catalase	1100
	Pepsin	0
	Lipase	1100
pH	2	1100
	4	1100
	6	1100
	8	700
	10	300
UV	1 min	1100
	2min	1100
	3min	1100

During storage at -20 ^{0} C, pediocin retained full activity for 20, 40 and 60 days. At 4 ^{0} C the activity was retained for 20 and 40 days while gradual reduction in activity was observed on storage for 60 days. At 37^0 C, for 20 days, the activity was reduced by 500 AU/ml, further decrease was observed by 300 AU/ml on storage for 40 days, while the activity was totally lossed during storage for 60 days (Table 4).

Table 4: ***Effect of storage time and temperature on activity of pediocin MF233***

Storage Time (days)	Temperature (^{0}C)	Activity AU/ml
control		1100
20	-20	1100
	4	1100
	37	600
40	-20	1100
	4	1100
	37	300
60	-20	1100
	4	900
	37	nil

Purification of pediocin MF233

The crude pediocin MF233 showed the activity as 1100 AU/ml. Purification with ammonium sulphate precipitation showed the activity of 2000 AU/ml, (81% increase), with dialysis technique showed, 2200 AU/ml (100% increase), while with ion exchange chromatography 2600 AU/ml (136 % increase), indicating protein concentration at each step (Table 5).

Table 5: *Purification of pediocin MF233*

Purification stage	Volume	Activity AU/ml	Total activity [a]	Protein (µg /ml) [b]	Specific activity [c]	Purification [d]
Culture supernatant	250 ml	1100	275000	300	3.66	1
Ammonium sulphate precipitation	20 ml	2000	40000	210	9.52	2.60
Dialysis	5 ml	2200	13200	80	27.50	2.88
Ion exchange chromatography	2 ml	2600	5200	12	216.60	7.87

a-Total activity determined by the multiplication of volume by activity, b -Total concentration determined by the Lowrys method, c- Specific activity is the activity units divided by protein concentration (AU/ µg /ml), d- Purification is the increase in the initial specific activity.

FTIR of crude pediocin

The FTIR spectrum of crude pediocin of *Pediococcus parvulus* MF233, is shown in Fig 2. Spectra clearly showed that crude pediocin contains CO-NH group (Peptide linkage). The bands of N-H stretching vibration at 3280 - 3311 cm^{-1} and C=O stretching vibration in the region 1635 - 1640 cm^{-1}. C=O stretching frequency decreases because it is in conjugation with NH group. This clearly indicates protein nature of the compound Fig 2.

FTIR of pediocin MF233

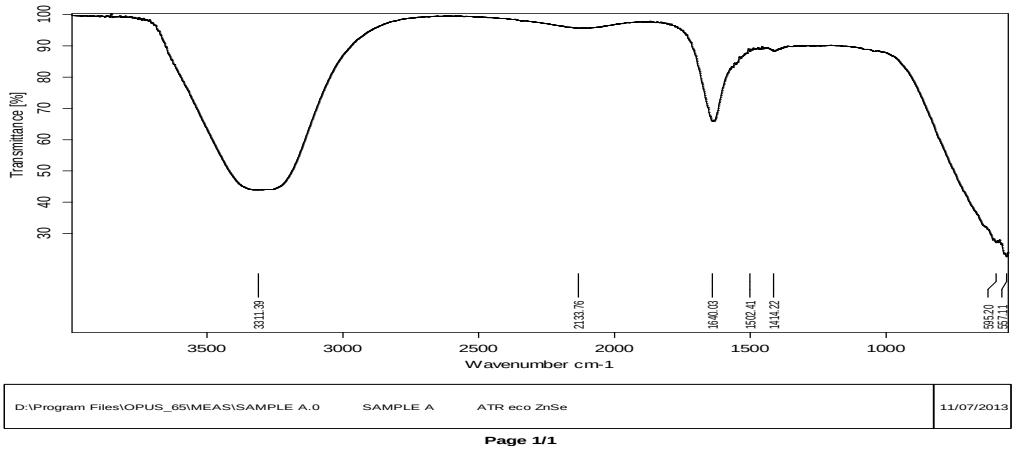

Fig -2

Electrophoresis

The molecular weight of purified protein pediocin was determined by SDS-PAGE electrophoresis and was found to be 3000 Da. [Fig 3]. The purified pediocin was isolated from *Pediococcoccus* sp. and therefore, it belongs to the class IIa of pediocins.

SDS-PAGE, Lane- 1 Protein markers as indicated. Lane- 2, Pediocin MF233

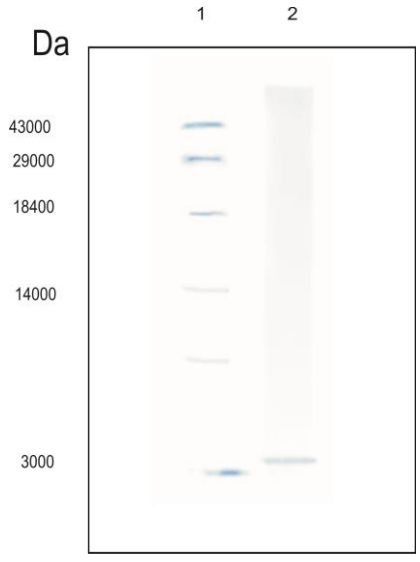

Fig. 3

DISCUSSIONS:

The choice of food source is important for preferred isolation of bacteriocin producing bacteria. Milk and meat products.[26], fresh cut vegetable products[27,] cheese and yogurt [28] are among the commonly used food sources. Idli batter used for preparing Indian cuisine idli used in present study can be a good source for pediocin production.

Bacteriocinogenic microbes produced wide array of antimicrobial compounds against food borne pathogens. Bifidin1 from *Bifidobacterium infantis*[29], thuricin 7 from *Bacillus thuringensis*[30] and pediocin PA1from *Pediococcus pentosasus*[31] are variously reported bacteriocin. Our results are in agreement with these studies for production of an antimicrobial compounds pediocin MF233 from *Pediococcus parvalus.* Narayanapillai et al.[32] isolated LAB from chick intestine, showed production of antimicrobial compounds against *E. coli, P. aeruginosa, S. typhi, S. aureus, B. cereus, P. mirabilis* and *K. Pneumoniae* by the agar well diffusion method. Messaoudi et al.[33] showed salivaricin SMXD51 inhibited the growth of Gram positive and Gram-negative bacteria but had no antimicrobial activity against fungi.

Ogunbanwo et al.[22] showed bacteriocin produced by *L. brevis* OG1 to be the most heat stable, as the activity (3200 AU/ml) remained constant after heating at 121ºC for 60 min , but declined thereafter, while bacteriocin produced by *L. plantarum* F1 activity (6400 AU/ ml) remained constant after heating at 121ºC for 10 min followed by subsequent decline. According to Miao et al.[34] pediocin F1 was found to be heat stable at 60, 80, and 100 °C for 60 min, and 121°C for 20 min. In the present work the partially purified pediocin MF233, was not affected by temperature at 121^0C for 10 min but get affected at 121^0C for 15 min. Thermostability of the all pediocin were important with regard to using pediocin in food and should be considered when thermal treatments are included in food processing.

Anastasiadou[35] demonstrated Tricine SDS-PAGE analysis confirmed a bactericidal peptide band of 5–6 KDa. Bacteriocin of *Pediococcus* sp. have antilisterial activity, are thermostable and fall within the size range from 2867 to 4685 Da [7]. Most of pediocins are plasmid encoded, post translationally modified, small, heat-stable, non-lanthionine-containing peptides, belonging to the class II of pediocins[4].The purified pediocin was isolated from various *Pediococcoccus* sp. and therefore, it belongs to the class IIa of pediocins. The results of present study showed the bacterial peptide band of 3 KDa with SDS-PAGE of purified pediocin MF233 and hence the pediocin belongs to the class IIa of pediocins. The FTIR spectrum of crude pediocin of *Pediococcus parvulus* MF233, clearly showed that crude pediocin contains CO-NH group (Peptide linkage). C=O stretching frequency decreases because it is in conjugation with NH group. This clearly indicates protein nature of the compound.[36]

The isolates of the present work were treated with the various enzymes viz. catalase, pepsin and lipase. The antimicrobial activity was adversely affected when treated with pepsin but not affected with the treatment with catalase and lipase, demonstrating the protein nature of pediocin MF233. The result of the present work agreed with result of Coventry et al.[37]

During the purification procedures, each step resulted in considerable loss of protein while specific activity was increased. The optimum pediocin MF233 recovery was achieved by including ammonium sulphate precipitation. The results agreed with the findings of Ivanova et al[38]. During salt precipitation various amount of the protein was fractionated as a surface pellicle, this might be due to the association of pediocin molecules with the hydrophobic globular micelle like structure in the supernatant fluid. Similar observations have also been recorded for lactocin S and lactacin F[39]. A considerable loss of the pediocin MF233 activity was observed during ultrafiltration which might be due to absorption of the pediocin on the membrane [40]. Dialysis experiment showed that the small molecular weight proteins and short peptides passed through the membrane dialysis tubing which is a cellulose material used in the removal of salts and low molecular weight compounds during the purification of biomolecules since the dialysis membrane of (MWCO 1000 D) was used. Coventry et al. [37] purified Pediocin PO_2 for bio preservation of meat products. The dialysed solution applied ion exchange resin activity was increased 3-fold after $(NH4)_2S0_4$ precipitation. The present work also demonstrated 2.60-fold increase in purification agreed with results of Coventry et al.

CONCLUSION:

In the present study novel pediocin MF233 was isolated from *Pediococcus parvulus* MF233 from idli batter. The pediocin demonstrated thermostability and stability

towards the storage and UV. These characteristics are very important with regard to using pediocin MF233 in food processing. Most of the pediocins produced by LAB are only active against Gram-positive organisms. The effect of pediocin MF233 produced *in situ* against Gram negatives is important since most of the foods borne pathogens are Gram negatives. Pediocin MF233 demonstrated antimicrobial activity against both Gram positive and Gram-negative organisms. These results suggest that, *Pediococcus parvulus* MF233 could be used as a natural biopreservative to preserve the food products.

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