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Isolation, Identification and Bacteriocin Production by Indigenous Diseased Plant and Soil Associated Bacteria

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Abstract: Five phytopathogenic strains namely, *Xanthomonas oryzae* NA1, *Xanthomonas citri* NA2, *Pseudomonas andropogonis* NA3, *Erwinia carotovora* NA4 (isolated from diseased fruits and vegetables) and *Agrobacterium radiobacter* NA5 (isolated from pepper rhizophere) were identified on morpho-cultural and biochemical considerations. API 20 E and API 20 NE kits were used to confirm the identification. All isolates were screened for bacteriocinogenic activity against phytopathogenic bacteria. Accordingly, only two strains i.e. *Erwinia carotovora* NA4, *Agrobacterium radiobacter* NA5 were found to produce bacteriocin. These bacteriocins are designated as erwiniocin NA4, agrocin NA5, respectively. Both the producer strains have shown antibacterial activity against closely related strains. The activity potential of erwiniocin NA4 was calculated as 160AU mL^{-1} while that of agrocin NA5 was 80AU mL^{-1}. The effect of temperature variation and pH on erwiniocin NA4 and agrocin NA5 was also checked and both were found activity resistant at 100°C for 10 min and pH range 2-14. Erwiniocin NA4 was also found resistant to autoclaving while bioactivity of agrocin NA5 was lost after similar treatment.

Key words: Bacteriocin, bacteriocin-like inhibitory substances (BLIS), phytopathogens, erwiniocin, agrocin

INTRODUCTION

Phytopathogenic bacteria have added to use the living plant cells as favoured food source. These bacteria are highly specialized to circumvent plant defenses and efficiently invade tissues and cause diseases[1]. As a result of the infection process, nutrient materials are elaborated which the pathogen is able to utilize. Once invasion of host tissues takes place, secondary responses are initiated in the plant which constitutes manifestations of the disease symptoms[2]. The use of bacteriocins has been acclaimed as one of the safest means to control the plant diseases of microbial origin. Bacteriocins have been described as extracelluar macromolecular protein/peptide antibiotics produced by certain bacteria, which exert their lethal effects on bacteria of the same or the related groups[3]. Current and future prospects for the control of these bacterial plant diseases with bacteriocins are immense and these extracelluar macromolecular antibiotics exert their lethal effects on bacteria of the same or related groups[4]. This is with particular reference to the risks in using broad-spectrum agro-chemicals and antibiotics to control the plant pathogens. Thus, bacteriocins have most of the attributes considered desirable for microbial control. They have been reported to inhibit a wide range of gram-positive and gram-negative bacteria. Bacteriocins and Bacteriocin-Like Inhibitory Substances (BLIS) are medically, industrially and agriculturally very important[5,6]. Many phytopathogenic bacteria including members of the corynebacteria, erwinia, pseudomonas, xanthomonas and agrobacterium produce proteinaceous bacteriocins[7]. These bacteriocins are highly specific, cost effective and are safe for the users and the environment and appear to be excellent candidates for agricultural use in controlling plant pathogens. Genetically Modified (GM) *Agrobacterium radiobacter* releases a bacteriocin (agrocin), active against *A. tumefaciens*. This agrocin is a novel nucleic acid derivative that prevents the formation of crown gall tumors in the infected plants[8].

MATERIALS AND METHODS

Collection of sample: Different infected fruits and vegetables were collected from local subzi mandi, bazaar, Murad memmon goth of Karachi distt. Malir and Mitchelle's farm-house (Renala khurd) and pepper-onion grown soil samples from Ayub Agriculture Research Institute, Faisalabad.

Isolation of bacteria from diseased plants: The phytopathogenic bacteria were isolated from plant materials of various types (this included rotten fruits,

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vegetables and diseased plant materials e.g. leaves, stem etc). The diseased plant material (leaves and skin of fruits) was washed with sterilized distilled water and cut into small pieces (with scalpel), then treated with 10% diluted hypochlorite bleach for 1-2 min to remove contaminants, rinsed with distilled water and sectioned. The water-soaked tissue at the lesion margin was streaked and stabbed across a sterile Yeast Peptone Glucose Agar (YPGA) and Nutrient Agar medium. Next day isolated colonies were gram-stained and streaked on different media. Biochemical tests for their identification were then performed as per Scoott[9].

Isolation of *Agrobacterium* from soil sample: The soil sample was collected from onion and pepper rhizosphere region. Dilution (1:10) of I g of soil was made in steriled distilled water. Then 10 uL from each dilution was spread on medium 79 (yeast extract manitol agar) and incubated at 29°C for over night. Next day isolated colonies were Gram-stained and streaked on medium 79. Different biochemical tests for their identification were then performed as per Gabriel[10].

Media and growth conditions: Yeast Peptone Glucose Agar (YPGA), King's agar, levans agar, Nutrient Agar (NA), medium 79 (yeast extract manitol agar) and MacConkey agar medium were used for the isolation and identification of phytopathogenic bacteria. All cultures were incubated at 29°C for over night to get maximum growth. All the cultures were maintained in vials by growing them in 3 mL of nutrient broth and after 24 h incubation overlaid with 3 mL 40% glycerol. Vials were stored at -70°C.

Identification of bacteria: Characteristics which were taken into consideration to identify the bacterial isolates from diseased fruits and vegetables include morpho-colonial bases (on particular media) e.g. pigment production, utilization of various carbohydrates and synthesis of enzymes like oxidase and catalase as per Holt[11]. Confirmation of the identification was based on the use of API 20 E and API 20 NE kits.

Bacteriocin production by phytopathogenic and soil associated bacteria: In order to check the bacteriocinogenic potential of phytopathogenic and soil associated bacteria following three methods were used:

Cross-streak method: Nutrient Agar plates were inoculated with the single producer strain as a streak across the surface of agar plate and incubated at 29°C for 24 h. Next day plates were exposed to chloroform vapours (Keeping the plates inverted and 9 cm diameter piece of Whatman filter paper No. 1 was introduced into the lid and impregnated with 1 mL of chloroform for 15-20 min) to kill the producer and sensitive/indicator cultures were cross-streaked perpendicular to the producer strain and incubated again for overnight. Next day plates were observed for inhibition of growth at each side of the producer culture[12].

Stab-overlay method: Nutrient Agar plates were stabbed with the producer plant pathogen and incubated at 29°C for 24 h. Next day plates were exposed to chloroform vapours to kill the producing strain for 15-20 min. Plate was then overlaid with 3 mL nutrient soft agar containing 0.1 mL of log phase indicator/sensitive organism. Plate was incubated at 29°C for overnight and observed for clear zone around the producer culture[13].

Agar-well diffusion assay: Nutrient Agar plates were overlaid with 3 mL NA soft agar containing 0.1 mL of log phase indicator/sensitive culture. Wells were bored into agar plates and 100 μL of bacteriocin (crude preparation of different dilutions) was placed into each well. The plates were incubated and zones of inhibition were measured in mm[14,15]. The bacteriocin activity was expressed as arbitrary units mL^{-1}. An arbitrary unit (AU mL^{-1}) is defined as 100 μL of the highest dilution of the preparation yielding a definite zone of inhibition on the lawn of the sensitive cells[16,17].

Physio-chemical characterization

Effects of temperature and pH range on bacteriocin bioactivity: Thermal stability of bacteriocins preparations was checked by exposing them to different temperatures i.e. 60°C (10 min), 80°C (10 min), 100°C (10 min) and autoclave (121°C 15 Lbs pressure for 15 min). Bacteriocin preparations were adjusted to different pH levels between 2-12 with 10 mM NaOH (Merck) or 10 mM HCl (Merck). Samples were maintained for 2 h at 37°C. All the samples were then adjusted to pH 7.0 with sterile 4.0 mM phosphate buffer and assayed for bioactivity[18,19].

RESULTS AND DISCUSSION

The present research findings pertain to the isolation of phytopathogens from different diseased fruits, vegetable (Fig. 1) and non-pathogen from soil. Five phytopathogenic bacteria were isolated from different diseased fruits, vegetables and rhizospheres soil. They were identified on the bases of morpho-cultural and biochemical considerations (Table 1, 2 and 3). API 20 E and API 20 NE kits were used for the final identification of the bacterial isolates. The isolated phytopathogenic and non-pathogen soil bacterial strains were screened for

Table 1: Source and disease of isolated organism

Sources	Isolated organism	Disease caused
Rice	*Xanthomonas oryzae* NA1	Bacterial leaf blight
Orange	*Xanthomonas citri* NA2	Citrus canker
Sorghum	*Pseudomonas andropogonis* NA3	Bacterial stripe
Potato	*Erwinia carotovora* NA4	Fire blight
Soil (pepper grown)	*Agrobacterium radiobacter* NA5	Soil borne

bacteriocin production potential by three methods i.e. stab-overlay, agar-well diffusion and cross-streak (Fig. 2). Out of five isolates, only two were found to be the bacteriocin producers i.e. *Erwinia carotovora* NA4, *Agrobacterium radiobacter* NA5 (their bacteriocins are designated as erwiniocin NA4 and agrocin NA5, respectively). Previously, bacteriocin production by other

Fig. 1: Infected potatoes (source for the isolation of *Erwinia carotovora* NA4) and oranges (source for the isolation of *Xanthomonas citri* NA2)

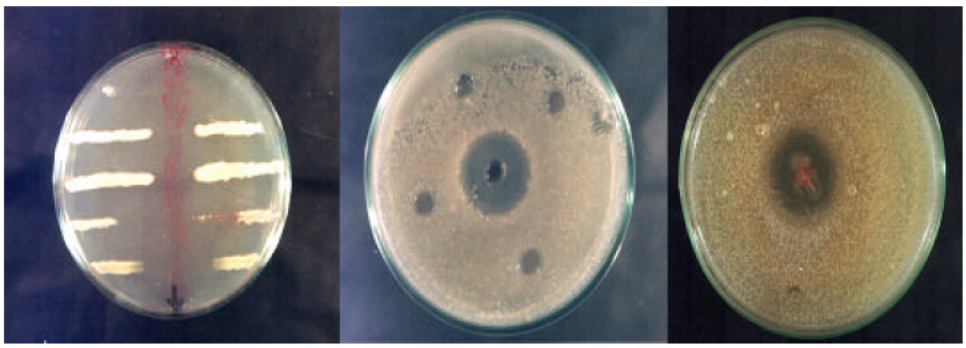

Fig. 2: Three methods (Cross streak, agar well diffusion and stab overlay) demonstrating bacteriocinogenic activity

Table 2: Morphological and cultural characteristics of isolated strains/bacteria

Sources	Isolated organism	Gram- reaction	Cultural characteristics on nutrient agar
Rice	*Xanthomonas oryzae* NA 1	Gram-negative rods	Cream to yellow colored, round and smooth colonies.
Orange	*Xanthomonas citri* NA2	Gram-negative rods	White colored round and smooth colonies.
Sorghum	*Pseudomonas andropogonis* NA3	Gram-negative rods	White colored round, smooth and pinpointed colonies.
Potato	*Erwinia carotovora* NA4	Gram-negative rods	Pink to orange pigmented, round, smooth and pin pointed colonies.
Soil (From pepper rhizosphere)	*Agrobacterium radiobacter* NA5	Gram-negative rods	White colored large, round, smooth and dry colonies. Red pigmented colonies on medium 79

Table 3: Biochemical characteristics of the isolates

Isolated organism	Catalase	Oxidase	Gelatin liquification	Nitrate reduction	Esculin hydrolysis	Urea hydrolysis	Growth at 37°C	Levan production
Xanthomonas oryzae NA 1	+	-	+	-	+	-	-	-
Xanthomonas citri NA2	+	+	-	+	+	+	-	-
Pseudomonas andropogonis NA3	-	+	-	+	+	-	-	+
Erwinia carotovora NA4	+	-	+	+	+	+	-	+
Agrobacterium radiobacter NA5	+	+	+	+	+	-	-	-

Key: + = Test is positive; - =Test is negative

Table 4: Cross bacteriocinogenic activity of isolated phytopathogenic and soil associated bacteria (by Stab-overlay and agar-well diffusion method)

	Sensitive strains					
Producer strains	*X. oryzae* NA1	*X. citri* NA2	*P. andropogonis* NA3	*E. carotovora* NA4	*A. radiobacter* NA5	*A. radiobacter* LMG
X. oryzae NA1	-	-	-	-	-	-
X. citri NA2	-	-	-	-	-	-
P. andropogonis NA3	-	-	-	-	-	-
Erwinia carotovora NA4	+	-	-	-	+	+
Agrobacterium radiobacter NA5	-	-	-	-	-	+

Key: + = zone of inhibition 15-30 mm; - = no zone of inhibition
A. radiobacter LMG is the isolate of Lab of Molecular Genetics

Table 5: Cross bacteriocinogenic activity of isolated phytopathogenic and soil associated bacteria (by Cross- streak method)

	Sensitive strains					
Producer strains	*X. oryzae* NA1	*X. citri* NA2	*P. andropogonis* NA3	*E. carotovora* NA4	*A. radiobacter* NA5	*A. radiobacter* LMG
X. oryzae NA1	-	-	-	-	-	-
X. citri NA2	-	-	-	-	-	-
P. andropogonis NA3	-	-	-	-	-	-
Erwinia carotovora NA4	+	+	+	-	+	+
A. radiobacter NA5	-	-	-	-	-	+

Key: + = zone of inhibition 15-30 mm; - = no zone of inhibition, *A. radiobacter* LMG is the isolate of Lab of Molecular Genetic

Table 6: Physico-chemical characterization of erwiniocin NA4 and agrocin NA5

Properties	Erwiniocin NA4	Agrocin NA5
AU mL^{-1}	160 AU mL^{-1}	80 AU mL^{-1}
Temperature treatments		
60°C	R	R
80°C	R	R
100°C	R	R
Autoclaving	R	S
pH treatments		
2-14	R	R

Key: R= resistant; S= sensitive

plant pathogens was reported by Heu *et al.*[7] and Chuang *et al.*[2]. Interestingly, all three isolates did produce the bacteriocin in all three screening procedures (Table 4 and 5). The two bacteriocin producing isolates so far have shown narrow antibacterial spectrum i.e. antagonising only closely related species. These results are in agreement with the findings of Nguyen[20], who also reported narrow-spectrum activity of carotovoricin ER. According to the present findings, cross-streak method was found to be more effective as erwiniocin NA4 possessed antibacterial activity against all isolated phytopathogens, while less active in stab-overlay and agar-well diffusion methods. This difference may be due to the difference in rate of diffusion of bacteriocin in agar medium in the three methods[19]. The activity potential of erwiniocin NA4 is calculated as 160 AU mL^{-1} while that of agrocin NA5 is 80 AU mL^{-1} (Table 6). The effect of different temperatures and pH on erwiniocin NA4 and agrocin NA5 and was also checked (Table 6). Both of the bacteriocin preparations were found resistant to 100°C for 10 min and pH range of 2-14. Erwiniocin NA4 was also found resistant to autoclaving while activity of agrocin NA5 was lost after autoclaving.

After proper field trials, these bacteriocin preparations can be used as prophylactic and therapeutic alternatives against phytopathogens responsible for a number of diseases in fruits, vegetables and cash crops.

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