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Isolation and Characterization of Pathogens Causing Disease in Pomegranate (*Punica granatum* L.), India.

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ABSTRACT: Pomegranate (*Punica granatum* L.) is a fruit crop with high nutritional and therapeutic values. Major problem in pomegranate productions is plant disease caused by various fungi and bacteria, CWDEs of phytopathogens are a key virulence factor in infection process and pathogenesis. Major aim of this study was: (I) to isolate and identify phytopathogens that cause disease in pomegranate fruit (II) to screen for hydrolyases. Pomegranate with spots and rotting symptoms collected from market of Ahmedabad, Gujarat. Four fungal, one yeast and one bacterial culture was isolated from it. These isolates further screened for hydrolytic enzyme (amylase, cellulase, chitinase, lipase, pectinase, protease) production. Result shows variety of enzyme production by isolates. The two fungal isolates selected namely, PGF1 and PGF4 were identified as *Xenoacremonium falcatum* (with 98.76% similarity) based on ITS sequencing, respectively; and one bacterial isolate PGB1 was identified as *Bacillus velezensis* (with 100% similarity) based on 16S rRNA sequence analysis by NCMR (NCCS), Pune, India. One yeast isolate PGF5 identified as *Candida freyschussii* (with 93% similarity) by Supratech Micropath Laboratories, Ahmedabad, India through VITEK2 system. The enzyme profiles may provide inputs for genetic manipulation to prevent disease, and also help define phytopathogens as bio-based resource of enzyme(s).

KEYWORDS: *Punica granatum* L., cell wall degrading enzymes, enzyme profile, *Xenoacremonium falcatum*, *Candida freyschussii*, *Bacillus velezensis*.

I. INTRODUCTION

Pomegranate (*Punica granatum* L.), a member of *Punicaceae* family, is an ancient high value fruit crop capable to cultivated in different agro-climatic conditions. Ancient traders in Central Asia and the Middle East referred to the pomegranate as the “fruit of paradise” [1]. Every part of pomegranate like root, bark, wood, sprouts, leaves, flowers, fruit, rind and seed have economic value [2]. Pomegranate has magical nutritional and therapeutic values due to the presence of several potentially bioactive compounds in its different parts like, ellagitannins, galloyl derivative in fruit, bark, wood and leaves [3]; Terpenoids [4], sex steroids [5], tocopherols [5] in seed and seed oil; Flavonols [6], flavanone glycoside [5], anthocyanidins [7] in fruit; Gallotannins [8], pyridine alkaloids [9] in bark and heartwood; Flavones [10], flavone glycosides [10], piperidine alkaloids [10] in leaves. Because of these properties of pomegranate, it is widely use in treatment of cardiovascular disease [11], oral diseases [12], breast cancer [13], Lung cancer [14], prostate cancer [15], skin tumorigenesis [16], colon carcinogenesis [17], Atherogenesis [18], AIDS [19], diabetes [20]. Growing awareness of innumerable medical and health benefits of pomegranate, stimulates demand for pomegranate and its by-products.

India is the world’s largest producer of pomegranates, followed by Iran, China and Turkey. The Indian pomegranate production is growing by 20 to 25% every year. India is the only country able to deliver pomegranates all year round because of its climate conditions. Among major pomegranate growing states, Maharashtra is the largest producer, occupies 62.91% of total production in country followed by Gujarat (16.23%), Karnataka (9.42%), Andhra Pradesh (4.91%), Madhya Pradesh (4.02%), Rajasthan (0.93%), Telangana (0.70%) and Tamil Nadu (0.46%) [21, 22].



Major problem in pomegranate productions is plant disease cause by various fungi and bacteria, result in yield loss and decrease market value of fruit. Plant disease also have large impact on local economies and rural community. Severe disease outbreaks can cause 60 to 80% yield losses of pomegranate [23].

These losses reflect incomplete knowledge relating to the causes and mechanisms behind disease development [24]. So, a better understanding of Plant-Pathogen relationship and its virulence factor is important to understand disease mechanism and help in development of disease management strategy. Linking plant pathology with ecology is very important to understand plant disease dynamics.

Plant cell wall functions as an important barrier against pathogen attack. So, initial step in infection is to pass across plant cell wall through degradation of plant cell wall components by producing several cell wall degrading enzymes such as amylase, cellulase, chitinase, cutinase, pectinases, proteases, xylanase, etc.

In view of this background, objective of this study was: (1) to isolate and identify phytopathogens that cause disease in pomegranate (2) to screen for hydrolyases.

II. MATERIALS AND METHODS

Isolation of Fungi and Bacteria from spoiled pomegranate fruit

Pomegranate with spots and rotting symptoms were collected from the local market of Ahmedabad, Gujarat. After surface sterilization of the fruit, microscopic examination of spoiled portion was carried out. Then spoiled portion of pomegranate fruit was placed on different microbiological media namely, Potato Dextrose Agar (PDA), Martin Rose Bengal Agar (MRBA), Glucose Yeast Extract agar (GYEA) medium for fungi at 26-28°C, 5.5-6.0 pH for 4-6 days, and on Nutrient Agar medium for bacteria at 37°C, 7.0-7.5 pH for 24-48 hours. Pure culture of fungi, yeast and bacteria obtained were preserved on slants of respective medium and stored at 4°C for further studies.

Screening of isolates for hydrolytic enzyme production

Screening of isolates for hydrolytic enzyme production done by using plate assay method. Fungal and bacterial isolates cultured on Starch agar medium for amylase, Carboxymethyl cellulose agar medium for cellulase, Chitin agar medium for chitinase, Tributyrin agar medium for lipase, Pectin agar medium for pectinase and Gelatin agar medium for protease activity. These plates were incubated at 26-28°C, 4-6 days for fungi and at 37°C, 24-48 hours for bacteria. After incubation 0.2% I₂ and 0.1% KI solution was added to chitin agar plate, pectin agar plate and starch agar plate. Congo red (0.5%) was added to carboxymethyl cellulose agar plate, after 15 min. solution was discarded and the culture were washed with 1M NaCl. Frazier's reagent added to Gelatin agar plate. A clear zone surrounding colony indicate production of respective enzyme by isolate. This clear zone directly observed in tributyrin agar plate.

Identification of isolates

Selected fungal and bacterial isolates were identified by National Centre for Microbial Resource (NCMR), NCCS, Pune, India through nucleotide BLAST analysis based on ITS and 16S rRNA gene sequencing, respectively. One yeast isolate was identified by Supratech Micropath Laboratories, Ahmedabad, India through VITEK2 system.

III. RESULTS AND DISCUSSION

Isolation, identification, colony characteristics and morphological study of isolates

The fruit with rot disease/symptoms were collected and observed externally, internally and microscopically (Fig. 1). Four fungal (PGF1, PGF2, PGF3, PGF4), one yeast (PGF5) and one bacterial (PGB1) culture isolated from infected pomegranate fruit. The colony characteristics of the isolates is shown in Fig. 2. PGF1 and PGF4 isolates were identified as *Xenoacremonium falcatum* with 98.76% similarity by NCMR, NCCS, Pune, India based on nucleotide BLAST

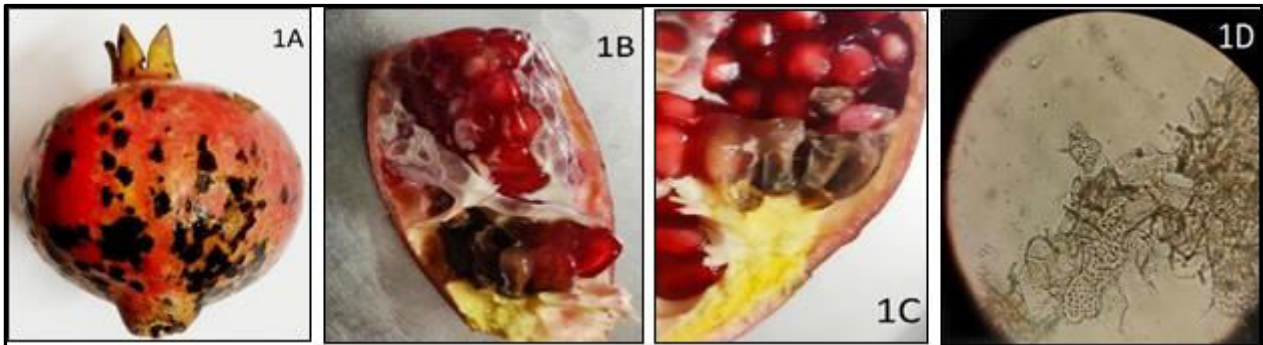


Fig.1. Photographs of external (1A) and internal (1B, 1C) symptoms in diseased pomegranate fruit sample. Fig. (1D) Photomicrograph of spoiled portion of pomegranate fruit, indicates microbial invasion of plant cell.

analysis of ITS and 16S rRNA sequencing. PGB1 isolate was initially identified as *Bacillus subtilis / amyloliquefaciens / atrophaeus* with 87% probability (Bionumber: 1262170715446671) by Supratech Micropath Laboratories, Ahmedabad with VITEK2 system, but later this isolate was identified as *Bacillus velezensis* with 100% similarity by NCMR, NCCS, Pune. PGF5 isolate was identified as *Candida freyschussii* with 93% similarity (Bionumber: 4545150275147111) with the VITEK2 system.

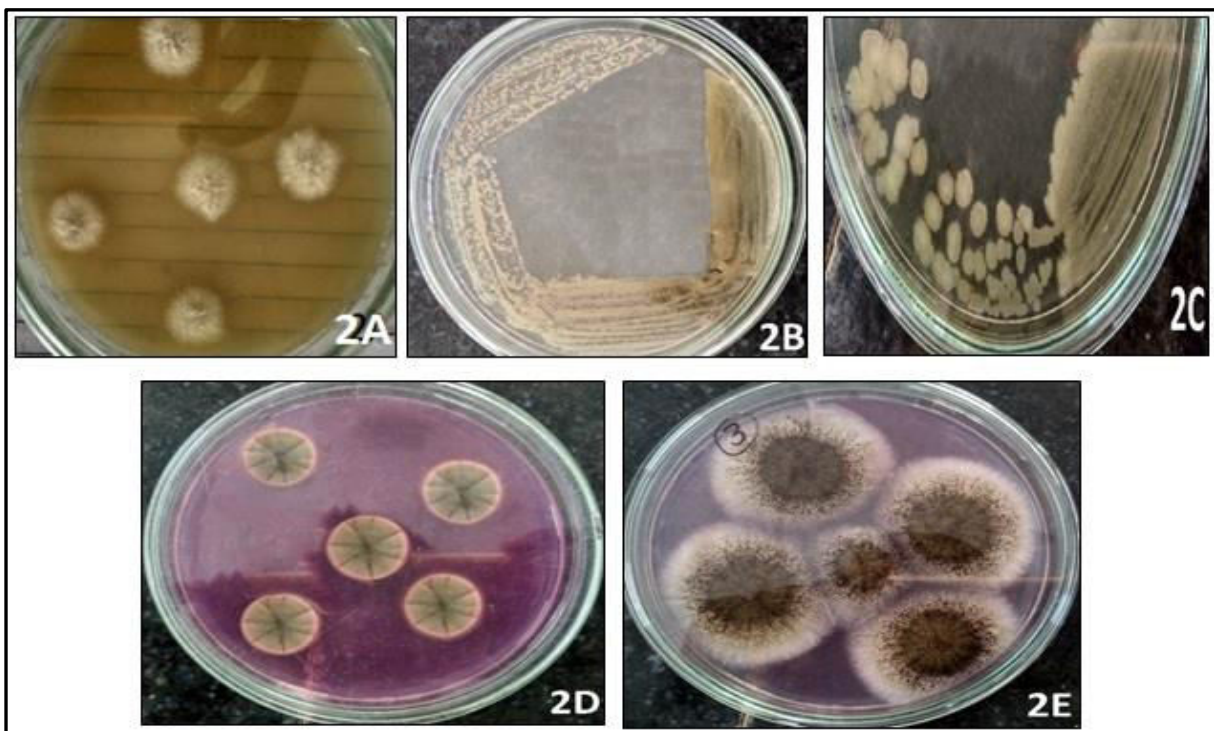


Fig.2. Colony characteristics of PGF1 (*Xenoacremonium falcatum*) on PDA plate (2A), *Candida freyschussii* on GYE agar plate (2B), *Bacillus velezensis* on Nutrient agar plate (2C), PGF2 and PGF3 on Rose Bengal Agar plate (2D, 2E).



PGF1 and PGF4 (*X. falcatum*); and PGB1 (*B. velezensis*) and PGF4 have been deposited at NCMR, Pune (India) which is an autonomous institute of Department of Biotechnology, Govt. of India, an affiliated member of the World Federation for Culture Collection and registered with the World Data Centre for Microorganisms; with Accession number MW376905, MW376895 and MW374949, respectively.

To the best of our knowledge, this is the first report of identification of *Xenoacremonium falcatum*, *Candida freyschussii* and *Bacillus velezensis* as pathogen causing rot disease/spoilage of pomegranate fruit.

Screening of isolates for hydrolytic enzyme production

All isolates were screened for hydrolytic enzyme production such as amylase, cellulase, chitinase, lipase, pectinase, protease (Fig. 3). The distribution of hydrolyase activities of different isolates is given in Table 1.

PGF1 produce chitinase, lipase and pectinase enzyme. PGF2 produce all enzymes except amylase and lipase. PGF3 produce all enzymes. PGF4 produce amylase, lipase and pectinase enzyme. PGF5 not shows lipase and pectinase activity. PGB1 produce all enzymes. These isolates screen for hydrolytic enzyme production and shows variety of enzyme activity. Different strains of *X. falcatum* produced amylase, chitinase, lipase and pectinase. *C. freyschussii* produced amylase, cellulase, chitinase and protease; while *B. velezensis* produced amylase, cellulase, chitinase, lipase, pectinase and protease. Thus, there is diversity in the hydrolyases produced by the isolates. A few isolates showed presence of all the hydrolytic enzymes tested.

Table. 1 Results of screening of isolates for hydrolytic enzyme(s) production.

No.	Isolate	Zone of hydrolysis					
		Amylase	Cellulase	Chitinase	Lipase	Pectinase	Protease
1.	PGF1	-	-	++	+	++	-
2.	PGF2	-	++	+++	-	++	+++
3.	PGF3	+++	+	+++	++	+	++
4.	PGF4	+++	-	-	+	++	-
5.	PGF5	+	+++	++	-	-	++
6.	PGB1	++	+++	+++	+	+	+++

Some of the phytopathogens that have been reported to cause fruit spot or rot in pomegranate include: *Alternaria alternata* [25], *Aspergillus spp.* [26], *Botrytis cinerea* [27], *Colletotrichum gloeosporioides* [28], *Penicillium spp.* (*P. implicatum*, *P. glabrum*) [29,30], and *Pseuercospora punicea* [31].

Polysaccharides such as cellulose, chitin, hemicelluloses, pectin and starch make up 90% of the plant cell wall [32]. The phytopathogens initially produce various hydrolyases that attack or hydrolyse the cell wall constituents. These CWDEs serve as key virulence factor in causing infection and pathogenesis [33, 34]. Several studies indicate that absence or decreased expression of genes encoding CWDEs can result in reduced virulence of phytopathogens [35,36]. Hence these hydrolytic enzymes can serve as potential targets for disease management therapy.

The global enzymes market size in, 2017 USD 7.082 billion, 2018 USD 8.18 billion, 2019 USD 9.9 billion and continuously growing [37]. There is an increasing demand of hydrolyases in various industries such as food, baking, detergent, oil, paper-pulp, textile, bioenergy etc. this has the potential to boost the market growth during the coming years. Microorganisms are becoming superior, most favoured, ecofriendly, bio-based source of enzyme in the global enzyme market. As mentioned above, phytopathogens produce various extracellular hydrolytic enzymes and emerge as promising source for commercial explorations. *Candida freyschussii* has been used to produce microbial oils from glycerol in biofuel industries [38]. The enzyme profile of phytopathogens demonstrated in this study may help to develop disease resistant transgenic plants [36].

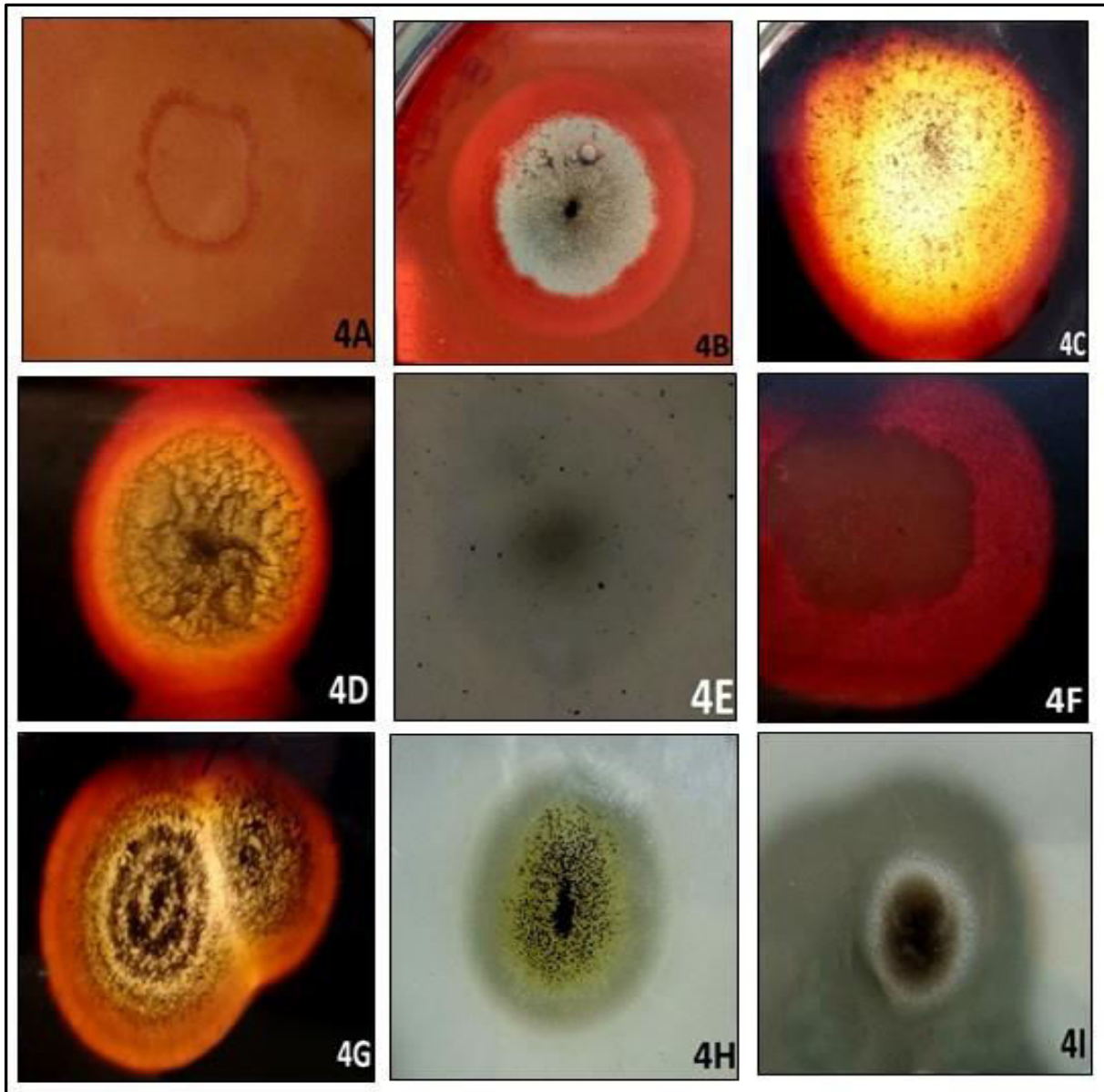


Fig.3. Results of isolates for hydrolytic enzymes: cellulase (4A-4B), chitinase (4C- 4D), lipase (4E), pectinase (4F-4G), protease (4H-4I) production.

To our best knowledge this is first report shows production of; amylase, chitinase, lipase, pectinase enzyme by *Xenoacremonium falcatum* and amylase, cellulase, chitinase, protease enzyme by *Candida freyschussii*. These enzyme profile of phytopathogen may help to develop transgenic plants which are resistant to such disease. These organisms may be useful as a bio-based resource of industrially important enzymes. *Candida freyschussii* is widely used to produce microbial oils from glycerol in biofuel industries [38].

IV. CONCLUSION

This is the first report of *Xenoacremonium falcatum*, *Candida freyschussii* and *Bacillus velezensis* as a causative agent of pomegranate fruit spot or rot, with the capability of producing CWDEs. Different strains of *X. falcatum* showed amylase, chitinase, lipase and pectinase activity. The yeast *C. freyschussii* showed amylase, cellulase, chitinase and protease activity. *B. velezensis* showed amylase, cellulase, chitinase, lipase, pectinase and protease activity.



Understanding the hydrolyases profile of phytopathogens may help in control of the rot disease in this fruit of economic and therapeutic value. These isolates can be useful for industrial production of these hydrolytic enzymes.

V. ACKNOWLEDGEMENT

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VI. CONFLICT OF INTEREST

Authors not have conflict of interest.

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