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BIO-PHYSICOCHEMICAL CHARACTERIZATION AND APPLIED STUDIES OF CAROTOVORICIN NA5 (CrNA5) ON BLB AFFECTED RICE PLANTS

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Abstract

Erwinia carotovora is a common soil borne plant pathogen, which generally infects plants of family Solanacea. In the present study, bacteriocin (CrNA5), produced by an indigenously isolated *E. carotovora* NA5 has been characterized and its possible anti phytopathogenic potential was shown in the field studies. CrNA5 showed its antimicrobial activity against many gram-positive and gram-negative bacteria including those associated with the plant diseases. The bacteriocin showed substantial stability against wide range of temperatures and pH. Additionally, it was also found resistant to the treatment of metal ions, organic solvents and non-proteolytic enzymes. Conversely, its inactivation by proteinase K and protease suggested its protein nature. Mode of action studies revealed that CrNA5 is bactericidal, particularly against *Xanthomonas oryzae oryzae*. The electron micrograph of CrNA5 revealed spherical particle (empty head) like structures implicating the vestigial bacteriophage based origin of carotovoricin. *In silico* analyses were also conducted in order to deduce the plausible ratio of the amino acids present in the protein. The *In vivo* experiments showed the efficacy of CrNA5 against *X. oryzae oryzae* (*Xoo*), the causative agent of bacterial leaf blight (BLB) of rice, both in controlled conditions (green house) as well as in field trials. To the best of our knowledge, the present study is the first of its kind with the bacteriocin of Erwinia origin (tested against the BLB infected plants in the field). It is expected that the present study will help visit new insights of the bacteriocins produced by *Erwinia carotovora* and their potential (application) as anti phytopathogenic agent.

Key words: Bio-physicochemical, CrNa5, BLB, Rice, *Erwinia carotovora*.

Introduction

Bacteria (under the influence of biotic and abiotic environmental pressure) have evolved many professional co-habitational antagonistic strategies to ensure their survival. Production of ribosomally synthesized peptides and/or proteins (with antimicrobial activity) namely bacteriocins carries a profound role among them, the sustainable survival related characteristics (Jack *et al*., 1995; Nes *et al*., 2007). Historically, antimicrobial spectrum of bacteriocin is generally ascribed to the bacterial species which are closely related to their producer, however, the recent findings have established that many bacteriocins are not only active against the bacterial species which are distantly related to their producer but also against certain viruses (Wachsman *et al*., 1999; Todrov *et al*., 2010).

Bacteriocins hold great promise for the treatment of certain bacterial diseases and could be the future alternative candidate to existing antibiotics (Hammami *et al.,* 2012). Although, a wealth of knowledge has been in hand regarding the bacteriocins of gram-positive bacteria, studies on the bacteriocins of gram-negative bacteria have comparatively been low profiled. Bacteriocins from the *Erwinia* spp. are no exception in this connection. *Erwinia carotovora* (an enterobacter) is the etiological agent of plant diseases like soft rot, black leg or stem rot of a variety of crops of economic and agricultural significance (Roh *et al*., 2009). This bacterial pathogenecity to plant capacity is the function of metabolites like pectate lyase, polygalacturonase and pectin lyase, which help these bacteria to degrade/ hydrolyse high profile plant cell walls (Yamada *et al.,* 2006). In addition to these metabolites, *Erwinia* spp. are known for the production (mostly) of high molecular weight bacteriocins (Nguyen *et al*., 1999 & 2001; Jabrane *et al*., 2002). The first bacteriocin (carotovoricin) from genus *Erwinia* (*E. carotovora* subsp. *carotovora*) was reported by Hamon and Peron (1961) and described as proteinaceous, narrow spectrum bactericidal substance(s). Lysak (1980) investigated eight different bacteriocins by *Erwinia* strains whereas, Tovkach (1998) reported two types of bacteriocins produces by *E. carotovora* i.e., colicin-like small and macromolecular carotovoricin (MCTVs). Yamada *et al.,* (2006) and Chuang *et al.,* (2007) also reported high and low molecular weight bactierocins, carotovoricin Er and carocin S1 respectively. Moreover, Roh *et al.,* (2009) reported the antibacterial substances produced by *Pectobacterium carotovorum* subsp. *carotovorum* and *P. betavasculorum* KACC10056 which were active against several strains of *P. carotovorum* subsp. *carotovorum* In addition to *Xanthomonas albilineans*. It is important to note here that *X. oryzae oryzae* (*Xoo*) is an etiological agent of leaf blight of rice (one of the major cereal crop of Pakistan). Exhibition of anti *Xanthomonas* bioactivity by bacteriocins of *Erwinia*, suggests their potential application in agriculture.

The present study has been designed to undertake a bio-physicochemical characterization of the bacteriocin produced by an indigenous strain of *E. carotovora*. Additionally, some applied studies were also carried out in this regard to illustrate the possible way forward agricultural usage of the understudy bacteriocin. It is anticipated; the findings will not only provide further insights to the attributes associated with the bacteriocins of *E. carotovora* but also demonstrate their applicability in order to counter atleast some of the economically irritant plant infections.

Materials and Methods

Isolation and identification: *Erwinia carotovora* NA5 (the producer strain), *Eewinia carotovora* NA8 and *Xanthomonas oryzae oryzae* NA1 (indicator/sensitive strains) were indigenously isolated from the diseased potato tuber and BLB affected rice plant(s).

Bacteriocinogenic potential and bacteriocin (CrNA5) preparation from *E. carotovora* NA5: The bioactivity of the producer strain was determined by stab and overlay (Cooper & James, 1984), cross streak method (Pugsley & Oudega, 1987) and agar-well diffusion assay [(AWDA) (Lambert & Pearson, 2000)]. For this *E. carotovora* NA5 was grown in nutrient broth at 29°C for 24 hours, cells were separated by centrifugation at 6000g at 4°C for 45 minutes. The cell free supernatant was filter sterilized by 0.45µm filter and concentrated (3-5 fold) using a prechilled (at 4°C) rotary evaporator. Further concentration of the (desired) protein was achieved by ammonium sulphate precipitation to the optimum saturation level. Precipitates were later recovered by centrifugation at 10000g for 60min at 4°C and the pellet was suspended in 50mM sodium phosphate buffer (pH 7.0). Protein concentration was measured by Biurett method (Muriana & Klaenhammer, 1991).

Physicochemical characterization of CrNA5: To detect the activity/stability of CrNA5 at different pH values, bacteriocin preparation was adjusted to varied pH values (2-12) with 1M NaOH or 1MHCl (Merck). The pH values remain maintained for 1 hour at 29°C, re-adjusted to neutral (7.0) pH by using same base/acid and assayed for bioactivity by AWDA. CrNA5 was also mixed with 10mM phosphate buffer of different pH range (1-14) and assayed for bioactivity (Bhunia *et al.,* 1988). To determine the temperature and time range stability, the bacteriocin (CrNA5) was kept at -20°C, 0°C, and 4°C and the activity was assayed after different time intervals for up to six months. Thermostability was also determined by after treatments at 60°C, 80°C, 100°C (for 30 minutes) and 121°C at 15p.s.i for 20 minutes and was assayed for bioactivity (Rasool *et al*., 1996). Biomolecular charecterization was determined by treating the CrNA5 preparation with enzymes: lipase, protease (Bacterial source) catalase, proteinase K (Fungal source) and lysozyme (Animal source, Sigma) at a final concentration of 1mg/mL (Muriana & Klaenhammer, 1991). Equal volumes of the bacteriocin CrNA5 were mixed with organic solvents (butane, propane, methanol, acetone, chloroform and formalin) in 1%, 5% and 10% concentrations (pre-chilled at 4°C) and with 1mM solutions of several metal ions ($BaCl_2$, $MnCl_2$, $CdCl_2$, $MgSO_4$, $CsCl_2$, $ZnSO_4$, $FeSO_4$ and $NiSO_4$). Mixture were agitated and incubated at 29°C for 2 hours and further processed through AWDA (Ahmad *et al*., 2004).

Kinetics of bacteriocin CrNA5 production and effect: Production of CrNA5 was monitored during the growth cycle by growing the *E. carotovora* NA5 cells for overnight; next day OD_{530} was measured and the calculated volume of the culture was transferred to 350mL of fresh nutrient broth maintaining OD_{530} not more than 0.01, followed by incubation in shaking incubator (200 rpm). The OD_{530} was recorded with one hour intervals, and supernatants were assayed for bacteriocin bioactivity by AWDA (Parrot *et al*., 1989). The efficacy of CrNA5 against the sensitive cells (at stationary and the logarithmic phases) were determined by harvesting the **s**tationary phase cells of *X. oryzae oryzae* NA1 by centrifugation (10000g) and resuspended in 50mM PBS (pH 7). Cell suspension (0.2mL) of the sensitive was added to 1.8mL of CrNA5, control constituted 0.2mL culture and 1.8mL nutrient broth followed by incubation; the samples were then drawn after 0, 0.5, 1.0, 2.0, and 4.0 hours (Biagi & Azevedo, 1992).

Transmission electron microscopy (TEM) of CrNA5: In order to elucidate the ultramicroscopic structure of CrNA5; 10µL of the bacteriocin preparation was applied on freshly carbon coated (300 mesh sized) copper grid followed by 1% uranyl acetate (Merck) for negative staining and observed under JOEL JEM-100SX Transmission Electron Microscope (Japan) after an acceleration of 80kV. Areas of interest were imaged at 40K–80K magnifications (Nguyen *et al*., 1999; Jabrane *et al.*, 2002).

Computational analysis for amino acid composition: For hypothetical amino acid composition of CrNA5, *in silico* approach was adopted. Complete protein sequencing (from NCBI genomics server) a low molecular weight hypothetical phage related protein was selected and by taking mean value of each amino acid it was presumed to be of low molecular weight (bacteriocin).

Screening of rice varieties: in field experiments: Seven entries/varieties from Rice Research Station, Kala Shah Kaku Lahore, two from rice breeding group, NIAB (Faislabad) and one from NARC (Islamabad) were grown in rice pathology nursery NIAB. Fresh inocula of *X. oryzae oryzae* (*Xoo*) were suspended in distilled water to get 10^8cells/mL; Plants were inoculated at pre panicle stage; scissors dipped in the inocula were used to cut one-fourth part of 10- 12 leaves per plant. After 24 hours of incubation, half of the concentration of (partially purified) bacteriocin was sprayed on *Xoo* inoculated (infected) plants in field, while $1/6^{th}$ dilution was used in control condition. The copper-oxychloride (0.3%) was applied as standard. Data were collected after three weeks of inoculation using BLB scale (Khan *et al.,* 2000) and were analyzed by one way analysis of variance followed by Annova multiple-comparisons test (where $p<0.05$ was accepted as indicating statistical significance).

Results and Discussion

Bacteriocinogenic potential: Detection of bacteriocin production potential from the isolated phytopathogens was monitored using three conventional approaches i.e. AWDA, stab-overlay and cross-streak to identify the best possible conventional method for the detection of bacteriocin bioactivity and to provided the useful preliminary information regarding the bacteriocinogenic organisms. Accordingly, *E. carotovora* NA5 did manifest the bacteriocin activity in all the three screening procedures and

was found to have a broad inhibitory spectrum against diverse groups of microrganisms like *S. epidermidis,* methicillin resistant *S. aureus* (MRSA)*, B. subtilis, M. luteus* (belonging to gram-positive) and those belonging to gram-negative bacteria; *E. coli, K. pneumoniae, S. flexneri* and *S. typhi para A,* phytopathogenic strains including *X. oryzae, E. carotovora*, *Ps. andropogonis, A. radiobacter*. The bacteriocin was designated as carotovoricin NA5 (CrNA5). Broad spectrum bioactivity of the *Erwinia* bacteriocin is not of uncommon occurrence as such the bacteriocin of *Erwinia* by Tovkach (1998) was reported to have bioactivity against *Agrobacterium, Pseudomonas, Klebsiella, E. coli, E. herbicola* and *E. chrysanthemi..* Another strains of *Erwinia* namely ENA49 also produced broad ranged bacteriocin(s) against several *Erwinia, Pseudomonas* and *Xanthomonas* strains (Lysak, 1980). The degree of antagonism (as per zones of inhibition) varied from organism to organism andvariation include the receptors/target sites on the indicator cell surface (targets for most of the bacteriocins may be modified to some extent), charge distribution on the cell, cell wall composition and diffusion of bacteriocin into medium. Additionally, magnitude of the inhibitory zones varies with the age of the culture(s) and amount of inocula stabbed into the solid medium, composition of the medium, agar concentration, depth of medium and conditions of incubation of the bacteriocinogenic strains. A group of researchers from Korea isolated *Pectobacterium carotovorum* showed diverse antibacterial activity against *P. carotovorum* subsp. *carotovorum*. The sensitivity of this bacteriocin varied, depending on the producer and the indicators (Roh *et al.,* 2009). Laukova & Marekova (1992) reported that bacteriocin production is affected (in most cases) by environmental milieu, including the characteristics of the culture medium and the incubation conditions. The AWDA is widely used to quantify and (partly) standardize for testing the bioactivity profile of the antimicrobial agents for further microbiological assays (Lambert and Pearson, 2000). A definition widely accepted for the titration of an inhibitory substance is the reciprocal of the highest dilution of the agent (bacteriocin) exhibiting a definite inhibition zone when the sample volume varies within a range of 5-100µL (Van Reenen *et al*., 1998). The titer of CrNA5 was found to be 160AU/mL against *E. carotovora* NA8 and *X. oryzae* NA1. Activity units (AU) were found different against different sensitive strains. The results in hand are supported by the ones reported by Nguyen *et al*., (2001) who showed the varied bactericidal titers of carotovoricin Er preparation against different strains of *E. carotovora* subsp. *carotovora* strains EC-2P7 and 645Ar. Roh *et al*., (2009) tested total of 29 different plant pathogens, representing 7 genera of *Brenneria, Pantoea, Pectobacterium, Erwinia, Xanthomonas, Pseudomonas* and *Burkholderia* (chosen to represent various regions and host plants). They were tested for the sensitivity against the antibacterial substances produced by 54 isolated strains (from Korea) of *P. carotovorum* subsp. *carotovorum* and were found mostly active against the strains of *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *atrosepticum* and *Xanthomonas albilineans.*

Table 1. Biophysical characterization of CrNA5 preparation.

Treatment	Activity units (AU/mL)
Temperature treatment	
-20°C to +4°C	160
60°C to 100°C (30 min)	160
121°C (15 psi, 15 min)	80
pH treatment (2 hours)	
2-9	80-160
10-14	80-40
Enzymes	
Protease & Proteinase K	0
Lipase, Catalase, Lysozyme	160
Metal ions (1mM)	
$BaCl_2$	160
$MnCl_2$	160
$CdCl_2$	160
$MgSO_4$	160
$CsCl_2$	160
$ZnSO_4$	160
$FeSO_4$	160
$NiSO_4$	160
Solvents	**1% & 10% concentrations**
Acetone	40&0
Butanol	160&160
Chloroform	160&160
Ethanol	40&0
Methanol	40&0
Propanol	60&160
Agar %	**Zone of inhibition (in mm) *X. oryzae* NA1 & *E. carotovora* NA8**
1.0&1.4	30&35
1.6&1.8	25&27
2.0&2.2	21&14

Physicochemical characterization of carotovoricin NA5: CrNA5 preparation remained stable at the pH range of 2-14 (Table 1). However, to some extent the loss of bioactivity was noticed. CrNA5 was found thermostable after several heat treatments and remained intact after several months of refrigeration. The present results could be confirmed by the finding reported by Tovkach (1998) regarding the thermostability of colicin-like carotovoricin CCTV. The protein nature of the bacteriocin CrNA5 was confirmed by enzymatic treatments. Accordingly, the bacteriocin was found stable against catalase, lipase and lysozyme while it lost the bioactivity after protease and proteinase K treatments thus it was confirmed to be a protein. Earlier bacteriocins from *Erwinia* were shown sensitivity to proteolytic enzymes and be referred as protein in nature (Shukin & Avdienko 1980; Chan *et al.,* 2011). These properties strongly suggested that the bacteriocin like activity of the strains could be defined as of a (typical) bacteriocin. Catalase enzyme also could not eliminate the bioactive potential of CrNA5 thereby, the possibility of hydrogen peroxide mediated antagonism by the producer strain was ruled out. Most of the plant pathogenic bacteria have been reported belonging, the gram-negative group, and almost all the known bacteriocins produced by these bacteria are proteins (Holtsmark *et al.,* 2008; Roh *et al*., 2010). Grinter *et al.* (2012) also reported the extended production of bacteriocins by the economically important gram-negative plant pathogens such as *Ps. syringae*, *Pectobacterium* spp., *D. solani, Agrobacterium, Brenneria* spp. and *Xanthomonas* spp. Similarly, CrNA5 was found resistant to number of solvents at a concentration of 1% (acetone, formalin, ethanol, methanol, propanol, butanol and chloroform) and 10% (propanol, butanol and chloroform). Treatment with 1mM solutions of several metal ions did not exerted an adverse effect on the bioactivity of the bacteriocin CrNA5 (Table 1).

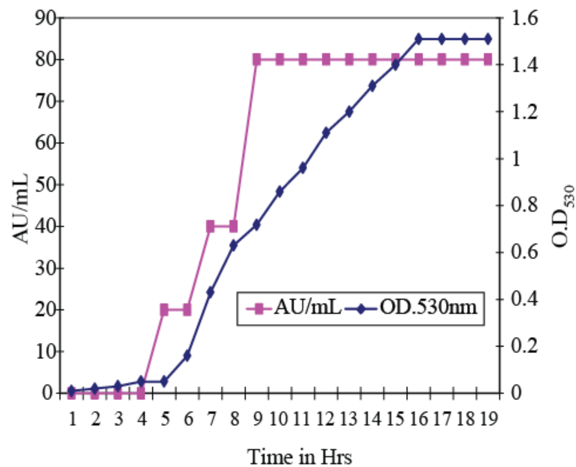

Fig. 1. Production of CrNA5 during growth cycle of *Erwinia carotovora* NA5, samples were taken at different time intervals and the absorbance was measured at 530nm while the production of CrNA5 was determined in terms of AU/mL by agar well diffusion assay.

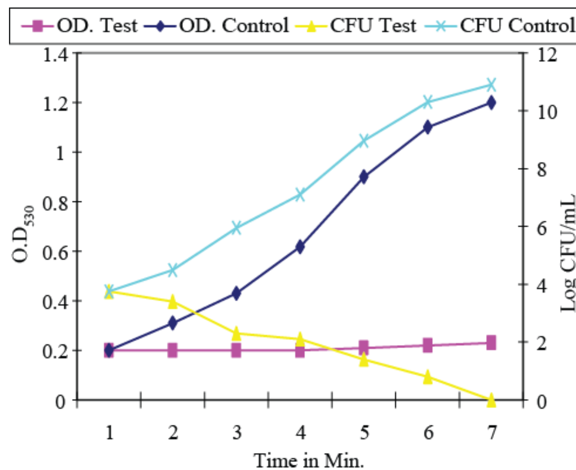

Fig. 2. Bactericidal effect of CrNA5 on log phase cells of *Xanthomonas oryzae* NA1.

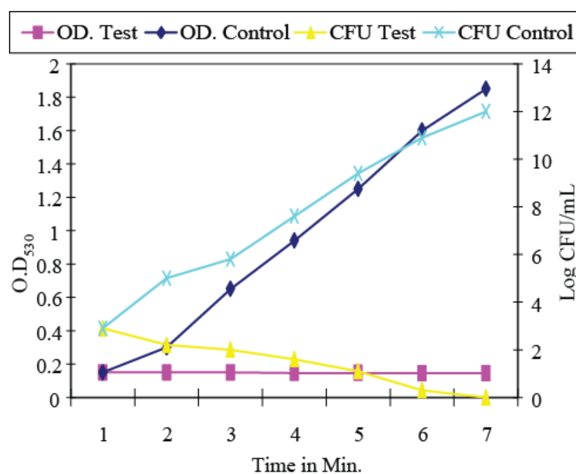

Fig. 3. Bactericidal effect of CrNA5 on stationary phase cells of *X. oryzae* NA1.

Growth kinetics & mode of action of CrNA5: According to the present study, the (extracellular) production of CrNA5 started in early logarithmic phase, its activity reached maximum after 8 hours of incubation and maintained the same bioactivity till late stationary phase. After reaching the maximum bacteriocin activity in the medium during the active growth phase, often a drastic decrease in soluble bacteriocin activity was observed which could be due to the auto proteolytic inactivation, protein aggregation and adsorption of the bacteriocin molecules to the cell surface of the bacteriocin producing cells (Callewaert & Vust, 2000) (Fig. 1). Treatment of CrNA5 during the logarithmic (Fig. 2) and stationary phase cells (Fig. 3) showed a decrease in the CFU/mL, while OD_{530} after the addition of CrNA5 remained the same in the test and in the positive control, thereby suggesting that CrNA5 exerted bactericidal rather than bacteriostatic effect on both the tested strains i.e. *E. carotovora* NA8 and *X. oryzae oryzae* NA1. Tovkach (1998) reported the lysis of *E. carotovora, E herbicola, E. coli* and *Ps. syringae* by the bacteriocins of *E. carotovora*.

Transmission electron microscopy: TEM helped visualizing the CrNA5 as molecules resembling empty bacteriophage /defective phage or it might be membranous vesicles because of their uneven size and shape (Fig. 4). These observations are in agreement with Nguyen *et al*., (1999); the spherical particles obtained from the crude preparation of carotovoricin Er seemed to be the membrane vesicles from the lysed cells of *E. carotovora* Er, because they were not homogeneous in size and shape. Crowely & DeBoer (1980) tested several *E. carotovora* serogroups for the bacteriocin activity; after ammonium sulfate fractionation and high-speed centrifugation followed by electron microscopy; bacteriocins from all the eight strains were morphologically similar to the bacteriophage tail, however the current observations are different from the previously reported findings, where these molecules were described as high molecular weight phage related bacteriocins (Nguyen *et al.*, 1999). However, these bacteriocins were mitomycin-C induced followed by several chemical treatments with several steps of low and high speed centrifugation (at 8000-120,000xg). In the present studies the un-induced crude and precipitated CrNA5 of low molecular weight bacteriocin (resembling the defective phage head or the membrane vesicles of the lysed cells. However, contractile tail like particles were also observed (Fig. 4).

In silico analysis: Considering the mean values of the bacteriocins from *E. carotovora*, it is plausible logical to conceive that the understudy bacteriocin may contain 7.9% alanine, 5.7% arginine, 4.28% asparagine, 5.28% aspartic acid, 1.37% cysteine, 4.27% glutamine, 7.16% glutamic acid, 6.75% glycine, 2.17% histidine, 9.7% leucine, 7.09% isoleucine, 5.7% lysine, 3.3% methionine, 3.2% phenylalanine, 2.9% proline, 7.46% serine, 4.42% threonine, 1.45% tryptophane, 3.4% tyrosine and 6.5% valine. Among these the acidic and basic amino acid components are respectively 12.44% and 13.57% with 49.8% polar and 50.16% non-polar amino acid components (Jeremy *et al.,* 2001). Holistically, the predominance distribution of the non-polar amino acid residues implies the hydrophobic nature of the bacteriocin.

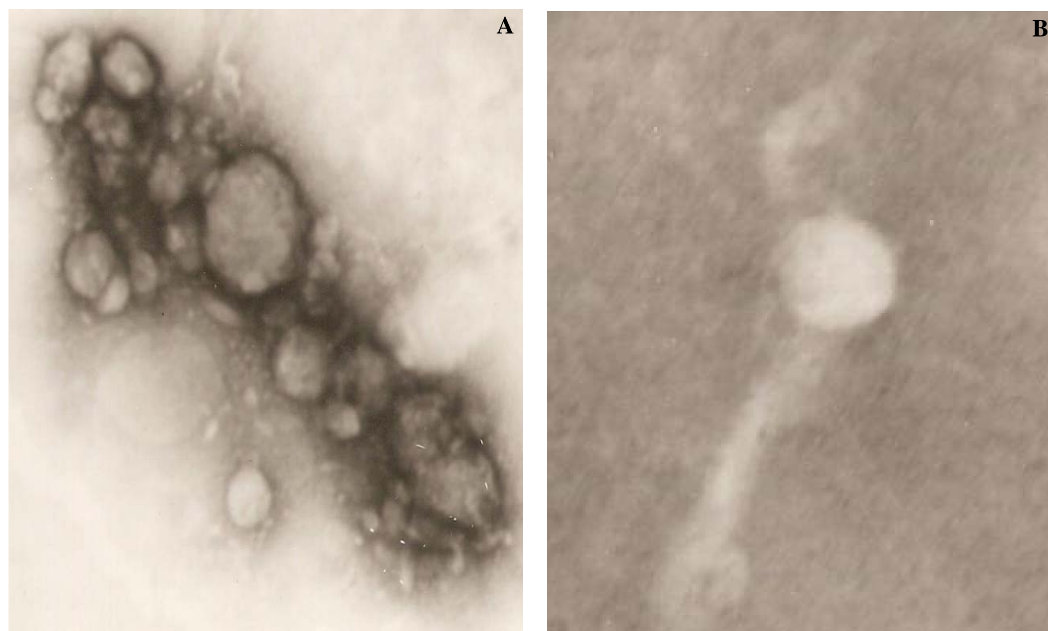

Fig. 4. Transmission electron microscopy of the CrNA5 preparation: Two morphological forms were observed at 80K magnification by negative staining (A) Circular (B) Contractile phage tail like particle(s) were also observed.

Fig. 5. *In-vivo* Assessment of The Efficacy of CrNA5 against *X. oryzae* challenged rice varieties under controlled conditions (A) and in field conditions (B).

Experiments/ trials under controlled & field conditions: Seeds of different varieties/ entries were used, data were recorded after ten days of inoculation using the BLB scale. Significant differences were found in disease scores among different varieties after treatment with CrNA5 (1/6th dilution) compared to the negative control (untreated). Some varieties did show reduction in disease score after bacteriocin/ carotovoricin NA5 application; for example reduction was observed in Bas-385, IRBB-62, Bas-370, Bas-super and Bas-2000 (Table 2, Fig. 5). While the copper-oxychloride treated plants (+ve control) resulted in almost the complete inhibition of infection. However, far reaching health hazards of such & similar chemicals need to be considered or such chemicals need to be replaced with biopesticides. While new biological control strategies are currently being sought, including the use of bacteriocins, bacteriophages, and attenuated plant pathogens (Hert *et al.,* 2005).

For field experiments, 10 rice varieties were used for monitoring the efficacy of CrNA5 (Table 3, Fig. 5). Reduction was observed after treatment with the bacteriocin (compared to the -ve control) in KSK-202, EF-1-20-6-10, EF-1-30-4-1, Bas-super, Bas-2000 and IR-6. The performance of CrNA5 may be rated “satisfactory” as 70% rice entries showed reduction in disease manifestation. Half of the concentration of the partially purified CrNA5 (ammonium sulphate precipitated) was used. A total of 180mL of diluted toxin was used to spray on 60 plants (two replications per plants), 10 entries/verities per replication (with three replications) were used (accordingly, three mL (per plant) diluted toxin was used. For optimum yield per

acre, 80,000 plants are recommended but usually 50,000 plants are grown by the farmers in Pakistan while 150 Litres of half of the concentration i.e 75 Litres of CrNA5 were required per acre (definitely not feasible). But if the results of the control room (where 1/6th dilution was found effective on relatively young plants) are analyzed, then the requirement of the CrNA5 is far low i.e only 1.5mL of CrNA5 (1/6th dilution) is required to spray on one plant and the requirement of the bacteriocin will be 12.5 Litre per acre (which still seems towards higher side). It is therefore, recommended that more concentrated form of partially purified CrNA5 should be used. After all, the use of the bacteriocin (the biocontrol agent) is better compared to the antibiotics or chemicals like streptomycin, cupric oxide, copper oxychloride and borex, which are frequently used for the eradication of plant diseases. Analysis of variance of the % of inhibition are significantly higher ($p<0.05$) in both (in field and in the control room). Gram-negative phytopathogens cause significant losses in a diverse range of economically important crop plants. The effectiveness of traditional countermeasures, such as the breeding and introduction of resistant cultivars, is often limited by the dearth of available sources of genetic resistance. An alternative strategy to reduce the loss involves the use of narrow spectrum protein antibiotics as biocontrol agent (Grinter *et al*., 2012). Sakthivel & Mew (1991) reported that treatment with nonpathogenic bacteriocin-producing strains of *X. compestris* pv. oryzae reduces the incidence and severity of the bacterial leaf streak in rice plants. *X. axonopodis* pv. glycines 8ra produces a bacteriocin called glycinecin A, which specifically inhibits the growth of bacteria belonging to *Xanthomonas* species. Glycinecin A was effective against *X. vesicatoria* on red pepper and *X. oryzae* pv. oryzae on rice. The optimum concentration of glycinecin A for the control in the greenhouse and in the field was 12800 AU/mL. The control efficacy was as high as (or even higher than) the chemical treatment of copper hydroxide. These results suggest that the bacteriocin (glycinecin A) is a potential controlling agent for bacterial diseases of plants (Jeon *et al.,* 2001). Many bacterial species produce peptide antibiotics, called bacteriocins that often have antimicrobial effect on closely related bacteria (Bizaani *et al*., 2005). These compounds (because of their potential application in agriculture, food and pharmaceutical industries) are extensively studied as natural bioperspectives. These bacteriocins are highly specific, cost effective (and even eco-friendly) and appear to be the excellent candidates for agricultural use in controlling the plant pathogens. Glycinecin A (by *X. axonopodis* pv. glycines) specifically inhibits the growth of bacteria belonging to *Xanthomonas* spp. The control efficacy was even higher than the chemical treatment with copper hydroxid; thereby suggesting that the bacteriocin is a potential control agent for the bacterial plant diseases (Fett *et al*., 1987). The bacterium *E. chrysanthemi* ENA49 was found to produce bacteriocin similar to tail fibers of bacteriophages and showed bioactivity against *Erwinia, Pseudomonas* and *Xanthomonas* strains (Lysak *et al*., 1988). It has been proposed that bacteriocins may play a key role in bacterial population dynamics (Riley, 1998). Two different bacteriocins i.e carotovoricin and carocin S1 were found in *Pectobacterium carotovorum* subsp. *carotovorum,* while Roh *et al*., (2010) also reported a third type of bacteriocin carocin D from *Pectobacterium carotovorum* subsp. *carotovorum*, which may have a high potential as a biological control agent in the field. Bacteriocin-producing, avirulent *E. carotovora subsp. carotovora* mutants (A-f-39 and B-e-19) were used as biological control agents against the pathogenic strains 2T-2 and TT-4, which cause soft rot of Chinese cabbage and this bacteriocin treatment was compared with an agrochemical dithianon-copper chloride which proved to be more efficient than the agro-chemical (Kyeremeh *et al.*, 2000). The present findings have served the purpose of prompting new interest in bacteriocins produced by plant pathogens that can convincingly be considered an alternative biocontrol etc. useful in reducing the hazards associated with the use of synthetic pesticides. Valid formulations of CrNA5 to ensure the adequate efficacy of the bactericide under natural environmental conditions should be pursued. While constructing the transgenic plants that express multiple bacteriocin(s) genes would be an acceptable and practicable strategy to bio fight against phytopathogens.

Table 2. Efficiency of CrNA5 to control BLB (Disease) of rice in controlled conditions (average disease scores of three replicates).

Entries/ varieties of rice (*Oryza sativa*)	Control (plants infected with *Xoo*)	Test (plants infected with *Xoo* and treated with CrNA5)
IR-6	8.5	8.5
Bas-370	8.5	5.5
Sup-Bas	8.5	6
IRBB-62	5	2.5
Bas-385	9	5.5
Bas-2000	9	6.5

Table 3. Efficiency of CrNA5 to control BLB (disease) of rice in field conditions (average disease scores of three replicates).

Entries/ varieties of rice (*Oryza sativa*)	Control (plants infected with *Xoo*)	Test (plants infected with *Xoo* and treated with CrNA5)
Pk-369943	6.16	7.00
KSK-203	6.08	6.50
KSK-202	6.93	6.73
KSK-201	6.93	7.03
IR-6	6.63	6.23
Bas-370	6.43	5.97
EF-1-20-6-10	3.63	3.47
EF-1-30-4-1	6.07	4.97
Bas-Super	6.80	5.70
Bas-2000	6.90	6.67

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