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BIOPHYSICOCHEMICAL CHARECTERIZATION OF BACTERIOCIN(S) FROM INDIGENOUSLY ISOLATED *AGROBACTERIUM RADIOBACTER* NA6

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Abstract

Agrobacterium radiobacter NA6 isolated from local agricultural soil of NIAB, Faislabad was found to produce antibacterial peptide designated as Agrocin NA6. The partial purification of bacteriocin was achieved by 70% Ammonium sulphate precipitation. These agrocin has a narrow spectrum of activity against different gram-positive and gram-negative organisms. It remained stable on boiling (100°C, 30min.) and autoclaving (121°C, 15 psi) up to 15 minutes and also showed stability at a wide 3-9 pH range. It was also stable and active in the presence of different heavy metal salts, surfactants and organic solvents. Molecular mass of the bacteriocin was estimated to be less than 12kDa as it passed through dialyzing membrane with cutoff point of 12kDa. The protein nature of the antibacterial substance agrocin NA6 was also confirmed by its sensitivity towards proteinase K. Agrocin NA6 showed bacteriostatic effect when mixed with log phase *Agrobacterium tumefaciens* B6 cells. The bacteriocin under study exhibit good potential to be used as a biocontrol agent.

Introduction

Bacteriocins are the most abundant of antimicrobial compounds produced by bacteria and are found in all major phylogenetic bacterial lineages (Riley & Wertz, 2002). Generally, these substances antagonize only those bacterial species that occupy the same ecological niche or closely related to the producer organism (Riley, 1998). Many phytopathogenic bacteria, including members of the *Corynebacterium, Erwinia, Pseudomonads* and *Xanthomonas* produce proteinaceous bacteriocins (Heu *et al*., 2001). In general, bacterial diseases of plants are very difficult to control owing to the lack of effective chemicals. Antibiotics could be used but they are expensive and pose a potential threat of emergence and subsequent dissipation of resistance. The most effective alternative is the use of copper, which is potentially phytotoxic. In this connection use of agrocin produced by *Agrobacterium* holds great promises. This notion is exemplified by the usage of *A. radiobacter* to control *A. tumefaciens* which causes crown gall disease in fruit and trees especially members of the rose family such as apple, pear, peach, cherry, almond, raspberry and roses. Kerr (1972) proved that the K84 strain of the non-pathogen *Agrobacterium radiobacter* could protect young plants, offering new perspective for the control of the disease. *A. radiobacter* (producing a nucleotide derivative-agrocin 84) had also been extensively used in Australia and California (USA) to control *A. tumefaciens* which causes crown gall disease in fruit trees and other dicots (Kerr, 1980). To date in Pakistan there is no evidence, for the use of bacteriocin as plant disease biocontrol agent. Considering this, *Agrobacterium radiobacter* NA6 (isolated from a local red pepper agricultural field) was tested for its ability to produce bacteriocin as a biocontrol agent and found to be a better producer against an ATCC culture *Agrobacterium tumefaciens* B6. The aim of this work was to investigate the spectrum of sensitive bacteria to agrocin NA6 (isolated from *Agrobacterium radiobacter* NA6) and to characterize its biochemical attributes.

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Materials and Methods

Isolation of *Agrobacterium* (for the bacteriocin production potential) from agriculture soil sample: The soil samples were collected from local agriculture rhizosphere (NIAB, Faislabad). Dilutions (1:10) of 1 gm of soil were made in sterile distilled water. Then 100 µL from each dilution was spread over medium 79 (yeast peptone mannitol agar) and incubated at 29ºC for over night. Next day isolated colonies were gram-stained and streaked on medium 79 for further purification (Gabriel *et al.*, 1989). Different biochemical tests for identification were then performed while final identification of *Agrobacterium radiobacter* NA6 was done by using API 20NE kit. *A. tumefaciens* B6 was used as indicator/sensitive strain. The producer *A. radiobacter* was maintained on Yeast Extract Mannitol Agar Medium 79. Nutrient agar was used for the growth and maintenance of *A. tumefaciens* B6 and for screening the bacteriocinogenic activity. All cultures were grown aerobically at 29°C for 24 hours and maintained at 4^oC. All the chemicals and reagents were obtained from Oxoid, UK.

Bacteriocin activity assays: The bacteriocinogenic activity of *A. radiobacter* was monitored against *A. tumefaciens* B6 with other phytopathogens and several gram-positive and gram-negative strains to check the inhibitory spectrum by: Stab and overlay method (Cooper and James, 1984), cross streak method (Pugsley & Oudega, 1987) and agar-well diffusion assay (Geis *et al*., 1983).

Preparation of crude bacteriocins: The strain that was selected as potential bacteriocin producer was grown in nutrient broth at 29°C for 24 hours. Cells were separated by centrifugation (6000g, 30 min, 4°C). The cell free supernatant (CFS) and was maintained at pH 7.0. Bacteriocin activity in the supernatant was then tested by agar well diffusion assay (Geis *et al.,* 1983) was further subjected to ammonium sulphate precipitation.

Ammonium sulphate precipitation: The cell-free supernatant was used as starting material for protein precipitation. Ammonium sulphate was gradually added to a final concentration (w/v) of 40%, 60%, 70% and 80% respectively and agitation continued for overnight at 4°C. The precipitate was collected (at every step) by centrifugation at 6000g for 45 min., and redissolved in 20 ml of nutrient broth (pH 7.0) and assayed for maximal bacteriocin activity (Harris, 1989).

Bacteriocin activity units: Two fold serial dilutions of cell free neutralized supernatant of the producer strain and of partially purified (70% ammonium sulphate precipitated) bacteriocin were prepared and followed for agar well diffusion assay. Plates were incubated overnight at 29°C and zones of inhibition around each well were measured in mm. The bacteriocin titer was expressed as arbitrary or activity unit/ml. One arbitrary unit (AU) of bacteriocin is defined as the reciprocal of the last serial dilution demonstrating significant inhibitory activity (Barefoot & Klaenhammer, 1983).

$$\text{AU/ml} = \frac{\text{Reciprocal of the highest dilution}}{\text{Volume of bacteriocin added}} \text{ X } 100$$

Effect of temperature: In order to test the heat resistance, 10 ml of partially purified bacteriocin preparation was heated for 15 minutes at 60°C, 70°C, 80°C, 100°C and 121°C (pressure, 15 psi) respectively. Residual bacteriocin activity was detected against *A. tumifacians* B6 at each of these temperatures (Ogunbanwo *et al*., 2003) by agar-well diffusion assay.

Effect of pH on bacteriocin activity: According to the method described by Karaoglu *et al*., (2003) sensitivity of partially purified bacteriocin preparation to different pH values was tested by adjusting the pH of the bacteriocin in the range of pH 3 to 9 with sterile 1N NaOH and 1N HCl. After 2 hours of incubation at room temperature, residual activity of each of the samples was determined against the indicator organism by agar-well diffusion assay.

Effect of proteolytic and lipolytic enzymes on bacteriocin activity: Action of proteolytic and lipolytic enzymes was tested on partially purified bacteriocin preparation by treatment with protease, lipase (Bacterial source), proteinase K (Fungal source) and trypsin (Animal source) (Sigma) each at a final concentration of 1mg per ml. It was then incubated at room temperature for 2 hours and residual activity of bacteriocin was assayed along with bacteriocin and salts (Nakamura *et al.,* 1983).

Effect of surfactants, organic solvents and metal ions on bacteriocin activity: The effect of surfactants on the bacteriocins was tested by adding SDS, Tween 20, and Tween 80 [(Merck) (0.5% v/v final concentration)], to the partially purified bacteriocin preparation. EDTA (Sigma) was added to the partially purified bacteriocin preparation to yield a final concentration of 1.0 mM and 2.5 mM, respectively. Partially purified bacteriocin preparations were mixed with organic solvents including acetone, butanol, chloroform, ethanol, methanol and propanol at a final concentration of 5.0%. In a separate experiment the effect of metal salts on bacteriocin was examined by adding 100 µl of 2mM $AgNO_3$, $BaCl_2$, $CdCl_2$, $CuSO_4$, $CsCl_2$, $FeSO_4$, $MgSO_4$, $MnCl_2$, $NiSO_4$ and $ZnSO_4$ (Merck) to 100 µl of partially purified bacteriocin preparation (1 mM final concentration). Untreated bacteriocin preparation (positive control), the detergents, organic solvents and metal ions at same concentrations were also used as negative controls. All samples were incubated at room temperature for 2 hours and then tested for residual antimicrobial activity (Muriana & Klaenhammer, 1991; Adinarayana *et al*., 2003) by agar-well diffusion assay.

Effect of different agar concentrations on the production and diffusion of agrocin NA6: Effect of different agar concentrations on the production and diffusion of bacteriocin was tested by stab and overlay method on nutrient agar with different 1.2%, 1.4%, 1.6%, 1.8%, 2.0%, 2.2%, 2.4%, and 2.6% agar concentrations (Rasool *et al*., 1996).

Bacteriocin size determination by diffusion through dialysis membrane: The diffusiblity of agrocin through dialysis membrane (cut off 12kDa) was tested to estimate its molecular mass. For this purpose, *A. radiobacter* NA6 was stabbed at two spots in a nutrient agar plate and incubated overnight. Sterile piece of dialysis membrane was used to cover a stab (for test) whereas control was left uncovered after treatment of overnight stabbed culture with chloroform vapours. Plates were then overlaid with 3 ml soft agar containing log phase (approximately $2x10^8$ CFU/mL) standardized indicator strain and observed for zone of inhibition after incubation at 29°C for overnight (Parrot *et al*., 1989; Ahmed & Rasool, 2003).

Table 1. Inhibition pattern of bacteriocin (agrocin NA6) producer strain against different indicator bacteria.

Indicator strains	Agrocin NA6 Activity
Phytopathogens	
Agrobacterium tumefaciens B6	++++
Agrobacterium radiobcter NA7	+++
Erwinia carotovora NA5	-
Erwinia carotovora NA8	-
Pseudomonas andropogonis NA4	-
Xanthomonas citri NA3	-
Xanthomonas oryzae NA1	-
Xanthomonas oryzae NA2	-
Gram negative bacteria	
Escherichia coli WT	-
Escherichia coli 5014	-
Escherichia coli	-
Klebsiella pneumoniae	-
Pseudomonas aeruginosa	-
Salmonella sv. typhi	-
Salmonella sv. *paratyphi* A	++
Salmonella sv. *paratyphi* B	-
Shigella dysenteriae	++++
Shigella flexenari	-
Gram positive bacteria	
Bacillus subtilis	++
Corynebacterium diphtheriae	+++
Corynebacterium pseudodiphtheriticum	-
Micrococcus luteus	++++
Staphylococcus aureus	+++
Staphylococcus epidermidis	-
Streptococcus fecalis	-
Streptococcus pyogenes	-

Key: ++++ = Very strong inhibition zone (30-35 mm), +++ = Strong Inhibition zone (22-25 mm)
++ = Moderate inhibition zone (15-20 mm), - = No zone of inhibition

Concentration determination of agrocin NA6 protein: Protein concentration of the crude and partially purified bacteriocin fractions was determined by the one of the reliable (Biuret) method using BSA (Sigma) as standard protein solution (Harris & Dunn, 1989).

Mode of action: To investigate whether the mode of action of crude bacteriocin on sensitive cells was bactericidal or bacteriostatic, *A. radiobacter* NA6 was grown in 100 ml nutrient broth at 29°C for 24 hours. Cells were separated by centrifugation at 6000 rpm for 30 minutes. The cell-free supernatant containing partially purified bacteriocin was added with sensitive (log phase) *A. tumefaciens* B6 cells and optical density change at O.D. 530 nm was determined every hour for 6 hours followed by colony forming units/ml (Bhunia *et al.*, 1991).

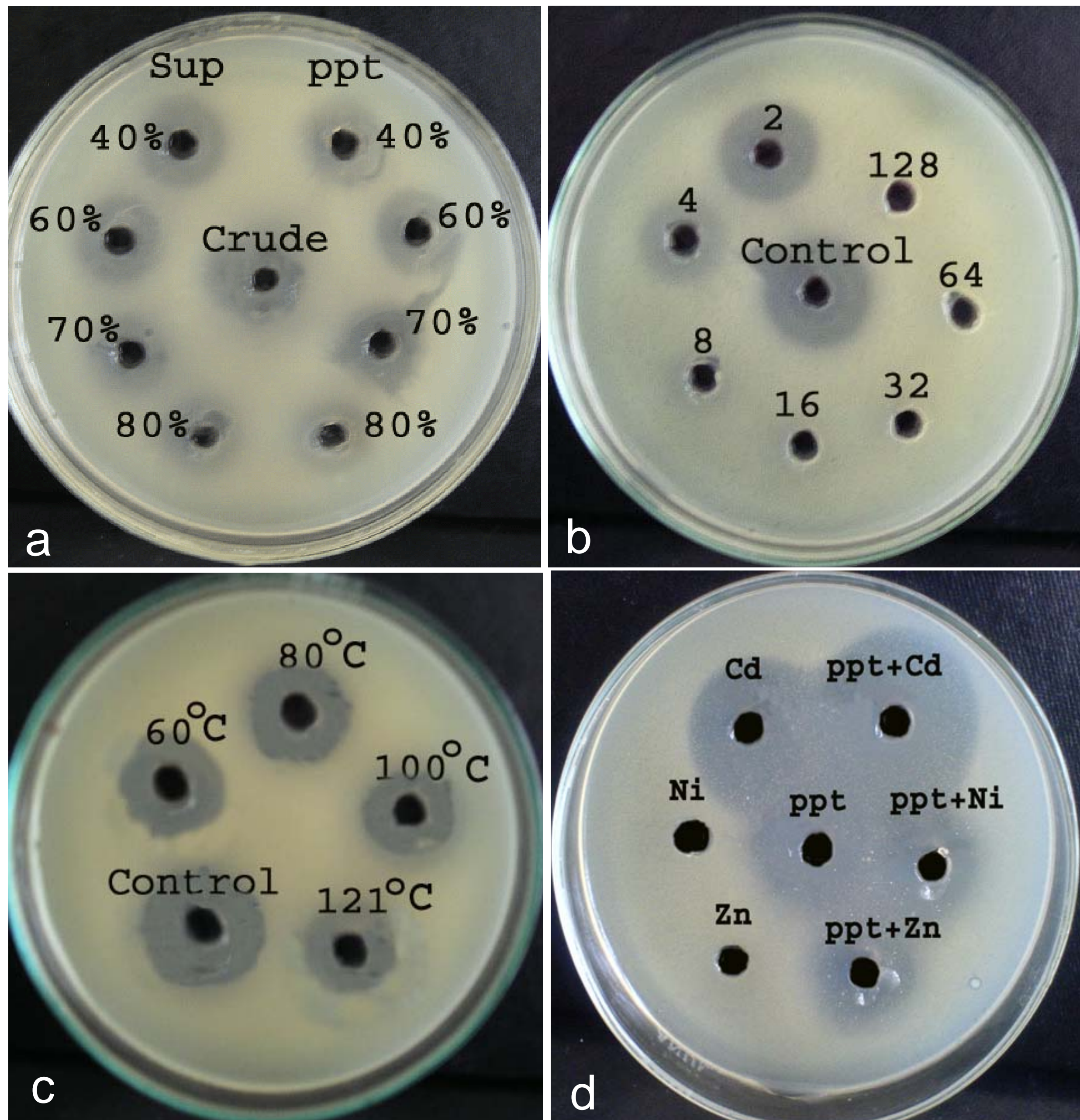

Fig. 1. Agar-well diffusion assay of agrocin NA6: a) purification patterns of bacteriocin by Ammonium sulphate precipitation at different concentrations; b) bioactivity of partially purified bacteriocin in terms of activity units (AU/ml); c) temperature stability of partially purified bacteriocin; d) Stability profile of partially purified bacteriocin in heavy metal salts against *Agrobacterium tumefaciens* B6.

Results

The agrocin NA6 was found to have a narrow spectrum, which can inhibit 23% and 50% of tested gram-negative and gram-positive bacterial cultures respectively (Table 1). Maximum activity of agrocin was observed at 70% ammonium sulphate precipitation (Fig. 1a). The most active crude supernatants contained 160 AU/ml whereas the most active partially purified agrocin after ammonium sulfate precipitation contained 80 AU/ml. The specific activity in crude culture supernatant was 66.66 (AU/mg) which was increased to 213.33 (AU/mg) after ammonium sulfate precipitation and the final

purification was 3.2-fold with a recovery of 50% (Table 2) and the activity was inhibited by proteinase K; however it remained resistant to other enzymes including protease, trypsin and lipase (sources given in material and method section). Isolated and purified agrocin NA6 was highly stable at wide pH range (4-9). Agrocin also showed resistance to high temperature (boiling and autoclaving up to 15min, Fig. 1c). Purified bacteriocin remained active at low temperature i.e. 4°C and -20°C up to 6 months (Table 3). Agrocin NA6 showed stability against the tested metal salts (Table 4, Fig. 1d), solvents and surfactants (Table 4) and was dialyzed through membrane of 12kDa cutoff, which roughly was suggestive of its low molecular mass. The best production/activity of agrocin NA6 was observed in 2.0% agar concentration (Table 5). Bacteriostatic effect of agrocin NA6 was observed against *A. tumefaciens* B6 (Fig. 2).

Table 2. Partial purification of agrocin NA6 from culture supernatant of *Agrobacterium radiobacter* NA6.

Sample	Volume (ml)	Activity[1] (AU/ml)	Protein (mg/ml)	Total protein (mg)	Specific activity[2] (AU/mg)	Yield[3] (%)	Purification factor[4]
Culture supernatant	500	160	4.8	2400	66.66	100	1
Ammonium sulfate precipitation (70%)	100	80	3.75	375	213.33	50	3.2

[1]Activity unit (AU/ml) = Reciprocal of the highest dilution x1000/volume of bacteriocin added.
[2]Specific activity (AU/mg) = Total activity of the subsequent purification step/Total protein of the same step.
[3]Recovery (%) = Total activity of subsequent step x 100/Total activity of crude preparation.
[4]Purification fold = Specific activity of subsequent step/ Specific activity of crude preparation.

Table 3. Effect of heat, pH and enzyme treatment on agrocin NA6 preparation

Treatment	Resistance/Sensitivity pattern
Temperature treatments	
-20°C	R
+4°C	R
60°C (30 min)	R
80°C(30 min)	R
100°C(30 min)	R
121°C (15 psi, 15 min)	R
pH treatments (2 hours)	
4-9	R
Enzymes	
Lipase	R
Protease	R
Proteinase K	S
Trypsin	R

R = No change in bacteriocin activity
S = Loss of bacteriocin activity.

Table 4. Factors affecting the activity of agrocin NA6.

Treatments	Activity (zone of inhibition)*		
	Positive control (ppt. only)	Negative control (salts only)	Test (Salts+ppt.)
Metal salts			
$AgNO_3$	+++	++	++
$BaCl_2$	+++	-	+++
$CdCl_2$	+++	+++	++++
$CuSO_4$	+++	-	+++
$CsCl_2$	+++	-	+++
$FeSO_4$	+++	-	+++
$MgSO_4$	+++	-	+++
$MnCl_2$	+++	-	++
$NiSO_4$	+++	-	++
$ZnSO_4$	+++	-	++
Surfactants			
SDS	+++	++	++++
Tween20	+++	-	++
Tween80	+++	++	++
EDTA	+++	++++	++++
Solvents 5%			
Acetone	+++	-	+++
Butanol	+++	-	+++
Chloroform	+++	-	+++
Ethanol	+++	-	+++
Methanol	+++	-	+++
Propanol	+++	-	+++

*Inhibitory activity determined by agar well diffusion method.
Key: ++++ = Very strong inhibition zone (30-35 mm), +++ = Strong Inhibition zone (22-25 mm)
++ = Moderate inhibition zone (15-20 mm), - = No zone of inhibition

Table 5. Effect of varried agar concentrations on agrocin NA6 diffusion.

Agar concentration%	Zone of inhibition (cm)
1.2	1.5
1.4	2.3
1.6	2.6
1.8	2.7
2.0	3.0
2.2	2.7
2.4	2.6
2.6	2.5

*Inhibitory activity determined by Stab & overlay method (Cooper & James, 1984).

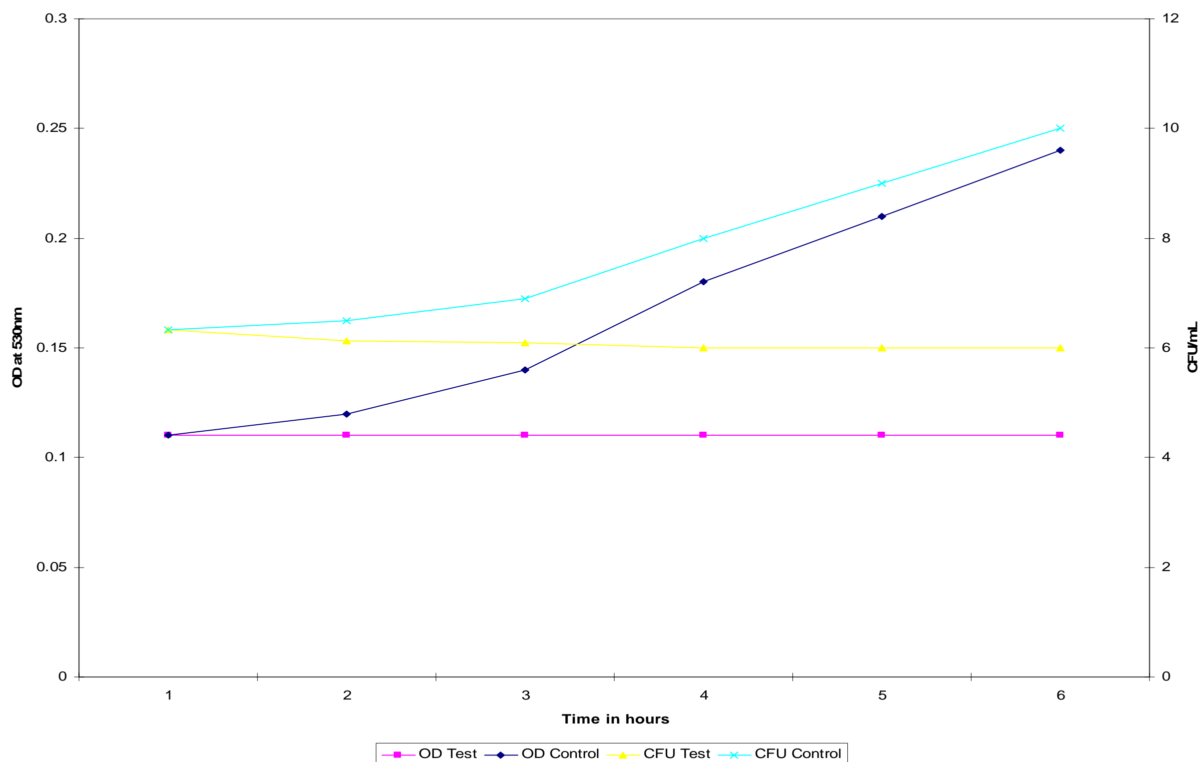

Fig. 2. Bacteriostatic effect of agrocin NA6 on log phase cells of *A. tumefaciens* B6

Discussion

The present study reveals the inhibitory effect of peptide bacteriocin (agrocin NA6) produced by *Agrobacterium radiobacter* NA6 (isolated from local agricultural field of NIAB Faislabad) against *Agrobacterium tumefaciens* B6. Antimicrobial spectrum of activity of agrocin NA6 was tested against various organisms. Agrocin NA6 produced by *A. radiobacter* NA6 was able to inhibit some gram-negative bacteria including *Salmonella* sv. paratyphi A, *Shigella dysenteriae* as well as some gram-positive organisms like *Bacillus subtilis, Corynebacterium diphtheriae, Micrococcus luteus* and *Staphylococcus aureus* (that belongs to distinct genera), but over all inhibitory spectrum of agrocin NA6 was narrow. The results have shown that the bacteriocin produced by gram negative bacteria usually has narrow activity spectrum but those produced by gram positive bacteria may have a wide range of action (Hardy, 1982). Bacteriocins differ greatly in their structure and spectrum of activity (Sablon *et al*., 2000). Fahim *et al*., (2007) reported interesting findings whereby a soil isolate *Bacillus brevis* Bravicin AFO1 (a bacteriocin like inhibitory substance) appreciably active against methicillin resistant *Staph aureus* (MRSA). In this study, we also confirmed the resistance of *Agrobacterium radiobacter* against its own bacteriocin, as the producer was not inhibited by its own protein product. Bacteriocin producers are generally immuned to their own bacteriocin because of a self-protection mechanism that is specific for the bacteriocin produced (Sahl & Bierbaum, 1998; Ennahar *et al*., 2000). Production of agrocin NA6 was also monitored in the presence of peptone in the medium. We observed that *A. radiobacter* is produced in peptone containing medium. However, Barbara & Pootjes (1976) demonstrated that agrocin 84 is not produced or inhibited in a peptone-containing medium and does not show activity against cells growing in a complex medium. The cell

free supernatant (CFS) was subjected to partial purification by Ammonium sulphate precipitation (70% saturation). Protein concentration and the activity unit (AU/ml) of the antimicrobial peptide were measured before and after Ammonium sulphate precipitation step. When CFS and crude (ammonium sulphate precipitated) agrocin was diluted and bioassayed, the inhibition zone was directly related to the log of the dilution, although, yields varied considerably. Protein nature of the inhibitory compound was established by Ammonium sulphate precipitation and sensitivity to proteinase K while resting trypsin, protease, lipase and heat resistance. Although the bacteriocin produced by *A. radiobacter* NA6 was stable above pH 3 (up to pH 9). Kerr & Htay (1974) and Heip *et al*., (1975) had shown that agrocin 84 was sensitive to beyond pH 8.0 and below pH 4.0. Agrocin NA6 remained stable on boiling and autoclaving up to 15 minutes at $4^{o}C$ and at $-20^{o}C$ on storage up to 6 months. These properties could be exploited for commercial applications of this bacteriocin. Other parameters for study include the resistance to heavy metal salts ($AgNO_3$, $BaCl_2$, $CdCl_2$, $CuSO_4$, $CsCl_2$, $FeSO_4$, $MgSO_4$, $MnCl_2$, $NiSO_4$ and $ZnSO_4$), organic solvents like acetone, butanol, chloroform, ethanol, methanol and propanol. Surprisingly, the bacteriocin of NA6 remained unaffected by the surfactant Tween 20 thereby confirming high stability to support for its commercial use. Kamoun *et al*., (2005) reported that the bacteriocin produced by *B. thuringiensis* strain BUPM4 responded to both criteria of thermostability and stability to low pH spectra. Hence, Kamoun *et al*., (2005) recommended their bacteriocin for the biocontrol of the related species of *Bacillus* (harmful for agricultural products). Indeed Saleem *et al*., (2009) have recently reported that soil bacteria *Bacillus bravis* Bb and *Pseudomonas aeruginosa* Pa produced bacteriocins against an indicator sensitive strain of *Bacillus* (isolated from the same ecological niche). A rough estimation of molecular weight of agrocin NA6 was done by placing dialysis membrane on stabbed producer culture and overlaying with sensitive culture. Presence of zone of inhibition around stabbed colonies with and without dialyzing membrane indicates that the agrocin has a molecular mass <12kDa; it was also confirmed by subjecting the crude agrocin NA6 in same dialysis tubing (cutoff 12kDa) where it passes through it. Previous reports on the estimation of molecular mass of agrocin have shown that it is a low molecular weight protein with an estimated mass of 2500 dalton (Barbara & Pootjes, 1976). Also, the ability of agrocin for greater diffusion in higher agar concentration (2.2%) suggests its low molecular weight. Bioactivity of crude bacteriocin was followed upto 6 hours by measuring the OD_{530} of sensitive cells (log-phase culture of *A. tumefaciens* B6) after adding the CFS of *A. radiobacter* NA6 followed by CFU/ml. Lopez *et al*., (1989) studied the agrocin-resistant *A. tumefaciens* strains and proved that other factors like substrate competition and colonization etc (*in vivo*) contribute to the efficacy of biological control besides agrocin 84 production and the sensitivity or resistance of *A. tumefaciens* to the bacteriocin. An earlier work with agrocin indicated that using *A. radiobacter* K84 as biological control agent has been very effective against crown gall on a number of hosts, but exceptions do exist (Utkhede & Smith, 1990).

This biological control is solely preventative for the control of crown gall disease of many plants including apple, pear, peach, cherry, almond, raspberry and roses. We conclude that agrocin NA6 can offer itself as a sustainable yet indigenous biocontrol product /agent. Thus, an appreciable economic loss and budget incurred on import of synthetic pesticides and their far reaching health hazards could be safeguarded.

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References

Adinarayana, K., P. Ellaiah and D.S. Prasad. 2003. Purification and characterization of thermostable alkaline protease from a newly isolated *Bacillus subtilis* PE-11. *AAPS Pharm. Sci. Tech*., 4: 56-64.

Ahmed, S. and S.A. Rasool. 2003. Isolation and biochemical characterization of Mutacin VSM43 isolated from human oral *Streptococcus mutans* VSM43. *Pak J. Pharm. Sci*., 16: 43-50.

Barbara, A.M. and C.F. Pootjes. 1976. Chemical Nature of Agrocin 84 and its effect on a virulent strain of *Agrobacterium tumefaciens*. *Antimicrob. Agents & Chemotherapy*, 7: 498-502.

Barefoot, S.F. and T.R. Klaenhammer. 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol*., 45: 1808-1815.

Bhunia, A.K., M.C. Johnoson and B. Ray. 1991. Mode of action of pediocin AcH from *Pediococcus acidilatici* H on sensitive bacterial strains. *J. Appl. Bacteriol*., 65: 261-268.

Cooper, C. and R. James. 1984. Two new E colicins E8 and E9 produced by a strain of *Escherichia coli*. *J. Gen. Microbiol*., 130: 209-215.

Ennahar, S., T. Sashihara, K. Sonomoto and A. Ishizaki. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol. Lett*., 24: 85-106.

Fahim, F., S. Saeed and S.A. Rasool. 2007. Studies of Brevicin AFO1: A bacteriocin like inhibitory substance active against methicillin resistant *Staphhylococcus aureus*. *Pak. J. Bot*., 39(4):1293-1302.

Geis, A., J. Singh and M. Teuber. 1983. Potential of lactic streptococci to produce bacteriocin. *Appl. Environ. Microbiol*., 45: 205-211.

Hardy, K.G. 1982. *Bacteriocins: Experimental Microbial Ecology*, Chapter 21, (Eds.): R.G. Burns and J.H. Slater. Black well Scientific Publishers, Edinburgh. pp. 368-379.

Harris, E.L.V. 1989. *Concentration of the extract: Protein Purification Methods*. In: *A Practical Approach*. (Eds.): E.L.V. Harris and S. Angal. IRL Press Oxford New York. pp. 125-172.

Harris, E.L.V. and M.N. Dunn. 1989. *Initial planning: Protein purification Methods*. In: *A Practical Approach Harris*. (Eds.): E.L.V. and S. Angal. IRL Press Oxford New York. pp. 1-13.

Heip, J., G.C. Chatterjee, J. Vanderkerckhove, M. Van Montague and J. Schell. 1975. Purification of the *Agrobacterium radiobaeter* 84 Agrocin. *Arch. Int. Physiol. Biochem*., 83: 974-976.

Heu, S., J., Y. Kang, S. Ryu, S.K. Cho, Y. Cho and M. Cho. 2001. gly gene Cloning, expression and purification of Glycinecin A, a bacteriocin produced by *Xanthomonas campestris* pv.glycines 8ra. *Appl. Environ. Microbiol*., 67: 4105-4110.

Iqbal, A., S.A. Ali, S. Ahmad and S.A. Rasool. 1999. Isolation and partial purification of Bac201: a plasmid associated bacteriocin like inhibitory substance from *Staphylococcus aureus* AB201. *J. Basic Microbiol*., 39: 325-336.

Kamoun, F., H. Mejdoub, H. Aouissaoui, J. Reinbolt, A. Hammami and S. Jaoua. 2005. Purification, amino acid sequence and characterization of Bacthuricin F4, a new bacteriocin produced by *Bacillus thuringiensis*. *J. Appl. Microbiol*., 98: 881-888.

Karaoglu, A., F. Aydin., S. Kilic and A. Kilic. 2003. Antimicorbial activity and characteristics of bacteriocins produced by vaginal lactobacilli. *Turk J. Med Sci*., 33: 7-13.

Kerr, A. 1972. Biological control of crown gall: seed inoculation. *J. Appl. Bacteriol.,* 35: 493- 497.

Kerr, A. 1980. Biological control of crown gall through production of agrocin 84. *Plant Disease*, 64: 25-40.

Kerr, A. and K. Htay. 1974. Biological control of crown gall through bacteriocin production. *Physio. Plant Pathol*., 4: 37-43.

López, M.M., M.T. Gorris., C.I. Salcedo., A.M. Montojo and M. Miró. 1989. Evidence of biological control of *Agrobacterium tumefaciens* strains sensitive and resistant to agrocin 84 by different *Agrobacterium radiobacter* strains on Stone Fruit Trees. *Appl. Environ. Microbiol*., 55: 741-746.

Muriana, P.M. and T.R. Klaenhammer. 1991. Purification and partial characterization of Lactacin F, a bacteriocin produced by *Lactobacillus acidophilus* 11088. *Appl. Environ*. *Microbiol*., 57: 114-121.

Nakamura, T., N. Yamazaki., H. Taniguchi and S. Fujimura. 1983. Production, purification and properties of a bacteriocin from *Staphylococcus aureus* isolated from saliva. *Inf. Imm*., 39: 609-614.

Ogunbanwo, S.T., A.I. Sanni and A.A. Onilude. 2003. Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1 *African J. Biotech*., 2: 179-84.

Parrot, M., M. Charest and M.C. Lavoie. 1989. Production of inhibitory substances by *Streptococcus mutans*. *Can. J. Microbiol*., 35: 366-372.

Pugsley A.P. and B. Oudega. 1987. *Methods for studying colicins and their plasmids*. In: *Plasmids: a practical Approach*, (Eds.): K.J. Hardy. Washington, D.C. IRL Press, Oxford. pp. 105-161.

Rasool, S.A., S. Ahmed and A. Iqbal. 1996. Streptococcins of indigenous hemolytic *Streptococci*. *Natural Product Letter*, 8: 67-74.

Riley, M.A. 1998. Molecular mechanisms of bacteriocin evolution. *Ann. Rev Genet*., 32: 255-78.

Riley, M.A. and J.E. Wertz. 2002. Bacteriocins: evolution, ecology and application. *Ann. Rev. Microbiol*., 56: 117-137.

Sablon, E., B. Conteras and E. Vandamme. 2000. Antimicrobial peptides of lactic acid bacteria: mode of action, genetics and biosynthesis. *Adv. Biochem. Eng. Biotechnol*., 68: 22-60.

Sahl, H.G. and G. Bierbaum. 1998. Lantibiotics: biosynthesis and biological activities of uniquely modified peptides from Gram-positive bacteria. *Ann. Rev. Microbiol*., 52: 41-79.

Saleem, F., S. Ahmed, Z. Yakoob and S.A. Rasool. 2009. Comparative study of two bacteriocins produced by representative indigenous soil bacteria. *Pak. J. Pharma. Sci*., 22(3):252-258.

Torreblanca, M., I. Meseguer and F. Rodriguez-Valera. 1989. Halocin H6, a bacteriocin from *Haloferax gibbonsii. J. Gen. Microbiol*., 135: 2655-2661.

Utkhede, R.S. and E.M. Smith. 1990. Effect of fumigants and *Agrobacterium radiobacter* strain 84 in controlling crown gall of apple seedlings. *J. Phytopathol*., 128: 265- 270.

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