STUDY ON *RALASTONIA SOLANACEARUM* CAUSING BACTERIAL WILT OF TOMATO AND ITS MANAGEMENT BY USING ANTAGONISTIC BACTERIA

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This is to certify that the thesis entitled**,** "**STUDY ON *RALSTONIA SOLANACEARUM* CAUSING BACTERIAL WILT OF TOMATO AND ITS MANAGEMENT BY USING ANTAGONISTIC BACTERIA"** submitted to the Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE IN PLANT PATHOLOGY** embodies the results of a piece of bona fide research work carried out by **SHAKILA ZAMAN** bearing Registration No**. 11-04703** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has been duly acknowledged.

Dated: 20 November, 2014
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STUDY ON *RALSTONIA SOLANACEARUM* CAUSING BACTERIAL WILT OF TOMATO AND ITS MANAGEMENT BY USING ANTAGONISTIC BACTERIA

ABSTRACT

The experiment was conducted in the Disease Diagnostic Laboratory and Net house of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka during the period of July 2012-June 2013 to find out suitable antagonistic bacteria against *Ralstonia solanacearum* causing bacterial wilt of tomato. Fourteen probable antagonistic bacterial isolates were isolated from rhizosphere soil of tomato and 6 isolates of *Rhizobium leguminosorum* collected from the department. *In-vitro* screening of these bacteria were done against *R. solanacearum* to detect the promising antagonistic bacteria. *Ralstonia solanacearum* was isolated from the wilt infected part of tomato plant and it's morphological, biochemical and cultural features were studied. The bacterium was gram negative, rod shaped and showed positive results in KOH solubility test, starch hydrolysis test, catalase test, oxidase test, citrate utilization test, motility indole urease agar (MIU) test, gelatin liquefaction test, and pectiolytic test. It produced highly fluidal, slightly raised and creamy white colonies with light pink or pinkish red centre and irregular margin after 48 hrs of incubation at 30^0C on TTC medium. Out of all antagonistic bacterial isolates, *Pseudomonas fluorescens (S1)* produced the highest inhibition zone (19 mm) against *R. solanacearum* at 72 hrs of incubation. In pot experiment, the efficacy of bacterial antagonist in controlling bacterial wilt severity in terms of PDI was found lowest in *Rhizobium leguminosorum* treated plants at each counting. Wilt severity also found lowest in *P. fluorescens* and *Bacillus subtilis* treated plants compared to control and the highest yield obtained from *P. fluorescens (S1)* treated plants. So bacterial wilt of tomato can be controlled by using antagonistic bacteria.

CONTENTS

LIST OF TABLES

LIST OF FLOW CHART

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF SYMBOLS AND ABBREVIATIONS

%	=	Percentage
et al.	=	And others
spp.	=	Species

J.	=	Journal
No.	=	Number
viz.	=	Namely
df.	=	Degrees of freedom
&	=	And
etc.	=	Etcetera
^{0}C	=	Degree Celsius
@	=	At the rate of
cm	=	Centimeter
cfu	=	Colony forming unit
ppm	=	Parts per million
NaCl	=	Sodium chloride
Kg	=	Kilogram
g	=	Gram
ml	=	Milliliter
WP	=	Wettable Powder
hr	=	Hour (s)
cv.	=	Cultivar (s)
i.e.	=	That is
T	=	Treatment
ft	=	Feet (s)
pv.	=	Pathovar
var.	=	Variety
mm	=	Milimiter
µl	=	Microliter
µm	=	Micrometer

LIST OF SYMBOLS AND ABBREVIATIONS (Cont'd)

SAU	=	Sher-e-Bangla Agricultural University
BAU	=	Bangladesh Agricultural University
BARI	=	Bangladesh Agricultural Research Institute
BBS	=	Bangladesh Bureau of Statistics
BER	=	Bangladesh Economic Review
USA	=	United States of America
NA	=	Nutrient Agar (media)
YDCA	=	Yeast Dextrose Calcium carbonate Agar (media)
PDA	=	Potato Dextrose Agar (media)
KB	=	King's B
YMA	=	Yeast Malt Agar (media)
PSI	=	Per Square Inch
ANOVA	=	Analysis of variances
LSD	=	Least Significant Difference
CV%	=	Percentages of Co-efficient of Variance

CHAPTER I
INTRODUCTION

Tomato (*Solanum lycopersicum*) is a widely grown nutritious vegetable in Bangladesh. It is one of the most popular and important commercial vegetable crops grown throughout the world. It is rich in vitamins A, B and C. Bangladesh produces 137 thousand metric tons of tomato per year from 47.96 thousand acres of land with an average yield of 2855 kg/acre (BBS, 2010). Many diseases and disorders can affect tomatoes during the growing season. This economically important vegetable crop suffers from several soil borne diseases. Bacterial wilt is also known as brown rot is caused by *Ralstonia (Pseudomonas solanacearum),* E. F. Smith, a soil borne bacterial species. An early crop of tomato is very profitable to farmers but its early production is badly hampered due to bacterial wilt. The loss in yield in tomato in India ranges from 10.83 to 90.60 percent while the plant mortality ranges from 10 to 100 percent (Ramkishun, 1987).

The bacterium can maintain infectious populations in soil over several years. It possesses a wide variability in environments, geographic spread and its host range, including several hundred plant species representing 50 families (Hayward 1992; Hayward and Hartman 1994 and Shekhawat *et al.,* 1992). The most severely affected crops as stated by Hayward and Hartman (1994) are tomato (*L. esculentum* L.), potato (*Solanum tuberosum* L.), eggplant (*S. melongena* L.), chilli (*Capsicum* spp.) and groundnut (*Arachis hypogaea* L.). Various methods have been introduced to control the bacterial wilt disease. The wide and indiscriminate use of chemical has been the cause of the appearance of resistant micro-organisms over and over, leading to the occurrence of emerging food borne diseases (Kaur and Arora, 1991 and Akinpelu, 2001). As a result of this, the interest to obtain alternative microbial agents for use against plant pathogen has increased. The use of microbial antagonists has been noted as a promising control strategy.

Many fungal and bacterial agents have been examined over a period of time for their potential as biocontrol agents (Khan *et al*., 2011). Several strains of *Pseudomonas fluorescens* have been reported to suppress soil borne diseases caused by pathogens (Sullivan and Gara, 1992; Weller, 1998). Shekhawat *et al*. (1993) observed that biological management of bacterial wilt with several bacterial and actinomycetes strains were possible. A large number of PGPR were reported to promote plant growth and to control plant diseases (Basan and de Basan, 2002). Biological control strategies may either help development of alternative management measures or be integrated with other practices for effective disease management at the field level. Biological control not only suppresses the disease and increases the crop yield but will be important in preventing the environmental pollution due to pesticides. There are some isolates of the genera *Bacillus* and *Pseudomonas* that are good bio-controllers, including some used as commercial products (Jacobsen *et al.*, 2004). The safety of the use of microorganisms for biocontrol was discussed by Cook *et al.* (1996), who showed that despite the existing risks, *Bacillus* and *Pseudomonas* do not present pathogenicity, allergenicity or toxigenic effect on people, domestic animals and wildlife. Besides this, the populations of microorganisms applied to the environment commonly often decline to undetectable levels (Cook *et al.*, 1996).

The antagonistic microorganisms through antibiosis, competetion and exploitation control the population of other microorganism including plant pathogenic ones i.e biological control may possibly become an ideal disease control method (Cook and Baker, 1983). For the bacterial wilt pathogen chemical control is arduous, uneconomical and not advisable owing to risk of ground water polution, death of non-terget bacterial flora and evolution of chemical resistant strains of pathogen. *B. subtilis* and *Bacillus* sp. completely controlled wilt throughout the growing period (Shekhawat *et al.*, 1992). They also found that two isolates of *P. fluorescens* inhibited *R. solanacearum in vitro*.

Considering the above facts, the present study was under taken with the following objectives:

- To find out suitable antagonistic microorganisms against *R. solanacearum*.
- To test the effectiveness of antagonistic bacteria to control bacterial wilt of tomato.

CHAPTER II
REVIEW OF LITERATURE

Ralstonia solanacearum constitutes a serious obstacle to the cultivation of many solanaceous plants in both tropical and temperate regions. The greatest economic damage has been reported on potatoes, tobacco and tomatoes. It can sometimes cause total crop losses. Disease severity mostly increases if *R. solanacearum* is found in association with root nematodes. In tobacco, nematode infestation changes the physiology of the plants, causing susceptibility to bacterial wilt. The bacterium is a quarantine organism. The occurence of different races and strains of the pathogen with varying virulence under different environmental conditions presents a serious danger to European and Mediterranean potato and tomato production.

Hence, the literature pertaining to the bacterial wilt of tomato along with information on related crops disease and pathogen are reviewed here as under.

Literature Related to Bacterial Antagonist

Tan *et al.* (2013) reported that bacterial wilt caused by *R. solanacearum* is a serious threat for agricultural production. In this study, *Bacillus amyloliquefaciens* strains CM-2 and T-5 antagonistic to *R. solanacearum* were used to create bioorganic fertilizers to control tomato wilt under greenhouse conditions. The possible mechanism of resistance inducement by the antagonistic bacteria was also evaluated.

Gilles Cellier *et al.* (2011) reported that the *R. solanacearum* species complex includes *R. solanacearum, R. syzygii,* and the blood disease bacterium. All colonize plant xylem vessels and cause wilt diseases, but with significant biological differences. *R. solanacearum* is a soil borne bacterium that infects the roots of a broad range of plants. *R. syzygii* causes Sumatra disease of clove trees and is actively transmitted by cercopoid.

Milling *et al.* (2011) reported that bacterial wilt caused by *R. solanacearum* is deemed to be one of the most important plant diseases in tropical agriculture.

Aliye *et al.* (2008) reported that *R. solanacearum* has a large host range of more than 200 species in 50 families.

Gou-Gen Hua *et al.* (2008) reported that bacterial wilt, caused by *R. solanacearum* (Smith) is an economically important disease on tomato in many provinces of China. Antagonistic bacterium *B. subtilis* strain AR12 was used to control bacterial wilt of tomato (*Lycopersicon esculentum*) Miller in the greenhouse. The biocontrol efficiency was as high as 90.18%.

Lwin and Ranamukhaarachchi (2006) reported a satisfactory suppression of the bacterial wilt pathogen by the application of a commercially available mixture of effective microorganisms (EM).

Doan and Nguyen (2005) reported that, in Vietnam, bacterial wilt causes significant damage on many important crops under disease-favorable weather conditions. Control is difficult due to high variability of the pathogen, limited possibility for chemical control, high capacity of the pathogen to survive in diverse environments and its extremely wide host range. The use of resistant varieties has been used to reduce disease.

Swanson *et al*. (2005) reported that bactrial wilt is a devastating disease worldwide, bacterial wilt limits the production of solanaceous crops such as tomato, pepper, eggplant, tobacco and potato as well as other important crops like peanut, banana, ginger and geranium. Approximately 450 crop species have been reported as hosts of this pathogen.

Bacillus species occur mainly in the soil, and because of spore forming the bacteria have the ability to survive in soil environment. *Bacillus* species produce many kinds of antibiotics which share a full range of antimicrobial activity such as bacitracin, pumulin and gramicidin (Todar, 2005).

The gram-positive *Paenibacillus* sp. has been shown to possess extracellular cellulolytic, proteolytic, chitinolytic, and pectinolytic enzyme activities (Budi *et al.*, 1999).

Seif *et al.* (2003) reported that the application of bioorganic fertilizers significantly reduced incidences of tomato wilt (by 63-74%), promoted plant growth and significantly reduced the *R. solanecearum* populations in rhizosphere compared with the control. Both strains CM-2 and T-5 applied with bioorganic fertilizers survived well in the tomato rhizosphere. Tomato seedlings treated with cell suspension of T-5 followed by challenge inoculation with *R. solanecearum* increased the activities of polyphenol oxidase, phenylalanine ammonialyase and peroxidase compared with the untreated control.

Van Overbeek *et al*. (2002) reported that biological control is still in its research phase with few studies reported for bacterial wilt. Biological control not only increases crop yield and suppresses disease but also avoids environmental pollution.

Raaijmakers and Weller (2001) reported that *Pseudomonas* possesses diverse mechanisms by which they can exert inhibitory activity towards phytopathogens and thereby mediate crop protection. One of the most effective mechanisms that antagonist employ to prevent proliferation of phytopathogens is the synthesis of antibiotics. Fluorescent *Pseudomonas* spp. has been reported to produce 2, 4-diacetylfluoroglucinol (2, 4-DAPG) by which they provide biological control of soil-borne pathogens on a wide range of crops and have a key role in the suppressiveness of some plant pathogens.

Weller *et al.* (2002) reported that the natural control of several phyto-pathogens is based on the presence of suppressive soils where several bio-control microorganisms belonging to *Trichoderma*, *Pseudomonas* and *Bacillus* genera are detected.

Bossis *et al.* (2000) reported that the genus *Pseudomonas* is a very large and important group of non-fermenting, gram negative bacteria, living as saprophytes in soils, sediments and fresh water.

Anith *et al.* (2000) reported that seed treatment with *P. flurescens* strain EM85 along with solarization decreased the wilt incidence by 7.42% and increased the yield up to 29.42% as compared to control.

Toyota and Kimura (2000) reported the suppressive effect of some antagonistic bacteria on *R. solanacearum*.

In a laboratory studies Abdulla *et al.* (1999) showed that the use of antagonistic bacteria *B. subtilis* (BI) and *Pseudomonas* spp. had a highly inhibitory effect against *R. solanacearum* on culture medium. Significant reduction in the number of wilted plants were achieved in greenhouse tests when the antagonistic bacteria were applied to tomato seedlings, which were then infested with *R. solanacearum*. They suggested that the antagonistic bacteria tested are promising candidates for the biological control of *R. solanacearum.*

The ability of an avirulent strain Tm3 to protect tomato plants from bacterial wilt investigated by Dong *et al*. (1999) in a greenhouse. The results indicated that strain Tm3 gave good biological control of tomato bacterial wilt. Antibiosis of bacteriocin and induced resistance of host are possible mechanisms for this protection.

Rhizoplane and root tissue of tomato were assessed against *R. solanacearum* by Moura *et al.* (1998). Eighteen actinomycetes showed 100% control of bacteria. The most efficient method of application was by dipping the seeds in a propagules suspention of the actinomycetes.

The antagonism of some soil bacteria to *R. solanacearum* was evaluated by Cartin and Wang (1997). Of 22 bacteria originally obtained, 12 showed antagonism to *R. solanacearum*. It is suggested that antibiotic substances may be involved in antagonism.

Ciampi *et al.* (1997) reported that a biological method to control bacterial wilt and to prevent latent infection in soil was developed based on a selected antagonist, strain BC8 of *P. flurescens*. The strain caused strong inhibition against *R. solanacearum in vitro* and prevented root and plant infection in growth chambers. Several carrier systems were developed for introduction of strain BC8 under field conditions and for testing protection of potato plant growing in *R. solanacearum* infested pots. The results showed that protection could be achieved even when soil infested with 10^6cfu of *R. solanacearum* per pot .

Li *et al.* (1997) conducted an experiment with bacterial strains those were isolated from samples of tobacco growing soils collected from 23 regions in Hunan, China. The inhibiting effect of these strains against *P. solanacearum* was examined. The results showed that 48 strains were effective, of which 10 strains were highly effective. Of those 10 strains, six had no inhibiting effects on one another. It is suggested that these may be used in combination to control bacterial wilt of tobacco.

Hayward (1994) reported that bacterial wilt is one of the most destructive plant diseases, which is predominantly distributed in the tropical, subtropical and warm temperate regions of the world.

Prior *et al*. (1994) reported that the common control measures employed in other countries include the use of resistant variety, crop sanitation, crop rotation, selection of disease free planting material and other cultural practices as single or integrated disease management. This is because such kind of resistance is strain specific and liable to break down by virulent and highly polymorphic strains of *R. solanacearum* at an ambient temperature and in nematode infested soil.

Grimault *et al.* (1993) reported that bacterial wilt disease is widely distributed, it has a wide host range and is mainly soil-borne; it is difficult to control with chemicals and cultural practices.

Ren *et al*. (1993) reported that control of bacterial wilt of tomato was investigated by using two avirulent, bacteriocin-producing strain of *R. solanacearum*, MA-7 and E104. Roots of tomato seedlings were dipped in pot suspensions containing 10^8cfu/ml for 30-50 minutes. After two months, 70% control was achieved and the yield of treated plot was 52.7-64.9% higher than that of control.

Anuratha and Gnanamanikam (1990) reported that utilization of antagonistic rhizosphere bacteria such as *Bacillus* spp., *P. fluorescens* and *P. putida* significantly increased the survival rate of tomato by 60–90% against bacterial and fusarium wilt disease.

Hartman (1992) evaluated the effectiveness of certain biological, organic and inorganic treatments in reducing population level of *P. solanacearum* and thus severity of bacterial wilt. They stated that *P. cepacia*, *P. flurescens* and *P. gladioli* caused large zones of inhibition against *P. solanacearum*. Bacterial wilt was controlled when *P. gladioli* was applied to the before inoculation of *P. solanacearum*.

The use of fluorescent pseudomonads (FP) has reported to be a promising measure of bacterial wilt control in tomato (Hsu *et al*. 1992). A study was carried out by them to determine the role of different soil amendments in increasing rhizosphere colonization of FP, which in turn play an important role in suppressing *P. solanacearum*. Among the 230 strains of FP were screened by the root tip method. Four strains, D-4, T-9-1, G-14 and 59 were more consistent in efficacy, reducing the disease with an average range of 50-70%.

Phae *et al*. (1992) reported that bacterial wilt caused by *R. solanacearum* was suppressed when a culture suspention of *B. subtilis* NB22 was poured into heavily infested soils and the percentages of dead plants were much reduced.

The results suggested that NB22 have antibacterial activity and can be used as a biological control against wilt disease of tomato.

Shekhawat *et al.* (1992) stated that potato bacterial wilt incidence reduced up to 79% by *Bacillus* spp. both under greenhouse conditions. Similarly *P. flurescens* isolates reduced incidence of bacteria by 43 to 75% and actinomycetes up to 79%.

Hara and Ono (1991) demonstrated the probability of biological control of bacterial wilt of tobacco by dipping the root system of seedlings in suspension of a weakly virulent bacteriocin-producing strain of *P. solanacearum* prior to transplanting.

Anuratha and Gnanamanickam (1990) reported that *Pseudomonas fluorescens* treated banana (*Musa balbisiana*), eggplant and tomato plants were protected from wilt upto 50, 61 and 95% in greenhouse and upto 50, 49 and 36%, respectively in field. Protection afforded by the *Bacillus* strains was lower. In bacteria-treated plants which were subsequently inoculated with *P. solanacearum* plant height and biomass values increased and were close to those of nontreated and noninoculated control plants.

Anuratha *et al.* (1990) reported that *R. solanacearum* disease affects a wide range of economically important crops such as tomato, potato, eggplant, chili and non *Solanaceous* crops such as banana and groundnut in India. The disease is called southern bacterial blight. *R. solanacearum* wilt another common name in countries where it occurs.

Tanaka *et al.* (1990) found that tobacco plants were protected from bacterial wilt with an avirulent strain M45 of *P. solanacearum* .

Ciampi-Panno *et al.* (1989) showed that antagonistic pathogens were effective in suppressing *R. solanacearum* under field conditions. Several antagonists have been evaluated with variable success.

Aspiras and De-la Cruz (1985) examined in an experiment to determine the extent by which *B. polymyxa* FU6 and *P. flurescens* could effectively protect tomato and potato from bacterial wilt by pre-emptive colonization of the plant roots. Rapidly growing bacteria such as *B. polymyxa* FU6 and *P. flurescens* have demonstrated great effectiveness in reducing the incidence of bacterial wilt in tomato and potato. *B. polymyxa* FU6 may be applied very conveniently as a spore preparation during seedling time.

Chen and Echandi (1984) studied the effect of avirulent bacteriocin producing strain of *P. solanacearum* for the control of bacterial wilt of tobacco and observed good control.

He *et al.* (1983) showed that bacterial wilt, caused by *R. solanacearum* E. Yabuuchi formerly known as *P. solanacearum* E. F. Smith is one of the most devastating, important and wide-spread bacterial diseases of crops in tropical environments

Ling (1977) tested a total of 106 fungi, actinomycetes and bacteria for their antagonistic activities against *P. solanacearum* and observed only 13 isolates were antagonistic to the pathogen *in vitro*. Of these, three fungi and one bacterium were selected and tested for effectiveness to tomato seedlings transplanted to infested soil. The three fungal isolates retarded seedling growth and failed to reduce wilt while the bacterial isolate identified as *Bacillus* sp. reduced wilting incidence by 46-70% using the “seedling-watering” method.

Directed evidence of the potential use of antagonist was reported by Celino and Gottlieb (1952). They found that by treating the infested soil with the broth cultures of *B. polymyxa* B3A, only 33% of the total population of tomato succumbed to bacterial wilt as against 70% on non-treated infested soil.

CHAPTER III
MATERIALS AND METHODS

3. 1. Experimental Site

The experiment was carried out in the Disease Diagnostic Laboratory and Net house of Department of Plant Pathology, Sher-e-Bangla Agricultural University, Dhaka.

3. 2. Time of Experiment

This study was conducted during the period of July 2012-Dec 2013.

3. 3. Collection of Seeds

Seeds of BARI tomato-2 (Ratan) were used throughout the investigation and seeds were collected from Bangladesh Agricultural Research Institute (BARI), Joydevpur, Gazipur, Bangladesh.

3. 4. Collection of Wilt Infected Plants

Bacterial wilt infected tomato plants were collected from different experimental plots of Sher-e Bangla Agricultural University, Dhaka, Bangladesh.

3. 4. 1. Isolation of *Ralstonia solanacearum* from wilt infected tomato plants

The wilt infected plants were selected based on visible symptoms of bacterial wilt as described by Shekhwat *et al*. (1992) and Ali and Dey (1998). To confirm bacterial wilt infection oozing test was performed (Shekhawat *et al*. 1992). For isolation of *R. solanacearum* from wilt infected plant specimens, streak plate technique was followed using a selective medium, Tetrazolium chloride agar (TZC) as described by Kelman (1954). The medium contained peptone 10g, casein hydrolysate 1 g, glucose 5 g, and agar 20 g in 1000 ml of

distilled water. The mixture was cooked, pH was adjusted to 7.0 using 0.1N KOH and autoclaved at 121^0C under 1.1 kg/cm^2 pressure for 20 minutes. Aqueous solution of 2, 3, 5- triphenyltetrazolium chloride (TTC) was prepared by dissolving 1g of the chemical in 100 ml of distilled water in an Erlenmeyer flask. The 1% stock solution of TTC solution was separately sterilized by passage through 0.45µm pore size filters (Millipore). The sterilized TTC solution was poured into the sterilized medium at the rate of 5 ml/1000 ml before solidification (45-45^0C) and mixed thoroughly. The medium was poured into Petri plates (9 cm) at the rate of 20 ml/plate. The TTC was kept in a colored bottle and was wrapped with aluminium foil to avoid light and preserved in a refrigerator at 4^0C for future use.

Diseased stem of tomato were washed under tap water and cut into small pieces ((2-3cm) from the base. The pieces of infected stem were surface sterilized with 5% chlorox for 1 minute and 70% ethanol for 1 minute and rinsed in sterilized distilled water. The surface sterilized pieces were immersed in 5 ml of sterilized distilled water in a test tube for oozing. The bacterial ooze released from the infected stem was thoroughly mixed in water after discarding the stem pieces. One loopful of suspension was streaked on the TZC agar medium in Petri plates and incubated at 30^0C for 48 hr. Virulent colonies of *R. solanacearum* were selected on the basis of characteristic colony characters on TZC medium (Kelman, 1954).

3. 4. 2. Preservation of cultures

For further study, virulent colonies were selected based on color (Kelman, 1954) and well-isolated fluidal colonies were restreaked on CPG (without the stock solution of TTC) plates because some strains are sensitive to the formazan pigment produced from TTC. Two loopful of bacteria from a composite of about six individual 48 to 72 hrs old colonies were transferred to screw capped test tubes containing 5 to 8 ml of sterilized distilled water for storage (Kelman and Person, 1961). The tubes with the cultures were preserved

at room temperature (25-30^{0}C). The isolates preserved in sterile water were recultured on TTC medium at every 3 months.

3. 4. 3. Pathogenicity of isolates on host species

The pathogenicity of selected six virulent isolates of *R. solanacearum* was tested by inoculating the tomato plants (var. BARI tomato 2). Seedlings of tomato were planted directly in polybag containing sterilized soils (well decomposed cow dung and silty soil at 1:1). Soil sterilization was done with formalin (40%) at the rate of 5 ml of formalin diluted with 20 ml of water for 4 kg soil (Dasgupta, 1988). The formalin treated soil was covered with polythene sheet for 48 hours and then exposed for 48 hrs aeration before setting the experiment. Fertilizers were added @ 50 g of TSP and 50 g of MP per polybag.

Stock cultures of the isolates in water were streaked on TTC medium and incubated at 30^{0}C for 48 hr. A single virulent colony of each isolates was transferred to individual culture plate containing casein peptone glucose (CPG) medium (casein 0.1%, peptone 1%, glucose 0.5% and agar 2%) in Petri plates for multiplication and incubated at 30^{0}C for 48 hr. After incubation, the bacterial cells were harvested in sterile distilled and inoculum suspension was adjusted to 10^{8}cfu/ml. Thirty days after sowing, the plants were inoculated with pure culture following stem puncture method by using sterile syringe (Winstead *et al.* 1952). Control plants were injected with sterile distilled water. The inoculated plants were placed in a net house and watered daily with tap water. Inoculated plants were checked every day to record the duration needed by individual isolates to develop the characteristic bacterial wilt symptoms (Devi 1982).

3. 4. 4. Biochemical tests for identification of *Ralstonia solanacearum*

For characterization of the isolates of *R. solanacearum* a series of biochemical tests were conducted. The tests were: Gram reaction (Suslow *et al.*, 1982), catalase test (Hayward, 1992), Oxidase test (Kovacs, 1956), motility test and nitrate reduction.

3. 4. 4. 1. Gram reaction test

It is a rapid method for gram differentiation of plant pathogenic bacteria without staining (Suslow *et al*., 1982). Two drops of 3% KOH solution were placed at the centre of a clean glass slide. One loopful colonies of *R. solanacearum* (grown on TTC medium) were added to the KOH solution and homogenized with a nichrome loop with rapid circular movement of about 10 seconds. Viscous strand formation was observed and on drawing it with a loop it formed a fine thread of slime, 0.4 to 2.5 cm in length. This indicated that the test was positive and confirmed gram negative nature of the bacterium. On the contrary the gram positive bacteria did not produce such thread.

3. 4. 4. 2. Catalase test

Hydrogen peroxide (H_2O_2) is a by product of aerobic respiratory metabolism in aerobic and facultative anaerobic bacteria. Hydrogen peroxide is a highly toxic oxidizing agent, which reacts with oxygen to produce potentially lethal oxygen free redicals such as superoxide anion. Bacteria are protected from these effects by the enzyme catalase, which catalyzes the following reaction:

$2H_2O_2 \rightarrow H_2O + O_2$.

Twenty four hour old cultures of *R. solanacearum* and 3% hydrogen peroxide solution were used to observe production of gas bubbles, which indicates positive reaction (Hayward, 1992). A loopful of bacterial culture (24 hr old on TTC medium) of each isolate was placed on a clean glass slide. A drop of H_2O_2 solution (3%) was added and mixed with the culture. Production of gas bubbles indicated positive reaction.

3. 4. 4. 3. Oxidase reaction

This test is particularly valuable for differentiating pseudomonalds from certain other Gram negative rods (Shekhawat *et. al.*, 1992). Aerobic or

facultative anaerobic bacteria, i. e., those with respiratory activity are divisible into two groups, those which are oxidase positive and those which are oxidase negative. An oxidase positive reaction transport is indicative of the presence of a cytochrome- C-Oxidase in the respiratory electron chain. Among pseudomonalds, the test has important differential value because isolates of *R. solanacearum* give positive reaction. Tetramethyl-p-phenyl diamine is oxidised by the cytochrome-cytochrome oxidase system of the bacterium to a purple compound. Aqueous solution of (1%) of tetramethyl-p-phenylenediamine is used as test reagent. A strip of Whatman filter paper (No 2) was soaked with 3 drops of 1% aqueous solution of freshly prepared tetra methyl- p- phenylene-diamine dihydrochloride (color indicator). A loopful of young bacterial culture (TTC medium) of each isolate was rubbed separately on the impregnated surface of the filter paper stripe by a platinum loop. Purple color develops within 10 seconds, which indicated positive reaction of oxidase test.

3. 4. 4. 4. Motility test

Motility agar was prepared in tubes without slanting. Sterile semisolid medium was inoculated by stabbing the centre of the medium without touching the bottom. The tube was incubated at 30^0 C for 18 to 24 hours. Motility of organism was shown by a diffused zone of the entire medium or only from one or two points.

3. 4. 4. 5. Nitrate reduction test

Nitrate reduction test was carried out in the nitrate broth. The freshly prepared cultures were inoculated in sterile nitrate broth containing tubes and incubated at 30^0 C for 24 hours. At the end of incubation 0.1 ml solution of A was added followed by solution B in equal volume. The appearance of deep pink color showed the positive results.

3. 4. 4. 6. Starch hydrolysis

A nutrient agar plate containing 2% soluble starch was inoculated with the bacterium isolate to be tested. Then incubated at optimum temperature for at

least 48 hours. After inoculation, the plate was flooded with Lugol's iodine and observed.

3. 4. 4. 7. Pecteolytic test

Potato tubers were disinfected with 99% ethanol, cut up into slices of about 7-8 mm thick, and then placed on moistened sterile filter paper in sterile Petri dishes. Bacterial cell suspension was pipetted into a depression cut in the potato slices. One potato slice pipetted with sterile water was treated as control. Development of rot on the slices was examined 24–48 h after incubation at 25°C. Examination was done for 5 days after inoculation. Two slices were inoculated for each isolate.

3. 4. 4. 8. Citrate utilization test

A portion of the test organism was picked up from the agar plate with a sterile inoculating loop and streaked into Simmon's citrate agar slants. Following incubation at 30^{o}C for 24 hours changing of the green bromothymol blue indicates positive results.

3. 5. Isolation and Purification of *Pseudomonas fluresencs*

3. 5. 1. Collection of soil (rhizosphere) sample

The soil samples were collected from the Plant Pathology Field, Sher-e-Bangla Agricultural University, Dhaka. Five soil samples were randomly collected from the rhizosphere of tomato field at different growth stages. Soil collection was made with the help of an augur of one inch diameter at the depth of six inches. At the time of collection, the surface of the top soil was scrapped to remove dry soil and other superficial plant debris. Each composite sample was kept in cellophane bag with proper labeling. After collection, the bags were kept under shade.

3. 5. 2. Preparation of working sample

For every dilution of soil samples, working sample was prepared from the composite sample that was made after the soil sample collection at the different growth stages of tomato plant.

3. 5. 3. Preparation of soil suspension

One gram of soil was placed in the test tube containing 9 ml of sterile water and stirred thoroughly for few minutes in order to obtain a uniform 1:10 dilute soil suspension. This was used as stock solution resulting 10^1 dilution. One ml of 1:10 stock suspension was transferred with the help of sterile pipette into the second test tube containing 9 ml sterile water and shaken thoroughly resulting 10^2 solution. This final dilution was made up to 10^4 dilution.

3. 5. 4. Preparation of King's B (KB) media and spreading of soil suspension

King's B media (Appendix-I) was prepared according to the method followed by Goszczynska *et al.* (2000). For the preparation of 1 liter King's B medium 20 g proteose peptone No. 3 (Difco.), 15 ml glycerol, 1.5 g K_2HPO_4 (anhydrus), 1.5 g $MgSO_4.7H_2O$ and 15 g bacto agar were taken in an Erlenmeyer flask containing 1000 ml distilled water and mixed well. It was then autoclaved at 121^0C under 15 PSI pressure for 15 minutes. Each dilution of soil suspension was then spread over on King's B (KB) medium and kept in an incubator at 30^0C temperature for two days. After incubation colony were selected on the basis of morphology and color and recultured on King's B medium for purification. Dilution plate technique was carried out as described by Islam (2009) for isolation of soil bacteria.

3. 6. Isolation of *Bacillus* spp.

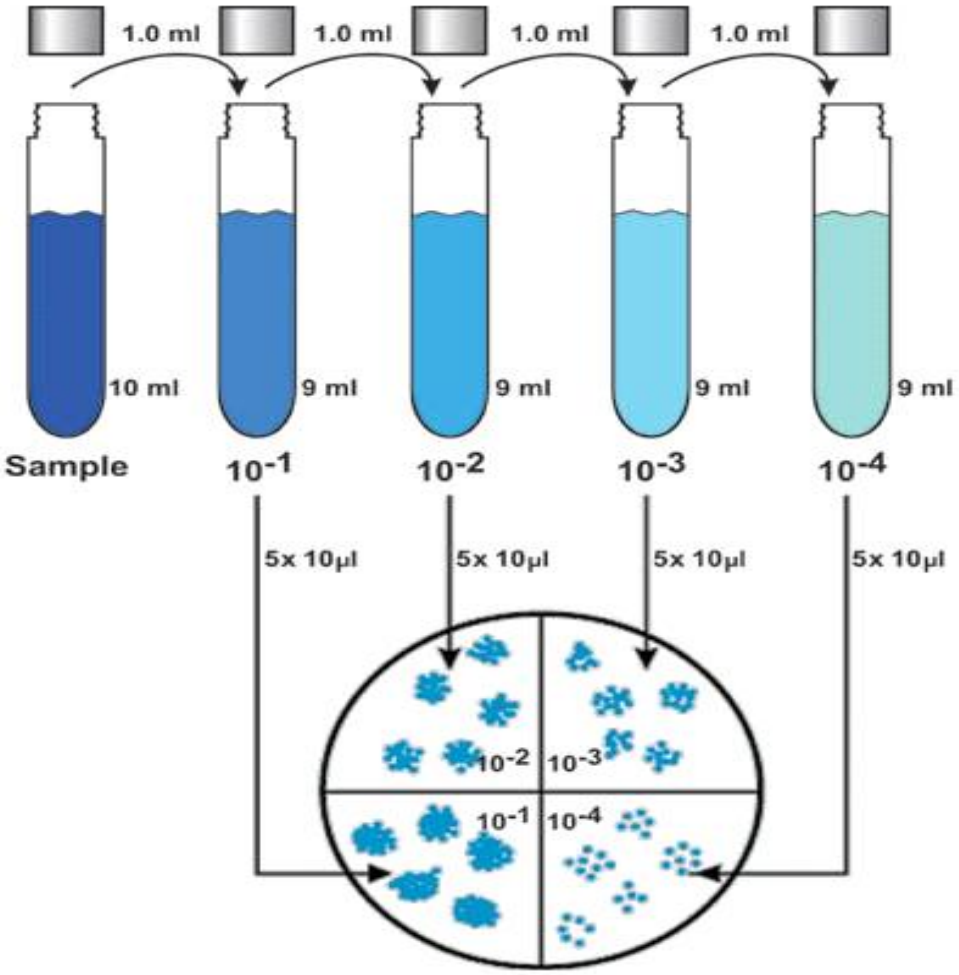
Probable Bacillus spp. Were isolated from rhizosphere soil on nutrient agar plates following serial dilution method. The plates were then incubated at 55ºC for 24 hrs.

3. 7. Collection of *Rhizobium leguminosorum* and *Bacillus subtilis*

Six isolates of *R. leguminosorum* were collected from Department of Plant Pathology, Sher-e-Bangla Agricultural University.

3. 8. *In-vitro* Antagonism

The efficacy of antagonists were evaluated against *R. solanacearum* following well method. At first the antagonists bacteria were cultured in nutrient broth and which were incubated in a shaker incubator at 150 rpm for 48 hrs. After incubation the culture broths were centrifuged and supernatant liquid was collected for further use. Simultaneously the nutrient agar plate was inoculated with *R. solanacearum* and a well was made at the center of each plate seeded with *R. solanacearum.* 80 µl of each supernatant liquid of antagonist was poured into each well under asceptic condition and incubated at 28 ± 2ºC. The plates with sterile water surved as control. The experiment was laid with completely randomized design with 3 replications. Observation were recorded by measuring the inhibision zone (clear zone around the well) upto 5 days of incubation.



Flow chart 1. Dilution plate technique for isolation of bacteria

3. 9. Characterization of Antagonist Bacteria

3. 9. 1. Morphology of the bacteria

For morphological characters, colony color, shape and surface textures were carefully studied as per the standard procedures described by Schaad *et.al* (1992) on NA plate.

3. 9. 2. Different biochemical tests

Different biochemical tests were done for each antagonistic bacterial isolate such as Gram's reaction, KOH solubility test**,** starch hydrolysis, citrate utilization test, catalase test, oxidase test, pecteolytic test and gelatin liquefaction test as per the methods described by Schaad (1992) and Salle (1961).

3. 10. Soil Sterilization and Preparation of Soil and Pot

Soil from the field of the field laboratory of the Department of Plant Pathology, SAU, Dhaka was collected and dried. Decomposed cow dung from the Dairy Farm of SAU was added to it. The soil was mixed uniformly with cow dung (2:1). The dried soil was sterilized with formalin (40%) at the rate of 5 ml formalin diluted with 20 ml of water for 4 kg soil (Dashgupta, 1988; Hossain, 2006). The formalin treated soil was covered with polythylene sheet for 48 hrs and then exposed of 48 hrs for aeration before setting the experiment.

3. 11. Raising of Seedlings

Seeds of tomato were sown in a plastic tray (50×35 cm) containing heat sterilized soil. The tray was kept in a net house. Proper soil moisture was maintained by watering whenever needed. Twenty five days after sowing healthy, uniform seedling were selected for the pot experiment.

3. 12. Seedling Treatment

Twenty five days old seedlings were then lifted from soil. Suspensions of antagonists were made. Then, 10 seedlings for each treatment were dipped in a separate beaker for 4 hrs. (Fig. 1).

3. 13. Preparation of Pots

The plastic pots were bought from the market which has the capacity to fill with 2 Kg of soil. Before preparation, soil was sterilized by 40% formaldehyde and used it as base soil. Then soil: Cow dung @ 2:1 was mixed and the pots were filled with that soil.

3. 14. Transplanting of Seedling

The seedlings were then transplanted in pots. It was then nourished for 5 days to put up with transplant injury (Fig. 2. A).

3. 15. Inoculation of *Ralstonia solanacearum*

Cell suspension of *R. solanacearum* was prepared in sterile water (10^8 cfu/ml). Ten plants were inoculated for each treatment, following stem puncture method (fig. 2. B). After inoculation, the pots were kept in a net house.

Fig.1. Seedlings root dipping in cell suspension of bacterial antagonists

Fig. 2. A. Transplanting of seedling in pots

Fig. 2. B. Inoculation of *R. solanacearum* by using sterilized syringe

3. 16. Computation of Disease Severity and Disease Incidence

Pot experiment for tomato plant was set following Complete Randomized Design (CRD) with five treatments. Ten plants were inoculated for each treatment. Data recorded on wilt incidence (%) and severity. To calculate the percent disease incidence, number of plants wilted per total number of plants was counted.

Percent disease incidence was calculated by the following formula as described by Mansoor *et al.* (2007):

$$\% \text{ Disease incidence} = \frac{\text{Number of plants wilted}}{\text{Total number of plants}} \times 100$$

Wilt severity was recorded based on a 0-3 scale, where 0= no wilt; 1= 1-50% leaves wilted; 2= more than 50% leaves wilted; 3= entire plant wilted or dead (Furuya *et al.* 1997). The percent disease index (PDI) was computed using a formula (Sing, 1984):

$$\text{PDI} = \frac{\sum (\text{Disease class} \times \text{Plant number in that class})}{\text{No. of Plants assessed} \times \text{highest score of the scale}} \times 100$$

3. 18. Statistical Analysis

Data collected during experimental period were tabulated and analyzed following Statistical package MSTAT-C. Treatment means were compared with Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1984).

CHAPTER IV

RESULTS

The results of the investigations undertaken on "Study on *Ralstonia solanacearum* causing bacterial wilt of tomato and its management by using antagonistic bacteria" during the study period are presented as below.

4. 1. Symptoms of Bacterial Wilts

The plants showed sudden wilting. The leaves of the infected plants lost turgidity, became flaccid, droop and finally died (Fig. 3).

Fig. 3. A. Bacterial wilt infected plant initial stage
B. Healthy plant

4. 2. Isolation and Purification of *Ralstonia solanacearum*

R. solanacearum produced highly fluidal, slightly raised and creamy white colonies with light pink or pinkish red centre and irregular margin after 48 hrs of incubation at 30^0C on TTC medium (Fig. 4). Colonies were purified by restreaking the isolated colony on TTC plate.

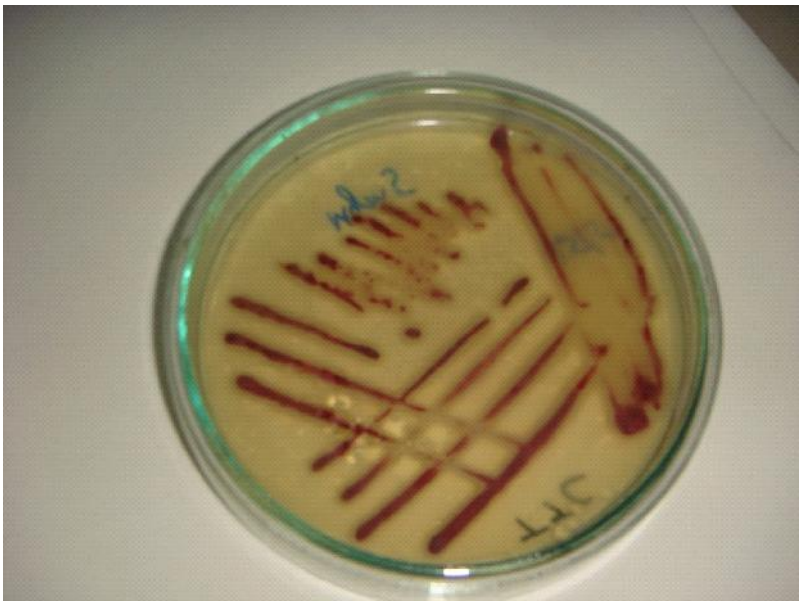


Fig. 4. Growth of *Ralstonia solanacearum* on TTC medium (Streaking method)

4. 3. Preservation of *Ralstonia solanacearum*

Purified bacterium was kept in refrigerator at 4^0 C in small screw-cap test tubes on NA slant, which served as a stock culture for further studies.

4. 4. Identification of *Ralstonia solanacearum*

The bacterium was identified as *R. solanacearum* on the basis of their pathogenicity test, morphological and biochemical characteristics.

4. 4. 1. Pathogenicity test of *Ralstonia solanacearum*

R. solanacearum produced characteristcs symptoms of bacterial wilt on inoculation into tomato plants. The infection of *R. solanacearum* was confirmed following oozing test. The variation of incubation period to manifest wilt symptoms among the isolates varied possibly due to variation in virulence.

4. 4. 2. Morphological characters

Under the compound microscope at 100x magnification with oil immersion, the bacterium was rod shaped with rounded ends, cells appeared singly and also in pairs, gram negative (red colour) and capsulated. (Fig .5. A).

In KOH solubility test, a mucoid thread was lifted with the loop (Fig. 5. B). Therefore the test was positive i.e., the bacterium was gram negative that supports the result of gram's staining test.

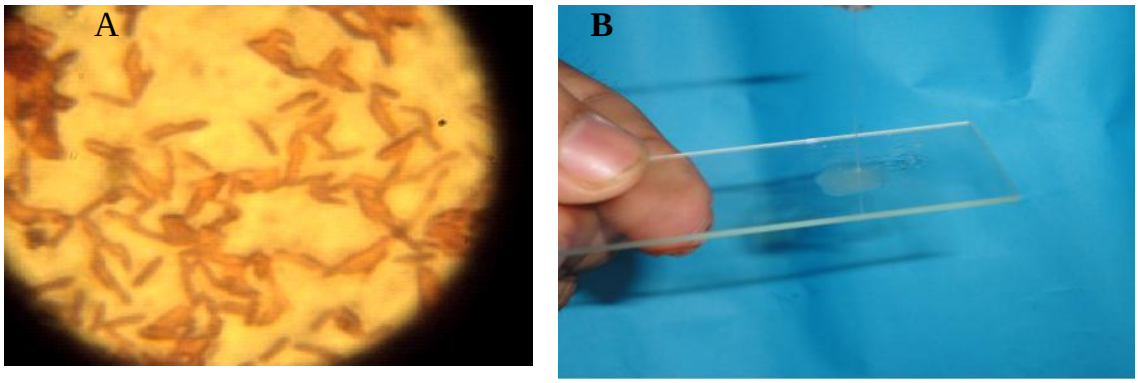


Fig. 5. Morphological characters of *Ralstonia solanacearum*

A. Microscopic view of *Ralstonia solanacearum* after gram's staining at 100x magnification
B. KOH solubility test for *Ralstonia solanacearum*

4. 4. 3. Biochemical analysis

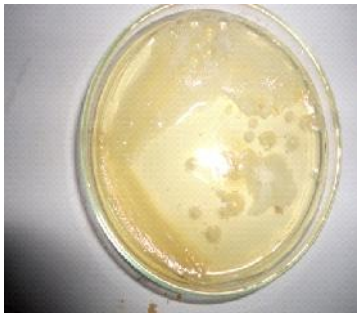
In catalase test, after adding 3% H_2O_2 onto the colony of the bacterium bubbles were formed within a few seconds (Plate1 B) which revealed that the test was positive. In oxidase test, after rubbing the bacterium onto the moistened oxidase disk, it form violet colour oxidase disk (Plate1 C) which revealed that the test was posetive. In motility indole urease agar (MIU) test, after 48 hours of incubation it was found the bacterium migrated away from the original line of inoculation (Plate1 E) thus the bacterium was motile (positive test). In starch hydrolysis test, after adding lugol's iodine a clear zone was formed around the bacterial colony indicated starch hydrolysis (amylase activity) i.e., the test was positive(Plate1 A). In citrate utilization test, after 24 hours of

incubation green colour of simmon's citrate agar slant changed into a bright blue colour indicated the test was positive i.e., the bacterium used citrate as a carbon source for their energy (Plate1 D). In petiolytic test, soft rotting caused by *R. solanacearum* (Plate1 G). Thus the bacterium showed the positive result. In gelatine liquefaction test, gelatin was liquefied after 15 minutes of refrigeration at 5^{o}(Plate1 F). Results obtained on various biochemical tests for the pathogen are presented in Table 1.

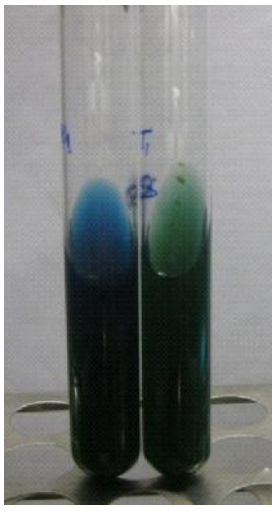
Table 1. Biochemical characteristics of *Ralstonia solanacearum*

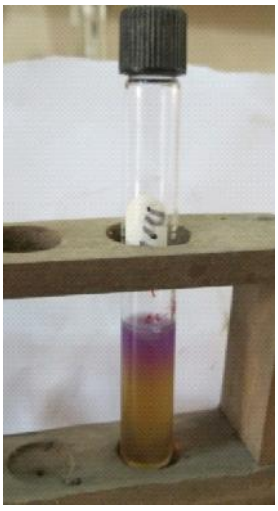
Biochemical tests	Results
Gram reaction test	-
Catalase test	+
Oxidase test	+
Motility test	+
Nitrate reduction test	+
Starch hydrolysis	+
Citrate utilization test	+
Pecteolytic test	+
Gelatin liquefaction test	+

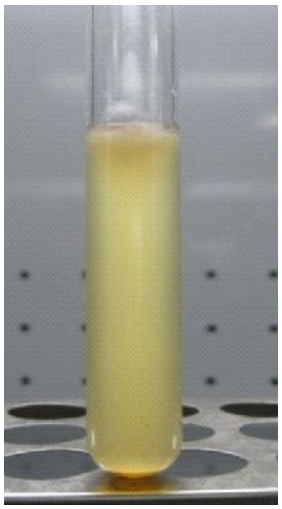
A B C



D E F







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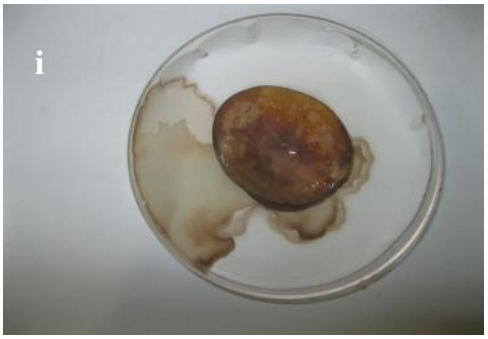


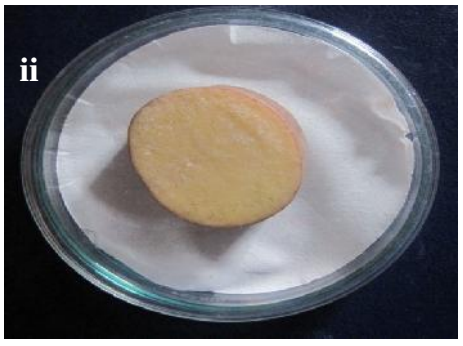


Plate1. Biochemical tests of *Ralstonia solanacearum*

A . Starch hydrolysis test. B . Catalase test. C . Oxidase test.

D . Citrate utilization test. E . Motility indole urease agar (MIU) test.

F . Gelatin liquefaction test. G.Pecteolytic test. i. Bacteria inoculated

ii. Control

4.5. Isolation and Purification of Antagonistic Bacteria

Ten *Pseudomonas fluorescens* and four *Bacillus subtilis* bacterial isolates were isolated from soil by employing dilution plate technique. *Pseudomonas fluorescens* produces pink pigmentation on King's B (KB) plate after 48 hours of incubation at 30^0C (Plate 2. A). Repeated isolation yielded creamy white, round colonies of *Pseudomonas fluorescens* whose margins were uneven on nutrient agar plate (Plate 2. B). *Bacillus subtilis* produced cream colour, flat and circular colonies with undulated margins (plate2. C), where *Rhizobium leguminosorum* produced white, fluidal and circular, convex colonies on nutrient agar plate (plate2. D).

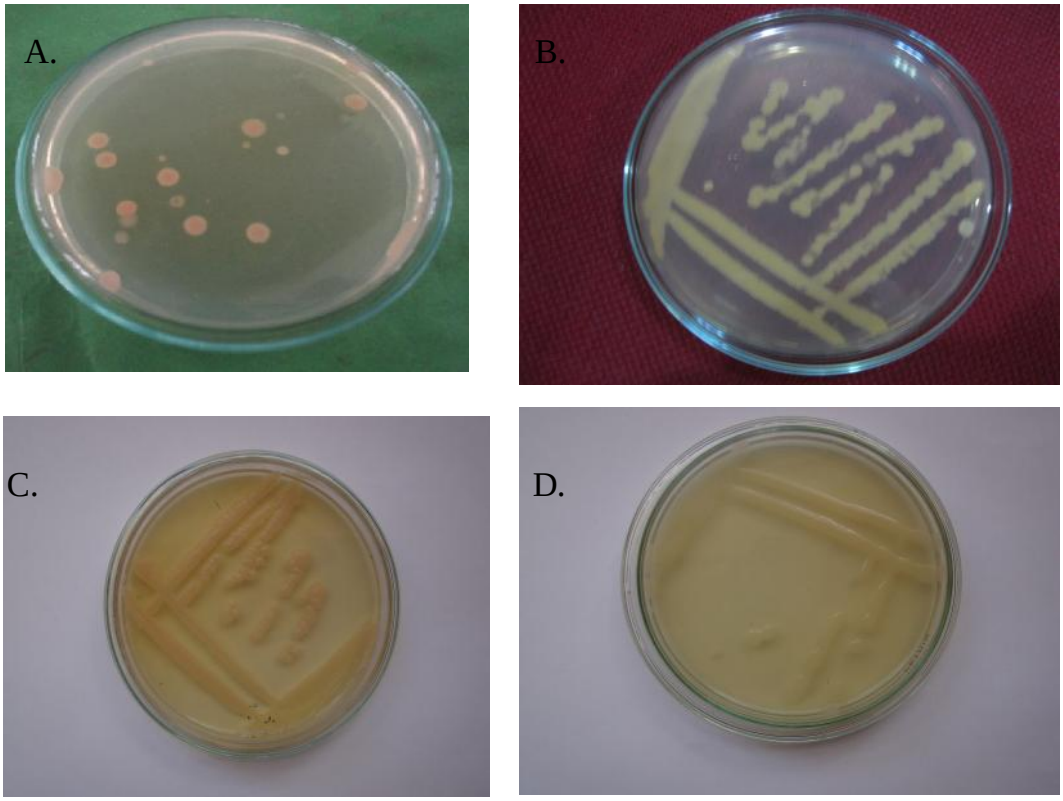


Plate 2**.** Pure culture of biocontrol agents

A. *Pseudomonas fluorescens* on King's B plate.

B. *Pseudomonas fluorescens* on NA plate.

C. *Bacillus subtilis* on NA plate.

D. *Rhizobium leguminosorum* on NA plate.

4. 6. Identification of Antagonistic Bacteria

Identification of antagonistic bacteria was done by conducting studies on their morphological and biochemical features as per standard microbiological procedures and growth on selective media. Schaad *et al* (2001).

4. 6. 1. Morphological characters

Under the compound microscope at 100x magnification with oil immersion, *Bacillus subtilis* was gram positive, straight to slightly bent rod that appeared singly or in chains; *Pseudomonas fluorescens* was gram negative, straight to curved rod that appeared singly or in short chains and *Rhizobium leguminosorum* was gram negative, rod shaped, pleomorphic that appeared singly or in cluster.

4. 6. 2. Biochemical characters

Results obtained on various biochemical tests for antagonistic bacteria are presented in Table 2.

Table 2. Biochemical characteristics of antagonistic bacteria

Biochemical tests	*Bacillus subtilis*	*Pseudomonas fluorescens* (Iso-1)	*Pseudomonas fluorescens*(Iso-2)	*Rhizobium leguminosorum*
Starch hydrolysis test	+	+	+	-
Catalase test	+	+	+	+
Oxidase test	+	+	+	+
Citrate utilization test	+	+	+	+
Motility indole urease agar (MIU) test	+	+	+	+
Gelatin liquefaction Test	+	+	+	+
Petiolytic test	-	-	-	-

+ = positive reaction

- = negative reaction

4. 7. Screening of Antagonistic Bacteria Against *Ralstonia solanacearum*

Among the ten isolates of *P. fluorescens* and four isolates of *B. subtilis* and six isolates of *R. ligominosorum*, only two isolates of *P. fluorescens* and one

isolates each of *B. subtilis* and *R. leguminosorum* were found antagonistic against *R. solanacearum.*

Significant effect of different bacterial antagonists against *R. solanacearum* were observed at different time of incubation. At 24 hrs of incubation the highest zone of inhibition produced by *P. fluorescencs (S1)* (Plate. 3 B) and *B. subtilis* (Plate. 3 A) followed by *R. liguminosorum* (Plate. 3 D). At 48 hrs *P. fluorescencs (S1)* produced highest zone of inhibition followed by *P. fluorescencs* (S2) (plate.3 C) which was similar with *B. subtilis.* After 72 hrs of incubation the highest zone of inhibition observed in *P. fluorescencs* and other antagonists showed statistically similar result. After 96 hrs of incubation *P. fluorescencs* (S1) produced highest zone of inhibition (16.67 mm). After 120 hrs of inhibition the highest zone of inhibition recorded in *P. fluorescencs* (S1) followed by *Pseudomonas fluorescencs* (S2). It was observed that inhibition zone was decreased with the increase of incubation time (Table 3).

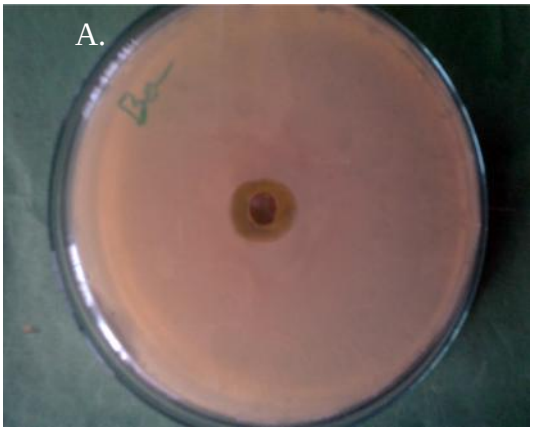
Table 3. Efficacy of bacterial antagonists against *Ralstonia solanacearum*

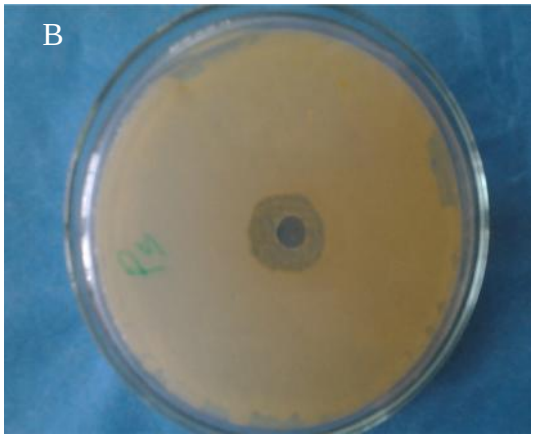
Treatments	**Inhibition zone (mm)**				
	24hrs	**48hrs**	**72hrs**	**96hrs**	**120hrs**
Pseudomonas	13.67 a	16.33 a	19.00 a	16.67 a	13.33 a

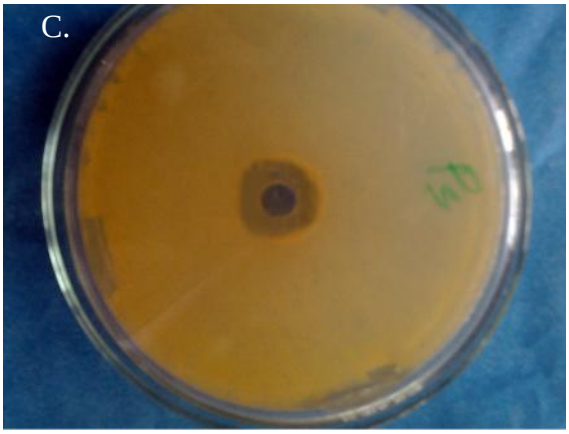
fluorescencs (S1)					
Pseudomonas fluorescencs (S2)	11.33 c	14.33 b	12.67 b	10.67 c	8.67 b
Rhizobium leguminosorum	12.67 b	12.67 c	12.33 b	13.00 b	0.00c
Bacillus subtilis	13.67 a	14.33 b	12.00 b	11.00 c	0.00 c
Control	0.00 d	0.00 d	0.00 c	0.00 d	0.00 c
LSD (0.05%)	0.8572	0.9205	1.197	1.229	0.5753
CV (%)	5.51	5.26	7.05	7.89	8.62

Each data represents the mean value of three replications.

Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.







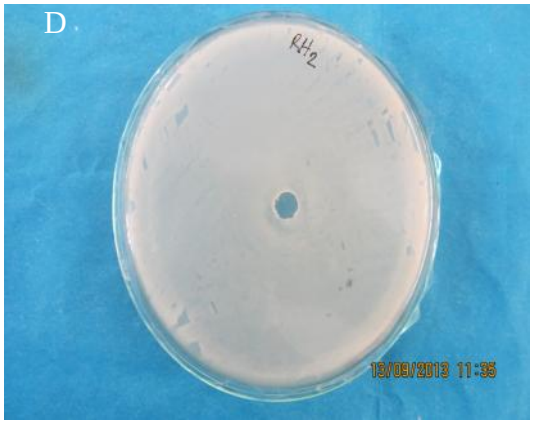


Plate 3. Effect of different bacterial antagonists against *Ralstonia solanacearum*

A. *Bacillus subtilis* at 24 hours of incubation

B. *Pseudomonas fluorescens (S1)* at 24 hours of incubation

C. *Pseudomonas fluorescens (S2)* at 48 hours of incubation

D. *Rhizobium leguminosorum* at 24 hours of incubation

4. 8. Efficacy of Root Dipping in Cell Suspension of Bacterial Antagonists on Wilt Incidence and Severity of Tomato

Significant effect on disease incidence and severity were recorded among different bacterial antagonist. At 15 days after transplanting the maximum disease severity in terms of PDI (15.40%) was recorded from untreated control and the minimum disease severity was observed in *R. leguminosorum* treated plants which was statistically similar with *P. fluorescens (S2)* treated plants (Table 4). At 25 days after transplanting the maximum disease severity in terms

of PDI (21.92%) was recorded from untreated control and the minimum disease severity was observed in *B. subtilis* treated plants. After 35 days after transplanting maximum disease severity in terms of PDI (32.92%) was recorded from untreated control and the minimum disease severity was observed in *B. subtilis* treated plants which was statistically similar with *P. fluorescens (S2)* treated plants (Table 4).

Table 4. Efficacy of root dipping in cell suspension of bacterial antagonists on wilt severity of tomato

Treatments	Wilt severity in percent Disease Index (%)		
	15 DAT	25 DAT	35 DAT
(T_1)	6.20 b	13.20 b	22.40 b
(T_2)	3.25 c	10.51 c	12.19 d
(T_3)	3.22 c	10.41 c	16.37 c
(T_4)	5.80 b	6.29 d	10.75 d
(T_5)	15.40 a	21.92 a	32.92 a

%LSD	1.236	1.972	2.889
%CV	9.91	8.40	8.11

Means within the same column with a common letter are not significantly different (p=0.05) by DMRT

T_1= Root dipping with *Pseudomonas* Isolate 1

T_2= Root dipping with *Pseudomonas* Isolate 2

T_3= Root dipping with *Rhizobium leguminosorum*

T_4= Root dipping with *Bacillus subtilis*

T_5= Control (Untreated)

4. 9. Effect of Antagonists on Yield of Tomato

The study conducted revealed that among the five treatments against *R. solanacearum*; the maximum yield per plant was (841.18 g) from *Pseudomonas fluorescens (S1)* treated plants which was statistically similar with *Pseudomonas fluorescens (S2*) and *B. subtilis* treated plants. Untreated control plants produced the lowest yield (720 g).

Table 5. Effect of Antagonists on Yield of Tomato

Treatments	**Yield (g)**

(T_1)	841.1 a
(T_2)	819.4 ab
(T_3)	782.1 b
(T_4)	822.3 a
(T_5)	742.0 c
%LSD	37.66
%CV	2.62

Each data represents the mean value of three replications.

Values followed by the same letter within a column are not significantly different ($p \leq 0.05$) according to Duncan's multiple range test.

T_1= Root dipping with *Pseudomonas* Isolate 1

T_2= Root dipping with *Pseudomonas* Isolate 2

T_3= Root dipping with *Rhizobium leguminosorum*

T_4= Root dipping with *Bacillus subtilis*

T_5= Control (Untreated)

CHAPTER V
DISCUSSION

Ralstonia solanacearum pathogen of tomato wilt disease was isolated from infected tomato plant collected from Horticultural Field of Sher-e-Bangla Agricultural University (SAU) campus by dilution plate method. Schaad *et al.* (2001) isolated the *R. solanacearum* from infected tomat plant by dilution plate method. For isolation of *R. solanacearum* from wilt infected plant specimens' streak plate technique was followed using a selective medium, Tetrazolium chloride agar (TZC) as described by Kelman (1954). Morphological and biochemical tests were done to identify *R. solanacearum.*

The capability of causing wilt disease by *R. solanacearum* was confirmed by artificial inoculation to tomato plant. The result is supported by the findings of Begum (2007) who observed that *R. solanacearum* able to produce wilting symptoms in tomato plants. The finding is also supported by the findings of Ling, 1977 who reported wilting in tomato plants in Turkey by *R. solanacearum.* Soil level inoculation method i.e. soil drenching method was followed to inoculate the tomato plant by *R. solanacearum*. Soil drenching method showed highly susceptible reaction for the inoculation of wilt causing organism of tomato plants. The reliability of soil drenching method is also supported by Sitaramaiah and Singh (1984) and Rahman (1992) who used the same method for inoculation of tomato.

Ten *Pseudomonas fluorescens* and four *Bacillus subtilis* bacterial isolates were isolated from the soil sample using dilution plate technique and 6 isolates of *Rhizobium leguminosorum* were collected from Department of Plant Pathology SAU, Dhaka to observe antagonistic behaviour against *R. solanacearum.* Morphological and biochemical tests were done identify these genera. Amomg the antagonists tested all showed positive result in starch hydrolysis except *R. leguminosorum* and all antagonists gave negative result in pectiolytic test. Suslow *et al.* (1982) discovered a quick method for Gram differentiation i.e

KOH solubility test. *P. fluorescens* and *R. leguminosorum* showed positive result in KOH solubility test and *B. Subtilis* showed negative result. *P. fluorescens* and *R. leguminosorum* were Gram negative rod shaped bacteria and *B. Subtilis* was Gram positive, endospore forming and coccobacilli shaped. Hayward (1992) conducted catalase test for *Pseudomonas fluorescens, Bacillus subtilis* which show similar reasult.

Among the isolates tested against *R. solanacearum*, only two isolates of *P. fluorescens*, one isolate of *B. subtilis* and one isolate of *R. leguminosorum* showed antagonistic activity. Thus it was assumed that, the four isolates have more or less ability to control *R. solanacearum*. In *in-vitro* screening, *P. fluorescens (S1)* showed best performance by reducing the growth of *R. solanacearum*, *B. subtilis* and *R. leguminosorum* also gave good result and produced inhibition zone against *R. solanacearum* . Weller *et al.* (2002) showed that *P. fluorescens* have antagonistic activity against *R. solanacearum*. Cartin *et al.* (1997) evaluated the antagonism activity of *P. fluorescens* and *B. subtilis.* Toder (2005) found that *B. subtilis* produced many kinds of antibiotics which suppress soil borne bacteria. *B. subtilis* and *P. fluorescens* had highly inhibitory effect on culture medium against *R. solanacearum* (Abdulla *et al.*, 1999). Fluorescent *Pseudomonas* spp. has been reported to produce 2,4-diacetylfluoroglucinol (2, 4-DAPG) by which they provide biological control of soil-borne pathogens on a wide range of crops and have a key role in the suppressiveness of some plant pathogens (Raaijmakers and Weller, 2001; Weller *et al.,* 2002). Ling (1977) tested 106 fungi, actinomycetes and bacteria for their antagonistic activities against *R. solanacearum* and observed only 13 isolates were antagonistic to *R. solanacearum.* The bacterial isolates were identified as *Bacillus* spp. Cartin *et al.* (1997), evaluated 22 soil bacteria against *R. solanacearum* and observed that 12 showed antagonism to *R. solanacearum.* They suggested that antibiotic substances may be involved in antagonism. 2001; Weller *et al.*, 20

Lowest wilt disease inceidence and severity at different days after transplanting were recorded in *P. fluorescens (S1)* treated plants. Chen and Echandi (1984) studied the effect of avirulent bacteriocin producing strain of *P. solanacearum* for the control of bacterial wilt of tobacco and observed good control. *Bacillus* sp. reduced wilting incidence by 46-70% stated by Ling, 1977. Shekhawat *et al.* (1992) reported that potato bacterial wilt incidence reduced up to 79% by *Bacillus* spp. both under greenhouse conditions. Similarly *P. fluorescens* isolates reduced incidence of bacteria by 43 to 75% and actinomycetes up to 79%.

P. fluorescens (S1) gave the highest yield per plant (841.1 g), also showed better performance compared to other antagonists. PGPR had been reported to directly enhance plant growth by variety of mechanism: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilisation of minerals such as phosphorus and synthesis of phytohormones (Glick, 1995). Anith *et al.* (2000) reported that seed treatment with *P. fluorescens* strain EM85 along with solarization decreased the wilt incidence by 7.42% and increased the yield up to 29.42% as compared to control. Root inoculation of *Bacillus* M3 alone or in combination with spraying *Bacillus* OSU-142 or *Pseudomonas* BA-8 have the potential to increase the yield, growth and nutrition content of strawberry plant under organic growing conditions (Esitken *et al.*, 2010).

The present study clearly supports that seedling root treatment with *P. fluorescens, B. subtilis* and *R. leguminosorum* have strong effect in reducing wilt incidence and severity and also increased the yield. So these antagonists could be used against *R. solanacearum* as seed or seedling root treatment.

CHAPTER VI

SUMMARY AND CONCLUSION

The present study was designed to study the managment of bacterial wilt of tomato by using antagonistic bacteria. *R. solanacearum* the bacterial wilt causing pathogen of tomato was isolated from the infected plant by following dilution plate technique using TTC medium. Causal organism of bacterial wilt of tomato was purified by restreaking on nutrient agar medium with single colony and confirmation was done by pathogenicity test. The pathogen was identified by it's morphological, biochemical and cultural features as per standard microbiological procedures. The bacterium was gram negative, rod shaped with rounded ends. It showed positive result in KOH solubility test, starch hydrolysis test, catalase test, citrate utilization test, motility indole urease agar (MIU) test, gelatin liquefaction test, salt tolerant test, oxidase test. The colonies of all isolates of *R. solanacearum* produced highly fluidal, slightly raised and creamy white colonies with light pink or pinkish red centre and irregular margin after 48 hrs of incubation at 30^0C on TTC medium .

Twenty bacterial isolates of rhizosphere bacteria were tested *in vitro* condition against *R. solanacearum.* Among these only four isolates showed antagonistic activity against *R. solanacearum* including one isolate of *Rhizobium leguminosorum.* These isolates were identified as *Pseudomonas fluorescens* 1 and 2 and *Bacillus subtilis* on the basis of morphological and biochemical characteristics. In *in vitro* test highest zone of inhibition (13.67 mm) of *R. solanacearum* was measured against *Pseudomonas (S1))* followed by *Pseudomonas fluorescens (S2)* and *Rhizobium leguminosarum.*

Seedlings were transplanted in pot having sterilized soil. Ten plants were planted for each treatments and the design of the experiment was Completely Randomized Design (CRD). Pretreated seedlings were inoculated following soil drenching method. Data were recorded on PDI at 15 days after transplanting following 0-3 scale (Furuya *et al.*, 1997). The highest wilt severity in terms of PDI was recorded from control plants at 15, 25 and 35

days after transplanting which was 15.40%, 21.92%, and 32.92%, respectively. The lowest disease severity recorded in *Pseudomonas fluorescens* treated plants which was 3.25%, 10.41%, and 12.19%, at 15, 25 and 35 days after transplanting respectively. In case of yield *P. fluorescens* (S1) treated plants gave the highest yield per plant (841.1 g) and lowest yield was recorded from control plants.

Considering the above results it can be concluded that, there is scope to use biological agent for management of bacterial wilt of tomato. *R. leguminosorum*, *P. fluorescences* and *B. subtilis* were found effective to control bacterial wilt of tomato. Further study is needed to test their efficacy under natural field condition and a comprehensive reseach should be undertaken to develop an effective method of controling bacterial wilt of tomato using antagonists.

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APPENDICES

Appendix I. Preparation of culture media and reagents

The compositions of the media used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved at 121^0C for 15 minutes at 15 lb pressure.

Nutrient Agar (NA)

Beef extract (Difco)	3.0 g
Peptone (Difco)	5.0 g
Bacto agar	15.0 g
Distilled water	1000 ml

Nutrient Broth (NB)

Beef extract (Difco)	3.0 g
Peptone (Difco)	5.0 g
Distilled water	1000 ml

Proteose peptone No. 3 (Dico)	20.0 g
Glycerol	15.0 ml
K_2HPO_4 (Anhydrous)	1.5 g
$MgSO_4.7H_2O$	1.5 g
Bacto agar	15.0 g
Distilled water	1000 ml

King's B (KB)

Proteose peptone No. 3 (Dico)	20.0 g
Glycerol	15.0 ml
K_2HPO_4 (Anhydrous)	1.5 g
$MgSO_4.7H_2O$	1.5 g
Bacto agar	15.0 g
Distilled water	1000 ml

Yeast Malt Agar (YMA)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Dextrose	10.0 g
Bacto agar	20.0 g
Distilled water	1000 ml

Gelatine Liquefaction Media

Beef extract	3.0 g
Peptone	5.0 g
Gelatine	120 g
Distilled water	1000 ml

Simmon's Citrate Agar

Magnesium sulphate	0.2 g
Sodium citrate	2.0 g
NaCl	5.0 g
Dipotassium Phosphate	1.0 g
Monopotassium Phosphate	1.0 g
Bromothymol blue	0.08 g
Bacto agar	20.0 g
Distilled water	1000 ml

Motility Indole Urease (MIU) Agar

Peptone	30.0 g
Urea	20.0 g
Monopotassium phosphate	2.0 g
NaCl	5.0 g
Phenol red	0.05 g
Bacto agar	4.0 g
Distilled water	1000 ml
pH	7.0

Starch hydrolysis media and reagent

Culture medium

Nutrient broth (Difco)	8.0 g
Soluble potato starch	10.0 g
Bacto agar (Difco)	15.0 g
Distilled water	1000 ml

Reagent (Lugol's iodine)

Iodine	5.0 g
Potassium iodide	10.0 g
Distilled water	100 ml

Gram's staining reagents

Gram's Crystal violet (Hucker's modification)

Solution A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol	20.0 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution A and B in equal volume to prepare crystal violate solution.

Gram's Iodine (Gram's modification of Lugol's solution)

Iodine	1.0 g
Potassium iodide (KI)	2.0 g
Distilled water	300.0 ml

Add iodine after KI is dissolved in water to prepare Gram's Iodine solution.

Gram's alcohol (decolorizing agent)

Ethyl alcohol (95%)	98 ml
Acetone	2 ml

Safranin (counter stain)

Safranin (2.5% solution in 95% ethanol)	10 ml
Distilled water	100 ml

KOH solubility reagent

3% aqueous solution of KOH was prepared from the KOH granules.

Catalase reagent

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution.

Oxidase reagent

1% aqueous solution of NNN'N-tetramethyl-p-phenylene-diaminedihydrochloride was prepared from the absolute solution.