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Research Paper

Biophysicochemical characterization of Pyocin SA189 produced by *Pseudomonas aeruginosa* SA189

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Abstract

Pseudomonas aeruginosa, in spite of being a ubiquitous organism (as it is found in soil, water, and humans), is also an opportunistic pathogen. In order to maintain its diversity in the community, it produces various toxic proteins, known as, bacteriocins. In the present study, pyocin SA189, which is a bacteriocin produced by *P. aeruginosa* SA189 (isolated from a clinical sample) was characterized. *P. aeruginosa* SA189, as identified by the conventional and 16S rRNA gene amplification, produced pyocin SA189 of molecular weight of 66 k Da. The pyocin showed antimicrobial activity against several clinically relevant Gram-positive and Gram-negative bacteria and was substantially stable for wide ranges of temperature and pH. Furthermore, the pyocin also retained its biological activity upon treatment with metal ions, organic solvents, and various proteolytic and lipolytic enzymes. The data from the growth kinetics indicated that the maximum bacteriocin production occurred in the late log phase. Overall, our results signify the potential of pyocin SA189 as a bio-control agent.

Key words: bacteriocin, pyocin, *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa, during the process of establishment in any habitat-soil, water, plants or human and animal systems synthesizes different antimicrobial weapons in order to dominate over the other competing organisms. Pyocin, among others, is a well-known weapon, which facilitates the bacterium to not only invade, but also to defend its ecological niche (Kerr *et al.*, 2002; Riley *et al.*, 2003). In the first report of its kind, Jacob (1954) described a protease resistant pyocin that was obtained from UV irradiated *P. aeruginosa.* Further investigations elaborated that it adhered to the cell surface of sensitive bacteria that led to their ultimate killing (Michel-Briand and Baysse, 2002).

Afterwards, it was revealed that pyocin is a high molecular weight bacteriocin, which is produced by certain strains of *P. aeruginosa* and aids in destruction of other *P. aeruginosa* strains (Ritchie *et al.*, 2011; Dingemans, 2014). However, it has been argued that the antimicrobial spectrum of pyocin exceeds such definition. Activities against other *Pseudomonas* species, such as, *P. fluorescens, P. putida*, *Burkholderia cepacia* complex as well as strains of *Neisseria* such as *N. meningitidis, N. gonorrhoeae, Haemophilus ducreyi* and *Campylobacter* spp. have also been reported (Williams *et al.*, 2008; Bakkal *et al.*, 2010).

Based on the structure and the mode of action, pyocins may be classified as R, F or S-types. A single strain of *P. aeruginosa* may produce more than one pyocin type at a particular time. Among 1400 strains of *P. aeruginosa*, isolated from different sources, more than 90% produced one or more types of pyocins (Riley, 1998). For instance, *P. aeruginosa* strain PA01 produces three types of pyocins, *i.e.*, R2, F2 and S2 (Parret and De Mot, 2002). Riley (1998) observed that the pyocin types, R and F, are produced by more than 90% of the clinical isolates, and the S-type is produced by more than 70% of the strains.

R-type pyocins are high molecular weight proteins, which are resistant to proteases and nucleases. Eight subtypes of R-type pyocin have been described, which are R-1, C-9, R-2, R-3, R-4, R-5, 21 and 430C (Michel-Briand and

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Baysse, 2002). These subtypes differ in specificities for their hosts, however, are similar in their morphological features, antigenic properties, and composition of proteins (Lee *et al.*, 1999). Based on the morphological features, as elucidated by transmission electron microscopy (TEM), and the genetic background, the R-type pyocins tend to bear a close resemblance to the T-even phages (Kageyama *et al.*, 1964). However, the physical and chemical stabilities of R-type pyocins, such as, resistance to acids and proteases, make them different from the phages (Scholl and Martin, 2009). The R-type pyocins destroy the target cells without lysing them, this is in contrast to the mode of killing of bacteriophages, where the phage infection results in lysis of the cells (Strauch *et al.*, 2001). The bactericidal activity of R-type pyocins is characterized by its rapidity and specificity, where every individual pyocin molecule is capable of killing a sensitive cell (Scholl *et al.*, 2009).

F-type pyocins are high molecular weight protease-resistant proteins, which were first described by Takeya *et al.* (1967). The electron microscopic examination revealed that they have a distinct morphological structure, however, are similar to the phage tails. S-type pyocins are soluble, and sensitive to proteases and heat, these features differentiate them from the R and the F- types. The S-type pyocins are composed of two protein sub-units which remain intact throughout the purification process, but they may be separated by gel-filtration in the presence of urea. The larger subunit is the effector component of the S-type pyocin and possesses the killing ability including a DNase activity. On the other hand, the smaller subunit of the pyocin is the immunity component, which provides immunity to the pyocin-producing strains from the destructive effects of the pyocin produced by themselves (Duport *et al.*, 1995; Ling *et al.*, 2010). The present study was designed to produce, purify and study bio-physicochemical characterization of a pyocin produced by an indigenously isolated clinical strain of *P. aeruginosa*, SA189, which also bears antagonistic activity against the bacterial pathogens.

Materials and Methods

Selection and identification of bacteriocin producing strain

P. aeruginosa SA 189 was originally isolated from a pus sample, which was obtained from a hospitalized patient. The strain has already been reported for its antibacterial activity (Naz and Rasool, 2013). The selected strain, for the current study, was identified by 16S rDNA gene amplification (Spilker *et al.*, 2004) using forward and reverse primers, PA-SS-F "GGGGGATCTTCGGACCTCA" and PA-SS-R "TCCTTAGAGTGCCCACCCG", respectively.

Inhibitory spectrum of Pyocin SA189

For determination of the inhibitory spectrum of pyocin SA189, several clinical bacterial strains were tested by agar well diffusion assay (Jabeen *et al.*, 2009; Naz and Rasool, 2013). Briefly in this method, the cell free supernatant (CFS) from a 24 hour culture of *P. aeruginosa* SA189 was filter sterilized using a membrane filter of 0.45μm pore size. A volume of 100 μL of the CFS was poured onto a 7-mm diameter well in nutrient agar plates that were previously seeded with the indicator bacteria (approximately 1 x 10^6 cfu/mL). The plates were incubated overnight at 37 °C. A clear zone of inhibition surrounding the well of test organism was considered to be positive for the test (Table 1).

Optimization of culture conditions and their kinetics

In order to achieve the maximum production of bacteriocin from *P. aeruginosa SA189*, the culture was inoculated in different growth media (*i.e.*, nutrient broth, trypticase soya broth, brain heart infusion broth (BHI), lactose broth, Luria-basal broth and pseudo agar base) with varied incubation periods (10, 15, 20, 24, 36, 48 and 72 h) and temperatures (29, 37 and 40 °C for 24 h). The bioactivity of the cell free supernatants of *P. aeruginosa SA189* was measured by agar well diffusion assay (AWDA) using *Staphylococcus aureus* SA 84 as the indicator strain. The inhibitory strength (bacteriocin titer) was expressed as arbitrary units (AU/mL) or activity units/mL (Rajaram *et al.*, 2010). The kinetics of bacteriocin production by *P. aeruginosa* SA189 was studied by growing the producer strain under optimum conditions for at least 24 h. The samples were col-

Table 1 - Inhibitory spectrum of Pyocin SA189.

Indicator strains	No of strains tested	Average Zone of inhibition (mm)
Acinetobacter lwoffi	6	0
Bacillus subtilis	5	12
B. cereus	7	10
Corynebacterium xerosis	4	11
Enterobacter aerogenes	8	0
Enterococcus faecalis	6	17
Escherichia coli	10	14
Klebsiella pneumoniae	5	14
Listeria monocytogenes	1	17
Proteus mirabilis	6	0
Pseudomonas aeruginosa	7	9
Salmonella typhi	8	0
Serratia marcescens	2	0
Shigella dysenteriae	8	0
Staphylococcus aureus	16	26
S. epidermidis	6	20
Streptococcus pyogenes	6	12
S. pneumoniae	2	10
Micrococcus luteus	5	22

lected after each hour to record the optical density at 600 nm. Thereafter, the samples were centrifuged and the cell free supernatant of *P. aeruginosa* SA189 was used to determine the activity in units/mL (Jabeen *et al.*, 2009)

Preparation and purification of Pyocin SA189

After optimization of culture conditions, *P. aeruginosa* SA189 was grown in BHI broth and was incubated at 37 °C for 24 hours. The following day, the culture broth was subjected to centrifugation at 10,000 x *g* for 30 min at 4 °C for separation of bacterial cells. The supernatant was filter sterilized by passing through 0.45 µm pore sized filter membrane (Millipore, MA, USA) and was concentrated to 3 to 5-fold using a pre-chilled (4 °C) rotary evaporator (Buschi, Germany). This cell free supernatant (CFS) was referred to as the `crude bacteriocin preparation'. This CFS was further partially purified by ammonium sulphate precipitation (Harris, 1989). In order to achieve the maximum saturation of pyocin SA189, different concentrations (50%, 60%, 70% and 80%) of ammonium sulphate were added by constant agitation at 4 °C and precipitates were recovered by centrifugation (10,000 x *g* for 30 min at 4 °C). The resulting pellets were re-suspended in 50 mM sodium phosphate buffer (pH 7.0) and were designated as `partially purified pyocin' (Jabeen *et al.*, 2009). For removal of the salt from the partially purified bacteriocin, ultra-filtration was done through a pre-treated dialysis tubing of 12 kDa cutoff size (Harris, 1989). The dialyzed bacteriocin was further subjected to gel chromatography on a Sephadex G-75 column of dimensions, 30 x 1.5 cm (Amersham Pharmacia Biotech, USA). The column was pre-equilibrated and the suspension was eluted with 50 mM Sodium phosphate buffer of pH 7.0. The flow rate was maintained at 0.2 mL/min and the eluates were subjected to absorbance measurement at 280 nm. The active fractions, thus obtained, were collected and pooled for assessment of inhibitory activity (DeCourcy, 2004). The bioactivity of pyocin SA189 was analyzed at each point of purification by agar well diffusion assay (AWDA), in terms of AU/mL. After every step of purification, protein concentration was measured (Lowry *et al.*, 1951).

Molecular weight estimation of Pyocin SA189 by SDS-PAGE and related antibacterial assay

The ammonium sulphate precipitate and their active fractions that were obtained after gel filtration chromatography were subjected to SDS-PAGE (10% polyacrylamide gel) analysis using the standard protein marker (range 14.5 kDa to 200 kDa (Sigma)) loaded on a vertical slab gel (BioRad, USA). After the complete run, the gel was cut into two halves. One half of the gel that contained protein sample and standard molecular weight marker was visualized by Coomassie blue staining to visualize protein bands (Jabeen *et al.*, 2007), while the other half of the gel, containing only the sample protein, was treated with a solution of 20% isopropanol (v/v) and 10% acetic acid (v/v) for 2 h followed by washing with distilled water for 4 hours. The gel was placed on a nutrient agar plate and overlaid with soft agar (0.6%) containing the indicator strain. The plate was incubated overnight at 37 °C and next day observed for the zone of inhibition (Bhunia *et al.*, 1988).

Physico-chemical characterization of Pyocin SA189

Thermostability of pyocin SA189 was determined by exposing the preparations to elevated temperatures, *i.e.*, from 40 °C to 80 °C for 30 min, and to 100 °C and 121 °C for 15 min. After treatment, the residual activity was determined by AWDA (Jabeen *et al.*, 2009; Benreguieg *et al.*, 2013). To determine the temperature and duration of the bacteriocin stability, the preparation was stored at temperatures, 0 °C, 4 °C or -20 °C, and their bioactivity was monitored till one year using AWDA. To assess the effect of different pH levels on its bioactivity, bacteriocin preparation was adjusted to different pH values, ranging between 1-14, with 1N NaOH (Merck) or 1 N HCl (Merck). The samples were incubated at 37 °C for 2 hours, followed by re-adjustment to neutral (7.0) pH and assessment of the bioactivity by AWDA (Vamanu and Vamanu, 2010). Biological stability of pyocin SA189 was performed after giving a treatment with different enzymes including proteases, proteinase K, pepsin, papain and lipase (Sigma, USA) at a final concentration of 1 mg /mL (Vamanu and Vamanu, 2010). Similarly, equal volumes of the pyocin SA189 were mixed separately with pre-chilled (at 4 °C) 10% concentrated preparations of various organic solvents, acetone, ethanol, methanol and chloroform, with 1 mM solutions of metal ions ($CaCl_2$, $FeSO_4$, $ZnSO_4$, $MgSO_4$, $BaCl_2$) and with different surfactants or detergents (at a final concentration of 1%) including sodium dodecyl sulphate (SDS) and ethylene diamine tetra acetic acid (EDTA), Tween 20, and Tween 80 (Sigma, USA). The mixtures were stirred and incubated at 37 °C for 2 hours and further analyzed by AWDA. The sets of respective positive and negative controls were also processed simultaneously in the same way (Rajaram *et al.*, 2010).

Results and Discussions

The present study basically focused on the bacteriocin, which is produced by a clinical strain *P. aeruginosa* SA189. This producer strain as well as the indicator strain, *S. aureus* SA84, were previously isolated and reported (Naz and Rasool, 2013). The identity of the producer strain (*P. aeruginosa* SA189) was confirmed by 16S rDNA gene amplification (conventional PCR, product size 956 bp).

Antimicrobial spectrum

Bacteriocins, in general, share a narrow spectrum of antimicrobial activity; however, there are certain bacteriocins that exhibit a broad spectrum antibacterial activity and are also capable of targeting viruses, protozoa and even

fungi (Rea *et al.*, 2011). The broad spectrum activity of bacteriocins from *Pseudomonas* spp. has widely been reported (Parret and De Mot, 2000). Pyocin SA189 also exhibited bioactivity against a number of sensitive organisms, notably the Gram-positive bacteria including *S. aureus, S. pyogenes* and *Listeria monocytogenes.* However, *Escherichia coli*, *Klebsiella pneumoniae, Shigella dysenteriae*, *P. aeruginosa* and *Proteus mirabilis* were also found to be susceptible to a certain extent (Table 1). These finding inspired another study where an isolated pyocin, JU-Ch, exhibited significant antagonistic activity against Gram-positive and Gram-negative bacteria along with different fungi (Grewal *et al.*, 2014). Such bio-active potential might be attributed to the presence of high number of bacteriocin adsorption receptors in the peptidoglycan-based cell wall of Gram-positive bacteria (Padilla *et al.*, 2002).

Optimization of conditions for bacteriocin production and their kinetics

Like other microbial products, bacteriocin production is a genetically regulated phenomenon and is influenced by a number of environmental and nutritional factors such, as the composition of medium, incubation time, temperature, and pH (Lucas *et al.*, 2006, Rajaram *et al.*, 2010). In order to obtain maximum production of the pyocin (bacteriocin) from *P. aeruginosa* SA189, the mentioned parameters were optimized in our current study. The results indicated that BHI broth (an enriched medium) enhanced the production of pyocin (data not shown). Enriched medium not only favors the growth of *P. aeruginosa* but also helps the bacterium to yield high titers of bacteriocins (MacKinnon, 2011). The maximum yield of pyocin SA189 was obtained when the organism was cultivated at 37 °C and pH 7.0. Our finding is in concordance with the previous study conducted by Scholl and Martin (2008). We recorded the maximum titer of pyocin SA189 as 640 AU/mL (data not shown).

The analysis of the growth and production kinetics revealed that the pyocin SA189 production varies in different phases of the growth cycle. The protein production is initiated in the early logarithmic phase, reaches the maximum level after 18 to 22 hours (late log phase) and remained constant till the late stationary phase (Figure 1).

Purification of Pyocin SA189

Production of pyocin is regulated in such a manner that only a few cells in a population actively produce pyocin. Further, the bacteriocin production can be enhanced by induction with DNA damage treatments, such as ultraviolet irradiation (Higerd *et al.*, 1967), mitomycin C (Kageyama, 1964) or by DNA gyrase inhibiting antibiotics (Brazas and Hancock, 2005). However, in the present study, the pyocin SA189 was harvested by propagation of the respective strain as per the optimum growth requirements followed by partial purification by ammonium sulphate precipitation, of the cell free supernatant (CFS). The salts, in fact, make the proteins resistant to denaturation, proteolysis or bacterial contamination (Harris, 1989). The maximum antagonistic activity, in case of pyocin SA189, was observed to be 1,280 AU/mL in the resolved precipitate with 70% saturation, which is in concordance with the previously reported findings of Scholl and Martin (2008). After ultra-filtration of partially purified pyocin SA189, it was noted that the pyocin almost retained their activity; however, a little loss might be attributed to the adsorption of the bacteriocin on the dialysis membrane. In the following step of conventional gel permeation chromatography, on Sephadex G-75 column, pyocin SA189 depicted the activity to reside in fraction 6, 7 and 8 and the chromatogram also showed a single peak of protein (Figure 2). These find-

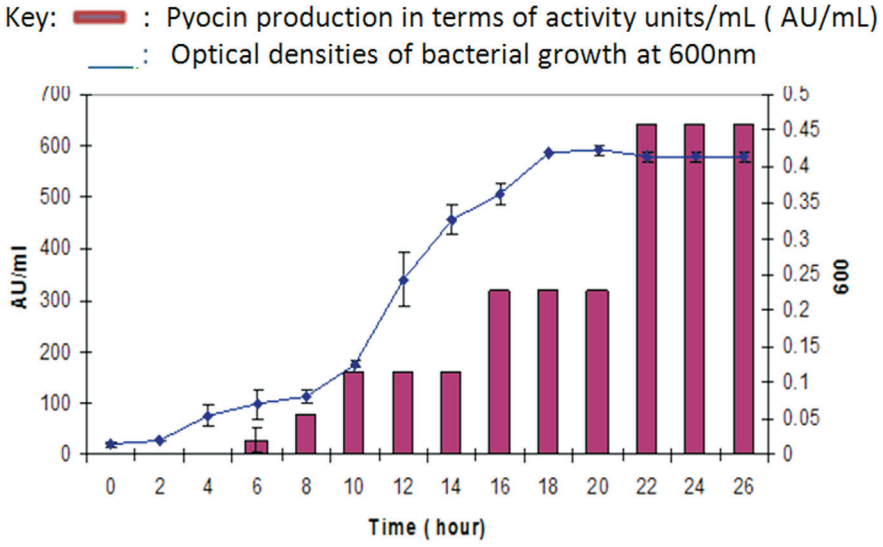

Figure 1 - Pyocin SA189 Production during the growth cycle of *P. aeruginosa* SA189.

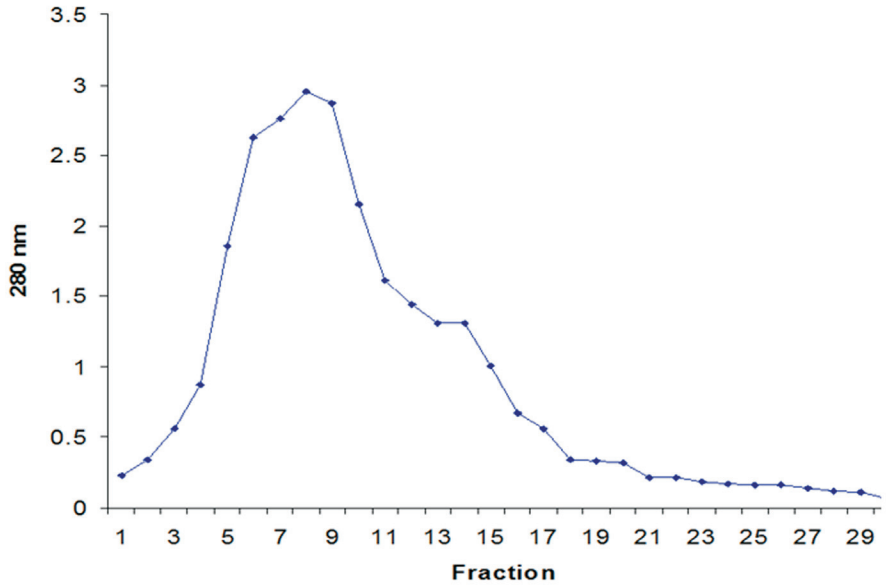

Figure 2 - Elution profile of pyocin SA189 by size exclusion chromatography (Sephadex G-75 gel filtration column).

ings correlate with the study, where pyocin was eluted from the CM Sephadex A-50 (pH 7.2) column as a single protein peak (Al-Shibib *et al.*, 1985). Furthermore, Duport *et al.* (1995) determined pyocin S3 as a single protein peak upon Sephacryl S-200 gel filtration (Mr of 90,000). In contrast to these results, a protease sensitive S-type pyocin AP41 was observed as a complex of two proteins that were observed as two separate peaks when subjected to gel filtration Sephacryl S 200 column in the presence of 6 M urea (Sano and Kageyama, 1981).

In the protein purification profile of pyocin SA189, the specific activity in CFS was found to be 220.7 AU/mg, which increased up to 752.9 AU/mg after ammonium sulfate precipitation and 1,828.6 AU/mg after gel filtration (Table 2). The final purification that was achieved after gel filtration chromatography was 8.2 folds with 2% recovery. These findings are in accordance with another study which also reported the purification of pyocin with an increase in the specific activity as well as purification up to 434-fold (Al-Shibib *et al.*, 1985). In another study, the specific activity of the pyocin after every step of purification was increased and final recovery after chromatography using Sephacryl S 200 was observed to be 13% (Sano and Kageyama, 1981). Increase in the specific activity was also demonstrated in each purification step for the pyocin 42A (Abdi-Ali et *al.,* 2004).

SDS-PAGE and related bioactivity

The purity of pyocin protein SA189 was further checked by electrophoresis and confirmed by gel overlay technique. Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulphate (SDS), an anionic detergent, is one of the important procedures, used successfully to characterize proteins with respect to the molecular sizes of their constituent polypeptides. Moreover, in bacteriocin studies, it has been a widely used technique, which is sensitive enough to detect the activity of the bacteriocin directly on the gel. This technique was employed to check the purity of pyocin SA189. Ammonium sulphate precipitates and the active fractions from gel filtration were subjected to 10% acrylamide denaturing gel. Electropherogram of pyocin SA189 revealed a single band, which corresponded to the molecular mass of approximately 66 kDa. This band also showed bioactivity by gel overlay method (data not shown). The high molecular weight pyocins, particularly the R-type pyocins are well-documented in literature (Ritchie *et al.*, 2011). A study in this relation demonstrated the isolation of five pyocins from clinical isolates, which were resistant to trypsin treatment and had molecular masses within the 54.9-282 Kg/mol. range (Al-Rubiee *et al.*, 1988). Similar protease-resistant pyocins with 20-135 kg/mol. molecular masses have been reported earlier (Al-Shibib *et al.*, 1985). In another study, R-type pyocin, resistant to all tested proteases, depicted a major band of about 30 kDa on SDS-PAGE (Fontoura *et al.*, 2009). On the other hand, a study on protease-sensitive pyocin showed a single band corresponding to molecular weight 62.4 kDa on the SDS-PAGE (Ling *et al.*, 2010).

Physicochemical characterization of pyocin SA189

Different physical and chemical factors may have significant impact on the bioactivity of bacteriocins. The determination of such impacts bears significance for possible application(s) of bacteriocins in food preservation, as probiotics or as chemotherapeutic agents. Interestingly, in the present study, pyocin SA189 was found to be resistant to

Table 2 - Purification Profile of Pyocin SA189.

Sample/steps	Vol./mL	AU/mL	Total AU	Total Protein	Specific activity	% recovery	Purification fold
Cell free Supernatant/ Crude extract	300	640	192 000	870	220.7	100	1
Ammonium Sulfate Precipitate (70%)	30	1 280	38 400	51	752.9	20	3.4
Size exclusion chromatography	3	1 280	3 840	2.1	1 828.6	2	8.2

the action of almost all the physical and chemical factors tested (Table 3). Thus, as per our analysis, pyocin SA189 could be stated as thermotolerant in nature. Our observations suggested that the pyocin retained its bioactivity even at the autoclaving temperature. Such high thermostability is in contrast to a previous report where R-type pyocin lost activity at a temperature above 60 °C (Al-Rubiee *et al.*, 1988). However, Padilla *et al.* (2002) found a pyocin to be resistant to proteolytic enzymes as well as high temperatures. Moreover, an antimicrobial peptide produced by *Pseudomonas* spp. also showed resistance to high ranges of temperature (Fontoura *et al.*, 2009). Regarding the influence of pH on the biological activity, pyocin SA189 remained stable within the pH range 2 to 11. Retention of bioactivity of the pyocins at various pH values has been reported earlier (Sano and Kageyema, 1981; Padilla *et al.*, 2002; Saleem *et al.*, 2009). However, in contrast to our findings, an R-type pyocin demonstrated stability only in a pH range of 6 to 8 (Al-Rubiee *et al.*, 1988).

Besides the physical agents, certain chemicals may modulate the bioactivity of pyocins. The most important finding was the resistance of pyocins SA189 to different proteolytic enzymes (Table 3). Although, this pyocin manifested an apparent proteinaceous nature in various experiments, such as ammonium sulphate precipitation, SDS-PAGE analysis and gel filtration, but when treated with proteolytic enzymes, like proteinase K, proteases, trypsin and papain, it survived digestion and retained its bioactivity. In addition, lipase also had no effect on the activity of this pyocin. These results were not surprising when compared with previous findings on pyocins, where protease resistant pyocins were obtained from the strains isolated from well water (Padilla *et al.*, 2002), pond water (Fontoura *et al.*, 2009) and clinical materials (Al-Rubiee *et al.*, 1988). Similarly, pyocins that are sensitive to proteases were also reported from different environments (Sano and Kageyama, 1981). The resistance to proteases might be due to the cyclic peptide nature of some of the bacteriocins, which may have unusual amino acids rendering them resistant to hydrolysis by proteolytic enzymes (Bizani and Brandelli, 2002). Exposure of pyocin SA189 to surfactants also resulted in complete retention of their activity. Incubation of pyocin SA189 with various concentrations of organic solvents and metal salts had no effect on its bioactivity. These findings are in accordance with a previously reported study where a pyocin was observed to be resistant to action of all the organic solvents (Saleem *et al.*, 2009).

Table 3 - Physicochemical characteristics of Pyocin SA189.

Treatments	Activity Units (AU/mL) Control (Pyocin alone) = 640 AU/mL	Treatments	Activity Units (AU/mL) Control ((Pyocin alone) = 640 AU/mL
Temperature treatment		Metal Salts (1 mM)	
40 °C to 100 °C (30 min)	640	$BaCl_2$	640
121 °C (15 psi 15 min)	320	$CaCl_2$	640
4 °C	640	$ZnSO_4$	640
0 °C-20 °C (till one year)	640	$FeSO_4$	640
pH treatment (2 hours)		$MgSO_4$	640
2-3	320	Surfactants (1%)	
4-9	640	SDS	640
10-11	160	EDTA	640
12-14	0	Tween 20	640
Enzymes		Tween 80	640
Protease	640	Solvents (10%)	
Proteinase K	640	Acetone	640
Lipase	640	Chloroform	640
Papain	640	Ethanol	320
Trypsin	640	Methanol	640

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