Production and Characterization of a Bacteriocin-Like Inhibitory Substance Produced by Indigenous Soil Associated *Pseudomonas putida* MAS-1

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Summary: Bacteriocins have been the subject of extensive research globally due to wide range applications. The aim of this research was to investigate the production of bacteriocin(s) or bacteriocin like inhibitory substance(s) by *Pseudomonas putida* MAS-1 strain. The bacteriocin produced (putidacin MAS-1) was found bioactive against clinical *Staphylococcus aureus* and *Enterococcus faecalis* strains. Bioactivity was observed by stab & overlay assay and multiwell antagonistic activity assay. Putidacin MAS-1 was sensitive beyond 70°C but stable at wide pH range (3 to 8). Bioactivity of putidacin MAS-1 was lost after treatment with trypsin and protease while partially lost after Proteinase K treatment. Treatment with ethanol, methanol, chloroform, acetone, Tween 20 and Tween 80 showed a partial decrease in bioactivity. SDS had stimulatory effect on putidacin MAS-1 bioactivity. EDTA however, showed no effect on the bacteriocin bioactivity. It was partially purified by ammonium sulphate precipitation. SDS-PAGE showed that putidacin MAS-1 had 15 kDa molecular weight.

Keywords: Bacteriocin, putidacin, SDS-PAGE, bioactivity, antagonistic, EDTA.

Introduction

Many soil bacteria are competent to degrade or reduce the efficiency of pesticides and enhance the process of biodegradation. Among bacterial populations, soil associated *Pseudomonas putida* was repeatedly isolated which could degrade a variety of complex substances and soil pollutants [1]. Infact, *Pseudomonas putida* has extensively been studied as a paradigm in environmental biotechnology. It manifestably contributes in the process of bioremediation due to its physiological status and catabolizing capabilities with reference to various aromatic compounds [2]. This is a fast growing, saprophytic, ubiquitous soil bacterium that is able to colonize soils because of metabolic potential to degrade various natural and man-made chemicals including, pesticides [3-5]. Besides, *Pseudomonas putida* can also metabolize different amino acids, sugars, fatty acids, organic acids and organo-sulfur compounds [6].

Pseudomonas putida is a soil bacterium, present in association with plant roots. It has been found to produce some antimicrobial substances including bacteriocin or bacteriocin like inhibitory substances (BLIS), also termed as "Putidacin" [7].

Bacteriocins, biologically active proteins or peptides, are antimicrobial in nature [8-10]. They are ribosomally synthesized polypeptides, produced by the strains of diverse bacterial species [11]. According to Smirnov and his co-workers, *Pseudomonas putida* produces BLIS that is broad spectrum and the production can be induced by DNA damaging agents including UV irradiation and Mitomycin C [12]. Parret and De Mot illustrated that *Pseudomonas putida* BWIIP2 produced bacteriocin showing characteristics that resemble S-type pyocins [13]. Parret *et al.* isolated *Pseudomonas putida* (strain BWIIMI) from rhizosphere [14]. The bacterium produced bacteriocin (putidacin) that was found to be sensitive to heat treatment and enzyme protease.

Investigation of bacteriocin production by bacteria dwelling in different ecological niches has been the subject of study for a long period. The present work was designed to study the production of bacteriocin(s) or bacteriocin like substance(s) by an indigenous soil associated *Pseudomonas putida* MAS-1 strain.

Results and Discussion

Assay of Bacteriocin Activity

Production of bacteriocin (or bacteriocin like inhibitory substance) was observed by stab and overlay assay and multiwell antagonistic activity assay.

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Optimization of Conditions for Bacteriocin Production:

Maximum bacteriocin production was observed in BHI medium as Cell free supernatant CFS obtained by growing the cells in BHI broth had maximum ability to inhibit the indicator strains, so BHI medium was used for further studies. Optimal temperature for the growth of *Pseudomonas putida* MAS-1 and the bacteriocin production was found to be 32 °C and 35 °C. Inhibition (60%) of the indicator strains occurred when the producer was grown at 32 °C. Inhibition of the sensitive strain was measured at wide range of pH but pH 7 and pH 8 were found optimal for maximum inhibition (Table-1).

Table-1: Optimization of conditions for bacteriocin production of *Pseudomonas putida* MAS-1.

Samples		(OD_{630})	Interpretation
Temperature	C^+	1.2	
(°C)	C^-	0.56	32° to 35°C is a suitable temperature range for the bacteriocin production by *Pseudomonas putida* MAS-1
	4°	1.2	
	25°	1	
	32°	0.6	
	35°	0.8	
	45°	0.95	
	55°	1.2	
	121°	1.2	
pH	2	0.95	Suitable pH for bacteriocin production lies in between 7 to 8
	4	0.95	
	5	0.925	
	6	0.9	
	7	0.8	
	8	0.7	
	9	0.8	
	10	0.8	
	11	0.85	
	12	0.85	
	14	0.85	

Key: C^+ = Positive control, C^- = Negative control

Characterization of Putidacin MAS-1

Bacteriocin was found stable and showed 40% inhibition at 4 °C, 40 °C, 50 °C, 60 °C and 70 °C while no activity was observed at high temperatures (80 °C, 90 °C, 100 °C) and 0 °C (Table-2). A 60% inhibition was observed at pH 8. It is a clear indication that 7-8 pH is optimal for the activity of the bacteriocin (Table-2). Bacteriocin activity was lost after treatment with trypsin. Bacteriocin was also found sensitive to fungal protease while the bioactivity was not lost after proteinase K and lysozyme treatments (Table-3). For this purpose, organic solvents including: ethanol, methanol, chloroform and acetone were used. Partial denaturation was observed with a decrease in bioactivity (Table-3). Bacteriocin of *Pseudomonas putida* MAS-1 was remained bioactive in the presence of EDTA (1%) and SDS (1%). A partial decrease in bioactivity was observed after treatment with Tween 20 (1%), and Tween 80 (1%) and urea (1%). This shows the partial sensitivity of the bacteriocin to urea (Table-4).

Table-2: Effect of Temperature and pH on the bioactivity of putidacin MAS-1.

Samples		(OD_{630})	Interpretation
Temperature (°C)	C^+	1.2	Putidacin MAS-1 is sensitive to extreme temperature. It is bioactive at 40° to 70°C.
	C^-	0.56	
	40	0.7	
	50	0.7	
	60	0.7	
	70	0.8	
	80	1.1	
	90	1.2	
	100	1	
	0	1	
pH	4	0.8	
	3	0.8	Optimal pH for putidacin MAS-1 activity is 7.
	4	0.78	
	5	0.75	
	6	0.74	
	7	0.7	
	8	0.6	
	9	0.9	
	10	0.9	
	11	0.85	

Key: C^+ = Positive control, C^- = Negative control

Table-3: Effect of enzymes, organic solvents and surfactants on the bioactivity of putidacin MAS-1.

Samples		(OD_{630})	Interpretation
Enzyme	C^+	1.2	-
	C^-	0.56	-
	Lysozyme	0.95	Resistant
	Proteinase K	0.9	Resistant
	Fungal protease	1.3	Sensitive
	Trypsin	1.2	Sensitive
Organic solvents	Methanol	0.9	Partial denaturation was observed with decrease in bioactivity
	Ethanol	0.85	
	Chloroform	0.9	
	Acetone	0.85	
Surfactants	SDS	0.15	Resistant
	EDTA	0.6	Resistant
	Tween 20	0.96	Partial denaturation
	Tween 80	0.72	
	Urea	0.9	

Key: C^+ = Positive control, C^- = Negative control

Results show that the putidacin is thermolabile as the bioactivity was completely lost at 100 °C. These findings are in agreement with the results of Muller and Radler [15]. Similarly, the bioactivity was retained at a wide pH range with respect to the bacteriocin of *Pseudomonas syringae* 4-A [16]. Bacteriocins stability at wide pH range carries importance in food industries as many food products are neutral or alkaline in pH [17]. The ability of this bacteriocin to be active at a wide pH range may be a useful factor in food preservation.

Table-4: Inhibitory spectrum of putidacin MAS-1 against clinical isolates.

Sensitive / indicator strain	No. of sensitive strains / no. of strains tested	zone of inhibition (mm)
Escherichia coli	**0/8**	**-**
***Klebsiella* sp.**	**0/3**	**-**
Staphylococcus aureus	**2/3**	**44**
Pseudomonas aeruginosa	**0/7**	**-**
Salmonella typhi	**0/1**	**-**
Enterococcus faecalis	**2/2**	**45**
Stenotrophomonas maltophilia	**0/1**	**-**
***Proteus* sp.**	**0/2**	**-**
***Acinetobacter* sp.**	**0/2**	**-**
***Acinetobacter* sp. (Multidrug-resistant)**	**0/1**	**-**
***Enterococcus* sp. (Vancomycin resistant)**	**0/1**	**-**
Pseudomonas	**0/1**	**-**
Haemophilus influenzae	**0/1**	**-**
Candida albicans	**0/1**	**-**

The presently reported bacteriocin may be "protein" in nature as no activity was found after treatment with trypsin and fungal protease. Proteinase K could partially decrease bioactivity but not completely as reported by Line *et al.* [18]. This may be due to the absence of the target amino acid sequences. Similar results were obtained by Lavermicocca *et al.* [19]. Accordingly, they reported a bacteriocin of *Pseudomonas syringae ciccaronei* exhibiting quite similar characteristics. Brien *et al.* isolated a bacteriocin producing *Pseudomonas* sp. CrCD21 from Antarctic soil [20]. The bacteriocin was found protein in nature. Parret *et al.* reported a lectin like putidacin produced by *Pseudomonas putida* BW11M1 showing analogous properties [21].

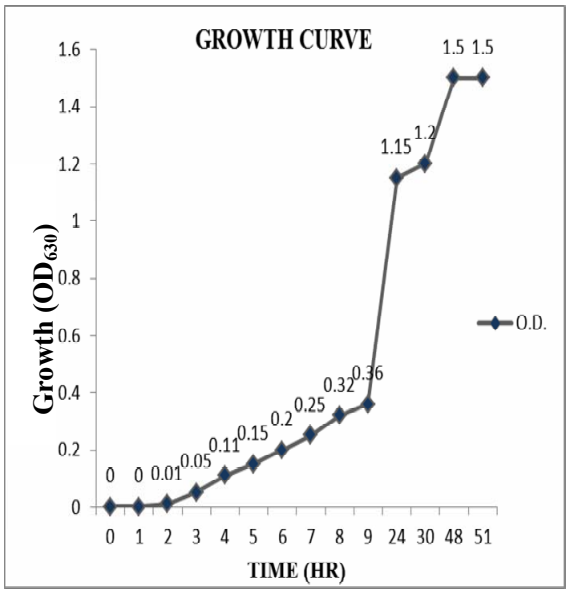
The bacteriocin (putidacin MAS-1) has shown partial sensitivity to organic solvents and some surfactants. Tween 80 and Tween 20 are non ionic surfactants that breakdown proteins into smaller peptides. Putidacin was partially denatured by Tween 20 and Tween 80. Bacteriocin under study was also found sensitive to urea that disrupts hydrogen bonds in protein molecules and the protein adopts highly unfolded conformation in a solution. SDS (Sodium dodecyl sulphate) is an anionic detergent that is responsible to breakdown hydrophobic bonds and hydrogen bonds as well. SDS was found to exert a stimulating effect in the killing of the sensitive cells. It indicates that SDS may have acted as a co-factor that increased the sensitivity of the indicator cells to putidacin. Moreover, resistance of the bacteriocin (putidacin) to EDTA (Ethylene diamine tetra acetate) indicates that metallic ion(s) may not be involved in the bacteriocin action. The results are in agreement with the findings of Saleem *et al.* [22].

Inhibitory Spectrum of Putidacin MAS-1

Several gram positive and the gram negative bacterial strains were tested against *Pseudomonas putida* MAS-1. Most of the indicator strains were isolated form clinical samples. The isolates included: *Staphylococcus aureus, Enterococcus faecalis,* vancomycin resistant *Enterococcus* sp. (VRE) (Gram positive) and *Escherichia coli, Klebsiella* sp., *Pseudomonas aeruginosa, Salmonella typhi, Stenotrophomonas maltophilia, Proteus* sp., *Acinetobacter* sp., *Haemophilus influenzae* (Gram negative) and *Candida albicans.*

Growth Curve

Bacteria remained in lag phase till two hours of incubation at 120 rpm in shaking incubator. Lag phase was followed by log phase. This phase lasted for 30 hours of growth. While growing *Pseudomonas putida* MAS-1, bacteriocin production was traceable during the late log phase or at the early stationary phase of the growth (Fig. 1).



Cells entered into the stationary phase after 30 hours of incubation. Similar results were obtained for EnterocinP, Ent Q, EntL50A and EntL50B by *Enterococcus faecium* L50 [23]. There may be possible reasons behind this decreased activity effect: (i) bacteriocin molecules may be instable; (ii) change in pH due to the production of a variety of matabolites or protease activity may degrade the bacteriocin molecules; (iii) bacteriocin may form complexes with media components and made themselves unavailable for performing antibacterial activity; (iv) possibly bacteriocin molecules

reabsorbed to the producer cells and hence no bioactivity was observed in CFS.

Bioactivity Assay

Bioactivity of the bacteriocin was monitored by multiwell antagonistic activity assay. Inhibitory activity against *Staphylococcus aureus* was observed after 48 hours of incubation (Table-5), indicating towards a delayed action of the bacteriocin.

Table-5: Bioactivity assay of putidacin MAS-1.

Indicator Strain → ↓Samples	OD_{630} *Staphylococcus aureus*		
(X)	**0 hr**	**24 hrs**	**48 hrs**
0 hr	**0.526**	**0.737**	**0.5**
1 hr	**0.518**	**0.844**	**0.824**
2 hrs	**0.585**	**0.824**	**0.81**
3 hrs	**0.585**	**0.756**	**0.755**
4 hrs	**0.526**	**0.702**	**0.76**
5 hrs	**0.572**	**0.915**	**1.689**
6 hrs	**0.551**	**0.878**	**1.16**
7 hrs	**0.566**	**0.704**	**0.75**
8 hrs	**0.542**	**0.74**	**0.688**
9 hrs	**0.544**	**0.72**	**1.74**
24 hrs	**0.49**	**0.78**	**0.7**
30 hrs	**0.6**	**0.839**	**0.602**
48 hrs	**0.59**	**0.845**	**0.842**
51 hrs	**0.575**	**0.926**	**1.478**

Key: samples taken at different time intervals during growth of *Pseudomonas putida* MAS-1.

Partial Purification of Bacteriocin of Pseudomonas putida MAS-1

During partial purification of the bacteriocin, several steps were followed. Each step was followed by the measurement of bacteriocin production. Accordingly, it was found that protein activity against the sensitive strains was significantly increased as bacteriocin fraction I was concentrated by rotary evaporation, followed by ammonium sulphate precipitation. To check if complete saturation had occurred, supernatant obtained after ammonium sulphate precipitation (i.e. fraction IV) was also analysed but bacteriocin activity was not traceable i.e. complete saturation had occurred at 60% of ammonium sulphate (Table-6). Hence, the protein (putidacin MAS-1) was completely precipitated at 60% saturation as no activity could be traced in the residual CFS. Concentration and the activity were increased after rotary evaporation and ammonium sulphate precipitation.

SDS-PAGE of Partially Purified Putidacin MAS-1

SDS-PAGE of partially purified putidacin MAS-1 showed that a 15 kDa protein was responsible for bacteriocin activity. A zone of inhibition was observed on the region where a band of 15 kDa protein was observed on a coomassie blue stained gel (Fig. 2).

Table-6: Partial purification of putidacin MAS-1.

Samples		(OD_{630})	Bioactivity
FRACTION I	**C^+**	**1.2**	+
	T	**1**	
	C^-	**0.5**	
FRACTION II	**C^+**	**2.5**	+
	T	**1.3**	
	C^-	**0.62**	
FRACTION III	**C^+**	**1.8**	+
	T	**1.2**	
	C^-	**1**	
FRACTION IV	**C^+**	**0.7**	-
	T	**1**	
	C^-	**0.11**	

Key: C^+ = Positive control (Culture+Broth)
C^- = Negative control (CFS+Broth)
T = Test (Culture+CFS+Broth)

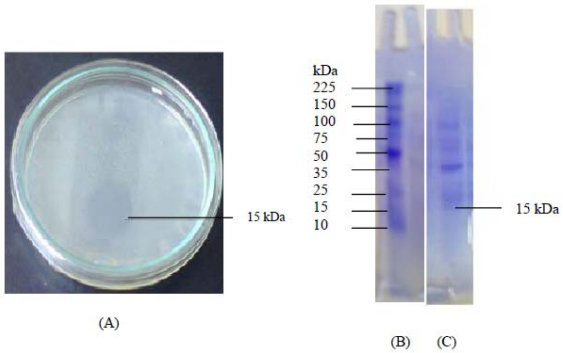


Fig. 2: SDS-PAGE of putidacin MAS-1. Lane A: Gel overlay assay showing zone of inhibition. Lane B: Ladder (Markers). Lane C: Band of partially purified sample.

Experimental

Bacterial Strains and Media

Pseudomonas putida MAS-1 was used to study bacteriocinogenesis. The producer strain was provided by Dr. Munazza of Federal University of Arts, Science and Technology, Karachi. All the indicator cultures (including gram positive and the gram negative) were procured from Liaquat National Hospital, Karachi and Reference Culture Collection Centre, Department of Microbiology, University of Karachi. All the cultures were grown and maintained in brain heart infusion medium. Strains were preserved in 20% (v/v) glycerol. Stocks were kept frozen until used [24]. Before use, strains were propagated in BHI broth for overnight at 32°C – 37°C [25].

Assay for Bacteriocin Activity

Spectrum of bacteriocin bioactivity was studied by standard procedures i.e. stab and overlay method, agar well diffusion assay and an advanced method i.e. multiwell antagonistic activity assay [25]. All the experiments were run in triplicate.

Stab and Overlay Method

Freshly prepared and pre-incubated BHI agar plates (containing 20-25ml of medium) were stabbed with fresh *Pseudomonas putida* culture followed by incubation. Next day 3ml molten BHI soft agar containing 5µl of the indicator strain was poured onto the plates (after chloroform treatment of the stabbed culture). Plates were again incubated at the same conditions for 24 hours and observed the zones of inhibition [26].

Agar Well Diffusion Assay

BHI soft agar was seeded with 5µl of indicator culture and poured into BHI agar plates (20-25ml of medium). It was allowed to solidify; wells were cut using the sterile borer; added with 100µl of cell free supernatant and kept at 4°C for 2 hours. It allows bacteriocin (or any metabolite) to diffuse through agar. Finally, plates were incubated (for 24 hours) to observe the antagonistic activity [27].

Multiwell Antagonistic Activity Assay

For this, the method of Yaakoubi *et al.* was followed (with certain modifications) [25]. Each of the sterile 96-wells microtiter plate well was filled with 200µl BHI broth. 5 µl indicator culture (24 hrs grown culture in 2ml BHI broth) and 100µl crude bacteriocin preparation (CFS) was added in 200µl BHI broth (test well). Positive control contained only 5µl of the indicator culture and 300µl of BHI broth. Negative control did not contain the indicator inoculum and added with 200µl of BHI broth and 100µl of CFS. OD_{630} was measured in ELISA reader and then incubated under optimal conditions for two days while OD_{630} was measured on each day.

Optimization of the Putidacin MAS-1

Effect of Media on the Bacteriocin Production

Pseudomonas putida MAS-1 was grown in 2ml BHI broth overnight at 30-32°C. 100µl inocula were transferred from broth culture to each of the 5ml broth tubes of different media including Nutrient broth (NB), Brain-heart infusion (BHI) broth, Luria-Basal (LB) broth and Lactose broth. Tubes were incubated at 32°C for 24 hours. After 24 hours, culture was spun at 5000 rpm for 25 minutes; supernatant was collected and assayed for the bioactivity.

Effect of Temperature on the Bacteriocin Production

A 24 hour grown culture of the producer strain was taken. 100µl of the freshly grown culture was transferred to 6 tubes containing 5ml BHI broth. Tubes were incubated at temperatures of 4°C, 25°C, 32°C, 35°C, 37°C, 45°C and 55°C for 24 hours and 121°C(15 psi) for 15 min, centrifuged (10,000g at 4°C for 15 minutes) and the supernatant was assayed for the bioactivity [28].

Effect of pH on the Bacteriocin Production

100µl inocula from the broth culture of *Pseudomonas putida* MAS-1 were transferred to 5ml BHI broth containing tubes, maintained at different pH values from 2.0 to 12. Tubes were incubated at optimal conditions (for 24 hours). Next day, samples were readjusted to neutral pH by sterile 1M HCl and 1M NaOH, centrifuged (10,000g at 4°C for 15 minutes) and CFS was checked for the bioactivity [28].

Characterization of the Putidacin MAS-1

Effect of Temperature on the Bacteriocin Activity

The effect of temperature on bacteriocin activity was tested by heat treatment at different temperatures. Cell free supernatant was treated at 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, 100°C for 1 min; 4°C and 0°C for 2 months. Thereafter, antagonistic activity was assayed [24].

Effect of pH on the Bacteriocin Activity

Crude bacteriocin preparation was adjusted at different pH values of 2.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10, and 11 by using sterile 1M HCl and 1M NaOH and incubated at 32°C for 2 hrs. Thereafter, the tubes were readjusted to pH 7.0 and the activity was checked [24].

Effect of Enzymes on the Bacteriocin Activity

Proteinase K enzyme solution (1mg/ml) was prepared by dissolving it in Tris-HCl buffer of pH 8.0 while fungal protease, trypsin and lysozyme were dissolved in 0.1M sodium phosphate buffer of pH 7.0. CFS was treated with enzymes by adding

enzyme solution in equal volume to CFS and keeping for 2 hrs at optimal conditions. Samples were boiled for 2 minutes to inactivate the enzymes and then tested for the antibacterial activity [22].

Effect of Organic Solvents on the Bacteriocin Activity

Equal volume of the bacteriocin and 1% of the organic solvent were mixed in sterile tube and left for 1 hr. Bacteriocin activity was then checked by the above stated procedure. Organic solvents included methanol, ethanol, acetone and chloroform.

Effect of Surfactants on the Bacteriocin Activity

The effect of EDTA, Tween 20, Tween 80 and Urea on bacteriocin activity was also checked. CFS was treated with these surfactants using 1% solutions in equal volume. Surfactants were allowed to react with the bacteriocin preparation for 1 hr and the antagonistic activity was assayed [28].

Inhibitory Spectrum of Bacteriocin of Pseudomonas putida MAS-1

Activity spectrum of *Pseudomonas putida* MAS-1 bacteriocin (against several Gram positive and the Gram negative bacteria belonging to different genera and species) was checked by using stab and overlay method as described by Saeed *et al*. [26].

Growth Curve and Bioactivity Assay

Method of Saleem *et al.* was followed [22]. A 2ml volume of BHI broth was taken, inoculated with the producer culture and grown overnight under standard temperature conditions. Culture (200µl) was transferred in 250ml fresh BHI broth, incubated at optimal temperature in shakobator at 120 rpm. Optical density (OD_{620}) was measured at different time intervals by spectrophotometer till cells reached the stationary phase; simultaneously a fraction of 1.5ml was taken in eppendorf tube, vortexed for 15 sec, spun in a microfuge at 10,000g for 10 mins, filter-sterilized and the supernatant was transferred to a clean sterilized tube and stored at 4°C [26]. All the samples were analyzed for bioactivity (i.e. production of bacteriocin) by multi-well antagonistic activity assay.

Partial Purification of Bacteriocin

The method of Fontoura *et al.* was followed with some modifications [29]. *Pseudomonas putida* MAS-1 was grown in 1000 ml BHI broth, centrifuged at 10,000g (4°C) for 15 mins. Cell free supernatant (Fraction I) was collected. Protein was concentrated by evaporation in rotary shaker at 125 cycles min^{-1} at 37°C for desired time, filtered through 0.45 µm membrane filter and termed as (Fraction II). Protein was precipitated with ammonium sulphate at 60% (w/v) saturation. Centrifuged and pellet was resuspended in 10mM Tris-buffer pH 7.5 (Fraction III). Supernatant was also saved (Fraction IV).

SDS-PAGE of Partially Purified Putidacin MAS-1

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to determine the molecular weight of the peptide responsible for bacteriocin bioactivity. Samples and markers were loaded in 12% gel placed in Tris-glycine buffer (pH 8.3) and run at 25 mA by using Mini-Protean 3 cell (Bio Rad) apparatus. Molecular markers ranged from 10 kDa to 225 kDa (Promega, USA).

Half of the gel was stained with coomassie blue dye while the other was washed with the solution of 20% isopropanol and 10% acetic acid. It was then overlaid with soft agar containing 10^6 cfu ml^{-1} *Staphylococcus aureus* culture [29].

Conclusions

Investigation of bacteriocin production by bacteria dwelling in different biological niches has been the subject of study for a long period. This may be due to its wide ranging applications. Bacteriocins play very important role in mediating the population dynamics [30, 17]. They limit the growth of neighboring cells by acting as anticompetitors (Professional competitive inhibition strategy). Importantly, the bacteriocins can be used as an alternative to therapeutics. They carry more target specificity compared to the antibiotics. Although antibiotics are generally considered as the life saving development, their mismanaged use has been a source for augmenting the bacterial resistance to multiple drugs. Antibiotics kill bacteria, the pathogens and the resident bacterial flora alike. Such a shift in the microbiome of body may allow the infectious agents to occupy the space and spread disease. Consequently, there is a dire need to develop such probiotics or narrow spectrum antibacterial agents (probably bacteriocins) that would be vulnerable to pathogens without affecting the resident flora [31].

Bacteriocins mostly kill close strains of their own species. Sometime, a non linear relationship is observed in a sense that these antibacterial peptides

may kill across the broad phylogenetically distanced strains but not their close relatives [32]. Similar results were observed in this study. Putidacin MAS-1 was found ineffective against closely related species but was found active against Gram positive *Staphylococcus aureus* and *Enterococcus faecalis* clinical strain. These results are in agreement with Pedron Moro *et al.* [33] and Messi *et al.* [34]. This may be due to the absence of receptors to which the bacteriocin may bind or adsorb and mediate the lytic activity or somehow strains have some mechanism of resistance to this particular bacteriocin [35]. Another fact ascribed by Ling *et al*. was the production of immunity protein by insensitive strains that would inactivate the bacteriocin molecules [9]. Sano *et al.* also reported a narrow spectrum pyocin of *Pseudomonas aeruginosa* [36].

Staphylococcus aureus and *Enterococcus faecalis* are among those bacteria that in recent years have been reported as multidrug resistant (MDR). Increasing resistance trend of *Staphylococcus aureus* against a number of antibiotics and the strategies it acquires to escape antibodies has been a potential source of causing a number of infectious diseases including the hospital acquired infections, epidemics etc. *Enterococcus faecalis* is a member of normal enteric flora. Although, it is considered as a harmless gut bacterium, it may cause serious life threatening infections. This organism produces a toxin that helps it to invade urinary tract surfaces to cause infection. Fortunately, the bacteriocin under study, (produced by *Pseudomonas putida* MAS-1) is capable of inhibiting *Staphylococcus aureus* clinical strain. Hence, it may be advantageous to use this bacteriocin as an alternative tool for the treatment of hospital acquired infections caused by *Staphylococcus aureus* and *Enterococcus faecalis*.

The bacteriocin of *Pseudomonas putida* MAS-1 has been found unique due to its characteristics and the mode of action. No effect was observed against *Staphylococcus aureus* after 24 hours incubation while clear inhibition of sensitive strain(s) was observed after 48 hours (a delayed lytic activity). This indicates towards a delayed action of bacteriocin as reported by Riley and Mee [37]. Similar effects of bacteriocin of *Aeromonas hydrophila* against *Staphylococcus* spp. was observed by Messi *et al.* [34].

More than one type of direct assays were done and two methods were selected for further studies i.e. Stab and overlay assay and Multiwell antagonistic activity assay. Multiwell antagonistic activity assay is a turbidity method using a microtitre plate that allows the efficient screening of bacteriocinogenic strains as the assay is done in 96 well plate, multiple producers can be tested against a variety of indicator strains in a single batch. This technique offers quick (within 24 hours) results and minimum volume of media and culture(s) are required to make the assay to be economically feasible.

In order to well and further define this antimicrobial compound (the putidacin MAS-1) produced by a soil associated *Pseudomonas putida* MAS-1 strain, further research is needed before more conclusive conclusions may be drawn about this bacteriocin.

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