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Plant Extract Regulating Pectate Lyase Production in Soft Rotting Bacterial Isolates

Nazmun Nahar[1], Md. Manjurul Haque[2], Md. Abu Ashraf Khan[3] and Md. Ashraful Haque[1]*

[1]Department of Biotechnology; [2]Department of Environmental Science; [3]Department of Plant Pathology, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh

**Corresponding author and Email: ashrafbiotech@gmail.com*

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Abstract

Soft-rotting bacteria caused diseases by secreting the plant cell-wall-degrading enzymes mainly pectate lyase (Pel). In this study, a total of 20 bacterial strains were isolated from soft rotted fruits and vegetables. Among them, 11 isolates produced soft rot symptom on potato tubers. All these isolates were partially characterized. When these isolates were tested for their Pel production in different media, the isolate P1 produced significantly higher amount of Pel than other isolates in M63 glycerol minimal medium. Interestingly, the isolate P1 grown on M63 glycerol minimal medium supplementing with 0.4% of polygalacturonic acid (PGA) and M63 glycerol minimal medium plus 0.4% of PGA and 1% of various plant extracts, Pel production was induced in M63 glycerol minimal medium containing 0.4% of PGA and hyper-induced in M63 glycerol minimal medium plus 0.4% of PGA and 1% of various plant extracts. However, Pel synthesis was not hyper-induced in high concentrations of potato extract. Thus, not only plant extracts but also concentrations of plant extract may be important for hyper-induction of Pel.

Keywords: Soft rotting bacterial isolates, pectate lyase, hyper-induction, plant extracts

1. Introduction

Bacterial soft rots are caused by different bacterial genera including *Erwinia*, *Pseudomonas, Bacillus* and *Clostridium*. The bacteria mainly attack the fleshy storage organs of their hosts (tubers, corms, bulbs, and rhizomes), and also affect the succulent buds, stems, and petiole tissues. Soft rot bacteria degrade pectate molecules that bind plant cells together, causing plant structure to eventually fall apart (Charkowski *et al*., 2012).

Among soft rotting bacteria, species of *Erwinia* causes soft rot in potato during storage, transportation and cultivation. Major pathogenicity determinants of *Erwinia* spp. are plant cell-wall-degrading enzymes such as pectinase [pectate lyase (Pel), polygalacturonase (Peh), pectin methyl esterase (Pme)], cellulases and proteases (Toth *et al*., 2003). Of these enzymes, pectinases are considered as the main exoenzymes which breakdown and utilize pectins in the middle lamella and plant cell walls, causing tissue collapse, cell damage and cell leakage. Among the pectinases, Pel has been shown to play a major role in virulence (Haque *et al*., 2009). Synthesis of Pel has been shown to be regulated by various physiological and environmental causes, such as pectin, nitrogen

starvation, iron and magnesium concentrations, the presence of metabolizable sugars, oxygen limitation, cell density, pH, temperature, osmolarity, organic acid and the presence of plant extracts (Enard *et al*., 1998; Hugouvieux-Cotte-Pattal *et al*., 1992; Haque and Tsuyumu, 2005; Haque *et al*., 2005; Nomura *et al*., 1998).

Among the environmental factors affecting the synthesis of Pel, plant signals other than pectate products are important. For example, in *Dickeya dadantii* (formerly *E. chrysanthemi*) 3937, Pel e synthesis is induced by 230-fold compared with the basal level by adding plant extract together with polypectate-Na (NaPP) into the bacterial growth medium (only a 9-fold induction occurred with NaPP alone (Bourson *et al*., 1993). Nomura *et al*., (1998) reported that pectate lyase is induced by the substrate NaPP and hyper-induced by the presence of plant extracts which is controlled by the pPIR (Plant Inducible Rgulator) in *D. dadantii* EC16. Morever, Pel production was shown to be hyper-induced in response to the concentrations of magnesium that controlled by the PhoP-PhoQ two component regulatory system (Haque and Tsuyumu, 2005) and SlyA, a MarR family transcriptional regulator of *D. dadantii* 3937 (Haque *et al*., 2009). In view of the above facts, the present study was undertaken to: (i) isolate and characterize soft rotting bacteria from diverse plant species affected by disease and (ii)) investigate the effect of plant extracts on the induction of Pel production in soft rotting bacteria.

2. Materials and Methods

1.1. Study area, sample collection and isolation of soft rotting bacteria

The experiment was conducted in the laboratory of Biotechnology and Plant Pathology, Faculty of Agriculture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur during June 2014 to May 2015. In order to isolate soft rotting bacteria, diseased potato tubers and plants (*Solanum tuberosum* L.), mango fruits (*Mangifera indica* L.), onion (*Allum cepa* L.), radish (*Raphanus sativus* L), brinjal (*Solanum melongena* L.) and apple (*Malus domestica)* were collected from local markets, experimental and farmer's fields. A portion of each sample was sliced with water, then a loop of sample was streaked on yeast extract peptone (YP) agar plates and incubated at 28°C for 48 h. Maceration tests were done using potato tubers. The isolate caused soft rot symptom on potato tubers was selected.

2.2. Biochemical characterization

2.2.1. KOH test

KOH test was done as described previously (Suslow *et al*., 1982) with a few modifications. In brief, two drops of 3% KOH solution were placed on a clean glass slide, then a loop full of 24-h old bacterial culture grown on YP agar plate was added and mixed properly. The viscosity of bacterial suspension was tested within 10 seconds. A loop was pushed in suspension and pulled out gently. The gram negative bacterial suspension produces a fine thread of slime while the gram positive bacteria do not produce such thread but are watery.

2.2.2. Catalase test

A loop full of 24-h old bacterial culture (grown on YP agar plate) was placed on a clean glass slide then a drop of 3% hydrogen peroxide (H_2O_2) solution was added and mixed with the culture. Production of gas bubbles indicated positive reaction (Hayward, 1992).

2.2.3. Oxidative-fermentative (OF) test

The test was performed as described by Hugh and Leifson (1953). The broth contained 2.0 g of peptone dissolved in 1L distilled water and the pH was adjusted to 7.0. The 5 mL of broth was dispensed into the test tubes, plugged and autoclaved. Test tubes were cooled to 45-50^{0}C, then 10% filter sterilized glucose solution was added aseptically. The medium was stab inoculated with a small loop containing 24-h old bacterial cells (two test tubes were inoculated). One test tube was covered with sterile liquid paraffin to a depth of 8-12 mm. The test tubes were then incubated at 28°C for 48 h and were

observed for production of acid manifested by yellow color. Fermentative bacteria produced acid in both test tubes while oxidative bacteria produced acid only in uncovered medium.

2.2.4. Production of indole acetic acid (IAA)

The production of indole-3-acetic acid (IAA) was determined as described by Hugh and Leifson (1953).

2.2.5. Media for growth and Pel production

To check the growth and Pel production, each isolate was grown in various growth media such as M63 glycerol minimal medium (per liter, 2.5 g of NaCl, 3 g of KH_2 HPO_4, 2 g of $(NH_4)_2SO_4$, 0.5 mg of FeSO4, 2 g of thiamine hydrochloride, and 0.2% (wt/vol) of glycerol), Luria-Bertani (LB) medium (1% of tryptone, 0.5% of yeast extract, 0.5% of NaCl, pH 7.0) and yeast extract-peptone (YP) medium (1% of peptone, 0.5% yeast extract, pH 6.8).

2.2.6. Measurement of growth and Pel production

For the growth of the bacterial isolates, 50 µL of each bacterial isolate was inoculated in glass test tube containing 5 mL of M63 glycerol minimal medium then incubated at the indicated temperatures. The optical density (OD) was measured after 24 h incubation with spectrophotometer at 660. Bar indicate error bar with standard error for data of three indipendent experiments. However, Pel specific activity was determined as described previously (Haque *et al*., 2009). In brief, isolates were grown in the medium until OD_{660} reached at 1.0 then 1 ml culture was centrifuged at 15000 rpm for 5 min to remove the cell debris. The supernatant was used for assaying Pel activity. A 10 µl of sample solution was added to 990 µl of the reaction buffer (0.05% PGA), 0.1 M Tris -HCl pH 8.5, 0.1 mM $CaCl_2$, pre-warmed to 30 ^{0} C. After a proper mixing of the solution, the increase in optical density at 230 nm was measured every minute. One unit of Pel activity was defined as the amount of the enzyme that produced a change in absorbance of 0.001 at 230 nm in 1.0 min. The mean of Pel activity from five independent experiments was expressed as the specific activity (U/OD_{660}).

2.2.7. Induction and hyper-induction of Pel

To study the induction of Pel synthesis, the isolate P1 was grown in M63 glycerol minimal medium containing 0.4% of polygalacturonic acid (PGA). To study the hyper-induction of Pel, the isolate P1 was grown in M63 glycerol minimal medium containing 0.4% of PGA and 1.0% of different plant extracts. Plant extracts were prepared as follows: potato tubers (*S. tuberosum* L), carrot roots (*Daucus carota*), radish (*R. sativus*), and pumpkin (*Cucurbita pepo*) were purchased from a local market. Crude juice was prepared from them, sterilized through a 0.45 µM nitrocellulose filter and was then used. This experiment was conducted in a complete randomized design (CRD) with 9 (nine) replications.

3. Results and Discussion

3.1. Isolation and characterization of soft rotting bacteria

A total of 20 bacterial isolates were isolated from different fruits and vegetable samples. Among them, 11 isolates (P1, P2, R1, A1, M2, M3, M6, M76w, M8, M9 and B1) showed positive for soft rot symptom on potato tubers (Table 1) suggesting that all these isolates may be soft rotting bacteria. Isolates were named based on initial capital letter of the host (P for potato, B for brinjal, R for radish, A for apple and M for mango). All the isolates were negative for Gram staining, KOH- and catalase tests. The isolates P1, R1, M2, M3, M6, M7, M8, M9 and B1 were also positive to oxidative and fermentation test, but the isolates P2, A1 and C1 (C for carrot) were negative to this test. Except P1 and R1, all the isolates were negative to indole acidic acid test. Collectively, these data suggested that at least some of the isolates may be different species of *Erwinia*. However, this hypothesis needs experimental validation by using 16S rRNA gene sequencing.

Table 1. Results of soft rot, Gram staining, KOH, catalase, O-F and indole test

Isolate name	Soft rot	Gram staining	KOH	Catalase	O-F test	Indole test
P1	+	-	-	-	+	+
P2	+	-	-	-	-	-
R1	+	-	-	-	+	+
A1	+	-	-	-	-	-
M2	+	-	-	-	+	-
M3	+	-	-	-	+	-
M6	+	-	-	-	+	-
M7	+	-	-	-	+	-
M8	+	-	-	-	+	-
M9	+	-	-	-	+	-
B1	+	-	-	-	+	-
C1	+	-	-	-	-	-

+: Indicates positive results and -: Indicates negative result

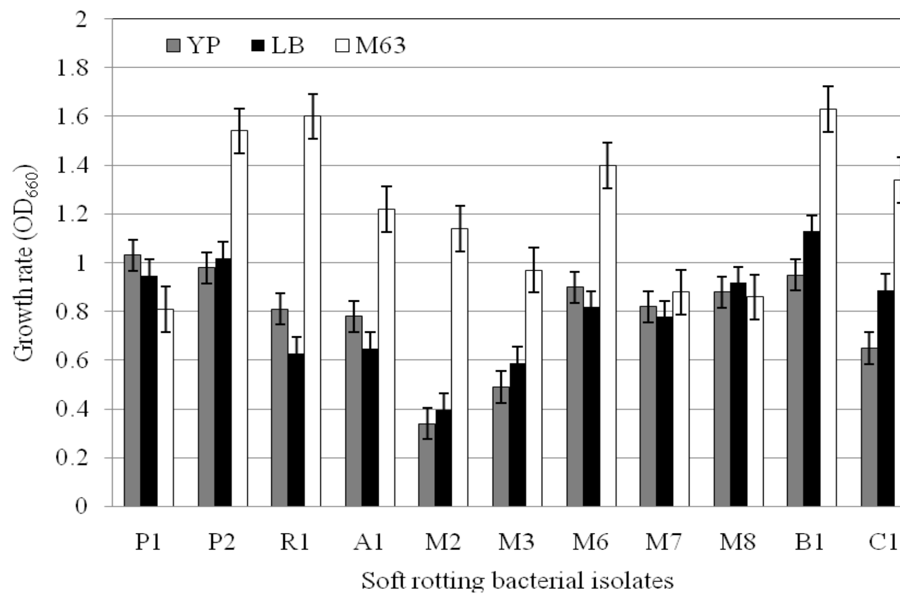

Figure 1. Growth of different soft rotting bacterial isolates on YP, LB and M63 glycerol minimal medium at 27ºC with shaking condition. The optical density (OD) was measured after 24 h incubation with spectrophotometer at 660. Bar indicate error bar with standard error for data of three indipendent experiments.

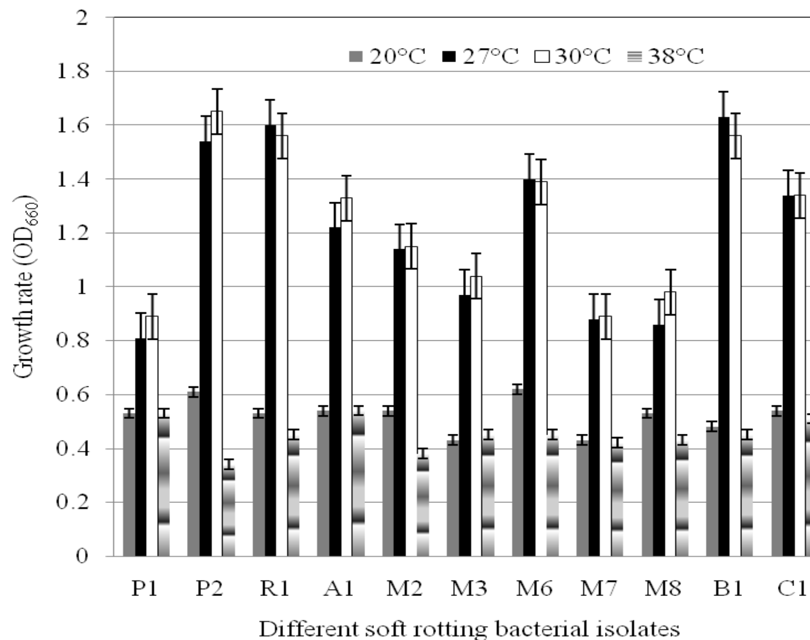

Figure 2. Effect of temperature on growth of different soft rotting isolates. The 50 µL of each bacterial culture was inoculated in glass test tube containing 5 mL of M63 glycerol minimal medium then incubated at the indicated temperatures. The optical density (OD) was measured after 24 h incubation with spectrophotometer at 660. Bar indicate error bar with standard error for data of three indipendent experiments.

3.2. Effect of media composition on growth of the isolates

When soft rotting bacterial isolates were grown in different media, all the isolates grew well in different media such as YP, LB and M63 glycerol minimal medium (Figure 1). However, the isolates R, B, M2, M3 and M6 grew better in M63 glycerol minimal medium than YP and LB medium after 24 h incubation at 27°C. These results suggest that media composition may be important for the growth of soft rotting bacteria. Normally, M63 glycerol minimal medium contains several salts. Among the salts, magnesium was shown to stimulate growth of *D. dadantii* 3937 (Haque *et al*., 2015). Thus, magnesium or other salts present in M63 glycerol minimal medium may be responsible for the enhanced growth of soft rotting bacterial isolates.

3.3. Effect of temperature on growth of soft rotting isolates

To address the role of temperature on growth, different soft rotting isolates were grown in M63 glycerol minimal medium and incubated at different temperatures such as 20, 27, 30 and 38^0C. Growth rate of soft rotting isolates were higher at 27^0C followed by 30, 20 and 38°C (Figure 2). Thus, 27 to 30°C may be the optimum temperature for the growth of soft rotting bacteria. Soft rotting *P. carotovorum* subsp. *carotovorum* and *D. dadantii* were also shown to better grow at 27 to 30°C (Toth *et al*., 2003).

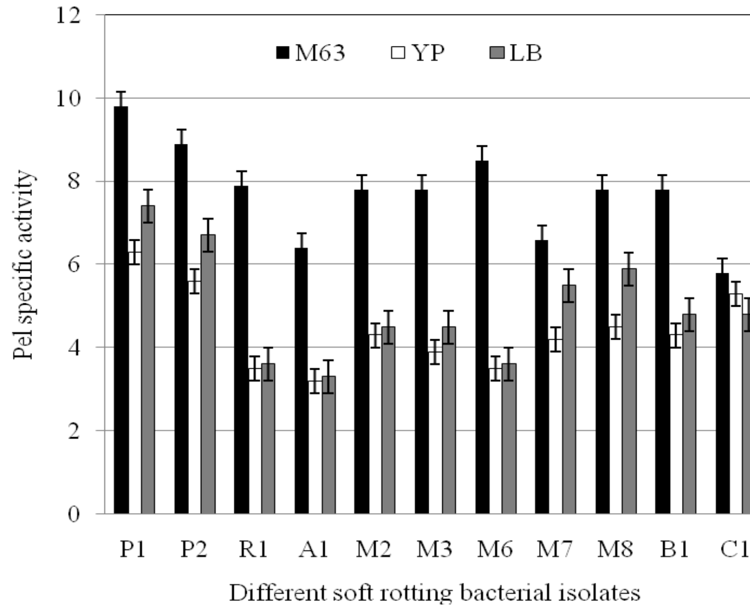

Figure 3. Pel specific activity. Different soft rotting bacterial isolates were grown in the indicated medium at 27°C with shaking condition. One milititer culture was collected and centrifuged. The supernatant was used to evaluate Pel production. The data was expressed as the mean of at least five independent experiments.

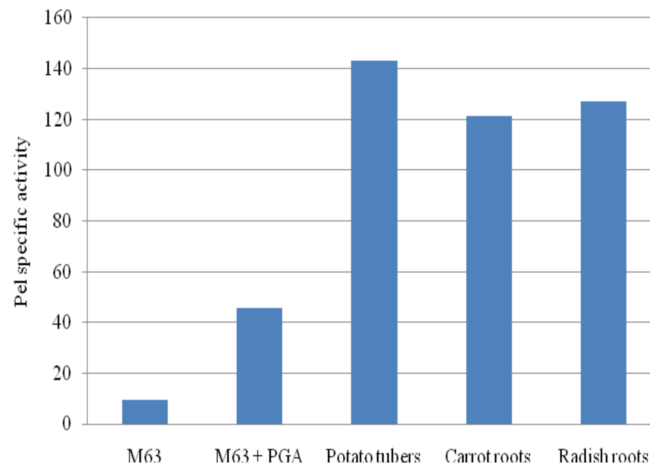

Figure. 4. Pel production in isolate P1 in different media. The isolate P1 was grown in M63 glycerol minimal medium, M63 glycerol minimal medium + 0.4% PGA and M63 glycerol minimal medium + 0.4% PGA + 1% of indicated plant extracts at 27°C with shaking condition until optical density reached at 1.5 then 1 mL of culture was collected and centrifuged. The supernatant was used to evaluate Pel production. The data was expressed as the mean of at least five independent experiments.

Table 2. Influence of various concentrations (%, v/v) of potato tuber extract (PTE) on hyper-induction of Pel

Condition	Pel specific activity (U/OD $_{660}$)	Induction ratio
M63 glycerol minimal medium	6.3 ± 2.0 h	-
M63 +0.4% PGA	47.6 ± 6.7 g	7.6
M63 + 0.4% PGA + 0.2% PTE	55.2 ± 4.5 f	8.7
M63 + 0.4% PGA + 0.5% PTE	62.4 ± 4.1 e	9.90
M63 + 0.4% PGA + 1.0% PTE	141.7 ±15.2 a	22.49
M63 + 0.4% PGA + 2.0% PTE	116.2 ± 15.8 b	18.44
M63 + 0.4% PGA + 4.0% PTE	92.5 ±15.5 c	14.7
M63 + 0.4% PGA + 8.0% PTE	90.5 ±11.5 c	14.4
M63 + 0.4% PGA + 10.0% PTE	72.3 ± 10.4 d	11.5

3.4. Pel specific activity of soft rotting isolates

The isolate P1 produced higher Pel than the other isolates (Figure 3). Among the media, Pel production was found to be higher in M63 glycerol minimal medium than YP and LB medium (Figure 3). Thus, media composition and bacterial isolates may be important for Pel synthesis. Matsumoto *et al.* (2003) also reported that Pel production was regulated by media composition such as YP and M63 glycerol minimal medium and different strains of *P. carotovorum* subsp. *carotovorum* and and *D. dadantii.*

3.5. Hyper-induction of Pel by the isolate P1

When the isolate P1 was grown in M63 glycerol minimal medium, M63 glycerol minimal medium containing 0.4% of PGA and M63 glycerol minimal medium containing 0.4% of PGA and 1.0% of various plant extracts, Pel production was induced (4.75-fold) in M63 glycerol minimal medium plus 0.4% of PGA and hyper-induced (12.5 to 14.9-fold) in M63 glycerol minimal medium containing 0.4% of PGA and 1.0% of plant extract (Figure 4). However, Pel specific activity was found to be slightly varied among the plant products (Figure 4). This result suggests that the amount of compound(s) responsible for hyper-induction of Pel may vary in different plant products. It was reported that the compound(s) responsible for hyper-induction of Pel is heat stable, low molecular mass and hydrophobic in nature (Bourson *et al.*, 1993; Haque and Tsuyumu, 2010).

3.6. Hyper-induction of Pel is concentration dependent

The effect of different concentrations (0.2, 0.5, 1.0, 2.0, 4.0, 8.0 and 10.0%) of potato tuber extract (PTE) on hyper-induction of Pel was analyzed in the presence 0.4% of PGA. Induction ratios were increased with the plant extract concentration to a maximum with 1.0% potato tuber extract (Table 2). However, with higher concentrations, the induction ratio was decreased. This could be due to catabolite repression exerted by sugars present in the extract of potato tuber or to the presence of Pel inhibitors in the extract, yet to be determined.

4. Conclusions

Different soft rotting bacterial isolates were isolated from different diseased fruits and vegetable samples. All the isolates were only partially characterized. Thus, 16S rRNA gene sequence is needed to confirm the genus and species of the bacteria. Among the isolates, P1 produced higher Pel than the other isolates. Media composition and concentration of plant extracts played an important role in hyper-induction of Pel synthesis but many crucial questions remains unanswered. The study will help us to know how bacterial soft rot pathogens cause disease and respond to the plant components.

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