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Characterization of onion soft rot bacteria in Bangladesh

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Abstract

A study was undertaken for characterization and identification of the soft rot causing bacterial pathogens of onion. Bacterial pathogens were isolated from soft rotted stored onions of different varieties and locations of Bangladesh. Altogether 73 bacterial isolates were isolated from soft rotted onions. Among them, twelve soft rot-positive isolates were selected for characterization and identification on the basis of more virulence. Physiological and biochemical tests were performed following standard methods for characterization and identification of selected soft rot bacterial isolates. Seven isolates namely O-03, O-18, O-69, O-72, O-130, O-156 and O-180 were identified as *Pectobacterium carotovorum* subsp. *carotovorum* (*E. carotovora subsp. carotovora*), two isolates O-101 and O-118 were identified as *E. chrysanthemi* and three isolates O-05, O-14 and O-15 were the members of *Burkholderia cepacia*.

Keywords : Characterization; Identification; Soft rot bacteria; Onion Bangladesh

Introduction

Bacterial soft rot commonly occurs in onion after harvest and during storage worldwide. Several bacterial species under different genera can enzymatically macerate parenchymatous tissues of a wide range of plants. Although many bacteria possess the ability to produce tissue macerating enzymes but only a few have been associated with rotting of living plant tissues. These include *Erwinia* spp., *Bacillus subtilis, B. polymixa, Pseudomonas marginalis* and pectolytic strains of *Pseudomonas, Clostridium* spp. and *Flavibacterium* spp. of these, soft rot erwinias are considered to be the most important pathogens of growing plants as well as harvested crops (Perombelon and Kelman, 1980). Toth *et al*. (2002) reported that the soft rot erwiniae, *E. carotovora* subsp. *carotovora (Ecc), E. carotovora* subsp. *atroseptica (Eca),* and *E. chrysanthemi (Ech)* are major bacterial pathogens of many crops world-wide. The soft rot of onion is caused by *E. carotovora* subsp. *carotovora* (Shing, 1985). This organism is a common cause of loss in storage (Sherf and Macnab, 1986). Choi and Han (1990) reported that *P. marginalis, P. syringae* and *P. cepacia* caused onion bulb rot in the field and in market places. A similar disease on onion bulbs stored at low temperature condition caused by *Burkholderia cepacia* was reported by Yi and Park (1999). It was also reported that *P. fluorescens* implicated in soft rot of purple variety of onions in Southern Nigeria by Aboaba (2007). A study confirmed that *P. marginalis* causes soft rot in potato, carrot and onion (Kim *et al*., 2002). Characterization and Identification of pectolytic erwinias are traditionally based on biochemical and phenotypic characteristics (De Boer and Kelman, 2000) and more recently molecular techniques have also been applied. Several methods have been employed to distinguish pectolytic erwinias. The most commonly used methods are biochemical tests (Dickey and Kelman, 1988) and pathogenicity tests (Smith and Bartz, 1990). A collection of 87 strains of the soft rot pathogen *E. carotovora* subsp. *carotovora (Ecc)* isolated from various host plants in Japan, Korea and Thailand was characterized by bacteriological, pathological and genetic properties (Seo *et al*., 2001). From above discussions it was understood that so many bacterial species/subspecies are involved with soft rot disease of onion. However, little is known about the variability of soft rotted bacterial strains, especially in Bangladesh. In order to improve control measures, detection systems and strategies of breeding for resistance to infection, it is important to improve understanding of their diversity. In Bangladesh research report on soft rot bacteria of onion are very scanty (Rasul *et al*., 1999; Islam, 2004). Considering the above facts the present study was undertaken for characterization and identification of soft rot bacteria isolated from onion in Bangladesh.

Materials and methods

Collection of soft rotted diseased samples of onion

Diseased plant samples of onion were selected based on visible symptoms of soft rot and characteristic odor described

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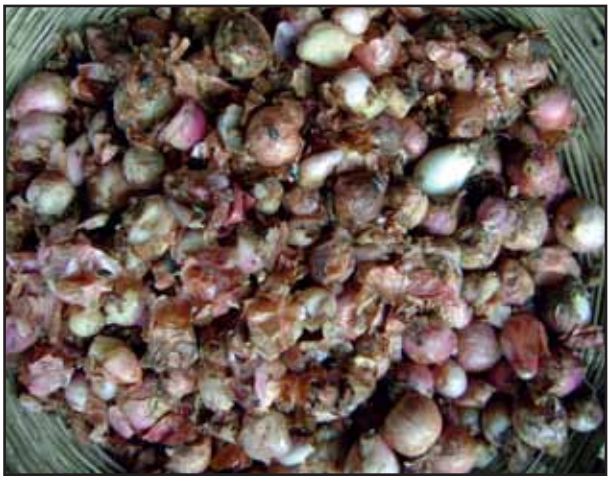
by Rich (1983) and Shing (2001). Symptoms were observed at various levels of post harvested onion bulbs during survey which were presented in Fig. 1. Soft rotted samples of onion were collected from farmers' homes and markets of different locations of Bangladesh. The onion varieties were Kalashnagari, BARI-1, Taherpuri, Faridpuri, Indian varieties and locations were Santhia (Pabna), Sujanagar (Pabna), Gazipur, Rangpur, Karwan Bazar (Dhaka) and Faridpur. After collection of rotted samples brought to the microbiology laboratory of Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Gazipur and the soft rotted bacteria were isolated.

Isolation of soft rot bacteria

Soft rot bacterial isolates were isolated from different sample of onion by "Streak plate" technique as described by Mortensen (1997) and Kim *et al*. (2002). A common bacteriological medium, yeast peptone dextrose agar (YPDA) was used for isolation of soft rot bacteria. YPDA was prepared by dissolving yeast 3.0 g, peptone 0.6 g, dextrose 3.0 g, and agar 15 g in 1000 ml of distilled water. The pH of the medium was adjusted to 7.0 using 0. 1 N KOH and cooked on hot plate. After cooking the medium was autoclaved for 20 min at 121° C under 1.1 kg/cm^2 pressures. The medium was poured into petridishes at the rate of 20 ml/plate and were cooled in a clean bench. To isolate the causal bacteria, small part from the margin of rotted tissues of the infected bulbs of onion samples were separated with a scalpel and were surface disinfected with 1% sodium hypochlorite (NaOCl) for 2-3 min. Sterilized samples were washed several times in sterilized water to remove the residual hypochlorite. The samples were placed in petridishes containing sterilized

A. Soft rotted Onion collected from farmers home during survey

B. Soft rotted onion collected from market during survey



C. Soft rotted onion collected from market during survey

D. Fresh onion collected during survey

Fig. 1. Soft rotted symptoms (A-C) and fresh onion (D) collected from different post harvest levels

water and were crushed with a sterile scalpel. After crushing, the petridishes were kept undisturbed for 10-15 min to release the bacteria associated with rotted tissues. One loop full of resulting suspension (water containing bacteria) was streaked on the solidified YPDA medium in each plate. The plates were incubated at 30^0 C for 48 hr. Characteristic individual bacterial colonies that appeared on YPDA medium were picked up using a bacterial loop and transferred to another plate. Purification of bacterial colony was done by re-streaking of single colony on another fresh plate.

Potato and onion soft rot test

All of the bacterial isolates originated from single colonies were tested for their ability to cause soft rot on potato tubers and onion bulbs following standard procedure (Lelliot *et al*., 1966). Potato tubers and onion bulbs were sterilized with 70% ethyl alcohol, rinsed in sterile distilled water and aseptically cut into slices (ca. 1 cm). The slices were put in petridishes containing sterilized filter paper impregnated with ca. 2 ml of sterilized distilled water. The soft rot tests were repeated at least twice for fulfilling the Koch's postulates. The slices were inoculated with needle pricking method. The inoculated slices were maintained in moistened petridishes (Togashi, 1988; Nabhan *et al*., 2006) and incubated at 30^0 C for 2-3 days. The bacterial cultures produced characteristic symptoms of soft rot on potato and onion slices were selected and preserved more virulence isolates for further studies.

Preservation of pathogenic bacterial strains

Pure single colonies of each of pathogenic soft rot bacterial isolates of onion were preserved in test tubes containing sterilized distilled water and in test tube with half slant culture containing YPDA overlapped with sterilized liquid paraffin. The tubes containing culture were preserved in a cool room (14^0 C).

Characterization of the pathogenic bacterial strains

A series of physiological and biochemical tests were performed for characterization of the isolated more pathogenic isolates. The physiological and biochemical tests were a. Potato soft rot test b. Fermentation of glucose (OF test) (Hugh and Leifson, 1953), c. Gram reaction (Suslow *et al*., 1982), d. Oxidase reaction (Kovacs, 1956), e. Catalase production (Hayward, 1992), f. Gelatin Liquefaction test (Schaad, 1988), g. Urease production (Schaad, 1988), h. Nitrate reduction test (Lelliott and Dickey, 1984), i. Indol test (Lelliott and Dickey, 1984), j. Lecithinase test (Clung and Tobae, 1947), k. Acetoin production (Dye, 1969), l. Methyl red test, m. Arginine dihydrolase (Thornley, 1960), n. Gas formation (Hugh and Leifson, 1953), o. Hypersensitive reaction (Klement and Goodman, 1967) and p. Utilization of carbon (Ayers *et al*., 1919).

Three soft rotting bacterial strains *Erwinia carotovora* subsp. *carotovora* ATCC-15713, *E. chrysanthemi* Ura-2 and *Burkholderia cepacia* ATCC 25416 were used as reference strains in this experiment.

A. Grayish white and raised colonies on YPDA

B. Creamy white and margins undulated to feathery colonies on YPDA

Fig. 2. Various types of bacterial colonies isolated from soft rotted onion (A-B)

Results and discussion

Collection and isolation of soft rot bacterial isolates

A total of 73 isolates from onion were isolated from different locations of Bangladesh (list not included). Colony morphology of most of the isolates on YPDA were white, creamy white, and grayish creamy white, smooth, round, glistening, slightly raised and some were flat to slightly raised, margins undulated to feathery and visible on isolation plates after about 24 h (Fig. 2, A-B).

Potato and onion soft rot test

Based on the results of soft rot test on onion and potato, the pathogenic isolates were selected from the isolated 73 isolates. 24 isolates produced soft rot on onion and potato slices among 73 isolates (Table-I and Fig. 3, A-B). All of the pathogenic isolates were preserved in test tube overlapping with liquid paraffin for further study. Twelve (12) soft rot-positive isolates were selected (on the basis of more virulence) for characterization and identification. More virulence isolates (12 isolates) were selected on the basis of aggressiveness and more soft rotted area produced on host samples.

Table I. List of potato soft rot positive isolates isolated from different soft rotted onion varieties and locations of Bangladesh

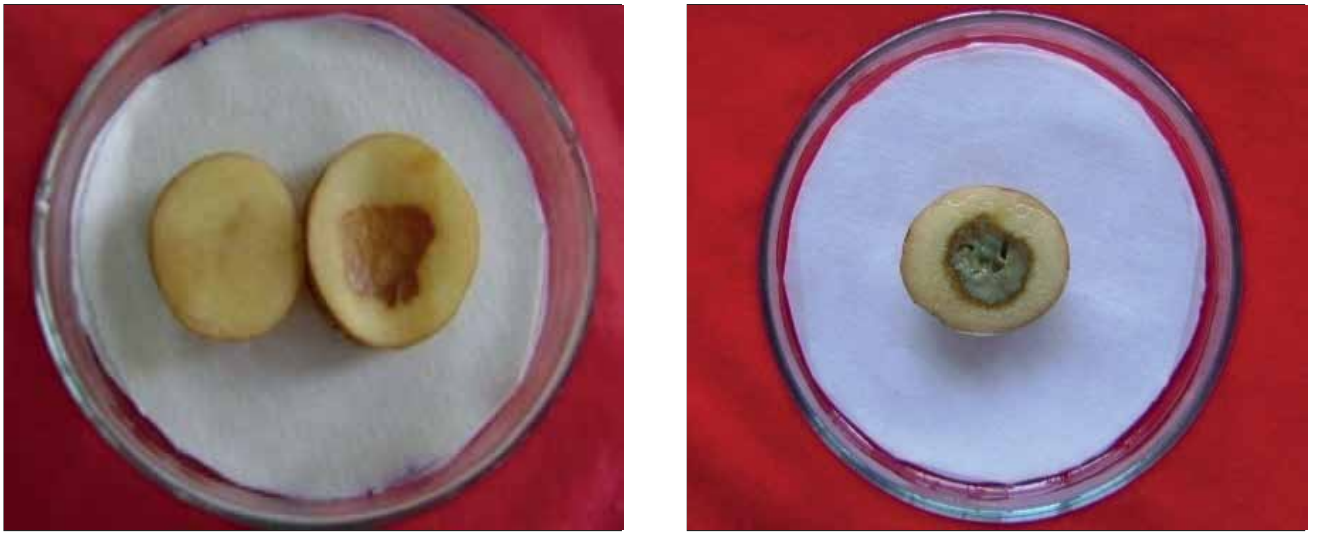
Sl. No.	Isolate no.	Colony characters	Locations	Potato soft rot test	Isolation time
1	O-03	Creamy white small	Santhia	+	2008
2	O-05	Creamy white	,,	+	,,
3	O-14	Creamy white	Rangpur	+	,,
4	O-15	,,	Dhaka	+	,,
5	O-18	Grayish white	,,	+	,,
6	O-42	Creamy white	Sujanagar	+	2009
7	O-52	White sticky	Sujanagar	+	,,
8	O-62	Creamy white	Gazipur	+	,,
9	O-69	White sticky	,,	+	,,
10	O-72	Creamy white	Sujanagar	+	,,
11	O-73	White small	,,	+	,,
12	O-75	Creamy white small	,,	+	
13	O-76	,,	,,	+	,,
14	O-77	Creamy white	,,	+	,,
15	O-78	White small	,,	+	,,
16	O-80	Creamy white small	Santhia	+	,,
17	O-101	White sticky	,,	+	,,
18	O-118	Grayish white	Gazipur	+	,,
19	O-120	Creamy white small	Santhia	+	,,
20	O-121	,,	,,	+	,,
21	O-129	Creamy white	,,	+	,,
22	O-130	Creamy white small	,,	+	,,
23	O-156	Creamy white small	Rangpur	+	,,
24	O-180	Grayish creamy white	Gazipur	+	,,

Characterization of onion isolates

Physiological and biochemical tests

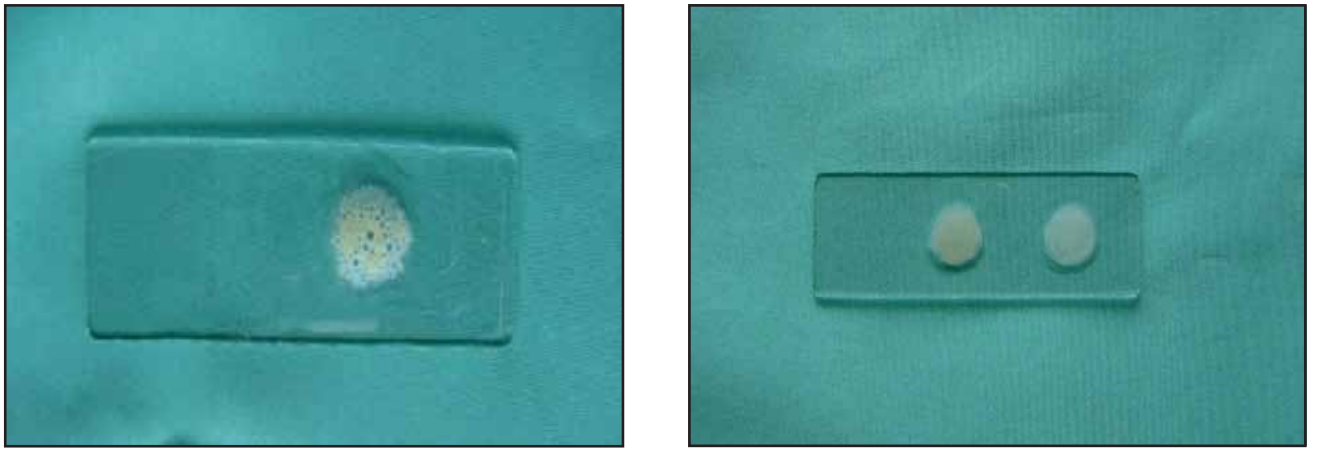
All the twelve bacterial isolates (O-03, O-05, O-14, O-15, O-18, O-69, O-72, O-101, O-118, O-130, O-156 and O-180) and two reference strains *E. carotovora* subsp. *carotovora* ATCC 15713 and *E. chrysanthemi* Ura-2 were found positive in potato soft rot, catalase (Fig. 4), oxidative, gelatin liquefaction, acetoin test, growth at 37^0 C and tobacco hypersensitivity tests (Fig. 9) but negative in gram reaction, oxidase test and arginine utilization tests and they differed in OF test, nitrate reduction, lecithinase test, methyl red test, indole test, in gas formaton and growth in 5% NaCl (Table II).

Nine isolates (O-03, O-18, O-69, O-72, O-101, O-118, O-130, O-156 and O-180) and two reference strains *E. carotovora* subsp. *carotovora* ATCC 15713 and *E. chrysanthemi* Ura-2 gave positive results in the OF test i.e., they produce yellow color in both liquid paraffin covered and uncovered tubes (Table II and Fig. 5). So these isolates belong to facultative anaerobic bacteria. Three isolates such as O-05, O-14, O-15 and a reference strains *B. cepacia* ATCC 25416 gave negative results in this test. So these isolates did not ferment glucose in covered tubes. i.e., these bacteria were strictly aerobic.



A. Soft rot positive (right, O-15), negative (left) B. Soft rot positive (O-118)

Fig. 3. Soft rot test on potato slices of different soft rot bacterial strains (A-B)



A B

Fig. 4. Catalase test of soft rot bacteria, positive-showing gas bubble (A), negative-not showing gas bubble (B)

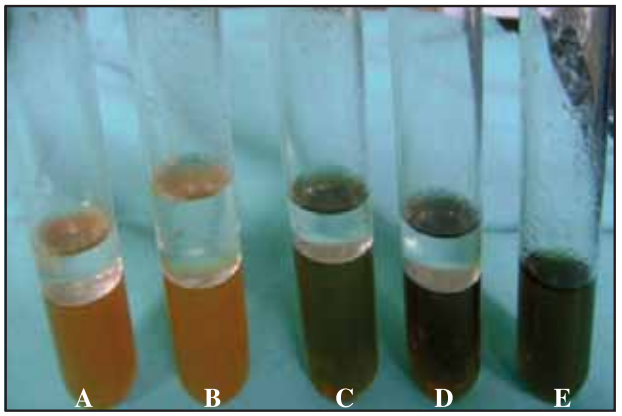


Fig. 5. Oxidative and fermentative test: A, B - positive reaction; C, D-negative reaction; E-control (no glucose added)

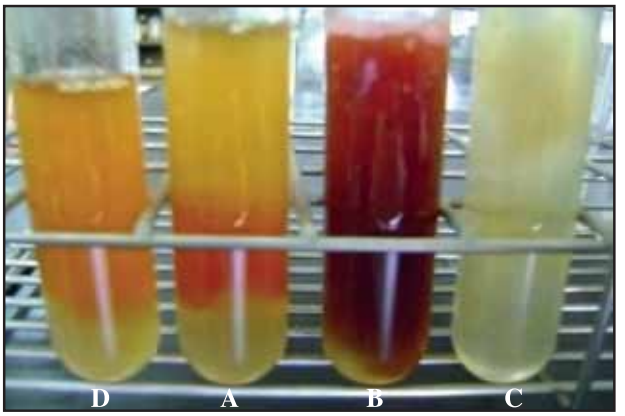


Fig. 6. Nitrate reduction test: A-weakly positive; B-strongly positive; C-negative; D-variable reaction/very weakly positive

Fig. 7. Methyl red test of soft rot bacteria: red color - positive reaction

Fig. 8. Gas formation test: A-positive, formation of bubbles/vacuum in the medium and the petrolatum seal being pushed upward; B, C, D-negative

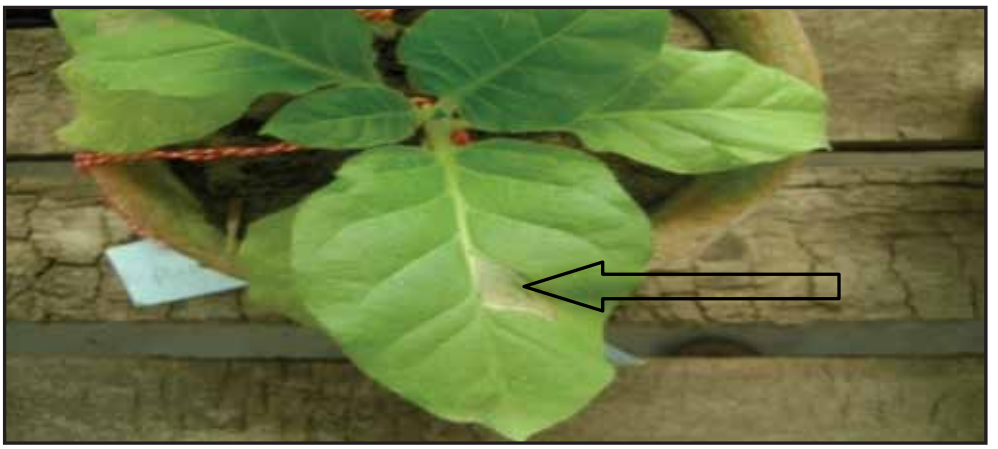


Fig. 9. Tobacco hypersensitivity test: A-positive, the tissues became collapsed within 24 h after inoculation and necrotic and the bacteria are trapped and die; B-negative

In case of nitrate reduction test, nine isolates (O-03, O-18, O-69, O-72, O-101, O-118, O-130, O-156 and O-180) and two reference strains *E. carotovora* subsp. *carotovora* ATCC 15713 and *E. chrysanthemi* Ura-2 were positive. But three isolates (O-05, O-14 and O-15) and a reference strains *B. cepacia* ATCC 25416 were negative in this test (Table II and Fig. 6).

Among the 12 virulence isolates, seven isolates (O-03, O-18, O-69, O-72, O-130, O-156 and O-180) and two reference strains *E. carotovora* subsp. *carotovora* ATCC 15713 and *E. chrysanthemi* Ura-2 were negative in the lecithinase test and three isolates (O-14, O-101 and O-118) and a reference strains *E. chrysanthemi* Ura-2 were positive and two isolates (O-05 and O-15) gave variable reaction in this test.

In case of methyl red test, 10 isolates (O-03, O-05, O-14, O-15, O-18, O-69, O-72, O-130, O-156 and O-180) and two reference strain *E. carotovora* subsp. *carotovora* ATCC 15713 and *B. cepacia* ATCC 25416 gave positive result while the two isolates (O-101, O-118) and a reference strains *E. chrysanthemi* Ura-2 gave negative results in this test (Table II and Fig. 7).

Table II. Physiological and biochemical characteristics of soft rot bacteria isolated from onion

Isolates No.	Name of Tests								
	Potato soft rot	Gram reaction	Catalase	Oxidase	OF- test	Gelatin liquefaction	Nitrate reduction	Lecithinase	Methyl red
O-03	+	-	+	-	+	+	+	-	+
O-05	+	-	+	-	-	+	-	v	+
O-14	+	-	+	-	-	+	-	+	+
O-15	+	-	+	-	-	+	-	v	+
O-18	+	-	+	-	+	+	+	-	+
O-69	+	-	+	-	+	+	+	-	+
O-72	+	-	+	-	+	+	+	-	+
O-101	+	-	+	-	+	+	+	+	-
O-118	+	-	+	-	+	+	+	+	-
O-130	+	-	+	-	+	+	+	-	+
O-156	+	-	+	-	+	+	+	-	+
O-180	+	-	+	-	+	+	+	-	+
Ecc ATCC 15713	+	-	+	-	+	+	+	-	+
Ech Ura-2	+	-	+	-	+	+	+	+	-
Bc ATCC 25416	+	-	+	-	-	+	-	-	+

Table II. Physiological and biochemical characteristics of soft rot bacteria isolated from onion (continued)

Isolates No.	Name of Tests						
	Arginine utilization	Acetoin	Indole	Gas formation	Growth at 37 °C	Growth in 5% NaCl	Tobacco hypersensitivity
-03	-	+	-	-	+	+	+
O-05	-	+	v	-	+	-	+
O-14	-	+	v	-	+	-	w+
O-15	-	+	v	-	+	-	+
O-18	-	+	-	-	+	+	+
O-69	-	w+	-	-	+	+	+
O-72	-	+	-	-	+	+	+
O-101	-	+	+	+	+	-	w+
O-118	-	+	+	+	+	-	+
O-130	-	+	-	-	+	+	+
O-156	-	+	-	-	+	+	+
O-180	-	+	-	-	+	+	+
Ecc ATCC 15713	-	+	-	-	+	+	+
Ech Ura-2	-	+	+	+	+	-	+
Bc ATCC 25416	-	+	v	-	w+	-	w+

Reference/standard isolates: Ecc ATCC 15713 (Ecc=E. carotovora subsp. carotovora), Ech Ura-2 (Ech= E. chrysanthemi), Bc ATCC 25416 (Bc=B. cepacia), ATCC= American Type Culture Collection; (+) = growth positive; (-) = negative; (w+) = weakly positive; (v)= variable reaction

In the indole test, seven isolates (O-03, O-18, O-69, O-72, O-130, O-156 and O-180) and a reference strain *E. carotovora* subsp. *carotovora* ATCC 15713 were negative while two isolates (O-101 and O-118)) and *E. chrysanthemi* Ura-2 were found positive. The remaining three isolates (O-05, O-14 and O-15) and the reference isolate *B. cepacia* ATCC 25416 showed variable reaction (Table II).

Two isolates (O-101 and O-118) and a reference strain *E. chrysanthemi* Ura-2 were positive in gas formation. The other 10 isolates and two reference strains *E. carotovora* subsp. *carotovora* ATCC 15713 and *B. cepacia* ATCC 25416 did not produce any gas bubbles in the test (Table II and Fig. 8).

The results of physiological and biochemical tests and carbon sources utilization tests also revealed that three isolates of onion O-05, O-14 and O-15 were identical with reference strain of *Burkholderia cepacia* ATCC 25416 and they were identified as *B. cepacia* (Table III, IV and V). Similar type

Table III. Utilization of different sugars as source of carbon by soft rot bacterial isolates of onion

Isolates No.	Name of carbon sources								
	Cellubiose	Lactose	Maltose	Maltose	D-Galactose	D-Xylose	Raffinose	Sucrose	Trehalose
O-03	+	+	-	+	+	+	+	+	+
O-05	+	-	-	+	+	+	+	+	+
O-14	+	-	-	+	+	+	+	+	+
O-15	+	-	-	+	+	+	+	+	+
O-18	+	+	-	+	+	+	+	+	+
O-69	+	+	-	+	+	+	+	+	+
O-72	+	+	-	+	+	+	+	+	+
O-101	+	-	-	+	+	+	+	+	-
O-118	+	-	-	+	+	+	+	+	-
O-130	+	+	-	+	+	+	+	+	+
O-156	+	+	-	+	+	+	+	+	+
O-180	+	+	-	+	+	+	+	+	+
Ecc ATCC 15713	+	+	-	+	+	+	+	+	+
Ech Ura-2	+	-	-	+	+	+	+	+	-
Bc ATCC 25416	+	-	-	+	+	+	+	+	+

Reference/standard isolates: Ecc ATCC 15713 (Ecc=E. carotovora subsp. carotovora), Ech Ura-2 (Ech= E. chrysanthemi), Bc ATCC 25416 (Bc=B. cepacia), ATCC= American Type Culture Collection; + = growth positive; - = negative; v= variable reaction

results of physiological, biochemical and carbon source utilization for *B. cepacia* were reported earlier (Kreig and Holt, 1984; Schaad, 1988; Alam *et al*., 1999; Khan, 2000).

Three soft rot bacteria such as *Pectobacterium carotovorum* subsp. *carotovorum (E. carotovora* subsp. *carotovora), E. chrysanthemi* and *Burkholderia cepacia* were identified as causative agents of onion soft rot disease in Bangladesh at the present study. Characterization and Identification of pectolytic erwinias are traditionally based on biochemical and phenotypic characteristics and more recently molecular techniques have also been applied by De Boer and Kelman (2000). Several methods have been employed to distinguish among pectolytic erwinias. The most commonly used methods are biochemical tests (Dickey and Kelman, 1988) and pathogenicity tests (Smith and Bartz, 1990). In the present study, characterization and identification of soft rot bacteria of onion were mostly based on the traditional methods. In Bangladesh, molecular based techniques yet not been performed for characterization and identification of soft

Table IV. Utilization of different alcohols and organic acids by soft rot bacterial isolates of onion

Isolates No.	Name of alcohols and organic acids					
	Dulcitol	Inositol	Manitol	Sorbitol	Benzoate	D-Tartrate
O-03	-	+	+	-	-	-
O-05	-	+	+	-	-	-
O-14	-	+	+	-	-	-
O-15	-	+	+	-	-	-
O-18	-	+	+	-	-	-
O-69	-	+	+	-	-	-
O-72	-	+	+	-	-	-
O-101	-	-	+	-	-	-
O-118	-	-	+	-	-	-
O-130	-	+	+	-	-	-
O-156	-	+	+	-	-	-
O-180	-	+	+	-	-	-
Ecc ATCC 15713	-	+	+	-	-	-
Ech Ura-2	-	-	+	-	-	-
Bc ATCC 25416	-	+	+	-	-	-

Reference/standard isolates: Ecc ATCC 15713 (Ecc=E. carotovora subsp. carotovora), Ech Ura-2 (Ech= E. chrysanthemi), Bc ATCC 25416 (Bc=B. cepacia), ATCC= American Type Culture Collection; + = growth positive; - = negative

Table V. List of identified soft rot bacterial isolates of onion from different locations of Bangladesh according to growth, physiological and biochemical characteristics

Sl. No.	No. of Isolates	Locations	Identified as
1	O-03	Santhia, Pabna	Erwinia carotovora subsp. carotovora
2	O-05	,,	Berkholderia cepacia
3	O-14	Rangpur	B. cepacia
4	O-15	Dhaka	B. cepacia
5	O-18	,,	E. carotovora subsp. carotovora
6	O-69	Sujanagar, Pabna	E. carotovora subsp. carotovora
7	O-72	,,	E. carotovora subsp. carotovora
8	O-101	Gazipur	E. chrysanthemi
9	O-118	Karwan Bazar	E. chrysanthemi
10	O-130	,,	E. carotovora subsp. carotovora
11	O-156	Rangpur	E. carotovora subsp. carotovora
12	O-180	Gazipur	E. carotovora subsp. carotovora

rot bacteria of onion due to lack of facilities. That's why molecular based techniques should be included in future for characterization and identification of soft rot bacterial strains of onion in Bangladesh.

Conclusion

Among the 12 selected virulent isolates, *Pectobacterium carotovorum* subsp. *carotovorum (E. carotovora* subsp. *carotovora)* was the major soft rot pathogen of onion in Bangladesh. These findings were based on traditional biochemical and physiological analysis, however, molecular analysis (De Boer and Kelman, 2000) is more reliable than the conventional biochemical and physiological analysis to draw a strong conclusion. Future work plan is suggested using molecular based techniques.

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