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# CHARACTERIZATION OF PLANT PATHOGENIC *RALSTONIA SOLANACEARUM* CAUSING BACTERIAL WILT OF POTATO

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**ABSTRACT**

After wheat, rice and maize, the potato (*Solanum tuberosum* L.) is considered one of the world's most significant food crops. Bacterial wilt or brown rot of potato, caused by *Ralstonia solanacearum*, is one of the most economically important diseases of potato. The purpose of this study was to characterize morphological, pathological and biochemical characterization of *Ralstonia solanacearum*. Total ten isolates of *Ralstonia solanacearum* were isolated on TZC agar medium from infected potato plant parts. All isolates of *R. solanacearum* showed fluidal, irregular and creamy white with pink center colony on TZC medium after 48 h of incubation were selected. The isolates of *R. solanacearum* showed a positive result for the pathogenicity test and produced wilting symptoms on potato plants (Kufri Pukhraj). The results of biochemical studies showed that all the ten isolates were gram-negative, rod-shaped and positive for KOH test, oxidase test, catalase test, production of hydrogen sulphide, casein hydrolization test and acid production test, but they showed negative reaction for indole test. *R. solanacearum* was identified as the pathogen causing potato wilt based on an isolation study (colony characteristics on TZC media), oozing test, pathogenicity test and various biochemical tests.

***Key words :*** Bacterial wilt, *Ralstonia solanacearum*, Potato crop, Biochemical characters, Morphological characters.

## Introduction

The potato (*Solanum tuberosum* L.) belonging to family Solanaceae is a vitally important starchy food crop of the world, commonly known as the "Poor Man's Friend" also "King of Vegetables". Potato is the staple food in many parts of the world and is an important part of the world's food supply after wheat, rice and maize. The potato crop is infested by many diseases caused by bacterial, fungal, viral, viroidal, nematodal diseases, *etc*. Bacterial wilt disease is responsible for yield loss in potato upto 70%, in India (Kuarabachew *et al*., 2007). Bacterial wilt or brown rot is caused by the pathogenic bacterium *Ralstonia solanacearum* (Smith, 1896; Yabuuchi *et al*., 1995). The plant pathogen *R. solanacearum* has extensively been distributed in tropical, subtropical and some warm temperate regions of the world and is one of the major constraints in the production of potato crop.

As a diverse species complex, *R. solanacearum* has developed an extremely broad host range throughout the world, including more than 450 host species representing 54 plant families (Wicker *et al*., 2007). In most cases, the bacterium enters plant roots from the soil through

wounds or naturally occurring openings, colonises the intercellular space of the root cortex and vascular parenchyma, and then eventually enters the xylem artery and travels up into the stem and leaves. Plants that are affected experience chlorosis, stunting, wilting, and typically die quickly. One of the most deadly potato diseases is bacterial wilt, which is caused by *Ralstonia solanacearum* (Liu *et al*., 2007).

Morphological and biochemical characterizations of the *Ralstonia solanacearum* are one of the most important areas of identification. *R. solanacearum* is a rod-shaped bacterium with an average size varying from 0.5 to 0.7 by 1.5 to 2.5 ìm and it is considered as an organism strictly aerobic (Denny and Hayward, 2001). The principal biochemical characteristics are catalase positive, oxidase positive and KOH positive. The pathogen is not capable of hydrolyzing starch or quickly destroying gelatin. In broth culture, the organism is inhibited by concentrations of sodium chloride (NaCl) greater than 2%. For bacterial culture, both liquid and solid (agar) growth mediums are frequently employed. On solid agar medium, individual colonies are usually visible after 36 to 48 hours of growth at 28°C and Kelman's tetrazolium chloride (TZC) agar is regularly used for its isolation (Kelman, 1954). Virulent wild-type colonies are big, raised, fluidal and either totally white or with a pale crimson centre after two days on TZC medium. For most strains, the optimal growth temperature is 28-32°C; however some strains that are pathogenic on potato have a lower optimal growth temperature of 27°C.

The pathogen exhibits wide variability and diversity. *Ralstonia solanacearum* was formerly known as *Pseudomonas solanacearum*, causing wilt on wide range of solanaceous crops (chilli, tomato, brinjal and potato), peppers (capsicum) and also bitter gourd and beans (O'Brien and Rich, 1967). The disease is becoming the major hurdle in successful cultivation of solanacious crops particularly potato hence it was felt to conduct the studies on different physiological and biochemical characteristics of the causal bacterium.

## Materials and Methods

### Collection of disease samples

The infected potato plant showing typical symptoms of bacterial wilt were identified and such diseased samples were collected from farmer's fields near by Sardarkrushinagar Dantiwada Agricultural University. Wilt infected plants were uprooted and brought to the departmental laboratory for the isolation and firther studies.

### Stem streaming test (Ooze test)

Wilted potato plant was cut from the stem at collar region and placed it into sterilized distilled water in test tube under laminar air flow cabinet for 5-10 min. Bacterial ooze coming out from the cut end of the infected plant parts into water and appeared like a smoke in clear water, which was the indication of presence of bacterium in infected plant part of potato.

### Isolation of *Ralstonia solanacearum*

With a flame-sterilized scalpel, the cut ends of surface-sterilized segments were picked up and put on petriplates containing triphenyl tetrazolium chloride (TZC). The bacterium was grown on the medium in the petri dishes by incubating them at 28°C for 48–72 hours. A single colony of the bacteria that displayed fluidal, erratic, creamy white with a pink centre was selected, maintained as pure culture and stored at 4°C for later use.

### Pathogenecity test

Pathogenecity test were carried out by soil dreanching method. The potato seed tubers of cv. Kufri Pukhraj which was procured from the Potato Research Station-Deesa, Sardarkrushinagar Dantiwada Agricultural University, Sardarkrushinagar, Banaskantha (Gujarat).

### Soil drenching method

Potato seed tubers were sown in plastic pots (disinfected with 4% formaldehyde) containing sterilized potting mixture, soil + sand + FYM (2:1:1). The pots were watered regularly to facilitate germination and plant growth. The bacterial suspension was prepared from 48 hours old bacterial cultures on nutrient broth medium and poured around the root zone of one month old potato plants by making slight injury on collar region to facilitate easy penetration of the pathogen. After inoculation, the plants were watered every alternate day. The uninoculated pots served as control. Both inoculated and uninoculated pots were placed under protected condition.

### Re-isolation of the pathogen

After development of typical symptoms of bacterial wilt on inoculated potato plants, the pathogenic bacterium was re-isolated on TZC agar medium; their cultural characteristics were studied and compared with the pure culture of *Ralstonia solanacearum* obtained earlier from naturally wilted potato plants.

### Characterization of the pathogen (*Ralstonia solanacearum*)

Different cultural, morphological and biochemical tests were carried out to characterize the *R.*

*solanacearum* are described below.

**Gram staining**

The microscopic examination of the isolates was done by Gram's staining procedure and the observations for cell shape and arrangement of cells in colonies were recorded using the method given by Cappucino and Sherman (1992).

**Cultural characteristics of the isolates**

Cultural characteristics of the isolates were studied after growing them on TZC agar medium plates as per methods described by Cappucino and Sherman (1992).

**Carbohydrate utilization profile**

The biochemical characterizations of *R. solanacearum* isolates were done based upon their efficiency to use various sources of carbohydrates. The efficiency of carbohydrate utilization is regarded one of the most important criteria for phenotypic characterization of the bacterial isolates. The carbohydrate utilization profiles for the *R. solanacearum* isolates were generated using Hicarbohydrate™ kit (KB009, HiMedia Laboratories, Mumbai) following standard protocol.

The result was observed in the form of color change of the medium in wells of the kit as per manufacturer's protocol. A binary matrix comprising of positive (1) and negative (0) values was generated based upon the carbon utilization profile of the isolates. The data were analyzed using a numerical taxonomy and multivariate analysis system NTSYSpc 2.02i software package (Rohlf, 2000). The dendrogram prepared was based on the proximity matrix obtained from the Jaccard's coefficient and Sequential Agglomerative Hierarchical Non-overlapping (SAHN) method and clustering was done using the UPGMA (Sneath and Sokal, 1973).

**KOH solubility test**

Inoculum of 48 h old bacterial culture was transferred on a clean glass slide with the help of sterilized inoculation loop. Few drops of 3 per cent KOH (potassium hydroxide) were added on the clean glass slide containing bacterial culture and mixed thoroughly for 5-10 seconds with the help of inoculation loop for formation of slime threads. When inoculation loop was raised from the glass slide, formation of strands of viscid material will indicate the positive reaction (Suslow *et al*., 1982).

**Acid production (Glucose)**

Nutrient broth containing 2 per cent glucose (adjusted to pH 7.0) was prepared and 48 h old bacterial cultures were inoculated in the test tubes containing nutrient broth. The inoculated test tubes were kept for incubation for 7 days at 28±2°C. After incubation, few drops of methyl red indicator was added in the test tubes. A discrete red or pink color indicates the positive reaction (Pawaskar *et al*., 2014a).

**Indole test**

The main requirement for indole test is presence of a suitable medium containing sufficient amount of tryptophan. Although, many media meet this criterion, tryptone broth was used in the present study. Tubes of tryptone broth were inoculated with a small amount of a bacterial pure culture and incubated at 35°C for 48 hours. 5 drops of Kovac's reagent was added directly to the tubes to test the bacterium's indole production ability. The reaction is positive, if there is a formation of a pink to red color in the reagent layer at the top of the medium within seconds of adding the reagent (MacWilliams, 2012).

**Production of hydrogen sulfide ($H_2S$)**

A nutrient broth with additional 3 per cent peptone was prepared and poured into test tube. The test tubes were sterilized in autoclave and inoculated with 48 h old bacterial culture. Then, filter paper strips were soaked in super saturated solution of lead acetate and were kept for drying. After drying, they were inserted into test tubes with cotton plug. The inoculated tubes were incubated for seven days at 28±2°C. If filter paper strips turns black in color, it indicates positive reaction for $H_2S$ production (Pawaskar *et al*., 2014b).

**Catalase test**

Take a clean glass slide and add a loopful bacterial culture from a petriplate containing 48 h old cultures of the test pathogen. Few drops of 3 per cent hydrogen peroxide ($H_2O_2$) was added to the clean glass slide, and mixed with the help of inoculation loop. The formation of gas bubbles indicates positive reaction (Schaad, 1980).

**Casein hydrolysis**

Skim milk agar medium was prepared, sterilized and poured into sterilized petriplates and allowed to solidify. A loopful of 48 h old bacterial culture was streaked on petriplates containing sterilized skim milk agar medium and plates were incubated in inverted position for two days at 28 ± 2°C. The formation of clear zone around bacterial colonies indicates positive reaction for casein hydrolysis.

**Oxidase test**

The Kovacs (1956) method was used to identify oxidase activity. Freshly grown (24 to 48 h) bacterial cultures from nutrient agar were picked with the help of sterilized inoculation loop and gently rubbed the colony on the oxidase disc (DD018, Himedia, Mumbai). A

reaction showing development of purple color in 30 seconds was recorded as oxidase positive reaction.

## Results and Discussion

### Isolation of *Ralstonia solanacearum* from potato

Total ten isolates of *Ralstonia solanacearum* were isolated on TZC agar medium plate from potato, which comprised of 2 isolates from Dantiwada, 5 isolates from Deesa, 2 isolates from Palanpur and 1 isolate from Amirgadh taluka sequencially named as RsSt1 to RsSt10.

### Pathogenecity test

The 48 hours old bacterial suspension of all the ten isolates was poured around the root zone of one month old potato plant in pots. After two weeks of inoculation, typical symptoms of bacterial wilt were developed on inoculated plants. The symptoms observed on inoculated plants were tender leaves which lost their turgidity, lower leaves pale yellow, drooping of infected leaves, the leaves became flaccid and sudden wilting of the whole plant was observed (Fig. 1). The wilted plants were further confirmed by ooze test (Fig. 2).

Re-isolation of test pathogen was done from artificially inoculated diseased potato plant parts on TZC agar medium. Cultural characteristics of re-isolated pathogen were compared with earlier obtained pathogen from naturally wilted potato plants and both cultures were found similar in their colony characteristics. Thus, pathogenicity of *R. solanacearum* causing bacterial wilt of potato was proved.

Similar work on pathogenicity of *R. solanacearum* causing bacterial wilt of potato and other solanaceous vegetables crops was reported earlier by several scientist; Kuarabachew *et al*. (2007) proved pathogenicity of *R. solanacearum* causing bacterial wilt of potato by stem puncture and seed dipping method. Murthy and Srinivas (2012) proved pathogenicity of *R. solanacearum* by root injury and soil drenching method on tomato plant. Ahmed *et al*. (2013) proved pathogenicity of *R. solanacearum* in potato by soil inoculation method. Shweta *et al*. (2018) proved pathogenicity of *R. solanacearum* by soil drenching and root dip method for potato and other host plants. Salvi (2020) proved pathogenicity of *R. solanacearum* causing bacterial wilt of brinjal by root inoculation method.


**Fig. 1 :** Pathogenicity test of *Ralstonia solanacearum* on potato plant (cv. Kufri Pukhraj). A: Before Inoculation; B: After Inoculation.

### Characterization of *Ralstonia solanacearum*

### Morphology and staining reaction of pathogen

The results of Gram staining revealed that all the tested isolates were Gram negative; rod shaped; appeared singly on the slide and monobacillus in nature. The length of *R. solanacearum* isolates ranged from 1.49 µm to 2.86 µm whereas cell width ranged from 0.31 µm to 0.83 µm. Several studies have noted similar morphological and staining reactions of *R. solanacearum*, including Rath and Addy (1977), Khetmalas (1984), Venkatesh (1988), Chaudhry and Rashid (2011).

### Cultural characters

Cultural characterization result revealed that the colonies of all the ten isolates were small to medium in size and appeared dull white or creamy coloured with slight pink or red center on TZC agar medium. Shape of the colony growing on TZC agar medium in petriplates indicate that all isolates produced circular type of shape and raised type of elevation. Appearance of the outer edge of the colony *i.e*. margin of the colony growing on TZC agar medium in petriplates revealed that all the

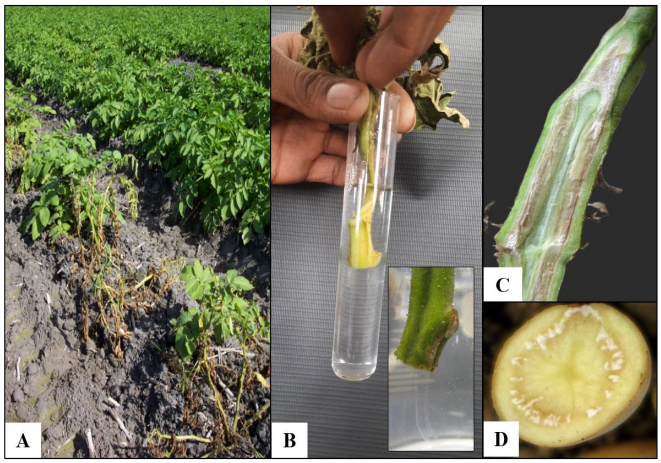


**Fig. 2 :** Symptoms of infection of *Ralstonia solanacearum*. A: Symptoms of bacterial wilt on potato plant; B: Bacterial ooze streaming in water from stem of infected plant; C: Vascular discoloration on the infected stem; D: Browning of vascular tissues of potato.

**Table 1 :** Characterization of *Ralstonia solanacearum* isolated from wilted potato plant.

| Isolate | KOH test | Acid production | Indole test | $H_2S$ production | Catalase test | Casein Hydrolysis | Oxidase test | Gram staining | Pathogenecity |
|---|---|---|---|---|---|---|---|---|---|
| RsSt1 | + | + | - | + | + | + | + | - | + |
| RsSt2 | + | + | - | + | + | + | + | - | + |
| RsSt3 | + | + | - | + | + | + | + | - | + |
| RsSt4 | + | + | - | + | + | + | + | - | + |
| RsSt5 | + | + | - | + | + | + | + | - | + |
| RsSt6 | + | + | - | + | + | + | + | - | + |
| RsSt7 | + | + | - | + | + | + | + | - | + |
| RsSt8 | + | + | - | + | + | + | + | - | + |
| RsSt9 | + | + | - | + | + | + | + | - | + |
| RsSt10 | + | + | - | + | + | + | + | - | + |

**Note:** '+' indicate positive result and '-' indicate negative result.

isolates produced 'irregular type' of colonies margin. The findings are in close conformity with Stanford and Wolf (1917), who described the colonies of *R. solanacearum* as white coloured with slight pink center, wet, shining, circular, raised and smooth. Khetmalas (1984) and Tahat and Sijam (2010) also recorded similar observations regarding colony characters of *R. solanacearum*.

## Biochemical characterization

### Carbohydrate utilization profile

All the ten isolates were positive for utilization of fructose, dextrose, trehalose, sucrose, ONPG, esculin hydrolysis, and citrate utilization. However, these isolates showed a varying degree of utilization of galactose, L-arabinose, mannose, glycerol, salicin, adonitol, arabitol, D-arabinose and malonate utilization. However, none of the ten isolates was tested positive for lactose, xylose, maltose, raffinose, melibiose, inulin, sodium gluconate, dulcitol, inositol, sorbitol, mannitol, erythritol, α-methyl-D-glucoside, rhamnose, cellobiose, melezitose, α-methyl-D-mannoside, xylitol and sorbose.

The numerical analysis of phenotypic characteristics based on ability of the isolates to metabolize various carbon sources revealed a high degree of metabolic polymorphism. The dendrogram based on proximity matrix obtained from the Jaccard coefficient and Sequential Agglomerative Hierarchical Non-overlapping (SAHN) and clustering using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) grouped these ten isolates of *R. solanacearum* into three phenons (Fig. 3). Overall, the similarity coefficient among the isolates ranged between 0.84 and 0.97. Phenon I (RsSt1, RsSt4, RsSt7 and RsSt9) and phenon II (RsSt2, RsSt5, RsSt8 and RsSt10) comprised of four isolates each and contains most of the isolates of *R. solanacearum* and show a similarity co-efficient of 0.90. However, rest two isolates RsSt3 and RsSt6 were grouped in the phenon III. The isolate RsSt1 and RsSt7 clustered together in the phenon I of the dendrogram and showed the highest similarity coefficient of 0.97.

### Potassium hydroxide test

The Gram negative test of *R. solanacearum* was also confirmed by the KOH test. The result of the test showed that the solution was viscous enough to stick to the loop causing a thin strand of slime, which was recorded

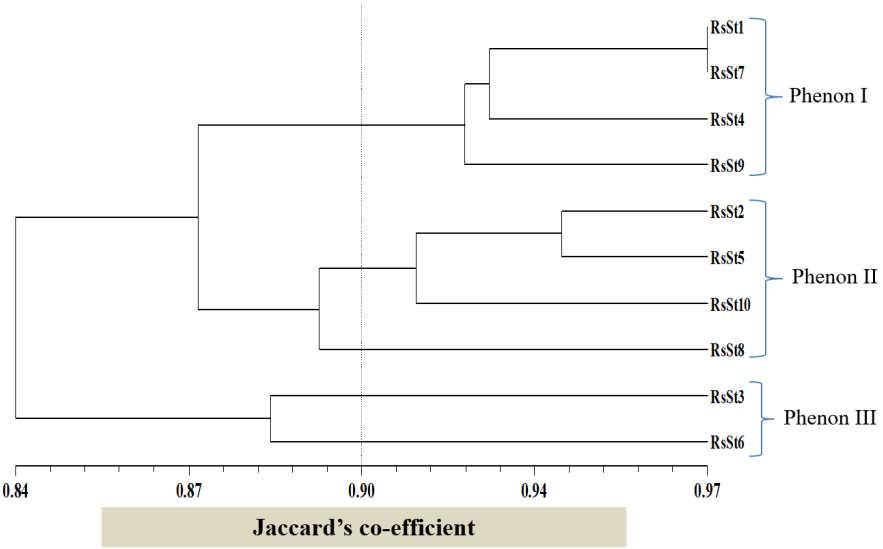


**Fig. 3 :** Dendrogram based on carbon utilization profile using Jaccard's co-efficient and Unweighted Pair Group Method with Arithmetic Average (UPGMA).

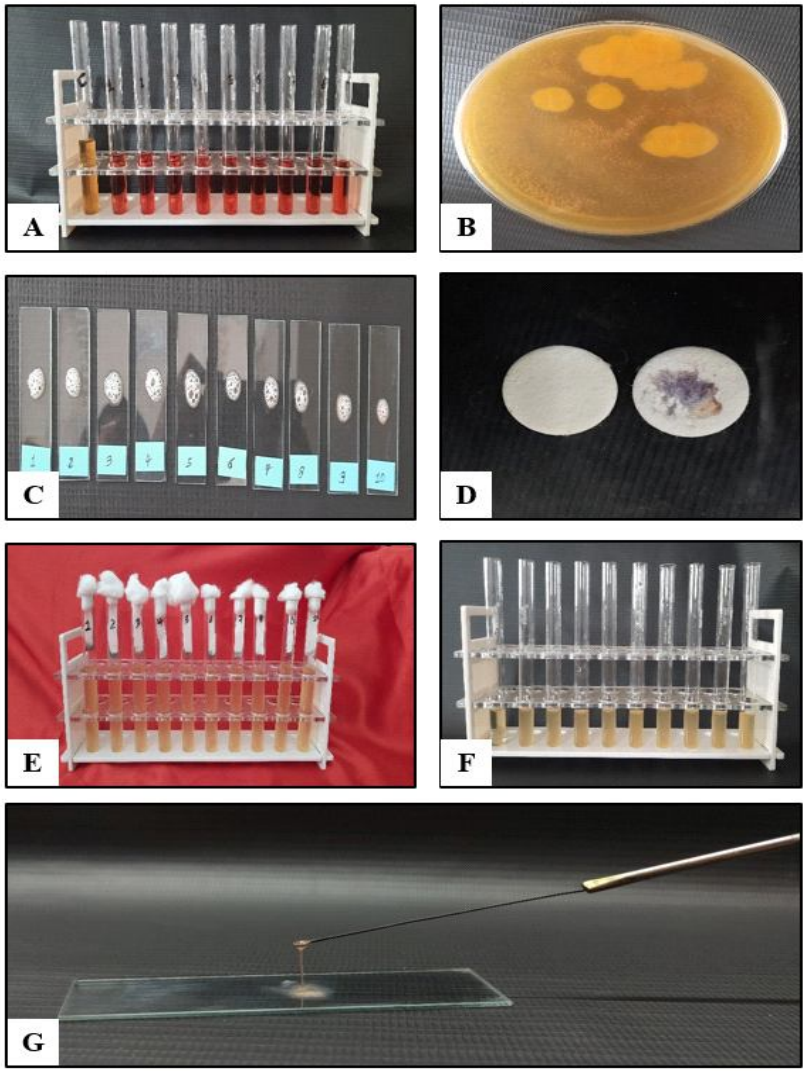


**Fig. 4 :** Biochemical characterization of isolates of *Ralstonia solanacearum.* A- Acid production test; B- Casein hydrolysis; C- Catalase test; D- Oxidase test; E- Production of hydrogen sulphide; F- Indole test; G- KOH solubility test.

as positive (Fig. 4g). Additionally, *R. solanacearum* strains have been shown to form slime thread, a sign of Gram-negativity, according to Popoola *et al*. (2015).

**Oxidase test**

Within 30 seconds of touching and spreading a well-isolated *R. solanacearum* colony on the oxidase disc marked as oxidase positive, a purple colour developed. This indicated that all the tested isolates (10) were positive for an oxidative test (Fig. 4d). Dhital *et al*. (2001), who also reported that all the strains of *R. solanacearum* were positive for oxidase.

**Catalase test**

Gas bubbles were produced within 60 seconds was recorded as a positive reaction for the catalase test. All the ten isolates were recorded positive for catalase test (Fig. 4c). Therefore, the test bacterium *R. solanacearum* is strictly aerobic. Dhital *et al*. (2001) who also reported that all the strains of *R. solanacearum* were positive for catalase test.

**Indole test**

Pink to red colour formation was absent. This indicated that all the ten isolates of *R. solanacearum* were negative for indole production test (Fig. 4f). Similar work also done by Murthy and Srinivas (2012) and reported that *R. solanacearum* isolates were negative for the indole test.

**Production of hydrogen sulphide**

All the isolates showed blackening at the lower end of strips within 72 hours. This indicated that all the ten isolates of *R. solanacearum* were positive for hydrogen sulfide production test (Fig. 4e). Kataky *et al*. (2017) reported that isolates of *R. solanacearum* were positive for $H_2S$ production.

**Acid production (Glucose)**

All the ten isolates of *R. solanacearum* was also confirmed by the acid production test. Results of test revealed that, bacterium produced acid when grown on nutrient broth containing 2 per cent glucose after addition of methyl red indicator and hence, showed positive reaction for the test (Fig. 4a). Anonymous (2004) reported *R. solanacearum* positive in acid production with 2 per cent glucose. The result are is also in conformation with Rath and Addy (1977), who reported that *P. solanacearum* produce acid from dextrose, glucose and salicilin. Khetmalas (1984) also reported *P. solanacearum* to be positive in acid production in presence of glucose.

**Casein hydrolysis**

All the ten isolates were grown on skim milk agar medium, it formed clear zone around the bacterial growth and this showed positive reaction for test. The formation of clear zone around bacterial growth is due to secretion of proteolytic exoenzyme caseinase, which hydrolyzed casein (milk protein) (Fig. 4b). This result is in agreement with the report of Bhide (1948), Das and Chattopadhyay (1955) and Hsu *et al*. (1993) and While Rath and Addy (1977).

## Conclusion

*R. solanacearum* was small straight rod shaped, measuring 1.49-2.86 µm × 0.31-0.83 µm and Gram negative in reaction with KOH positive test. The colonies of *R. solanacearum* apperied dull white or creamy coloured with slight pink or red center on TZC agar medium. The bacterium was positive in acid production test, casein hydrolysis, catalase test, oxidase test, production of $H_2S$, KOH solubility test and negatively responded to indole test.

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