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Full Length Research Paper

Antifungal activity of *Comamonas acidovorans* isolated from water pond in south Jordan

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A bacterial strain identified as *Comamonas acidovorans* NB-10II was isolated from water pond in South Jordan. It was found to have an antifungal activity against filamentous fungi (*Aspergillus niger* SQ 40, *Fusarium oxysporium* SQ 11, *Verticillium dahliae* SQ 42), yeasts (*Saccharomyces cerevisiae* SQ 46, *Candida albicans* SQ 47). All gram-positive bacteria (*Bacillus megaterium* SQ 5, *Bacillus cereus* SQ 6, *Staphylococcus aureus* SQ 9, *Streptococcus pyogenes* SQ 10) and gram-negative bacteria (*Escherichia coli* SQ 22, *Klepsiella* spp SQ 33, *Pseudomonas mallei* SQ 34) were found to be resistant. The isolate NB-10II was found to accumulate the main portion of the antimicrobial substances in their cells. In batch culture, the active antimicrobial substances accumulated at the late growth cycle, reaching their maximum at 42 h of growth. The data clearly show an antifungal activity of *C. acidovorans* against filamentous fungi and yeasts, and indicated the possibility of using NB-10II as a source of antimicrobial substances or as a biocontrol agent of some plant diseases in Jordan.

Key words: Antifungal activity, *Comamonas acidovorans*, *Pseudomonas*.

INTRODUCTION

The genus *Pseudomonas* has been heterogenous since Migula first named it in 1894 (Migula, 1894). He designnated and described the species associated with the genus in 1895 (Migula, 1895). Pseudomonads are gram-negative, strictly aerobic, polarly flagellated rods. They are aggressive colonizers of the rhizosphere of various crop plants, and have a broad spectrum of antagonistic activity against plant pathogens, such as antibiosis (the production of inhibitory compounds) (Cartwright et al., 1995; Rosales et al., 1995), siderophore production (iron-sequestering compounds) (Winkelmann and Drechsel, 1997) and nutrition or site competition (Bull et al., 1991). Some species of *Pseudomonas* can also produce levels of HCN that are toxic to certain pathogenic fungi (David and O'Gara, 1994). These characteristics make *Pseudomonas* species good candidates for use as seed inoculant and root dips for biological control of soil-borne plant pathogen.

Among *Pseudomonas* species, *Pseudomonas acidovorans* which has been renamed *Comamonas acidovorans* (Tomaoka et al., 1987) is a non-glucose-fermenting, gram-negative, non-spore forming rod. It is a ubiquitous bacterium in soil and water (von Gravenitz, 1985), and has been isolated from clinical specimens and the hospital environments (Horowitz et al., 1990). *C. acidovorans* supposed to have potential for biological control against fungal pathogens. The production of an antifungal compound by *C. acidovorans* will be regarded as one of the mechanisms involved in antagonism. Thus, the goal of this investigation was to screen a new bacterial strain for the production of antimicrobial substances which could be used as a biocontrol agent of soil-borne plant pathogen.

MATERIALS AND METHODS

Isolation of antibiotic producing bacteria

Both antagonistic and non antagonistic strains were isolated from water pond in south Jordan. Appropriate serial dilution from the water samples in sterile normal saline solution were spread on nutrient agar (g/l of distilled water) (peptone 5, sodium chloride 5, yeast extract 1.5, beef extract 1.5 and agar 15) (HiMedia Laboratories Pvt. Limited, Bombay, India) in sterile plastic, 9 cm diameter Petri-dishes. Six plates were used per dilution and dried in a lami-

nar flow-cabinet for 60 min before incubation at 27°C in the dark for 48-72 h. Bacterial colonies were sub-cultured and transferred onto nutrient agar plates. Single colonies were isolated and screened for antimicrobial activity using the Petri plate assay (El-Banna and Winkelmann, 1998).

Petri plate assay

All bacterial isolates were preliminary screened for their ability to inhibit fungal growth on nutrient agar plates using *Fusarium oxysporium* SQ 11 as a preliminary test organism. Single bacterial colonies were selected and patched along the perimeters of plates on which 30 µl of spore suspension of *F. oxysporium* SQ 11 was placed at the center and spread over the entire surface of the plate. The plates were incubated at 27°C for 48 h, and the antifungal activity was determined by measuring zones of fungal growth inhibition (Jayaswal et al., 1990).

Identification of the producing strain

The isolate was identified with API 20 NE diagnostic strips (bioMerieux sa, 60280 Marcy / Etoile - France).

Shake flasks culture

All experiments dealing with the growth and antimicrobial substances production were carried out in 500 ml Erlenmeyer flasks containing 100 ml nutrient broth containing (g/l) 5 peptic digest of animal, 5 sodium chloride, 5 yeast extract and 1.5 beef extract (HiMedia Laboratories Pvt. Limited, Bombay, India) and inoculated with 1 ml of 24 h preculture of the bacterial isolate suspension. Inoculated flasks were incubated at 27°C on a rotary shaker (Sanyo Gallenhamp PLC, Leicester, LE 3 2uz, UK) at 180 rpm for 42 h.

Growth measurements

The growth of bacteria was measured spectrophotometrically at 600 nm (Roitman et al., 1990).

Agar diffusion test

Extraction of the active substances from the supernatants and cells grown in liquid cultures (100 ml) was carried out (El-Banna and Winkelmann, 1998). Cells were isolated by centrifugation at 8000 rpm for 20 min. and extracted with acetone, and the supernatant was extracted with ethyl acetate, both extracts were evaporated by a rotary evaporator (Heidolph instruments, GmbH and Co KG Vertrieb, Kelheim, Germany) at < 50°C, and the dry substances were dissolved in 0.5 ml methanol. The antimicrobial activities of these extracts were carried out against a range of bacteria, filamentous fungi and yeasts by agar diffusion test (El-Banna and Winkelmann, 1998).

Biotest plates preparation

Using gram-positive and gram-negative bacteria (*Bacillus megaterium* SQ 5, *Bacillus cereus* SQ 6, *Staphylococcus aureus* SQ 9, *Streptococcus pyogenes* SQ 10, *Escherichia coli* SQ 22, *Klepsiella* spp SQ 33 and *Pseudomonas mallei* SQ 34) and yeasts (*Saccharomyces cerevisiae* SQ 46 and *Candida albicans* SQ 47) as test microorganisms, cell suspension of 24 h precultures were prepared ($O.D_{578}$ = 1), and 0.5 ml of this suspension was used to inoculate 250 ml soft agar medium (Arab Food and Media Applicances Co ltd. Zarka industrial area, Jordan). In case of spore forming fungi (as test microorganisms), plates with potato dextrose agar containing (g/l) 200 potatoes infusion, 2 dextrose and 15 agar (HiMedia Laboratories Pvt. Limited, Bombay, India) were inoculated with fungi and incubated at 27°C for 10 days. After sporulation, the spores were harvested using tween 80-saline suspension (0.1% tween, 0.9% NaCl). The spores were then washed and resuspended in normal saline. Aliquots (250 ml) of test media (soft agar) were inoculated with 1 ml of spores suspension (10^7spore/ml).

Table 1. Taxonomic identification of strain NB-10II*.

Tests	Strain NB-10II
NO_3 reduction	+
Indole formation	-
Acid from glucose	-
Arginine dihydrolase	-
Urease	-
Esculin hydrolysis (ß-glucosidase)	-
Gelatine hydrolysis	-
ß-galactosidase	-
Glucose assimilation	-
Arabinose assimilation	-
Mannose assimilation	-
Manitol assimilation	-
N- acetyl - glucosamine assimilation	-
Maltose assimilation	-
Gluconate assimilation	+
Carpate assimilation	-
Adipate assimilation	+
Malate assimilation	+
Citrate assimilation	+
Phenyl - acetate assimilation	+
Oxidase	+

*Identification by API 20 NE diagnostic strips.

RESULTS

In the present study, emphasis was laid on the isolation of antimicrobial substances producing bacteria from water pond. The screening of antimicrobial substances producing bacteria isolated from water pond in south Jordan yielded a number of bacterial isolates. The antimicrobial activity of these bacterial isolates was tested by Petri plate assay against *F. oxysporium* SQ 11.

Bacterial strain that inhibited the growth of the fungal strain was then tested against other known test microorganisms. A zone of inhibition (mm) indicated the antimicrobial activity, and the active strain (NB-10II) was selected and used for further study. The isolate NB-10II was identified with API 20 NE diagnostic strips (bioMerieux sa, 60280 Marcy/Etoile - France) (Table 1).

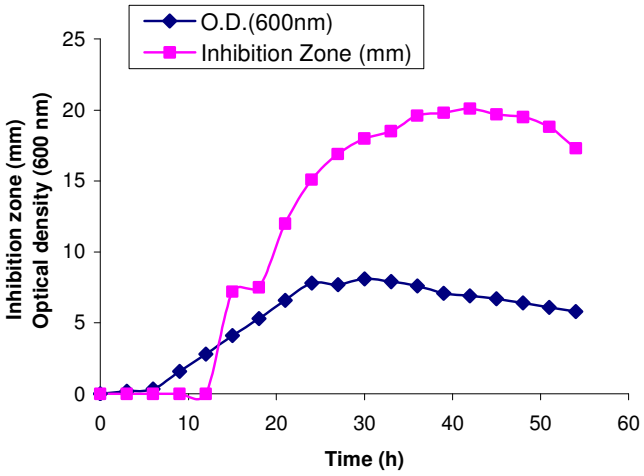

Figure 1. Time course of antifungal substance produced by *Comamonas acidovorans* NB-10II.

Strain NB-9 is gram-negative, aerobic rod shaped bacterium and was identified as *C. acidovorans*.

At the end of fermentation, the cells and the supernatant were extracted with acetone and ethyl acetate, respectively. Agar diffusion test showed that *C. acidovorans* NB-10II accumulated the antifungal substances within the cells. To determine and monitor the time course for the production of the antifungal substances in batch culture, agar diffusion tests were employed. The antifungal activity was first detected after 15 h of incubation, corresponding to the late exponential growth phase, and continued to increase during stationary growth phase reaching maximal activity at 42 h (Figure 1).

The active substances produced by *C. acidovorans* NB-10II exhibited an antifungal activity against filamentous fungi (*Aspergillus niger* SQ 40, *Fusarium oxysporium* SQ 11, *Verticillium dahliae* SQ 42) and yeasts (*Saccharomyces cerevisiae* SQ 46, *Candida albicans* SQ 47) (12.5-18.6). While all tested gram-positive bacteria (*Bacillus megaterium* SQ 5, *Bacillus cereus* SQ 6, *Staphylococcus aureus* SQ 9, *Streptococcus pyogenes* SQ 10) and gram-negative bacteria (*Escherichia coli* SQ 22, *Klepsiella* spp SQ 33, *Pseudomonas mallei* SQ 34) were resistant (Table 2).

DISSCUSSION

Since the discovery of penicillin (1929) and its use in chemotherapy in 1941 as a response to the great fatalities in the Second World War, a great number of important antibiotics have been found. Besides *Streptomyces*, members of the genus *Pseudomonas* have been proven to be fruitful in the order Eubacteriales (Lancini and Parenti, 1982).

Table 2. Antimicrobial spectrum of the active substance produced by *Comamonas acidovorans* NB-10II.

Test microorganisms***	Inhibition zone (mm) *
Bacillus megaterium SQ 5	n.a.
Bacillus cereus SQ 6	n.a. **
Staphylococcus aureus SQ 9	n.a.
Streptococcus pyogenes SQ 10	n.a.
Escherichia coli SQ 22	n.a.
Klepsiella spp SQ 33	n.a.
Pseudomonas mallei SQ 34	n.a.
Aspergillus niger SQ 40	17.1
Fusarium oxysporium SQ 11	20.1
Verticillium dahliae SQ 42	16.2
Saccharomyces cerevisiae SQ 46	14.7
Candida albicans SQ 47	14.7

*Agar difusion test.
**No activity.
***All microorganisms were obtained from Jerash Culture Collection of Microorganisms.

In the screening program for antimicrobial substances producing microorganisms, a bacterial strain isolated from water pond in south Jordan, was identified as *C. acidovorans* NB-10II. *C. acidovorans* (*Psedomonas acidovorans*) is a gram-negative rods which are motile by polar flagella. It is one of three species of the genus *Comamonas*, which belongs to the beta subdivision of the class *Proteobacteria*. It is a soil and water bacterium, but also has been observed in clinical isolates (Faude and Hoefle, 1997).

In batch culture, some processes leading to the production of antibiotics are sequential, i.e, they exhibit a distinct growth phase (trophophase) followed by a production phase (idiophase). In other processes, trophophase and idiophase overlap (Martin and Demain, 1980). *C. acidovorans* NB-10II seems to accumulate the antimicrobial substances within their cells. Under the conditions used in the present investigation, the active substances accumulated in the late growth cycle, (i.e., in stationary phase) in the laboratory media reaching a maximum at 48 h. Mahoney and Roitman (1990) reported that 98% of phenylpyrroles produced by *Pseudomonas cepacia* are contained in the cell extracts and the broth contains only 1% of the pyrroles produced by *Pseudomonas cepacia* cells during fermentation.

The fermentation time needed for maximal yield of the antimicrobial substances production seems to be different among bacterial strains, 36-40, 72, 120, 144 and 168 h were respectively reported (Zheng and Slavik, 1999; Janisiewicz and Roitman, 1988; El-Banna and Winkelmann, 1998; Meyers et al., 1973; Moyne et al., 2001).

The antimicrobial spectrum of the active substances

produced by *C. acidovorans*, determined by agar diffusion method, exhibited an antifungal activity against filamentous fungi and yeasts, while all tested gram-positive and gram-negative bacteria were resistant. Although antibiotics from pseudomonads are not used for medical purposes, they have potential value in plant protection. The practical use of antibiotics from pseudomonads dates back to the period before the antibiotic era. Emmerich and Löw (1899) reported that the cell free culture of *Pseudomonas aeruginosa*, concentrated to one tenth of its original volume, killed several kinds of bacteria. Due to the lytic action of culture broth on suspensions of some kinds of bacteria, they ascribed the inhibition to an enzyme termed pyocyanase. Later investigations, however, showed that several factors were present, none of which had enzymatic activity, and that the major active principle was pyocyanine. It has been used extensively in the therapy of diphteria, influenza and meningitis during the first two decades of this century (Leisinger and Margraff, 1979).

C. acidovorans NB-10II may participate actively in establishing the microbiological equilibrium in the nature (soil and water), and may be a factor in affecting the incidence of certain soil-borne plant pathogens. The data presented in this study showed that *C. acidovorans* NB-10II produced antimicrobial substances active against filamentous fungi and yeasts. The presented data exhibit the antifungal and antiyeast activity of *C. acidovorans* and indicate the possibility of using *C. acidovorans* NB-10II as a source of antimicrobial substances production or as a biological control agent of some plant diseases in Jordan. However, this requires further screening of a large number of pseudomonads strains isolated from different regions in Jordan.

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