Journal of Advances in Microbiology

14(1): 1-9, 2019; Article no.JAMB.45835
ISSN: 2456-7116

Characterization of Bacterial Isolates Associated with Postharvest Decay of Potato in Bangladesh

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Authors' contributions

This work was carried out in collaboration between all authors. Author SMT designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors IJ and NR managed the analyses of the study. Authors SM and SB managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMB/2019/45835
Editor(s):
(1) Dr. Foluso O. Osunsanmi, Department of Biochemistry and Microbiology, University of Zululand, South Africa.
Reviewers:
(1) Balogun Olalekan Blessing, Joseph Ayo Babalola University, Nigeria.
(2) Liamngee Kator, Benue State University, Nigeria.
(3) Nabin Sharma Poudel, Agriculture and Forestry University, Nepal.
Complete Peer review History: http://www.sciencedomain.org/review-history/28057

Original Research Article

Received 26 November 2018
Accepted 13 December 2018
Published 01 January 2019

ABSTRACT

A study was conducted in order to assess the bacteria associated with postharvest 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, Bangladesh. Four different bacteria (species) were isolated from the different rotten potato tubers using standard bacteriological analysis or Standard dilution method. The isolated bacteria were *Pectobacterium carotovorum, Ralstonia solanacearum*, *Pseudomonas* spp. and *Bacillus* spp. 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%). Bacteria were identified based

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on morphological and biochemical tests. All the isolated bacteria showed positive results except *Bacillu*s 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.

Keywords: Bacterial characterization; potato; postharvest decay; soft rotting disease.

1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is an 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 [1]. It is produced and recognized as popular vegetable throughout the entire tropical and subtropical region of the world [2]. Potato production in Bangladesh began in the later part of the 19th century [3]. The total world potato production is estimated at 364,808,768 tonnes in 2012 [4]. The yield growth was measured at 2.33 per cent over that of FY'15, according to provisional estimates by Bangladesh Bureau of Statistics [5]. There are 30 Government cold storage under BADC and about 400 nongovernment cold storage facilities in Bangladesh. A few studies on economic aspect of cold storage have been conducted so far in Bangladesh. Nasif et al. [6] reported that 90% farmers of Bangladesh face potato diseases as major threat in potato production and storage. Due to diseases, potato fetches lower market price than usual [7].

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 [8]. Fakir [9] 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 [10]. 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 [11,12]. 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 [13].

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*, is one of the most important and widespread bacterial disease of a variety of plants either in the field or storage [14,15]. 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 [16]. 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 65 million tones and bacterial soft rot alone accounts for 30-50% of this huge loss [13]. Stevenson et al. [17], Rahman [18] and Kamaluddin [19] 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 [20]. Bacterial soft rot leads to losses of potato that may reach up to 60 % in field, transit, and during storage [21].

Considering all things, the present research was under taken to identify bacteria associated with rotten potatoes in selected cold storage of Comilla.

2. MATERIALS AND METHODS

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. The experiments were conducted during the period of November 2016 to August 2017.

2.1 Collection of Samples

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

room. Total 5 kg diseased sample were collected randomly from 1250 kg potato.

2.2 Preservation of Samples and Isolation of Bacteria on NA Media

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. For Isolation of bacteria on NA media, 15g bacterial agar powder was mixed in 1000ml distilled sterilized water. Then 3 gm 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 [22]. The inoculated NA plates were kept in an incubation chamber at 30ºC. 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.

2.3 Preparation of Triphenyltetrazolium Chloride (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.

2.4 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. 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.

2.5 Preparation and 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 [23].

2.6 Preparation and Growth of Bacteria on Cetrimide Agar

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

2.7 Growth of Bacteria on *Bacillus agar*

Holbrook and Anderson developed *Bacillus Cereus* Agar, which is a highly specific and selective medium for isolation [24]. For *Bacillus* identification, 20.5 grams of *Bacillus Cereus* Agar was mixed in 475 ml distilled water. The mixture was heated to boil to dissolve it completely. Then the media was sterilized by autoclaving at 15 lbs pressure (121℃) for 15 minutes. Then it was cooled at 40-50℃ and rehydrated contents of 1 vial of Polymyxin B selective supplement and 25 ml of sterile Egg Yolk Emulsion were added aseptically. Then the mixture was mixed well and poured into sterile petridishes. This media contains 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 15.00 gm/ litre, respectively.

2.8 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 [25]. 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.

2.9 KOH Solubility Test or Gram Differentiation Test

KOH test was done for gram differentiation of plant pathogenic bacteria without staining [26]. 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.

2.10 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.

2.11 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.

2.12 Oxidase Test

For oxidase test, aqueous solution of (1%) of tetra methyl-p-phenylenediaminedihydrochloride 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.

2.13 Gelatin Liquefaction Test

For 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.

2.14 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.

2.15 Potato Soft Rotting Test

For Potato soft rotting test, 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.

3. RESULTS AND DISCUSSION

Several cultural, physiological and biochemical tests were conducted and some selective and semi-selective media were also used to identify and differentiate the bacteria.

In this investigation four genera (Table 1) 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 Amadioha and Adisa [11]. 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 [14]. *Erwinia* and *Ralstonia* present in the test samples have been implicated in potato bacterial soft rot diseases. The disease can be found on crops in the field, in transit, in storage and during marketing in great economic losses [27].

Brown rot is caused by the bacterium *Ralstonia solanacearum* and is widely distributed in warm temperature areas of the world. *Pectobacterium* produced (Table 1) circular, convex, creamy, white shiny colonies on NA medium. In biochemical tests (Table 2), it showed positive result in KOH solubility test, gelatin liquefaction test. Similar results have been also supported by several authors [26,28]; 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 [29]. 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 [30]. 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 [13].

Ralstonia produced (Table 2) typical characteristics fluidal colonies with pink center on TTC medium, which was supported by Rahman et al. [31]. *Ralstonia solanacearum* was Gram-negative, rod shaped anaerobic bacteria. In biochemical tests (Table 2) 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 many researchers [25,32,33]. In gram staining reaction (Table 2) *Ralstonia* produced negative result that has supported by Schaad [34]. *Pseudomonas* is a rod shaped and Gram-negative bacteria.

Pseudomonas produced (Table 1) large, circular, opaque, convex and white or creamy colonies with entire margins on NA medium. In biochemical tests (Table 2) *Pseudomonas* showed positive result in growth of bacteria on cetrimide agar, catalase, KOH solubility, gelatin liquefaction, starch hydrolysis test. These results have been partially supported by many researchers [26,35,36]. It also produced negative result in oxidase test (Table 2) and gram staining reaction. These observations were partially supported by many researchers [37,38].

Bacillus spp. is facultative anaerobic, endospore-forming bacteria and ubiquitous in nature. *Bacillus* spp. Produced (Table 1) 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 (Table 2) *Bacillus* spp. showed positive results in catalase, Gram staining, gelatin liquefaction and starch hydrolysis test. These results have been partially supported by Ash et al. [39]. 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 several authors [12,40] in their separate works reported that *Erwinia* spp and *Bacillus* spp could be found

Table 1. Cultural characterization of different bacteria on NA plates

Sl.	Isolates	Size	Form	Elevation	Margin	Pigmentation	Appearance
1.	*Pectobacterium*	Large	Circular	Convex	Entire	Whitish	Shiny
2.	*Pectobacterium*	Large	Circular	Convex	Entire	Whitish	Shiny
3.	*R. solanacearum*	Large	Irregular	Convex	Entire	Yellowish	Shiny
4.	*R. solanacearum*	Moderate Large	Irregular	Convex	Entire	Whitish	Shiny
5.	*Pectobacterium*	Large	Circular	Convex	Entire	Yellowish	Shiny
6.	*R. solanacearum*	Large	Circular	Convex	Entire	Whitish	Shiny
7.	*R. solanacearum*	Moderate	Circular	Convex	Entire	Whitish	Dull
8.	*Pectobacterium*	Moderate	Circular	Convex	Entire	Whitish	Shiny
9.	*Pectobacterium*	Moderate	Circular	Convex	Entire	Whitish	Smooth
10	*Bacillus* spp.	Large	Spindle	Convex	Undulate	Creamy	Dull
11	*Psudomonas* spp.	Small	Circular	Raised	Entire	Whitish	Shiny
12	*Psudomonas* spp.	Small	Circular	Raised	Entire	Creamy	Shiny
13	*Pectobacterium*	Moderate	Irregular	Raised	Undulate	Creamy	Shiny
14	*Pectobacterium*	Moderate	Circular	Convex	Entire	Creamy	Smooth
15	*Bacillus* spp.	Large	Circular	Raised	Undulate	Whitish	Shiny

Table 2. Biochemical tests for identification of bacteria

Isolate no.	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
1.	*Pectobacterium*	-	-	-	+	-	-	-	-
2.	*Pectobacterium*	-	-	-	+	-	-	-	-
3.	*Ralstonia Solanacearum*	+	+	-	+	+	+	+	-
4.	*Ralstonia Solanacearum*	+	+	-	+	+	+	+	-
5.	*Pectobacterium*	-	-	+	+	-	-	-	-
6.	*R. solanacearum*	+	+	-	+	+	+	+	-
7.	*R. solanacearum*	+	+	-	+	+	+	+	-
8.	*Pectobacterium*	-	-	-	+	+	-	-	-
9.	*Pectobacterium*	+	+	+	+	+	+	+	-
10	*Bacillus*	+	+	+	-	+	+	-	+
11	*Pseudomonas*	+	-	-	+	+	+	+	-
12	*Pseudomonas*	+	-	-	+	+	+	+	-
13	*Pectobacterium*	-	-	-	+	+	-	-	-
14	*Pectobacterium*	-	-	-	+	+	-	-	-
15	*Bacillus*	+	+	+	-	+	+	-	+

'+' indicates the positive result in respective test and '-' indicates the negative result.

in association with bacterial soft rot in potato. Wilson et al. [41] 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.

4. CONCLUSION

This research study was conducted based on the bacteria responsible for rotten of potatoes in cold storage. 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. Such results assist us to understand the types and abundance of bacteria available in local cold storage. Potato growers and middlemen will be able to take necessary precautionary measures to get rid of severe soft rotten disease. However, more research work is needed to identify the causes of rotten of potato in the cold storage.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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