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BIO-CHEMICAL CHARACTERIZATION OF *XANTHOMONAS AXONOPODIS* PV. *CITRI:* A GRAM NEGATIVE BACTERIUM CAUSING CITRUS CANKER

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ABSTRACT
Bacterial citrus canker is one of the major causes of yield losses in citrus growing areas of world. *Xanthomonas axonopodis* pv. *citri* (*Xac*) is the cause of this disease, which is a gram negative bacterium. Biochemical analysis helps to differentiate between gram positive and gram negative. Gram staining, Starch hydrolysis, Tween 80 hydrolysis, Gelatin Liquefaction, KOH test, Kovacs' Oxidase and Fluorescent Pigmentation tests were performed to characterize the *Xac*. The results of all biochemical tests confirmed the *Xac* a gram negative bacterium.

KEYWORDS: Citrus canker, *Xac*, Gram negative and Biochemical assays.

INTRODUCTION

Citrus is a juicy fruit having best nutritional values. It is rich source of vitamins and mineral with addition of carbohydrates (Khan *et al*., 1992). The yield and poor quality of citrus fruits is threatened to attack of many diseases caused by fungal, viral, bacterial and nematode pathogens. *Alternaria* brown spot, Citrus wither tip, Citrus nematode, citrus gummosis, Citrus canker, Citrus greening and Citrus tristeza virus are commonly prevalent in citrus orchards of Pakistan. Among these diseases, citrus canker has its own importance, which badly affects the plant health and fruit quality. Canker has been distributed in all citrus growing areas of the world (Koizumi, 1985). *Xanthomonas axonopodis* pv. *citri* (*Xac*) causes this bacterial disease, probably originated in South-East Asia. Subsequently, the pathogen was disseminated throughout Asia and then to Africa, Oceania and South America (Rossetti, 1977). Grapefruit, sweet oranges like pineapple, hamlin, mexican limes, lemons, trifoliate orange and their hybrids are severely affected by *Xac* (Gottwald *et al.*, 2002). All plant parts are affected by this disease. Raised corky lesions surrounded by oily or water-soaked margins are characteristic symptoms. Early fruit drop is major economic impacts of canker. *Xac* is gram -ve, Proteobacteria belongs to family *Xanthomonadaceae.* Most of plant pathogenic bacteria are gram negative except Bacillius species, Clavibactor, Streptomyces. Biochemical assays play an important role to distinguish between gram positive and gram negative bacterium. Management of the disease will not increase the production of citrus but also earns a fair amount of foreign exchange. Etiology is one of the key factor for management of plant disease. Though the research has been conducted on its management of this disease but scarcity of literature is available on the biochemical characterization of this bacterium. Therefore studies on biochemical analysis of *Xac* were carried out. In this experiment, we isolated and purified the *Xac* stains from kinnow and further characterized on the basis of biochemical assays like, Starch hydrolysis, Tween80 Hydrolysis, Gelatin Liquefaction, Kovacs' Oxidase, Fluorescent Pigment and KOH tests. Although these tests are of basic nature but still important for the characterization of gram negative bacterium.

MATERIALS & METHODS

Isolation and purification of *Xanthomonas axonopodis* pv. *citri (Xac)*

Leaves samples of citrus infected with bacterial canker were collected from declining orchard and cut into small pieces and placed on Nutrient agar (NA) media plates and incubated for 24 hours at 30ºC. One of single yellow colony was picked by wire loop and streaked on another media plate for pure culture.

Biochemical characterization of *Xanthomonas axonopodis* pv. *citri*

Gram staining: Gram's reagents were prepared by taking crystal violet, Logol's iodine, Acetone (pure) and counter stain Safranin. Bacterial smears of two isolates were made from pure *Xac* culture on clean slides in a drop of distilled water. The smears were stained by placing one drop of crystal violet hold it 30 seconds and washed with distilled water. One drop of Lugol's iodine was placed for 30 seconds followed by washing with distilled water followed by pure acetone. Finally one drop of Safranin was added, holds it for 30 seconds prior washing with water. The slides were dried using blotter paper and observed under 100X microscope along with one drop mineral oil to examine shape, size, arrangement and staining reaction of bacterial isolates (Bradbury, 1970).

Starch Hydrolysis Test: Two gram rice starch enriched NA media was autoclaved and pured into petriplates. After cooling each isolate was transferred into media and incubate at 27ºC for seven days. The plates were dispensed with 3% Lugol's iodine after scraping and without scraping the *Xac* culture on the media (Cowan, 1974).

Tween 80 Hydrolysis Test: Purified *Xac* culture was subjected to tween 80 hydrolysis test (Sierra, 1957). 5g NaCl, 0.1g $CaCl_2.2H_2O$, 10g Peptone and 16g agar to distilled water (1 L) with pH 7.4. Tween 80 was added to the molten media. The media was poured into petriplates. Each isolate of *Xac* was streaked on a medium. The culture was incubated at 27ºC for seven days to observe opaque milky precipitate/milky crystal formation.

Kovacs' Oxidase Test: A drop of 1% Kovacs' reagent (1g Tetramethyl-p-phenylenediamine Dihydrochloride in100 ml distilled water) was placed on the center of Whitman filter paper no.1 and platinum loop full of *Xac* inoculum was gently rubbed on the filter paper. Positive control was also maintained (Kovacs, 1956; Bradbury, 1970).

Gelatin Liquefaction Test: Beef extract (3g), peptone (5g) and gelatin (120g) in 1L of water was poured into the test tubes 5mL/test tube- plugged and autoclaved. The 24 hours old *Xac* culture of each isolate was stab inoculated and incubated at 27ºC. After 72 hours, tubes were placed at 40ºC for 30 minutes prior to record the results. The same procedure was followed after 7, 14 and 21 days (Cowan, 1974).

Fluorescent Pigment Test: King's medium B (Proteose Peptone 20g, K_2HPO_4 1.5g, $MgSO_4.7H_2O$ 1.5g, Agar 15g, Glycerol 10g, distilled water 1.0 L) prepared and $MgSO_4.7H_2O$ was added when temperature become lowered and autoclaved. Overnight bacterial culture was streaked on the KB medium and incubated at 30ºC for four days. The plates were observed under UV-pro light for fluorescent. Positive control was maintained by inoculating unknown *Pseudomonas* culture on KB medium (King *et al*., 1954).

KOH Test: *Xac* aseptically removed from the agar medium with a toothpick placed on a glass slide into a drop of 3% KOH, and stirred for 10 seconds using a quick circular motion (Suslow *et al*., 1982).

RESULTS

Isolation and Purification

The leaves samples placed on Nutrient Agar plates showed the bacterial colonies after 48 hours of incubation at 30ºC temperature. The visual observation was identified the colony morphology of bacterium *Xac.* The colony color of two isolates was variable from yellow to light yellow. The size and shape of colonies were found to be small to medium, convex and mucoid (Fig. 1, A).Purification was done by streaking method. From a single colony a loop was streaked on slants to preserve the isolate for a longer period of time (Fig.1, B, and C).

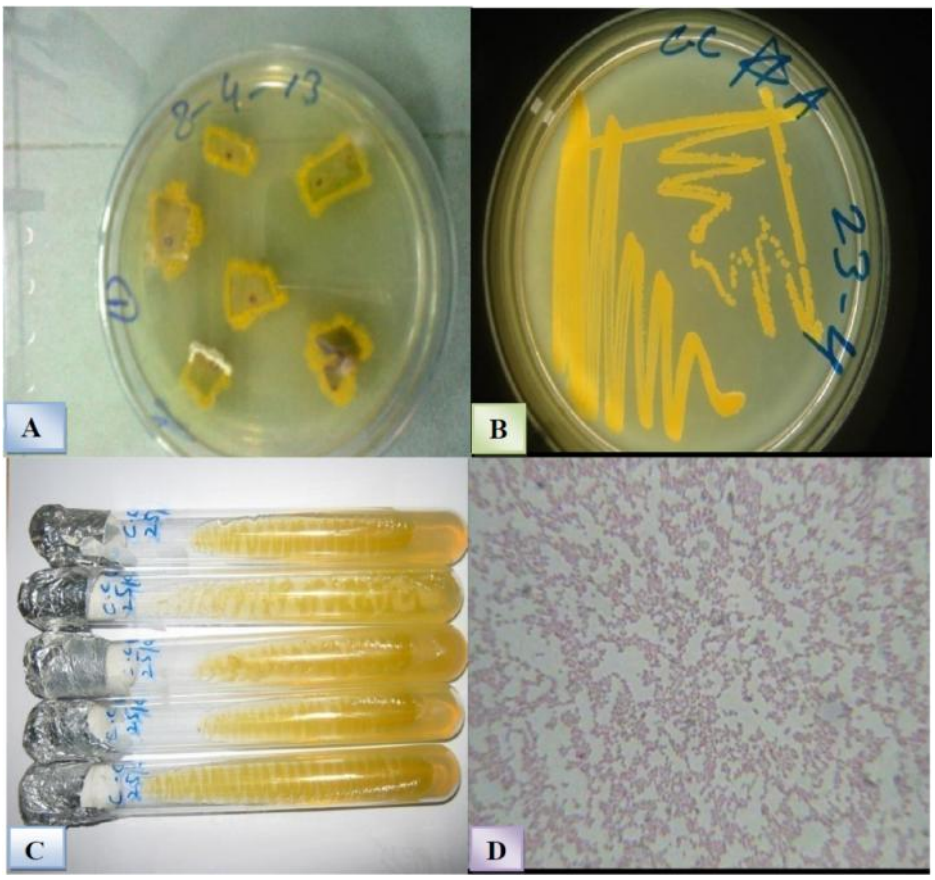

FIGURE 1: Leaf samples on NA plant (A), pure colony of *Xac* on NA plate (B) and NA slants (C).After Gram staining pinkish colony under micro scope (D)

Biochemical characterization of *Xac*

The results for the biochemical characterization have been presented in a table.1. In gram staining bacterial stained mounts were observed under light microscope at 100X using oil immersion. *Xac* isolates were found to be gram negative, rod shaped and pinkish in color when stained with counter strain Safranin which was the confirmation for gram negative bacterium (Fig.1.D).

TABLE 1: Biochemical characteristics of Gram negative bacteria

Test	Reaction	Appearance	Remarks
Gram reacion	-ve	Small, Rod, pink color colony	Gram staining is negative showing confirmation of gram negative bacteria
Starch Hydrolysis	+ve	Clear zone in Iodine Stained medium	Gram negative bacteria positively hydrolyzed the starch
Tween 80 Hydrolysis	+ve	Milky White Precipitate	Gram negative bacteria positively hydrolyzed the tween 80
Kovacs' Oxidase	-ve	No color	Gram negative bacteria did not produce any characteristic color
Gelatin Liquefaction	+ve	Liquefy the gelatin medium	Liquefying of gelatin indicated the presence of gram negative bacteria
Fluorescent Pigmentation	-ve	No fluorescent of any color	Gram negative bacteria characteristically produced no fluorescent of any color
KOH test	+ve	Thread like slime	Gram negative bacteria formed thread like slime which is absent in gram positive bacteria

Starch Hydrolysis test*: Xac* produced clear golden zones around bacterial colonies on starch medium when stained with Lugol's iodine after 7 days of incubation at 27ºC (Fig.2.,A).Similarly bacteria hydrolyzed the starch present in the medium. Presence of milky white precipitate/opaque zones around the *Xac* colonies confirmed the characteristically hydrolysis of tween 80 (Fig.2. B).

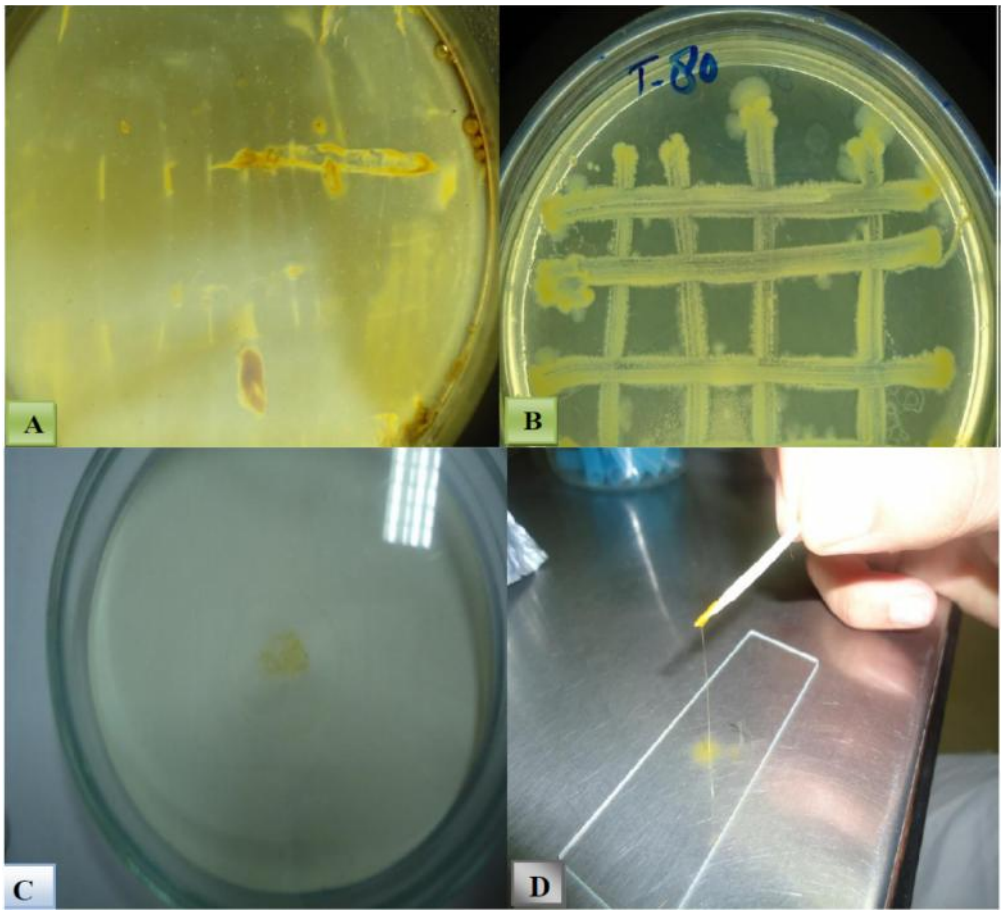

FIGURE 2: Clear golden zones (A), presence of milky white precipitate/opaque zones around the *Xac* colonies (B), no color after 60 seconds(C) and gram negative bacteria form thread like slime(D).

Kovacs' oxidase test

Characterized the *Xac* as gram negative bacterium that gave no color after 60 seconds (Fig. 2.C).

Gelatin Hydrolysis

The flow of gelatin medium upon the tilting of test tube indicated the positive reaction of *Xac* isolates that hydrolysis gelatin medium. King's B medium inoculated with *Xac* isolates did not show fluorescent of any color when observed under UV- light as compared with the control, inoculated with the unknown Pseudomonas spp. that give greenish yellow fluorescence under UV-pro light. The fluorescent pigment test was found to be negative (-ve) in all isolates of *Xac*.

KOH Test

Gram negative bacteria, the suspension becomes viscous and form thread like slime when picked up with a toothpick during KOH test (Fig. 2.D).

DISCUSSION

In our study canker infected leaves samples from kinnow were collected and *Xac* was isolated and purified. Different biochemical tests such as Gram reaction, Starch hydrolysis, Tween 80 hydrolysis, Kovacs' oxidase, Gelatin liquefaction, Fluorescent pigmentation and KOH characterized the *Xac* as gram negative bacteria. Our results confirmed the work of Vernière *et al*. (1998) who used several biochemical tests to identify and differentiate different pathotypes of citrus canker bacteria. The tests included hydrolysis of gelatin and casein, in addition to the growth on 3% NaCl. Kishore and Chand (1972; 1975) also observed a remarkable decrease in amino acid contents in canker infected leaves as compared to healthy ones. Similarly Mohan and Schaad (1987) observed non-fluorescent pigmentation occurred in gram negative bacteria on KB media as compared to *pseudomomas syringae* pv. *syringae*. Moreover Suslow *et al*. (1982) performed KOH test to accurately characterized gram negative bacteria of wheat. However, this test is not reliable for Bacillus megaterium and some gram positive strains of Clavibacter, Therefore it is concluded that biochemical assays can successfully characterized the gram positive and gram negative bacteria. We can formulate different strategies for the management of citrus canker using this biochemical information.

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