ISOLATION AND IDENTIFICATION OF BACTERIA ASSOCIATED WITH ROTTEN POTATOES IN SELECTED COLD STORAGE OF COMILLA

BY
SHAHRIN MAHBUBA TANNI

DEPARTMENT OF PLANT PATHOLOGY
SHER-e -BANGLA AGRICULTURAL UNIVERSITY DHAKA-1207

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BY
SHAHRIN MAHBUBA TANNI
Reg. No.: 15-06913

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APPROVED BY:

Dr. Nazneen Sultana **Professor** **Supervisor**	**Md. Ziaur Rahman Bhuiyan** **Assistant Professor** **Co-Supervisor**

Associate Prof. Dr. Md. Belal Hossain
Chairman
Examination Committee
Sher-e-Bangla Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

Sher-e-Bangla Agricultural University
Sher-e-Bangla Nagar, Dhaka-1207

CERTIFICATE

This is to certify that the thesis entitled," ***ISOLATION AND IDENTIFICATION OF BACTERIA ASSOCIATED WITH ROTTEN POTATOES IN SELECTED COLD STORAGE OF COMILLA"*** *submitted to the 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* ***SHAHRIN MAHBUBA TANNI****, Registration No. 15-06913 under my supervision and my 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 duly been acknowledged.

Dated: 25th September, 2017

Dhaka, Bangladesh

Prof. Dr. Nazneen Sultana

Supervisor

Dedicated to my
Beloved
Parents

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ABSTRACT

A study was conducted in order to assess the bacteria associated with post-harvest decay of cold storage potato. Rotten potatoes were collected from cold storage of Homna upazilla under Comilla district of Bangladesh. The experiment was conducted in the MS Laboratory of Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University during the period of November 2016 to August 2017. Four different bacteria (species) were isolated from the different rotten potato tubers following dilution plate method on NA medium. The isolated bacteria were *Pectobacterium carotovorum,Ralstonia solanacearum, Pseudomonas* spp. and *Bacillus* spp. Out of these four bacteria isolated, *Pectobacterium* and *Ralstonia* were more abundant. Bacteria were identified based on morphological and biochemical tests. All the isolated bacteria showed positive results except *Bacillus* spp in potato soft rotting test. *Pectobacterium*, *Ralstonia solanacearum* and *Pseudomonas* spp. were pathogenic for storage potato while *Bacillus* spp. was possibly a secondary micro flora or saprophyte that manifested as contaminants.

CONTENTS

CHAPTER	TITLE	Page no.
	ACKNOWLEDGEMENTS	I
	ABSTRACT	II
	CONTENTS	III
	LIST OF FIGURES	V
	LIST OF TABLES	V
	LIST OF APPENDICES	V
	LIST OF ABBREBIATIONS	VI
I	**INTRODUCTION**	1
II	**REVIEW OF LITERATURE**	4
	2.1 Present status of potato in Bangladesh	4
	2.2 Occurrence and host range of soft rot enterobacteria	5
	2.3 Potato diseases and their effect on post harvest	6
	2.4 Epidemiology of soft rot of Potato	8
	2.5 Occurence and Host Range of Brown Rot of Potato	9
	2.6 Epidemiology and Survival of the Pathogen	10
	2.7 Symptomology of different potato diseases	12
	2.8 Isolation and Identification of Bacteria	15
III	**MATERIALS AND METHODS**	19
	3.1 Experimental Site	19
	3.2 Experimental Period	19
	3.3 Collection of Samples	19
	3.4 Measurement of prevalence of disease occurrence in cold storage of Comilla	19
	3.5 Preservation of samples	20
	3.6 Isolation and Identification of Bacteria from diseased potato	20
	3.6.1 Isolation of bacteria on NA media	20
	3.7 Preparation of Triphenyl tetrazolium Chloride (TTC) medium	21
	3.7.1 Preparation of Triphenyl tetrazolium Chloride(TTC)	21

CONTENTS (CONTD.)

CHAPTER	TITLE	Page no.
	3.7.2 Preparation of CPG media	21
	3.7.3 Preparation of TTC medium	21
	3.7.4 Growth of bacteria on TTC medium	21
	3.8 Preparation of Cetrimide Agar	22
	3.8.1 Growth of bacteria on Cetrimide Agar	22
	3.9 Growth of bacteria on Bacillus agar	22
	3.10 Biochemical test	23
	3.10.1 KOH solubility test or Gram differentiation test	23
	3.10.2 Gram staining	23
	3.10.3 Catalase test	24
	3.10.4 Oxidase test	24
	3.10.5 Gelatin liquefaction test	24
	3.10.6 Starch hydrolysis test	25
	3.10.7 Potato Soft Rotting test	25
IV	**RESULT AND DISCUSSION**	26
	4.1. Visual symptoms of collected samples	26
	4.1.1 Symptoms of soft rot of potato	26
	4.1.2 Symptoms of Brown rot of potato	26
	4.2 Isolation and Identification of Different Bacteria	27
	4.2.1 Cultural Characterization of different bacteria on NA	27
V	**DISCUSSION**	35
VI	**SUMMARY AND CONCLUSION**	38
VII	**REFERENCES**	40
	APPENDICES	56

LIST OF FIGURES

FIGURE NO.	TITLE OF THE FIGURE	PAGE NO.
1	Symptoms of soft rot of potato	26
2	Symptoms of Brown rot of potato	26
3	Growth of *Pectobacterium* on NA medium (By Spread Plate method)	28
4	Growth of *Pectobacterium* on NA medium (By Streak Plate method)	28
5	Growth of *Ralstonia solanacearum* on TTC medium	31
6	Growth of *Pseudomonas* spp. on Cetrimide agar medium	31
7	Growth of *Bacillus* spp. on Bacillus agar medium	32
8	Biochemical tests	32-34
9	Soft rotting test	32
10	Catalase test	32
11	Oxidase test	33
12	KOH Solubility test	33
13	Starch Hydrolysis test	33
14	Gelatin liquifaction test	34

LIST OF TABLES

TABLE NO.	TITLE OF THE TABLES	PAGE NO.
1	Cultural characterization of different bacteria on NA plates	29
2	Biochemical test for identification of different bacteria	30

LIST OF APPENDICES

APPENDIX	TITLE OF THE APPENDIX	PAGE NO.
	Preparation of culture media and reagents	56

LIST OF SYMBOLS AND ABBREVIATIONS

No.	=	Number
%	=	Percentage
et. al.	=	And others
	=	Degree Celsius
@	=	At the rate
J.	=	Journal
&	=	And
ppm	=	Parts per million
Kg	=	Kilogram
G	=	Gram
ml	=	Milliliter
PSI	=	Pound per Square inch
Ibs	=	Pound
FAO	=	Food and Agricultural Organization
Hr	=	Hour (s)
SAU	=	Sher-e-Bangla Agricultural University
BBS	=	Bangladesh Bureau of Statistics
NA	=	Nutrient Agar (media)
NB	=	Nutrient Broth (media)
CVP Medium	=	Crystal violet Pectate Medium
CPG Medium	=	Casamino acid-Peptone-Glucose Medium
TTC	=	Triphenyltetrazolium Chloride
Pa	=	*Pectobacterium atrosepticum*
Pcc	=	*Pectobacterium carotovorum* subsp. *carotovorum*
EPPO	=	European and Mediterranean Plant Protection Organization

CHAPTER I

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a herbaceous tuber crop belonging to the family Solanaceae. It is one of important food crops next to rice and wheat and is of course the most important vegetable grown in Bangladesh and also in the world (Ahmed and Talukder, 1978). It is produced and recognized as popular vegetable throughout the entire tropical and subtropical region of the world (Hayward, 1991).Potato production in Bangladesh began in the later part of the 19th century (Islam *et al*., 1983). Bangladesh ranks 11th in the world in terms of potato production in 2008 (Hossain and Miah, 2010). Annual potato production in Bangladesh is 6648 thousand metric tons (WFP, 2013).Bangladesh is the third greatest potato producer in Asia and standing sixth in the world (FAO, 2010). The yield growth was measured at 2.33 per cent over that of FY'15, according to provisional estimates by Bangladesh Bureau of Statistics (BBS, 2016).

There are 30 Government cold storage under BADC and about 400 non-government cold storage facilities in Bangladesh. A few studies on economic aspect of cold storage have been conducted so far in Bangladesh. In a preliminary survey of the diseases of potatoes in cold storage in Bangladesh it was observed that 2-9 percent of cold stored potatoes were lost in every year due to disease (Khan *et al*., 1973). Fakir (1979) stated that an amount of Taka 8 crores approximately was lost annually due to storage disease in Bangladesh.

Potato tubers suffer from post harvest losses because of some physical, pathological factors and physiological or a combination of all three factors (Booth, 1974).The principal factors responsible for losses during storage has

been reported to be because of infection caused by microorganisms resulting in tuber decay mainly bacteria causing rots in potato have been reported to produce a wide range of hydrolytic enzymes such as cellulases, pectinases, Xylanses and proteases. These enzymes are also responsible for tissue maceration and cell death (Amadioha and Adisa 1993, Olivieri *et al*.,2004).Potato tubers being nearly 80% water, they are especially susceptible to bacterial pathogens that cause Soft rot resulting to losses up to 90% in the field and in storage(Czajkowski *et al*., 2011a).

Bacterial soft rot is one of the most common potato disease in the tropics and induces quick and heavy spoilage losses. Its causal agent *Erwinia carotovora*, is one of the most important and widespread bacterial disease of a variety of plants either in the field or storage (Hajhamed *et al*.,2007; Harrison and Nielson, 1990). It causes substantial losses in transit and storage, particularly in the warm regions where temperatures are high and there are no facilities available for cold storages (Cromarty and Easton, 1973).Approximately 22% of potatoes are lost per year due to viral, bacterial, fungal and pests attack to potato tuber potato plant, incurring an annual loss of over 65million tones and bacterial soft rot alone accounts for 30-50% of this huge loss (Czajkowski *et al*., 2011). A recent report indicated that0.187 million tons of potatoes were spoiled in Bangladesh due to diseases (Anonymous, 2006).Stevenson *et al*. (2001); Rahman, (1969) and Kamaluddin (1970) reported that 2 to 9% losses of tubers occur every year in each storage due to diseases.

Bacterial soft rot is considered as one of the most destructive diseases of vegetable in storage and transit conditions (Hossain, 1986). A survey was carried out by Masum *et al*., (2011) to determine the losses due to storage diseases reported that maximum loss within the three months namely July,

August and September were found in cultivar Cardinal (5.55%), where losses were caused by soft rot (3.97%), dry rot (0.88%) and scab (0.70%).

In Dhaka district, the survey revealed that 3.27, 0.96 and 0.81% of tubers were affected with soft rot and dry rot(Masum *et al*.,2011).Bacterial soft rot leads to losses of potato that may reach up to 60 % in field, transit, and during storage (Mantsebo *et al*., 2014).Considering all things the present thesis are under taken the following objective:

Objectives:

1. To identify bacteria associated with rotten potatoes in selected cold storage of Comilla.

Chapter II

REVIEW OF LITERATURE

2.1. Present status of potato in Bangladesh

Potato is the third most significant crop in Bangladesh. Bangladesh is the third largest potato producer in Asia and standing sixth in the world (FAO, 2010).Potato is cultivated in about 4.61lac hectares of land to produce 84lac tons (Chowdhury and Hasan, 2013). The average yield of potato is 13.32-18.08 ton/ha (BBS, 2012).

Hossain *et al.,* (2008) reported that the national approximate yield of potato is very low (19.07 tha^{-1}) compare to its potential yield 30-40 tha^{-1},due to deficiency of quality seed, cultivation of indigenous potato (yield 5-7 tha^{-1}) and high price of quality seed. Bangladesh experienced much development in its potato production in the past decades; it has increased by 5 percent per annum. In the scenario of Bangladesh, after the rice (Boro-irrigated rice, Aman-rainfed rice, Aus-short duration rice), potato is the second most important crop in terms of consumption in some parts of the country such as Munshigonj district.

The potato is now a beneficial crop October-March winter crop in Bangladesh, with a production value - estimated at $560 million in 2005 - second only to that of paddy rice. In February 2016 Bangladesh potato production hit an all-time-high of 9.47 million tons. The yield growth was measured at 2.33 per cent over that of FY'15, according to provisional estimates by Bangladesh Bureau of Statistics (BBS). The BBS data showed that each hectare yield of potato also increased to 19.9 million tons in the last FY from 19.6 million tons in FY'15.

While potato production has increased by 1.26 million tons in the last five years, cold storage facility increased by only 0.4 million tons during the period, official data revealed. According to the Department of Agricultural Marketing (DAM), the country had 375 cold storage facilities with 2.7 million tons of retention in FY'15, which was 2.3 million tons in FY'11.Assistant Director of DAM TM Rashed Khan mentioned that over 2.02 million tons of potatoes were stored in the cold storehouses in 2015 against the capacity of 2.7 million, as many facilities low in Rangpur and Rajshahi regions.RANGPUR, April 12, 2017 (BSS) - The farmers of all five districts in the northern region achieved a bumper production of over 22lakh and two thousand tons of potato this year BBS(2016).

Storing of potato in the cold storage plants certainly reduces the excessive losses of potato but all farmers can not avail the facility of cold storage due to several reasons, such as high cold storage charge, uncertainty of future market price, financial insolvency, bad communication and inadequate transport facilities and lack of any provision in getting compensation for damage of potato in the cold storage plants (Hajong *et al*., 2014).

2.2. Occurrence and host range of soft rot enterobacteria:

Dickey (1979) described that Soft rot *Pectobacteria* are pathogens with a worldwide distribution, but with aberrant host ranges and host specificities. These characteristics are shown by their serological response. *Pa* is a pathogen that mainly invades potato grown in cool climatic conditions, but strains similar to *Pa* have been identified on other crops.

De Boer *et al*. (1979) stated that most *Pectobacterium atrosepticum* strains irrespective of origin form a serologically homogenous group and do not grow at temperatures above 30˚C.

Graham (1964) studied that *Pectobacterium carotovorum* Subsp.*carotovorum* prefer higher temperatures and have wide host ranges. *Pcc* strains affect soft rots in many crops in the temperate and tropical regions and cause diseases in many plant species than *Dickeya dadantii.*

Ma *et al*. (2007) reported that although *Pectobacterium* and *Dickeya* spp. are responsible for causing disease in many identical host species, their reported host ranges do not completely overlap. Of all these reported plant family hosts of *Pectobacterium* spp., only 6 dicot and 4 monocot families have been listed as hosts for both genera.

Ma *et al.* (2007) found no reports of soft rot enterobacteria causing disease under natural conditions in non angiosperms, the basal angiosperm orders.

2.3. Potato diseases and their effect on post harvest

Hajhamed *et al*. (2007) stated that Bacterial soft rot disease of potato caused by *Erwinia carotovora* subsp. *carotovora* can cause great reduction of potato yield, especially during storage.

Pérombelon (2002) found that Bacteria belonging to the *Pectobacterium* and *Dickeya* genera are causal agents of blackleg and tuber soft rot of potato, respectively.

van der Wolf and De Boer, (2007) studied that in seed potato production, these diseases are next in economic importance to bacterial wilt caused by *Ralstonia solanacearum*, forward to ring rot and common scab caused by *Clavibacter michiganensis* subsp. *sepedonicus* and *Streptomyces scabies*, respectively .

Czajkowski *et al*. (2015) reported that Soft rot bacteria *Pectobacterium* spp. and *Dickeya solani* cause important disease on potato and other arable and horticulture crops, and are responsible for causing tuber soft rots in storage.

Mantsebo *et al*. (2014) observed that Bacterial soft rot leads to losses of potato that may reach up to 60 % in field, transit, and during storage.

ChigumirawaNgwerume(2002) and Manzira (2010) stated that Soft rot causes great economic losses estimated to be between 40-80% depending on climatic conditions.

Nadia *et al.* (2013) reported that in 2011 the highest wilt occurrence was recorded in Munshigonj (22.65%), followed by Nilphamari (19.98%) and the lowest occurrence was recorded in Jamalpur (9.07%).

Nadia *et al.* (2013) estimated that the highest bacterial wilt intensity was recorded in Munshigonj (3.80), while the lowest wilt intensity was recorded in Jamalpur (2.90).

Yabuuchi *et al*. (1995) and Champoiseau *et al*. (2010) studied that Bacterial wilt, caused by *Ralstonia solanacearum*, is the second most important potato disease in tropical and sub-tropical regions of the world after late blight.

Champoiseau *et al*.(2009) estimated that globally, the disease has been estimated to affect about 1.7 million hectares of potatoes in approximately 80 countries, with global damage estimates of over USD 950 million per annum.

Bradbury, (1986) and Beaulieu *et al*. (2008) reported that In Mali, the commercial viability of potato has been threatened by the occurrence of bacterial soft rot in storage, a disease of potato which is generally reported to be caused by the phytopathogenic bacterium *Erwinia carotovora* var. *carotovora*.

2.4. Epidemiology of Soft Rot of Potato:

Rangarajan and Chakravarti (1970),Lim (1975),Pérombelon& Hyman, (1988) stated that the soft rot bacteria do not overwinter in soil. Survival in soil is restricted to 1 week to 6 months, depending on environmental conditions such as soil temperature, moisture and PH. Survival can be longer in association with plant material, including volunteers. In any event, the bacteria cannot live in the soil in a crop rotation system of 3–8 years.

Harrison *et al.*(1987) and Pérombelon,(1992)described that Crop contamination can also occur from airborne sources .Soft rot bacteria can be spreaded from diseased plants by airborne insects over long distances to contaminate other potato crops. Also, they can be exist in aerosols produced by rain impaction on blackleg plants and by haulm pulverization prior to harvest. Air sampled in Scotland, even away from potato crops, contained both *Pcc* and *Pa*, more on rainy than dry days. Although they remain viable

for 5–10 min only, they can be blown away for several hundred meters before deposition, mainly by the scrubbing action of rain.

McCarter-Zorner *et al.*(1984) and Harrison *et al.*(1987) stated that Surface water in the USA and Scotland was found to be contaminated with *Pcc* and to a lesser extent with Pa.

Laurila *et al.* (2010) studied that recently, in Europe, a biovar3*Dickeya* spp. genetic clade, not earlier described, was found in river water and at the same time in seed potatoes in Finland. Hence, surface water used for sprinkling purposes is likely to be a source for the pathogen, and may also be a source of new variants of the pathogen.

2.5 .Occurrence And Host Range of Brown Rot of Potato:

Champoiseau *et al*. (2009) reported that Globally, the bacterial wilt of potato has been estimated to affect about 1.7 million hectares of potatoes in approximately 80 countries, with global damage estimates of over USD 950 million per annum.

Hayward, (1991) studied that in addition to potatoes, the disease also attack 200 plant species from more than 50 families.

Bacterial wilt is widely distributed in tropical, subtropical, and warm temperate climates of the world, and it is noticed in about 45 countries in the southern hemisphere. In Africa, it is observed in Angola, Burkina Faso, Burundi, Cameroon, Congo, Ethiopia, Gabon, Gambia, Kenya, Madagascar, Malawi, Mauritius, Mozambique, Nigeria, Reunion, Rwanda, Senegal,

Seychelles, Sierra Leone, Somalia, South Africa, Swaziland, Tanzania, Tunisia, Uganda, Zaire, Zambia, and Zimbabwe (EPPO, 2004).

2.6. Epidemiology and Survival of the Pathogen

Champoiseau *et al.*(2009) stated that in potatoes, the bacteria is tuber borne, and is mainly disseminated through infected seed tubers.

Kelman (1954) and Sunaina *et al.* (1989) reported that Potato seed tubers carry the bacterium in the vascular tissue, lenticels and on the surface.

Martin & French (1985) and Pradhanang (1999) described that in cool conditions, such as tropical elevations above 2500m; infected but symptomless plants may harbour the bacterium and then spread the pathogen to progeny tubers as latent infection, leading to severe disease outbreaks when grown at warmer locations. The other source of inoculum is the infested soil; the bacterium is native in many tropical soils. Bacterial wilt is further extended through infected run-off water or soil adhering to tools and shoes.

Kelman (1954) described that under field conditions, plant infection usually occurs through the root system, especially through wounds.

The pathogen can also enter through stem wounds or stomata (EPPO, 2004).

Martin & French(1985); Shekhawat and Chakravarti(1993) described that Wounds can occur due to cultivation practices, natural growth of secondary roots, attack by nematodes or other pests .

Kinyua *et al*. (1998) stated that Once introduced, the pathogen endure at soil depths of 1m or more, where microbial competition is less, or as slimy masses in the upper soil layers .

Martin & French (1985), Hayward (1991) and Milling *et al.* (2009) stated that the pathogen can survive in soil (mostly on plant debris) and in the rooting system and rhizosphere of many hosts (weeds, other host crops, potato volunteers). Survival of the pathogen in the soil is reduced by extreme cold, and the presence of antagonistic microorganisms, while volunteer host plants enable bacterial survival across seasons.

Champoiseau *et al.* (2009) studied that bacterial Survival depends also on the race involved.

Harris (1976); Martin & French (1985) described that the aggressiveness of the pathogen is affected mainly by temperature and moisture; high temperature, and high soil moisture promote survival, reproduction, infectivity, and spread of the bacterium, and hence disease development.

Graham and Lloyd (1979) reported that long-term survival in deeper soil layers is likely to be a function of lower soil temperatures and decreased microbial activity owing to a paucity of indigenous soil microorganisms.

Granada and Sequeira (1983) found that Survival of up to 673 days in naturally infested soils stored in plastic bags at 4 has been reported.

Milling *et al*. (2009) reported that Although R3bv2A survives poorly at 4 in water or in field soil; it survives in potato tubers at this temperature indicating that the pathogen is adapted to endure constant low temperatures when sheltered in the host tissue.

Harris (1976) found that the optimum temperature for R3bv2A was 27 and the minimum was 12-15 .

French(1994) reported that a cold climate with average soil temperatures of 14 or below in the tropical Kenyan highlands impeded the soil survival of R3bv2A.

2.7. Symptomology of different potato diseases

Champoiseau *et al*. (2009) described that Brown rot of potato is disseminated due to the bacterium *Ralstonia solanacearum*, previously known as *Pseudomonas solanacearum*. It is one of the most damaging plant pathogens.

Muthoni *et al.* (2013) reported that Bacterial wilt has spread to all potato growing areas in Kenya, affecting over 70% of potato farms and causing yield losses of between 50 to 100%.

Champoiseau *et al*. (2009) cited that Brown rot of potato is caused by either race 1 or race 3 of *R. solanacearum*.

Champoiseau *et al*. (2009) described that Brown rot symptoms may also be present in potato tubers at the later stages o disease. Cross-section of infected potato tubers may reveal a grey-brown discoloration of vascular tissues, also called the vascular ring.

Champoiseau *et al*. (2009) Bacterial ooze may also be visible at the eyes or at the point where the stolon attached to the tuber. These signs may not be visible early in disease development.

Champoiseau *et al*. (2009) observed that another common sign of the disease is seen when the stem cut sections are placed in clear water. It consists of a viscous white spontaneous slime streaming from the cut end of the stem. This streaming represents the bacterial ooze exuding from the cut ends of colonized vascular bundles. This “stem-streaming” test is easy to conduct and can be used as a valuable diagnostic tool for quick detection of brown rot in the field.

On tubers, symptoms may be visible in the later stages of disease development (EPPO, 2004).

Martin & French (1985) stated that the symptoms include bacterial ooze at the tuber eyes or at the point where the stolon attach to the tuber; and soil may adhere to the tubers at the eyes.

Champoiseau *et al*. (2009) observed that a milky-white sticky exudate usually appears spontaneously on the vascular ring a few minutes after cutting the tuber. Martin and French (1985); EPPO, (2004) observed that the streaming test is of presumptive diagnostic value in the field.

Dye (1969) and van der Wolf & De Boer (2007) stated that bacteria responsible for tuber soft rot of potato are the soft rot *Pectobacterium atrosepticum(Pa), Pectobacterium carotovorum subsp. carotovorum (Pcc)*and *Dickeyas* spp. all formerly belonging to the genus *Erwinia* (*E. carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica* and *E. chrysanthemi*).

Charkowsky (2006) stated that they are pectinolytic Gram-negative, facultative anaerobic, non-sporing, motile, straight rods with peritrichous flagella. They belong to the γ-*Proteobacteria* subdivision and are clustered in the Enterobacteriaceae family.

Barras *et al.* (1994) stated that they characteristically produce a variety of cell-wall-degrading enzymes that allow infiltration and maceration of plant tissues on which they feed.

Rosenzweig *et al.*(2016) stated that soft rot caused by *Pectobacterium* on infected potato exhibits small, cream to tan, water-soaked surface spots. This decay can increase rapidly, resulting in rotting tissue that is mushy, slimy and water soaked. Infected areas of the tuber can become puffy, creating blister-like lesions that often ooze a watery substance. The bacteria that inaugurate the disease are odorless, although secondary infection by other organisms can cause an unpleasant odor.

2.8. Isolation and Identification of Pathogen

Yabuuchi *et al*. (1995) stated that the genus *Ralstonia* was proposed in 1995 to accommodate the generically misplaced species *Burkholderia pickettii*, *Burkholderia solanacearum* and *Alcaligenes eutrophus*.

Kelman (1954) observed that the virulent (colonies with pink or light red colour or characteristic red center and whitish margin) and avirulent (smaller, off-white and non-fluidal colonies) strains of *R. solanacearum* were identified in TTC medium containing 0.005% TTC.

Balabel *et al.* (2005) suggested that plating bacterial suspensions of *R. solanacearum* from different sources revealed virulent and avirulent forms, the virulent form appeared as milky, white, flat, irregular and fluidal with red coloration in the center whereas, the avirulent form was less fluidal or a fluidal colony which is completely pink to red.

Priou *et al*. (1999) described that Two drops of 3% potassium hydroxide (KOH) is placed on the ooze and mixed using a laboratory loop or a wooden toothpick for 10 seconds. The formation of a milky thread upon lifting the toothpick indicates the presence of *R. solanacearum* (a Gram-negative bacterium), whereas with *C. michiganensis* subsp. *Sepedonicus*(a Gram positive bacterium) the thread is not produced.

Czajkowski *et al*. (2015) reported that soft rot *Pectobacterium* spp. and *Dickeya solani* cause important disease on potato and other arable and horticulture crops, and are responsible for causing and tuber soft rots in the field, storage and in transit.

De Boer and Kelman, (2001) identification of pectolytic *Erwinia* and differentiation species and subspecies were made simply based on pectolytic activity, colony characteristics on crystal violet pectate (CVP) medium and limited number of morphological, biochemical and physiological tests.

Hauben *et al*. (1998) observed that molecular methods based on 16S rDNA sequence analyses supported the revival that soft rot *Erwinia* species placed in a separate genus called *Pectobacterium*.

Zhu *et al*., (2010); Nabhan *et al*. (2013); and Gasic *et al*. (2014) studied that recently, 16S rDNA sequences analysis is used to confirm identification, genetic relationships and taxonomic classification of soft rot bacteria .

De Boer and Kelman (2001) reported that identification of pectolytic *Erwinia* is traditionally based on morphological, physiological and biochemical characteristics.

Bacteriological characteristics of *Bacillus* were identified by using the methods described by Lelliott and Stead (1987) and Palleroni (1984). (Bergy's Manual).

Thiery and Frachon (1997) stated that the bacteria belong to *Bacillus* spp. are rod-shaped, usually Gram-positive, catalase-positive, and aerobic or facultatively anaerobic.

Identification of the spore of the genus bacillus was performed using the chart developed by Elmer *et al*. (1997).

Franco *et al*. (2002) colony pattern is a primary criteria to differentiate isolates in a population.

Cappuccino and Sherman (2002) stated that a loopful of fresh bacterial culture (24 hours) of each isolate was rubbed separately on the surface of the filter paper by a platinum loop.By soaking with oxidase reagent Purple color develops within 10 seconds, which indicated positive reaction of oxidase test.

Ash *et al*. (1991) stated that the isolates of *Bacillus* did not retain crystal violet strain that indicated negative result of Gram staining.

Cappuccino and Sherman (2002) observed that after washing the fresh bacterial growth on NA medium with lugol **’s** iodine no clear zone is produced that indicated negative test result.

Guttmann, D. M. (2000) described that *Bacillus* identification is based on the morphological characteristics. After 48hr white to creamy white growth on NA medium was described as *Bacillus* Spp.

Holbrook *et al*. (1980) stated that Bacillus cereus agar medium is used to differentiate the pathogen from *pseudomonas*. Development of deep yellow colour on the medium indicated positive result.

Fahy *et al*. (1983) stated that a loopful of bacteria from a well grown colony was mixed with 1-2 drop of 3% KOH solution was placed on microscope slides, bacterial isolates was slowly stirred in the KOH solution by means of a loopfull and the loopfull was slowly raised from the solution. If the solution was mucoid and it was stretched as fibres it was recorded as positive. If the

solution was watery, not mucoid and fibres formation was not occured while the loofull was raised it was recorded as negative.

Kovacs, N. (1956) observed that a filter paper was impregnated with 1% aqueous solution of Nitrogen, (Nitrogen tetra methyl-p-phenolin-diaminedihydrochloride). Then, a loopful inoculum from pure culture was picked up by sterilized platinum loop. The inoculum was smeared over the area of filter paper containing oxidize reagent to develop deep blue or purple color within ten seconds indicating the oxidation of the reagent.

Bradbury reported that *Pseudomonas* are Gram-negative, aerobic rods, straight or curved and range 0.5-1.0 × 1.5-4.0 µm in size.

Chapter III

MATERIALS AND METHODS

3.1. Experimental site

The experiment was conducted in the MS Laboratory of Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, and Bangladesh.

3.2. Experimental Period

The experiments were conducted during the period of November2016 to August 2017.

3.3. Collection of Samples

The samples were collected from the cold storage of Comilla one time. The cold storage authority store the potato samples on month of March and discard the diseased sample in the month of November. The capacity of the cold storage is 1250kg.The diseased sample were collected randomly from every sac of the storage room. Total 5kg diseased sample were collected randomly from 1250kg potato.

3.4. Measurement of prevalence of disease occurrence in cold storage of Comilla

According to Rai and Mamatha, (2005), Disease incidence of infected potato was determined by the following formula

$$\overline{\qquad\qquad\qquad\qquad} \times 100$$

3.5. Preservation of samples

The collected samples were cleaned by washing properly to remove the soils and dust from the potatoes then dried and kept in a poly bag and stored in the refrigerator at 50-60 for further study.

3.6. Isolation and identification of Bacteria from diseased potato

3.6.1. Isolation of bacteria on NA media

In a conical flask 15g bacterial agar powder was mixed in 1000ml distilled sterilized water.Then3gm Beef extract, 5gm peptone and 5gm sodium chloride were added to make 1000ml NA media. Then the mixture was shaken carefully to mix the components in the distilled water. It was then autoclaved at 121 under 15 PSI pressure for 15 minutes.The diseased potatoes were washed properly with water. Then they were cut into small pieces. For surface sterilization of the diseased samples they were kept in 95% ethanol solution. Then they washed three times with distilled water. After surface sterilization, the cut pieces were kept in a petridish containing 3-4 ml of distilled water. Then the cut pieces were chopped into very small pieces using a sterile sharp blade. One ml of this stock solution was transferred with the help of sterile pipette into a test tube containing 9ml distilled water and shaken thoroughly resulting 10^{-1}, 10^{-2},10^{-3},10^{-4},10^{-5},10^{-6}. Then 0.1 ml of each dilution was spread over NA plate at three replications as described by Goszczynska and Serfontein (1998). The inoculated NA plates were kept in an incubation chamber at 30° C. The plates were observed after 24 hrs and 48 hrs. Then single colony grown over NA plate was restreaked on fresh NA plate with the help of a loop to get pure culture.

3.7. Preparation of Triphenyltetrazolium Chloride (TTC) medium

3.7.1. Preparation of TriphenyltetrazoliumChloride (TTC)

Aqueous solution of 2, 3, 5- triphenyltetrazolium chloride (TTC) was prepared in an erlenmeyer flask by dissolving 1g of the chemical in 100 ml of distilled water The 1% stock solution of TTC solution was separately sterilized by passage through 0.45µm pore size filters (Millipore). The TTC was kept in a colored bottle and was wrapped with aluminum foil to avoid light and preserved in a refrigerator at 4 for future use.

3.7.2.Preparation of CPG media

CPG media was prepared by adding 1g casamino acid, 10g peptone, 5g glucose, and 17g agar in 1000 ml distilled water. The mixture is than taken in an erlenmeyer flask and was then autoclaved for 20 minutes at 121 under 15 PSI pressure.

3.7.3. Preparation of TTC medium

The sterilized TTC solution was poured into the sterilized CPG medium at the rate of 5 ml/1000 ml before solidification and it was mixed thoroughly. For solidification, the CPG media with TTC was poured in to several petridish.

3.7.4. Growth of bacteria on TTC medium

The pure colony that grew over NA medium was transferred on TTC medium by streak plate method. The plates were kept in an incubation chamber at 300 after inoculating them on TTC medium. Virulent colonies of *Ralstonia solanacearum* were selected on the basis of characteristic colony charter on TTC medium (Kelman, 1954).

3.8. Preparation of Cetrimide Agar

In a Erlenmeyer flask 46.5g cetrimide Agar was taken in 1000 ml water. Then 10ml glycerin was added in it. The mixture was boiled for to mix the elements properly. After that, it was autoclaved at 121 under 15PSI pressure for 20 minutes.

3.8.1. Growth of bacteria on Cetrimide Agar

The pure colony that grew over NA medium was transferred on cetrimide agar medium by streak plate method. After inoculation the plates were kept in an incubation chamber at 300 .Virulent colonies of *Pseudomonas* were selected on the basis of growth of bacteria on Cetrimide Agar medium.

3.9. Growth of bacteria on Bacillus agar:

Holbrook and Anderson developed Bacillus Cereus Agar, which is a highly specific and selective medium for the isolation (Holbrook *et al*., 1980).For *Bacillus* identification 20.5grams of Bacillus Cereus Agar was mixed in 475ml distilled water. The mixture was heated to boiling to dissolve it
completely.Then the media was sterilized by autoclaving at 15Ibs pressure (121) for 15 minutes. Then it was cooled at 40-50 and rehydrated contents of 1 vial of Polymyxin B selective supplement and 25ml of sterile Egg Yolk Emulsion were added aseptically. Then the mixture was mixed well and poured into sterile petridishes. This media contain the ingredients peptone, Mannitol, Sodium Chloride, Magnesium sulphate, Disodium phosphate, Monopotassium phosphate, Sodium Pyruvate, Bromothymol blue and Agar 1.00, 10.00, 2.00, 0.10, 2.50, 0.25, 10.00, 0.12 and 15.00gm /litre, respectively.

3.10. Biochemical test

Single colonies of each isolate were used for biochemical tests. Many diagnostic and identification tests are based upon structural and chemical properties of bacteria (Lelliott and Stead 1987). The chemical compositions of certain substances in bacterial cells can be detected with specific staining techniques. Information about the presence or absence of such substances is used for identification of bacteria (Agrios, 1997).

3.10.1. KOH solubility test or Gram differentiation 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 bacterial pathogen (grown NA 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.

3.10.2. Gram staining

At first on a clean microscope slide a small drop of distilled water was mounted. Small Part of a young colony (24 hrs old) was removed with the help of a sterile loop from the nutrient agar medium and then the bacterial smear was made on the slide. The thinly spread bacterial film was air dried. Underside of the glass slide was heated by passing it two times through the flame of a sprit lamp for fixing the bacteria on it. Then the slide was flooded with crystal violet solution for 1 minute. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then it was flooded with lugol’s iodine solution for 1 minute. After that it was decolorized with 95% ethanol for 30 seconds and again rinsed with running

tap water and air dried. Then it was counterstained with 0.5% safranine for 10 seconds. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then the glass slide was examined at 40x and 100x magnification using oil immersion.

3.10.3. Catalase test

A few drops of freshly prepared 3% H_2O_2 (Hydrogen peroxide) was added with 48 hours old pure culture of bacterium grown on NA plate and observed whether it produced bubbles within a few seconds or not.

3.10.4. Oxidase test

For this test aqueous solution of (1%) of tetra methyl-p-phenylene-diaminedihydrochloride 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-diaminedihydrochloride (color indicator). A loopful of young bacterial culture (24 hours) of each isolate was rubbed separately on the surface of the filter paper by a platinum loop. Purple color develops within 10 seconds, which indicated positive reaction of oxidase test.

3.10.5. Gelatin liquefaction test

One loopfull bacterial culture was stab inoculated into the tube containing 12% (w/v) gelatin with the help of a sterile transfer loop. Then it was incubated at 300 for 24 hours. Gelatin liquefied microorganism was determined by the formation of liquid culture after keeping it at 50 in refrigerator for 15 minutes.

3.10.6. Starch hydrolysis test

For starch hydrolysis test, pure colony of bacterium was spot inoculated on nutrient agar plate containing 2% soluble starch. After that it was incubated at 300 for at least 48 hours in incubation chamber. After incubation the plates were flooded with lugol'S iodine solution and observed whether a clear zone appeared around the colony or not.

3.10.7. Potato Soft Rotting 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 24-48h after incubation at 25 °C. Examination was done for 5 days after inoculation. Two slices were inoculated for each isolate.

Chapter IV

RESULTS

4.1. Visual symptoms of collected samples

Soft rot of potato

After cutting the rotten potato tuber foul odor came out from the decayed tissue. When the rotten sample was pressed with finger water soaked lesion smeared with the hand. (Fig-1)

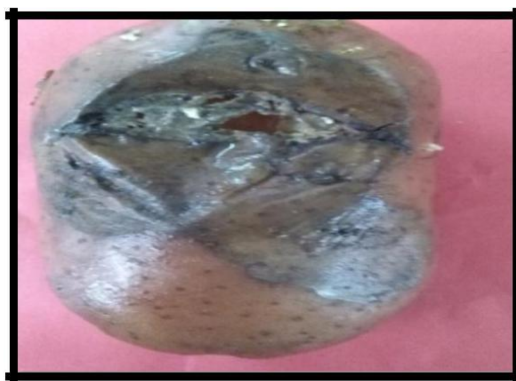


Figure No 1: Symptoms of Soft Rot of Potato

Brown rot of potato:

After cutting the diseased tuber a milky-white sticky exudate usually appeared spontaneously on the vascular ring within a few minutes.(Fig.-2)

Figure 2: Symptoms of Brown rot of potato

4.2. Isolation and Identification of Different bacteria

Several cultural, physiological and biochemical tests were conducted and some selective and semi-selective media were also used to identify and differentiate the bacteria. Four different bacteria (species) colonies were isolated from the different rotten potato tubers. The bacteria species were *Pectobacterium, Ralstonia solanacearum, Bacillus* spp. and *Pseudomonas* spp. Out of these four bacteria species tested, *Petobacterium* (46%) and *Ralstonia* (26%) were more abundant. Pectobacterium were produced creamy white, slighty raised and glistening colonies on NA plates (Figure 3 & Figure 4) and was found Gram negative rod shaped. *R. solanacearum* produced large, irregular whitish fluidal colonies with a red centre on TTC medium (Figure 5. *Pseudomonas* spp. (Figure 6) and *Bacillus* spp. (Figure 7) were identified by observing their growth over Cetrimide agar and Bacillus agar, respectively.

4.2.1. Cultural Characterization of different bacteria on NA plates

A colony is defined as a visible mass of microorganisms all originating from a single mother cell, therefore a colony constitutes a clone of bacteria all genetically alike. In the identification of bacteria and fungi much weight is placed on how the organism grows in or on media. This research study will help to identify the cultural characteristics of a bacterium on an NA plate---called colony morphology.

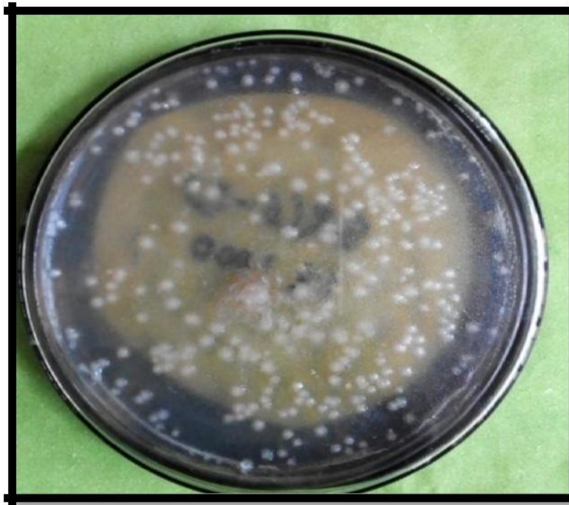


Figure 3. Growth of *Pectobacterium* on NA medium (By spread plate method)

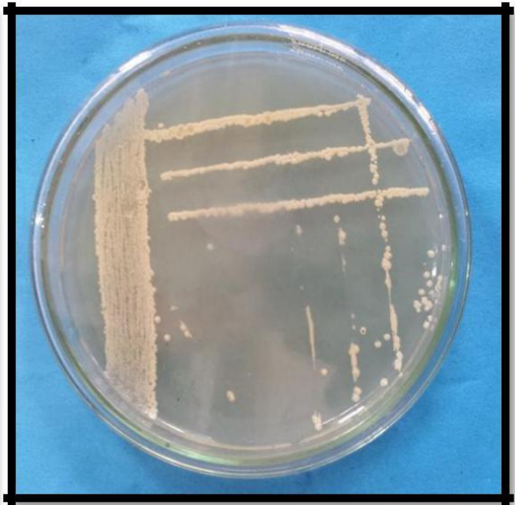


Figure 4. Growth of *Pectobacterium* on NA medium (By streak plate method)

Table 1. Cultural Characterization of different bacteria on NA plates

Isolates	Size	Form	Elevation	Margin	Pigmentation	Appearance
Pectobacterium	Large	Circular	Convex	Entire	Whitish	Shiny
Pectobacterium	Large	Circular	Convex	Entire	Whitish	Shiny
R.solanacearum	Large	Irregular	Convex	Entire	Yellowish	Shiny
R. solanacearum	Moderate Large	Irregular	Convex	Entire	Whitish	Shiny
Pectobacterium	Large	Circular	Convex	Entire	Yellowish	Shiny
R. solanacearum	Large	Circular	Convex	Entire	Whitish	Shiny
R. solanacearum	Moderate	Circular	Convex	Entire	Whitish	Dull
Pectobacterium	Moderate	Circular	Convex	Entire	Whitish	Shiny
Pectobacterium	Moderate	Circular	Convex	Entire	Whitish	Smooth
Bacillus spp.	Large	Spindle	Convex	Undulate	Creamy	Dull
Psudomonas spp.	Small	Circular	Raised	Entire	Whitish	Shiny
Psudomonas spp.	Small	Circular	Raised	Entire	Creamy	Shiny
Pectobacterium	Moderate	Irregular	Raised	Undulate	Creamy	Shiny
Pectobacterium	Moderate	Circular	Convex	Entire	Creamy	Smooth
Bacillus spp.	Large	Circular	Raised	Undulate	Whitish	Shiny

Isolate No.1. *Pectobacterium*
Isolate No.2. *Pectobacterium*
Isolate No.3. *R. solanacearum*
Isolate No.4. *R. solanacearum*
Isolate No.5. *Pectobacterium*
Isolate No.6. *R. solanacearum*
Isolate No.7. *R. solanacearum*
Isolate No.8. *Pectobacterium*
Isolate No.9. *Psudomonas* spp.
Isolate No.10. *Bacillus* spp.
Isolate No.11. *Psudomonas* spp.
Isolate No.12. *Psudomonas* spp.
Isolate No.13. *Pectobacterium*
Isolate No.14. *Pectobacterium*
Isolate No.15. *Bacillus* spp.

Table 2. Biochemical tests for identification of bacteria

Isolates	Catalase test	Oxidase test	Gram Staining test	KOH Solubility Test	Gelatin Liquefaction Test	Starch Hydrolysis Test	Growth of Bacteria on Cetrimide Agar	Bacillus Agar Test
Pectobacterium	-	-	-	+	-	-	-	-
Pectobacterium	-	-	-	+	-	-	-	-
Ralstonia Solanacearum	+	+	-	+	+	+	+	-
Ralstonia Solanacearum	+	+	-	+	+	+	+	-
Pectobacterium	-	-	+	+	-	-	-	-
R. solanacearum	+	+	-	+	+	+	+	-
R. solanacearum	+	+	-	+	+	+	+	-
Pectobacterium	-	-	-	+	+	-	-	-
Pectobacterium	+	+	+	+	+	+	+	-
Bacillus spp.	+	+	+	-	+	+	-	+
Psudomonas spp.	+	-	-	+	+	+	+	-
Psudomonas spp.	+	-	-	+	+	+	+	-
Pectobacterium	-	-	-	+	+	-	-	-
Pectobacterium	-	-	-	+	+	-	-	-
Bacillus spp.	+	+	+	-	+	+	-	+

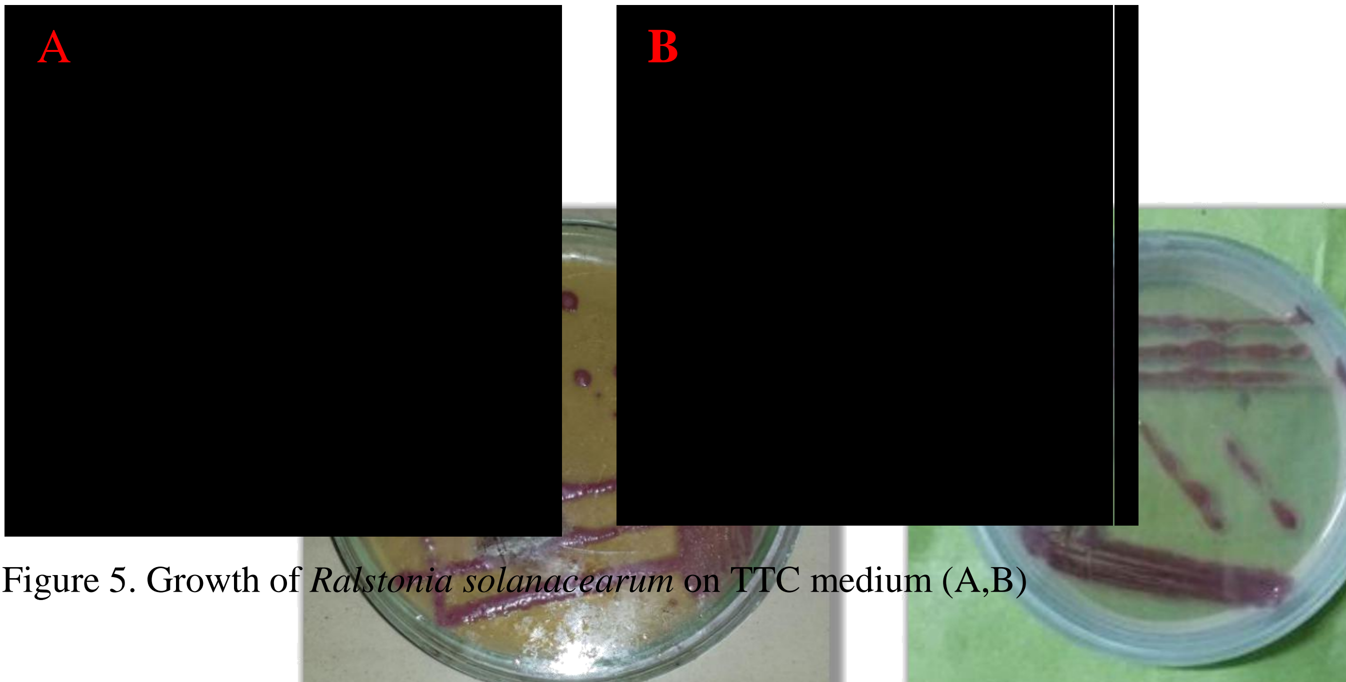


Figure 5. Growth of *Ralstonia solanacearum* on TTC medium (A,B)

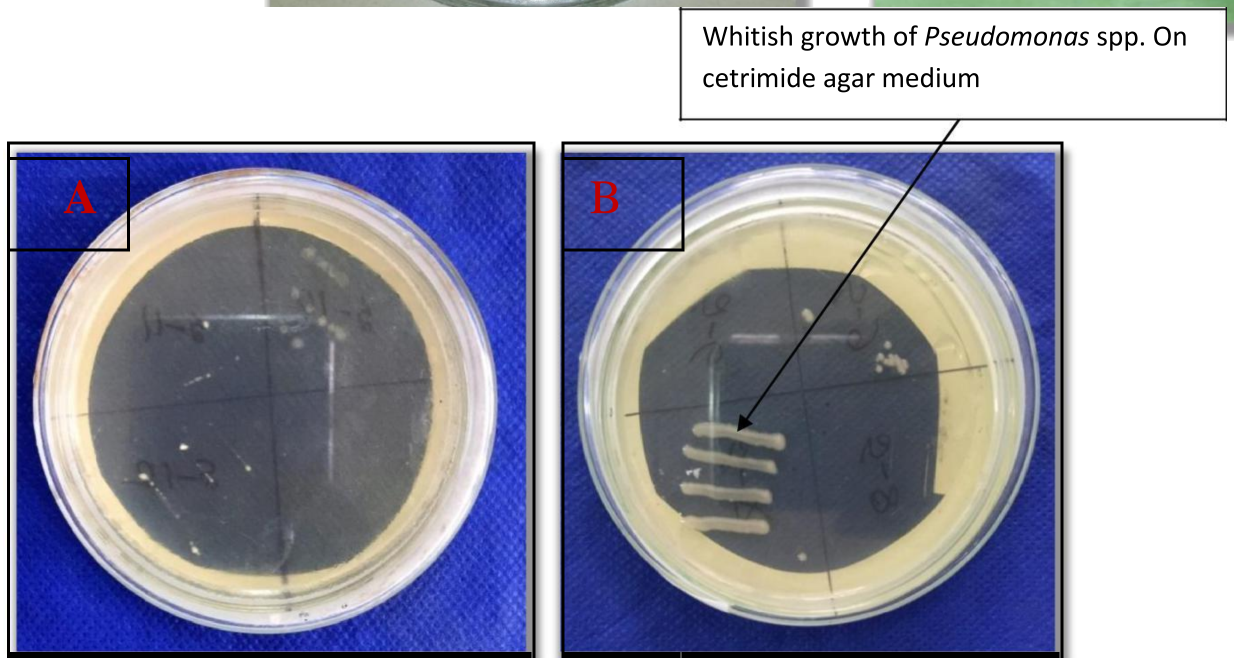
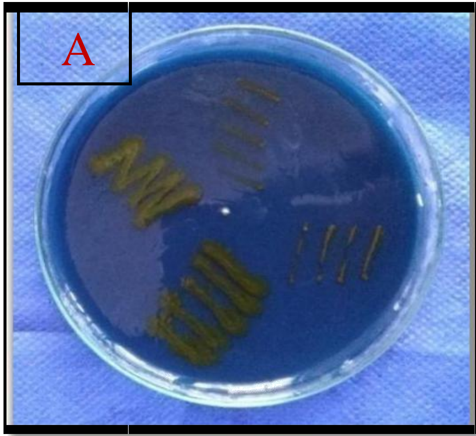


Figure 6. Growth of *Pseudomonas* spp. on Cetrimide agar medium (A,B)



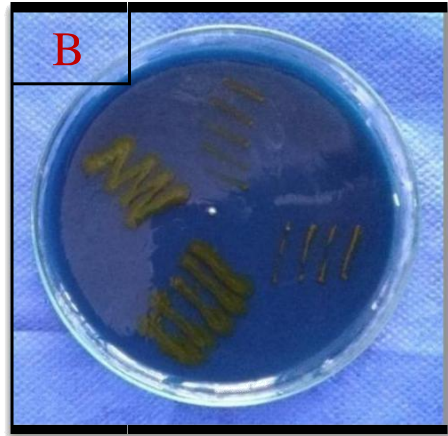


Figure 7. Growth of *Bacillus* spp on Bacillus Agar medium (A,B)

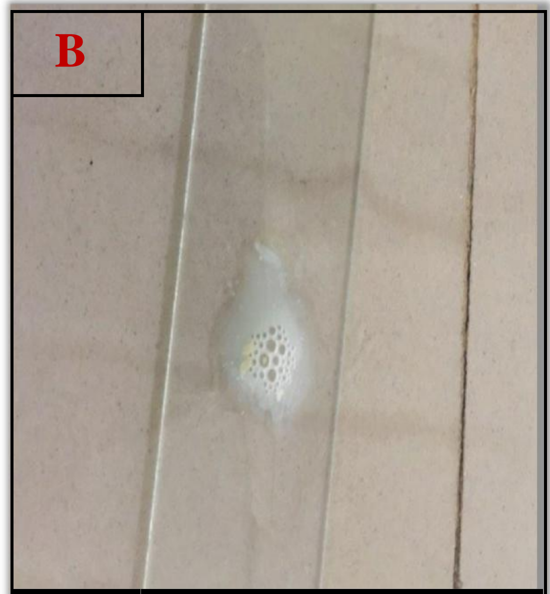


Figure 8. Soft rotting test

Figure 9. Catalyse test (Positive for *Ralstonia* and *Bacillus* spp)

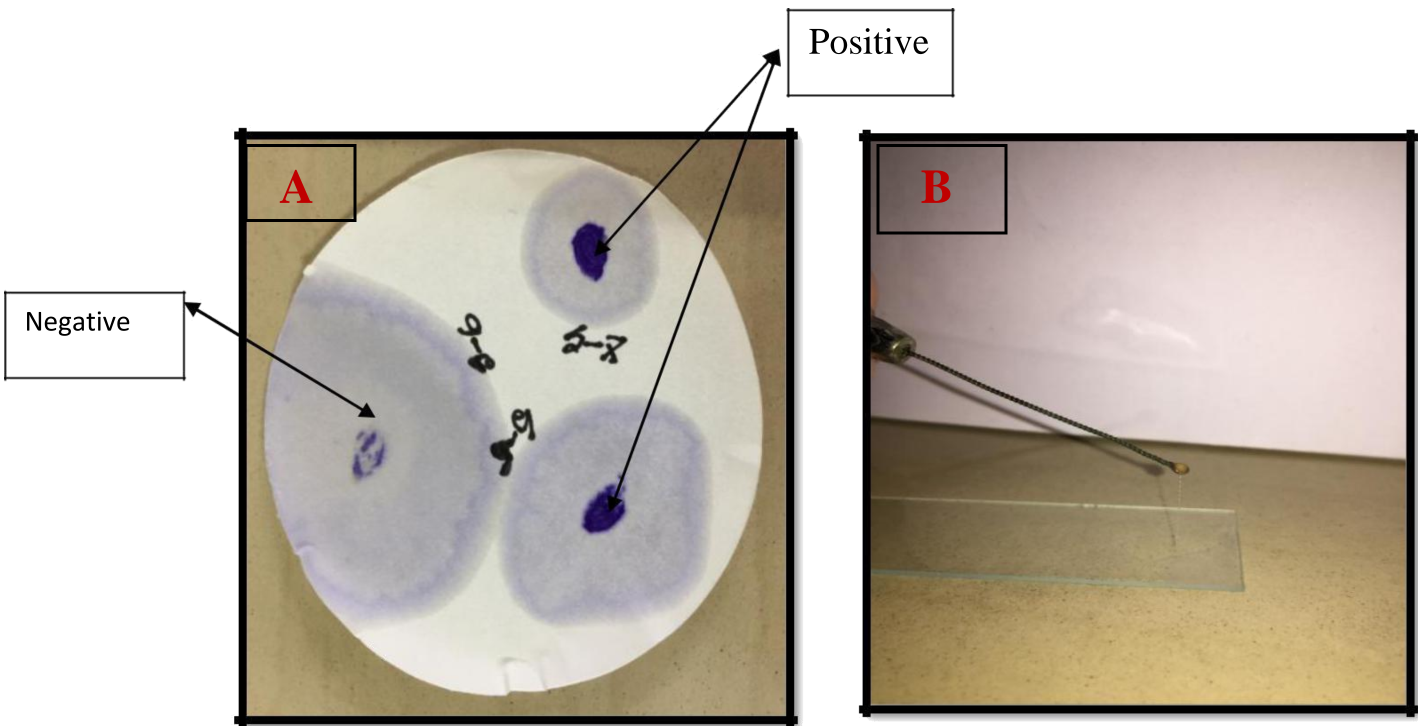


Figure 10. Oxidase test (Positive for *Ralstonia* and *Bacillus* spp.)

Figure 11. KOH Solubility test (Positive for *Pectobacterium*)

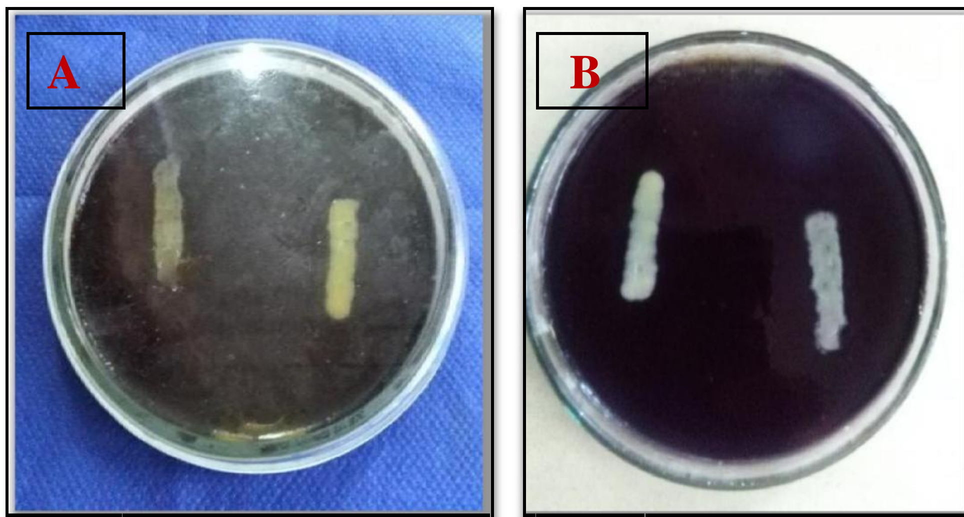


Figure 12. Starch Hydrolysis test (Negative for *Pectobacterium*)

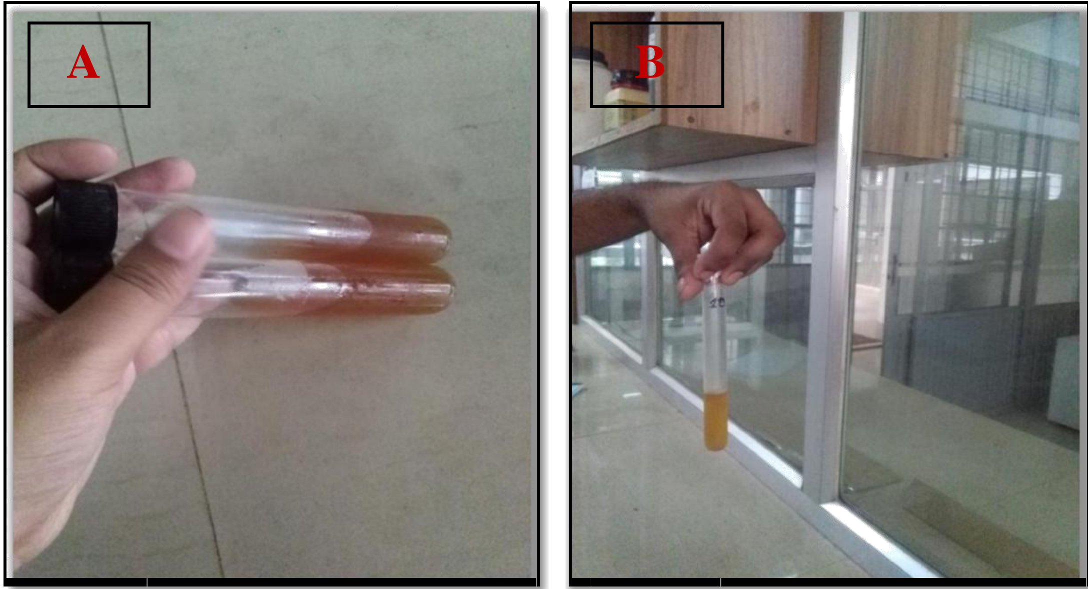


Figure 13. Gelatin liquefaction test Showing Negative (A) and Positive Result (B)

Chapter V

DISCUSSION

The present study was carried out to isolate, identify and characterize with rotten potato collected from cold storage. Soft rot and brown rot were the most common symptoms that found on rotten potatoes. In this investigation four genera of bacteria were found to be associated with stored rotten potatoes. The bacteria were *Pectobacterium carotovorum*, *Ralstonia solanacearum*, *Pseudomonas* spp. and *Bacillus* spp. This result partially supported by Umunna and Anselem (2014).They isolated *Erwinia*,*Ralstonia*, *Flavobacteria* and *Bacillus* spp. from rotten sweet potato. Bacterial soft rot is one of the most common potato diseases in the tropics and induces quick and heavy spoilage losses.Its causal agent, *Erwinia carotovora* sub spp. *carotovora* (Van Hall) Dye, is one of the most important and widespread bacterial disease of a variety of plants either in the field or in the storage (Hajhamed *et. al*., 2007).*Erwinia* and *Ralstonia* present in the test samples have been implicated in potato bacterial soft rot diseases (Agrios, 2006). The disease can be found on crops in the field, in transit, in storage and during marketing in great economic losses (Agrios,2006,Bhat *et. al*., 2010).Brown rot is caused by the bacterium *Ralstonia solanacearum* and is widely distributed in warm temperature areas of the world.

Pectobacterium produced circular, convex, creamy, white shiny colonies on NA medium. In biochemical tests it showed positive result in KOH solubility test, gelatin liquefaction test.Similar results have been also supported by Suslow *et al*. (1982) and Dye (1968); it gave negative result in Gram staining reaction, catalase test, oxidase test and starch hydrolysis test. *Erwinia* and its sub generic members usually are motile rods bearing

peritrichous flagella and are able to ferment glucose leading to the formation of acid. Their fermentative pathway yields mixed acids and 2, 3-butanediol. They are unable to utilize starch as a carbon source (Feistner *et al*., 1983). They are catalase positive and negative for exocellular cytochrome oxidase activity. It causes substantial losses in transit and storage, particularly in the warm regions where temperatures are high and there are no facilities available for cold storage (Cromarty and Easton, 1973). Approximately 22% of potatoes are lost per year due to viral, bacterial, fungal, and pests attack to potato tuber and potato plant, incurring an annual loss of over 65 million tones and bacterial soft rot alone accounts for 30–50% of this huge loss (Czajkowski *et al*., 2011).

Ralstonia produced typical characteristics fluidal colonies with pink center on TTC medium, which was supported by Rahman *et al.,* (2010).*Ralstonia solanacearum* was Gram-negative, rod shaped anaerobic bacteria. In biochemical tests it was found that all isolates of *Ralstonia* were positive in catalase, oxidase, starch hydrolysis, gelatin liquefaction test and KOH solubility test. Similar results also observed by Hilderbrand *et al*.,(1988);Lelliott *et al*., (1987); Zubeda and Hamid (2011) and Stead (1987).In gram staining reaction *Ralstonia* produced negative result that has supported by Schaad (1980).

Pseudomonas is a rod shaped and Gram negative bacteria.*Pseudomonas* produced large, circular, opaque, convex and white or creamy colonies with entire margins on NA medium. In biochemical tests *Pseudomonas* showed positive result in growth of bacteria on cetrimide agar, catalase, KOH solubility, gelatin liquefaction, starch hydrolysis test. This result have been

partially supported by Forbs *et al*., (2007); Phillip (1969);Suslow *et al*., (1982); York *et al*., (2004) and Karkalas (1985).It also produced negative result in oxidase test and gram staining reaction.This observations were partially supported by Kovacs (1956) and Meyer *et al*. (2002).

Bacillus spp. is facultative anaerobic, endospore-forming bacteria and ubiquitous in nature. *Bacillus* spp. produced large, spindle, raised and white or creamy shiny colonies with undulate margins on NA medium and grew on growth of bacteria on Bacillus cereus Agar.In biochemical tests *Bacillus* spp. showed positive results in catalase, Gram staining, gelatin liquefaction and starch hydrolysis test. This results have been partially supported by Can (1980); Cappuccino and Sherman (2002);Ash *et al*.,(1991).It showed negative result in KOH solubility test.

The tests further showed that *Pectobacterium*, *Ralstonia solanacearum* and *Pseudomonas* spp. were all pathogenic since those gave positive results in potato soft rotting test while *Bacillus* spp. were found negative. *Bacillus* was possibly a secondary micro flora or saprophyte that manifested as contaminants. Though Olivieri *et al*., (2004) and Mahmoud *et al*., (2008) in their separate works reported that *Erwinia* spp and *Bacillus* spp could be found in association with bacterial soft rot in potato. Wilson *et al.,* (1978) stated that bacterial soft rot of potato can be caused by *Erwinia carotovora* (L.R.Jones) Holland and *E. atroseptica* (van Hall) Jennison. Since these organisms can liquify pectin materials, it has been suggested that they be placed in newly created genus *Pectobacterium* (Waldee) instead of *Erwinia*. Species of *Pseudomonas* and *Bacillus* also cause soft rot of potatoes.

Chapter VI

Summary and Conclusion

The present study was conducted to isolate and identify the bacteria associated with rotten potatoes in cold storage of Bangladesh. The diseased samples were collected from potato cold storage of Homna Upazilla under Comilla district. Bacterial soft rot and brown rot were identified by visual observation of the symptoms. The bacteria were isolated following dilution plate method on NA medium. Total fifteen isolates were isolated and cultural characteristics were recorded. Out of all isolates only two were found negative in KOH solubility test. These were separated and identified as *Bacillus* spp. Further this was confirmed by growing them in *Bacillus* agar medium and found that they grew well in *Bacillus* agar media which was a selective media for *Bacillus* spp. Rests of the isolates were Gram negative and KOH solubility test positive. Among them seven isolates were identified as *Pectobacterium carotovorum*, four were *Ralstonia solanacearum* and two were *Pseudomonas* was found positive in selective medium cetrimide agar. On nutrient agar *Pectobacterium* produced circular, convex, creamy, white and shiny colonies whereas, *Ralstonia* produced large more or less circular, convex and yellowish colonies on NA medium. Biochemical tests result revealed that *Pectobacterium* gave negative results in starch hydrolysis and oxidase test and positive result in potato soft rotting test. *Ralstonia* produced large, irregular whitish fluidal colonies with a red centreon TTC medium. It was positive in catalase, oxidase and, starch hydrolysis and potato soft rotting test.

Among the bacteria isolated and identified from rotten potatoes the highest frequency was found in *Pectobacterium*(46%) followed by *Ralstonia*(26%) and *Pseudomonas* (13.3%) and *Bacillus* (13.3%).*Pectobacterium, Ralstonia, Pseudomonas* were found pathogenic and *Bacillus* spp. was non-pathogenic that was found associated with rotten potatoes.

This research study was conducted based on the bacteria responsible for rotten of potatoes in cold storage. More research work is needed to identify the causes of rotten of potato in the cold storage.

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APPENDICES

Preparation of culture media:

The compositions of the media used in this thesis work are given below: Unless otherwise mentioned all media were autoclaved at 121°C for 20 minutes at 15 lb pressure.

Nutrient Agar (NA)

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

Nutrient Broth (NB)

Beef extract	3.0 g
Peptone	5.0 g
Distilled Water	1000 ml

Triphenyltetrazolium Chloride (TTC)

2, 3,5 triphenyltetrazolium chloride (Soluble)	10.0 g
Distilled water	1000 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.

Gram 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

Bacillus **Spp. Identification Test:**

Peptone	1.00 Gms/litre
Mannitol	10.00 Gms/litre
Sodium Chloride	2.00 Gms/litre
Magnesium sulphate	0.10 Gms/litre
Disodium phosphate	2.50 Gms/litre
Monopotassium phosphate	0.25 Gms/litre
Sodium Pyruvate	10.00 Gms/litre
Bromothymol blue	0.12 Gms/litre
Agar	15.00 Gms/litre