

## PRODUCTION AND PURIFICATION OF PYOCIN FROM A SOIL ASSOCIATED *Pseudomonas aeruginosa* STRAIN SA 188

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Bacteria associated with rhizosphere are recognized as highly diverse bio control agents. Production of antagonistic substances by such bacteria has significance in the control of phytopathogenic microorganisms. *Pseudomonas aeruginosa* SA 188, an indigenous isolate from rhizosphere was found to produce a high molecular weight pyocin which was resistant to proteolytic enzymes (protease, proteinase K, pepsin, papain and lipase). This pyocin exhibited antibacterial activity against both the Gram-positive and Gram-negative bacterial strains including *Corynebacterium xerosis*, *Listeria monocytogenes*, *Micrococcus luteus*, *Staphylococcus aureus*, *Streptococcus pyogenes* and *Escherichia coli*. The production of this pyocin by *P. aeruginosa* SA 188 started in the early logarithmic phase of the growth cycle and reached maximum at the late log phase. A single peak of protein was demonstrated by size exclusion chromatography (using Sephadex G-75 column). There was an increase in the specific activity after each step of purification and the final purification of pyocin SA 188 was found to be 4.6 folds. Electrophorogram of this protein revealed a single band of molecular mass approximately 45 kDa by SDS-PAGE (10% acrylamide denaturing gel). The band was found bioactive in the direct gel overlay.

**Keywords:** Bacteriocin, pyocin, *Pseudomonas aeruginosa*, phytopathogen

### INTRODUCTION

Rhizosphere (Plant root-soil interface) is a highly competitive environmental niche for harboring bacterial flora. A constant competition does exist between these bacteria for ecological space and nutrients (Parret *et al.*, 2003). For this professional cohabitational antagonism, the bacteria may exploit different offensive and defensive biological weapons such as bacteriolytic enzymes, antibiotic substances, bacteriocins etc. Among all, bacteriocins have gained interestful attention because of their abundance and diversity (Beneduzi *et al.*, 2012; Kuo *et al.*, 2013; Zineb *et al.*, 2013; Ahmad *et al.*, 2014).

Bacteriocins are proteinaceous bioactive toxins produced by all major groups of bacteria and they are antagonistics to closely related bacterial strains (Todorov, 2009; Rajaram *et al.*, 2010; Fatima and Mebrouk, 2013; Inglis *et al.*, 2013). However, in contrast to earlier concept of narrow spectrum of these peptides, they have been reported to have broad spectrum activities against wide range of bacteria, fungi, viruses and protozoa (Rea *et al.*, 2011). Another important feature of these antimicrobial peptides is their limited resistance from bacteria because development of resistance might limit their application in biocontrol (Xue *et al.*, 2005). Although the multiplicity and diversity of bacteriocins and their interactions with the targeted bacteria from different biological niches has been well established but their potential as an alternative to conventional antibiotics make them a rapidly expanding field (Cavera *et al.*, 2015).

*Pseudomonas* spp. are well recognized for their abundance in rhizosphere of tropical and temperate soil. Among them, the bacteriocinogenic activity of *P. fluorescens*, *P. putida* and *P. syringae* have been reported in literature (Parret and De Mot, 2000). Further, *P. syringae* pv.ciccaronei has been reported to produce a bacteriocin which can inhibit the growth of *P. syringae* sub sp. savastanoi, the causative agent of olive knot disease (Lavermicocca *et al.*, 2002). Another *Pseudomonas* sp. BW11MI isolated from rhizosphere of banana roots has also been reported to have antibacterial activity against rhizosphere colonizing strain *P. putida* as well as some other phytopathogenic *Pseudomonas* spp. including *Ps. syringae* (Weller, 1994). A similar finding has been reported regarding *P. fluorescens* SF4c, which was isolated from rhizosphere of wheat plants. This strain revealed production of high molecular weight bacteriocin having antimicrobial activity towards closely related strains (Fischer *et al.*, 2012).

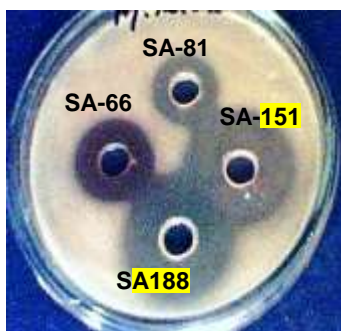
*Pseudomonas aeruginosa*, a ubiquitous *Pseudomonas* sp., has been well recognized for its extraordinary abilities to survive and adapt in wide range of environments including soil, plants, water, sewage, hospitals and humans etc. (Hare *et al.*, 2012). Pyocin (a bacteriocin) produced by *P. aeruginosa* might constitute as one of the factors responsible for its diverse adaptations in different environments. These pyocins can be classified (on the basis of their structure and mode of action) in three types i.e. R, F and S type pyocins. However, even a single strain of *P. aeruginosa* may produce more than one type of pyocin at a time. More than 90%

strains of *P. aeruginosa* have been reported to produce one or more types of pyocin (Ling *et al.*, 2010; MacKinnon, 2011).

In view of the importance of bacteriocinogenic potential of bacterial strains in agriculture as biological control of phytopathogens, the present study was designed to purify and characterize pyocin from a rhizosphere associated *Pseudomonas aeruginosa* SA 188.

## MATERIALS AND METHODS

**Isolation and screening of *Pseudomonas* spp. for bacteriocin production:** Sixty eight strains of *Pseudomonas* (from different sources) were isolated and characterized. These isolates were screened for bacteriocin production against twelve bacterial strains (comprising both Gram-positive and Gram-negative strains) by agar well diffusion method (Fig.1) as described elsewhere (Naz and Rasool, 2013).



**Figure 1. Screening of different *Pseudomonas* spp. for bacteriocin production by agar well diffusion method (*Staphylococcus aureus* 84 as indicator strain)**

**Selection of representative *Pseudomonas* strain:** On the basis of bioactivity, a rhizosphere associated *P. aeruginosa* SA 188 was selected as pyocin producer strain while *Staphylococcus aureus* SA 84 was used as indicator strain in the follow up study. The producer strain was identified earlier by both the conventional and API 20 NE kit methods (Naz and Rasool, 2013). The identification was confirmed by PCR amplification as described by Spilker *et al.*, 2004. The sequence of set of the forward and reverse primers used in this process was as follows:

PA-SS-F GGGGGATCTTCGGACCTCA

PA-SS-R TCCTTAGAGTGCCACCCG

**Kinetics of bacteriocin production by *P. aeruginosa* SA 188:** The production kinetics of bacteriocin by *P. aeruginosa* SA 188 was monitored by growing the representative culture in BHI broth (5ml) for overnight at 29°C. After incubation, OD<sub>600</sub> was measured and 100µl of the culture was transferred to 500ml of fresh BHI broth maintaining its

OD<sub>600</sub> at 0.01. After every hour, OD<sub>600</sub> of the culture broth was recorded. Simultaneously, 1.0 ml of the sample was collected and centrifuged at 7000× g at 4°C for 30 min. and the bioactivity of the supernatant (obtained after centrifugation) was detected by agar well diffusion method using *Staphylococcus aureus* SA 84 as indicator strain (Nespolo and Brandelli, 2010; Alam *et al.*, 2011).

### **Molecular mass estimation of pyocin by dialysis tubing:**

The molecular mass (rough estimate) of pyocin SA 188 was determined by using Dialysis membrane of 12 k Da cut off size. *P. aeruginosa* SA 188 was stabbed into BHI agar plate and incubated at 29°C for overnight. The test (stabbed) culture was then exposed to chloroform and covered with dialysis membrane, while the control (stabbed) culture was left uncovered. Sensitive culture of *S. aureus* SA 84 was overlaid on the agar and incubated for overnight to observe the zone of inhibition (Parrot *et al.*, 1989; Ahmad and Rasool, 2003).

**Preparation and purification of Pyocin SA 188:** Crude preparation of pyocin SA 188 was obtained by ammonium sulfate saturated precipitation (70%) of culture supernatant of *P. aeruginosa* SA 188, grown in BHI broth of pH 7 at 29°C. The crude preparation was subjected to ultra-filtration and size exclusion chromatography for purification.

**Ultra-filtration of crude pyocin by dialysis:** The salts were removed from crude pyocin SA 188 ammonium sulfate precipitates by dialysis using dialysis tubing of 12 k Da cut off size. To ensure the removal of the heavy metals and more uniform size, a piece of dialysis membrane was pretreated by submerging it in a solution of 2% sodium bicarbonate and 0.05% EDTA, boiled for 10 min. and the solution discarded. The tubing was then boiled and immersed in 0.2% (w/v) sodium azide solution and stored at 4°C. After boiling, one end of tube was sealed by double knot and then the crude pyocin preparation was transferred in it. The other end was tied by thread after expelling the air. This bag of tubing was immersed in buffer with gentle agitation on magnetic stirrer for more than 6 hours at 4°C. This bag was left in buffer in refrigerator for an overnight. The tube was cut open next day and the dialysate was checked for its bioactivity (Harris, 1989)

**Size exclusion chromatography:** Dialysed pyocin preparation was further subjected to size exclusion chromatography using Sephadex G-75 column of 30 x 1.5 cm (Amersham Pharmacia Biotech, USA) equilibrated and eluted with buffer of pH 7.0 (50mM sodium phosphate). The flow rate of elution was maintained at 3ml/15 min. and the elutes obtained were monitored for absorbance at 280nm. The fractions obtained after chromatography were screened for bioactivity by agar well diffusion method, the active fractions showing bioactivity were pooled and stored at 4°C (DeCourcy, 2004).

**Protein estimation:** At each step of purification of the pyocin SA 188, the protein was estimated as suggested by Lowry *et al.* (1951) and Perbal (1988).

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):** The purity of the protein (Pyocin) SA188 was determined by subjecting it to SDS-PAGE involving 10% polyacrylamide gel (Laemmli, 1970; Chung, 1987). The molecular weight markers ranging from 14.5 kDa to 200 kDa (Sigma) were used for comparison.

**Direct detection of antimicrobial activity by gel overlay assay:** The antibacterial activity of the purified protein band in gel after SDS-PAGE was detected by gel overlay assay (Bhunja *et al.*, 1991). This was achieved by soaking the gel after SDS PAGE (without staining) in 10% acetic acid (v/v) solution and 20% isopropanol for 2 hours and then washed in distilled water for 4 hours. The gel after washing was transferred on to nutrient agar plate and overlaid with *S. aureus* SA 84 (sensitive strain) suspension. The zone of inhibition around protein band was observed after 24 hours of incubation at 37°C.

## RESULTS AND DISCUSSION

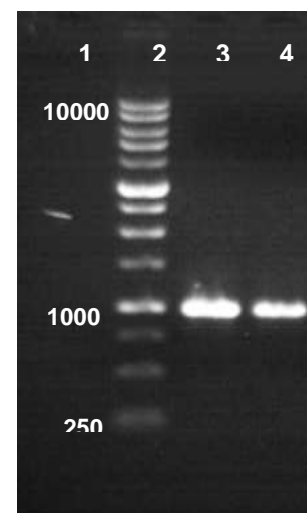
Soil comprises five major groups of organisms including bacteria, fungi, actinomycetes, algae and protozoa as microbial population. Bacteria are the predominant among them and have marked significance in decomposition of wastes. In addition to decomposition, they also produce certain useful substances which enhance soil health, plants growth and generally keep balance in natural ecosystem (Pavithra *et al.*, 2015). Rhizosphere is a rich reservoir of nutrients and energy for bacteria because of the accumulation of plant exudates like amino acids and sugar (Gray and Smith, 2005). The bacteria colonizing in these zones are called rhizobacteria and their numbers are generally 10 to 100 times higher compared to those found in soil (Weller, 1994). The bacteria associated with plants may prove beneficial, deleterious or neutral and the beneficial free living are known as plant growth promoting rhizobacteria (PGPR), and they constitute only 1 to 2% of the total bacterial load. These PGPR belong to different bacterial genera, however, *Bacillus* spp. and *Pseudomonas* spp. are the predominant ones (Podile and Kishore, 2006). The PGPR indirectly promote the growth of plants by reducing or even inhibiting the deleterious effects of plant pathogenic microorganisms by releasing certain antagonistic substances (Glick, 1995; Shaharoon *et al.*, 2006).

The wide range capability of bacteriocins positions them as one of the most promising choice in the next wave of antibiotics (Cavera *et al.*, 2015). Bacteriocins are ribosomally synthesized antagonistic proteins produced by wide range of bacteria. The spectrum of their activity is not limited to bacterial species but recent findings established their bioactivity against viruses and fungi. Being low

molecular weight proteins, they are mostly non-immunogenic which suggests their possible application as a biocontrol agent (Jabeen *et al.*, 2014). *P. aeruginosa* strains produce bacteriocins called pyocins for the sake of invasion and defense of their ecological niche (Riley, 2003). Pyocin production has been reported in more than 90% of *P. aeruginosa* strains therefore, it can be hypothesized that its survival in any environment may be associated with wide distribution of pyocin production (Heo *et al.*, 2007; MacKinnon, 2011).

The present study was conducted to determine the potential of indigenous *Pseudomonas* strains (isolated from diverse environments) for their bacteriocin production. One of these soil associated bacteriocinogenic *P. aeruginosa* SA 188 was selected for detailed focused study keeping in view of the possible application of such potential strains as biological control agents against phytopathogenic bacteria.

**Identification of the selected strain by 16SrDNA analysis:** The selected *Ps. aeruginosa* SA 188 strain was identified earlier by conventional methods including the use of API 20 NE kit. However, problem of the taxonomic complexity was eliminated by using PCR approach based on 16S ribosomal DNA sequence analysis (Spilker *et al.*, 2004). The identification was confirmed by the presence of a specific DNA product of 956 base pairs detected by agarose gel electrophoresis (Fig. 2).

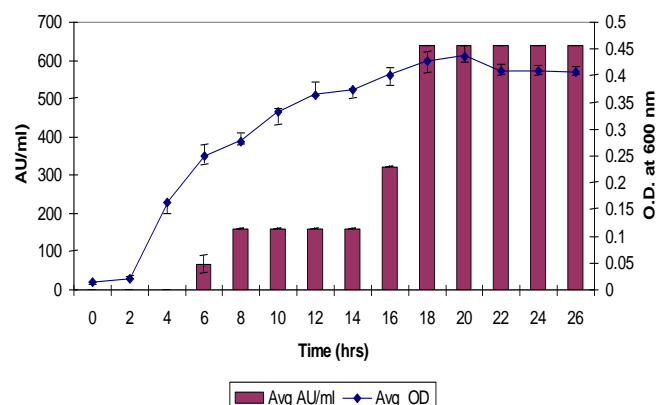


**Figure 2. Identification of *P. aeruginosa* SA 188 by PCR using *P. aeruginosa* specific primers.**

Lane 1, negative control (Master mix without DNA); Lane 2, DNA reference markers 1kb (Gene Direx); Lane 3, *P. aeruginosa* ATCC- 27853; Lane 4, *P. aeruginosa* SA188

**Kinetics of bacteriocin (Pyocin) production:** The production of bacteriocin varies in different phases of bacterial growth cycle. This variation was determined in pyocin production by *Ps. aeruginosa* SA 188. Accordingly,

the pyocin production started in the early logarithmic phase and reached the production peak after 18 to 22 hours i.e. late log phase after which it became constant till onset of the stationary phase (Fig. 3). This decrease in production of bacteriocin after stationary phase could be due to auto proteolytic inactivation, aggregation of protein and adsorption of the bacteriocin molecule to the cell surface of the bacteriocin producing cells (Jabeen *et al.*, 2014).

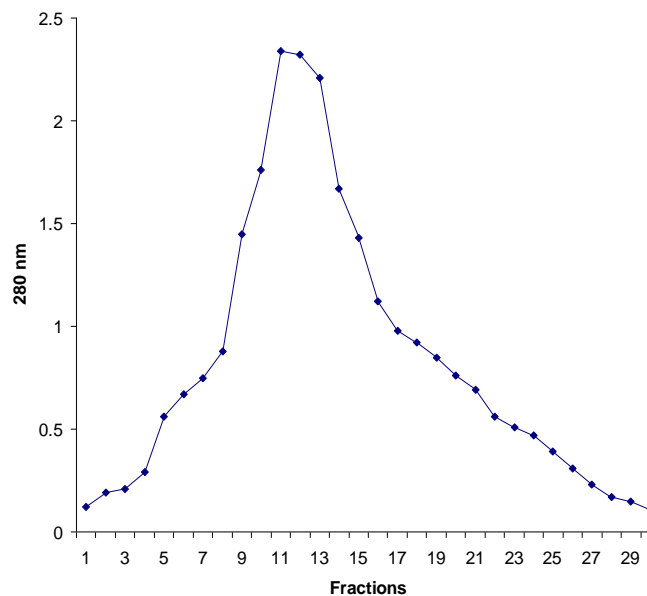


**Figure 3. Production of Pyocin SA 188 during growth cycle of *P. aeruginosa* SA 188.**

**Molecular mass determination by diffusion through dialysis membrane:** A rough estimate of molecular mass of bacteriocin molecules of *Ps. aeruginosa* SA 188 was carried out by using dialysis membrane of 12 kDa cut off size. While the inhibition zone was absent in the stabbed area covered with dialysis membrane, there was a clear and marked inhibition zone in the uncovered stabbed area. These observations suggest the high molecular mass of the bacteriocin i.e. >12 kDa. The high molecular mass of bacteriocins produced by *Ps. aeruginosa* strains is frequently documented in the literature (Kageyama *et al.*, 1964; Scholl *et al.*, 2009).

**Preparation and purification of Pyocin SA 188:** The crude bacteriocin preparation obtained from cell free supernatant of *P. aeruginosa* SA 188 after ammonium sulfate saturated precipitation (70%) was subjected to purification. The ultrafiltration of the crude pyocin was achieved by using dialysis tubing of cut off size 12 kDa. The retention of bioactivity in the dialysate was indicative of high molecular weight of pyocin although, there was a marginal reduction in the bioactivity. This little loss of bioactivity might be because of adsorption of the pyocin molecule on the dialysis membrane (Mojgani *et al.*, 2009). Size exclusion chromatography is an important technique used for protein purification, analysis of purity and determination of interaction among proteins and it is considered to be one of the final steps of purification (Harris, 1989). The pyocin SA 188 after ultrafiltration was further purified by conventional gel permeation chromatography using Sephadex G-75

column. Bioactivity of the (collected) fractions was determined and was found to reside in fractions 11, 12 and 13. The chromatogram of the pyocin SA 188 demonstrated a single peak of protein (Fig. 4). These findings correlate with the observations of Al-Shibib *et al.* (1985) where a single peak of protein was observed when pyocin was eluted from CM Sephadex A-5.



**Figure 4. Elution profile of Pyocin SA 188 by size exclusion chromatography (Sephadex G 75 Gel filtration column).**

The protein purification profile of pyocin SA 188 demonstrated an increase in specific activity of cell free supernatant from 304.7 AU/mg to 457 AU/mg in ammonium sulfate precipitate, whereas, the activity was further increased up to 1422 AU/mg after gel filtration chromatography (Table 1). Findings with similarity were reported in earlier studies where specific activity of pyocin was increased after every step of purification (Sano and Kageyama, 1981; Al-Shibib *et al.*, 1985; Abdi-Ali *et al.*, 2004).

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE):** Proteins can be characterized with respect to their molecular size of the constituent polypeptide by SDS-PAGE (Li, *et al.*, 2014). Further, the bioactivity of a bacteriocin can be directly detected on gel by using this technique (Bhunja *et al.*, 1991). The purity of pyocin SA 188 was determined by employing SDS-PAGE (10% acrylamide denaturing gel). The electrophorogram of the purified pyocin SA 188 revealed a single band of molecular mass of approximately 45 kDa. The band also demonstrated bioactivity by gel overlay method (Fig. 5). The high molecular weight pyocins (particularly R type pyocins)

**Table1. Purification of pyocin SA 188 from culture supernatant of *P. aeruginosa* SA 188.**

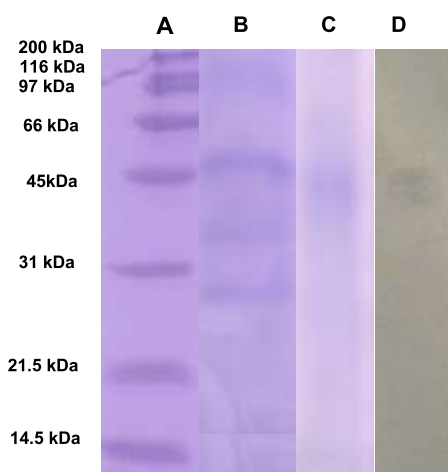
Sample/Step	Volume (ml)	Activity Units (AU/ml)	Total Activity (AU)	Total Protein (mg)	Specific Activity (AU/mg)	Recovery (%)	Purification (fold)
Cell free Supernatant/ Crude extract	300	640	192000	630	304.7	100	1.0
Ammonium Sulfate Precipitate (70%)	30	640	19200	42	457	10	1.5
Size exclusion chromatography (Sephadex G75)	3	1280	3840	2.7	1422	2	4.6

Activity units (AU/ml) = Reciprocal of the highest dilution X 1000 / volume of bacteriocins added; Total Activity (AU) = Activity units X volume of bacteriocin; Total Protein (mg) = Lowry *et al.*, (1951); Specific Activity (AU/mg) = Total activity of subsequent purification/Total protein of the same step; Recovery (%) = Total activity of subsequent step X 100 /Total activity of the crude preparation; Purification (fold) = specific activity of subsequent step/ specific activity of the crude preparation.

have been well documented (Williams *et al.*, 2008; Scholl *et al.*, 2009; Ritchie *et al.*, 2011).

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**Figure 5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) of Pyocin SA-188.**

(A) Lane 1; Molecular weight markers, (B) Lane 2; Partially purified pyocin (Ammonium sulfate precipitate), (C) Lane 3; Purified pyocin (active fractions after gel filtration chromatography), (D) Lane 4; Direct detection of pyocin bioactivity against *S. aureus* SA84.

**Conclusion:** The ability of bacteriocin production by rhizosphere associated bacteria could be of agronomic importance because of their possible use as biocontrol agent. These natural biocontrol agents may improve crops and help to create eco-friendly systems. The purified bacteriocin (produced by *P. aeruginosa* SA 188) reported in the present study might carry importance in this regard, because of its bioactivity (against different bacteria) and its sustainability under certain physico- chemical conditions.

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