

PENICILLIUM IMRANIANUM, A NEW SPECIES FROM THE MAN-MADE SOLAR SALTERN OF PHETCHABURI PROVINCE, THAILAND

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Abstract

Penicillium imranianum was isolated along with other halophilic and halotolerant microorganisms from a man-made solar saltern in Phetchaburi province, Thailand. Morphological and molecular identifications were performed along with characterization studies, which proved it to be new as well as halophilic species. The name was obtained and released from mycobank (MB812859) after getting the accession ID (KP780293) from NCBI. The species is indexed in index fungorum and available as EMCB-HF08 and TISTR 3655 for public access. The species is found to have immense biotechnological potential.

Key words: *Penicillium imranianum*, Hypersaline habitats, Halophilic Fungi, Extremophiles.

Introduction

Species from *Penicillium* genus have generally been found as soil inhabitants (Pitt, 1979). They have mostly been reported as decomposers of dead material and also as food spoiling fungi (Lamsal *et al.*, 2013). *Penicillium* has not been much famous of being extremophile. We report a new halophilic fungal species *Penicillium imranianum*, isolated from a man-made solar saltern, located at Phetchaburi province Thailand. To the best of our knowledge, it is the most extremely halophilic *Penicillium* ever reported.

Materials and Methods

Habitat information and sample collection: The man-made saltern is located at Ban Leam district (13°13'14.61"N, 99°58'32.92"E), Phetchaburi province, Thailand (Ali *et al.*, 2013). Temperature of the area varies from 24-32°C throughout the year with an average 85 mm of rain in dry season and 950 mm of rain in rainy season (Ali *et al.*, 2015b). Soil samples were collected in the end of dry season in April, 2010. Soil samples for fungal isolation were diluted in 13% of NaCl concentration (w/v) in accordance with salinity test performed on site. The soil analysis showed mild alkalinity, poor nutrients and high salinity (Ali *et al.*, 2013). By serial dilutions the fungal species were grown and isolated on Potato Dextrose Agar (PDA) supplemented with 13% of NaCl concentration (w/v). Based on morphospecies observations the fungal colonies were purified as single colonies.

Morphological identification: Morphological studies were aided by previous available literatures and keys as described by Ali *et al.*, (2013). Microscopic observations were performed under stereomicroscope (SZ30; Olympus, Tokyo, Japan) and by fluorescent microscopy (Olympus BX60). For morphological observations the fungus was

grown on Czapek Yeast extract Agar (CYA), Malt Extract Agar (MEA), Potato Dextrose Agar (PDA) and Yeast Extract Sucrose agar (YES) media. The growth on solid cultures were recorded as diameter in mm after 7, 14 and 21 days.

For scanning electron microscopic studies the fungus was grown for a week on PDA at 30°C. The structure was examined by using a JEOL JSM-7610F scanning electron microscope (SEM) at Scientific and Technological Research Equipment Centre, Chulalongkorn University.

Molecular identification: Genomic DNA was isolated by phenol-chloroform extraction method by following methodology explained by Davis *et al.*, (1986). DNA of internal transcribed spacer (ITS) region was amplified with polymerase chain reaction (PCR) by using the ITS4 (5'-TCCTCCGCTTATTGATATGC-3') /ITS5 (5'-GGAA GT AAAAGTCGTAACAAGG-3') primer pair (White *et al.*, 1990). Chroma Taq DNA polymerase (Denville Scientific, Metuchen, NJ, USA) was used in 25 µl. The PCR cycles were at 94°C for 2 min, followed by 25 cycles at 94°C for 10 s, 55°C for 30 s, 72°C for 1 min, and at 72°C for 10 min. QIA quick PCR Purification Kit (Qiagen, Hilden, Germany) were used for the purification of PCR products. PCR products of 20 ng were sequenced by dideoxy chain termination method (GENEWIZ DNA Sequencing Service, NJ).

The ITS sequence obtained was edited and assembled by using Editseq (DNASTAR Lasergene; DNASTAR, Madison, WI) and Clustal X ver. 1.81 (Thompson *et al.*, 1997).

Characterization studies: *P. imranianum* was characterized by checking its growth at different pH (4-8), temperature (20-40°C) and salinity (0-20 % NaCl) by dry weight estimation method, following the methodology explained by Ali *et al.*, (2013).

Screenings of biotechnological potentials: The species was screened for its antibacterial activity (against *Staphylococcus aureus*, *Escherichia coli* and *Acinetobacter Spp* by plate screening method), antioxidant [by Total Phenolic Content (TPC) screening] and enzymatic (amylase, cellulase, invertase, lipase, protease and xylanase by plate screening method) potentials by methodology explained by Ali *et al.*, (2014c).

Results and Discussion

Morphological observations: After 7 days the colonies were found growing approximately: 8 mm in CYA and PDA, 6 mm in MEA and YES, after 14 days growing approximately 17 mm in CYA, 17-18 mm in PDA, 11 mm in MEA and 9 mm in YES and after 21 days they were found growing 24-25 mm in CYA and PDA, 18 mm in MEA and 15 mm in YES. There were not much growth differences in CYA and PDA.

The colonies were observed growing slowly, appearing plane, slightly raised in central area, fimbraite margin, bluish gray-green shades, exudate and lacking odour. Colonies in reverse plates were found in pale yellow shades. Penicilli were found strictly monoverticillate with slender conidiophores, 1.5-2 μm in diameter, smooth walled; Phialides were in loose to compact clusters, 5-10 in verticil, 6-7 x 2.5 μm ; conidia were globose, conspicuously roughened, 2-2.5 μm in diameter, in chains with loosely parallel columns to tangled (Fig. 1). The SEM images show the conidiophores arising from basal hyphae with a terminal vesicle. At the swollen end of a hyphae the conidiogenous cells produced the chain of spherical conidia (Fig. 2).

These observations confirmed the affiliation of *Penicillium* genus to the fungus under investigation and it was found most closely related with *P. aeneum* on the basis of morphology.

Molecular identification and phylogenetic analysis:

The sequence similarity was found 88% with most closely related *Penicillium* species by using Basal local alignment tool (BLAST) from National Center of Biotechnology Information (NCBI). The sequence was submitted in NCBI as new species and the accession number KP780293 was obtained.

Most of *Penicillium* species in BLAST search from NCBI were found having 80-88% similarity with *P. imranianum*. *P. aeneum* which was found most closely related in morphological analysis was found having 83% similarities with *P. imranianum*. Closely related *Penicillium* species were compared with *P. imranianum* for its phylogenetic analysis by Neighbor joining method, using MEGA 6 v 5.10 (Tamura *et al.*, 2013). Bootstrap value of 1000 replicates was used. *P. imranianum* (KP780293) was found to be originating from a separate clade from the parental node far distant from its related species (Fig. 3). This shows the unique genetic makeup of this species. This position of *P. imranianum* can provide a genetic niche for more unexplored *Penicillium* species especially for the extremophilic ones that can be found in the years to come. The phylogenetic tree was submitted in the TreeBASE (www.treebase.org) with an ID 17869.

Etymology: As suggested by mycobank, following the ICN Rec. 60C.1(d) the epithet (*imranianum*) was derived

from the name of its collector, isolator, identifier and reporter: Imran Ali. Mycobank ID: MB812859 was allotted to the fungus and its name was released after getting it passed from curators. *P. imranianum* has also been accepted as new *Penicillium* species by index fungorum (www.speciesfungorum.org).

The strain name (PT 2015) was provided, representing 'P' for Phetchaburi, 'T' for Thailand and 2015 represents the depositing year for NCBI, mycobank and index fungorum.

Holotype

EMCB-HF08

Taxonomy: Cellular organisms; Eukaryota; Opisthokonta; Fungi; Dikarya; Ascomycota; saccharomyceta; Pezizomycotina; leotiomyceta; Eurotiomycetes; Eurotiomycetidae; Eurotiales; Aspergillaceae; *Penicillium*; *imranianum*; PT 2015.

Characteristic observations: The species was found growing best at 7.5 pH, 30°C and at 5% of NaCl concentration (w/v). *P. imranianum* falls in the definition of halophilic fungi because of being isolated from the hypersaline environment and was able to grow over 3 Molar concentration of salt (upto 20% of NaCl) (Ali *et al.*, 2015 a,b).

Biotechnological potentials of *P. imranianum*: Increasing salt concentration of soil is one of the effects of climate change in the world (Ali *et al.*, 2014b; Vu *et al.*, 2015). *P. imranianum* can be taken as model species for understanding the halophilic characteristics of plants, which can later help in improving the drought tolerance for food crops (Bukhari *et al.*, 2015; Parveen & Nazeer, 2018). Like its fellow halophilic fungi (Ali *et al.*, 2014 a,c; 2015a), *P. imranianum* has been found to have promising applications in biotechnology. The glimpse of its biotechnological potential has been expressed in figure 4.

The plate screening results of antibacterial screenings shows that it was found having more antibacterial potential against gram negative bacteria (*A. spp* & *E. coli*) than gram positive one (*S. aureus*). This result corresponds to our previous report of antibacterial screening of halophilic fungi (Ali *et al.*, 2014c). Similarly, the TPC screening for antioxidant was found positive expressing the strength of biotechnological potential of *P. imranianum*.

Amongst the six enzymes tested by plate screening method, four (amylase, cellulase, invertase and xylanase) were found to be secreted in significant amount (creating clear zones) by *P. imranianum*. We have already reported that the enzymes from halophilic fungi are mostly been found to have polyextremophilic characteristics which can be used at various industrial processes as well to counter against post global warming effects (Ali *et al.*, 2014a, 2015a).

In addition to the above mentioned potential applications, *P. imranianum* has been found to produce salt induced red pigment. The growing need of natural colorants in food and textile industry can be fulfilled by this pigment, which can be more efficient than pigments from normal microbes because these pigments can work better in low water activity operations.

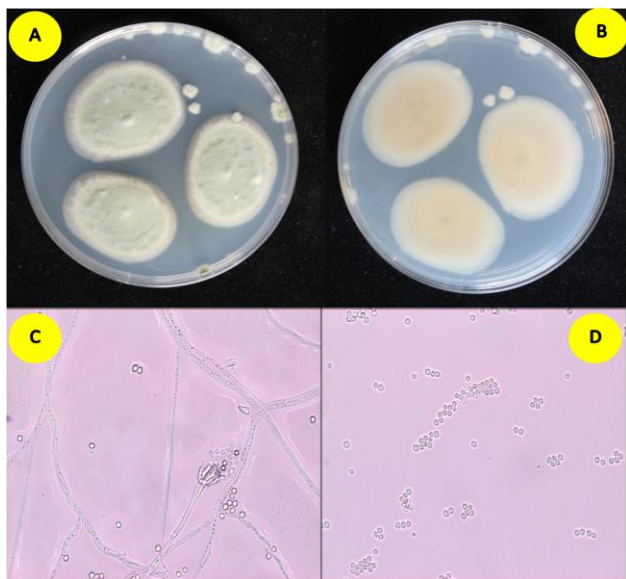


Fig. 1. A: front plate; B: Reverse plate culture characteristics on CYA (20 days) with conidia sporulate in culture; C,D: Penicilli, oonidiophores ,and phialides of Conidia of *P. imranianum* grown on PDA (4 Days).

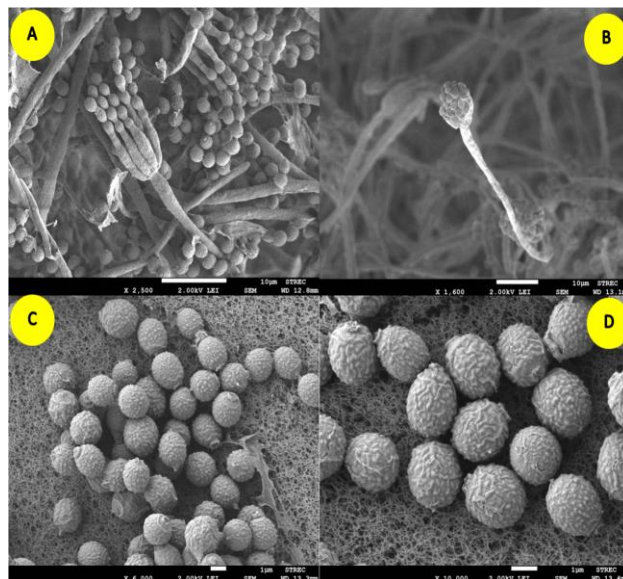


Fig. 2. A, B: SEM showing the stalk and conidia, mature conidiophore grown on PDA at 30°C SEM showing the stalk and conidia, measurement bars are at 10 µm; C, D: SEM showing ascospores of *P. imranianum* Bars are at 1 µm.

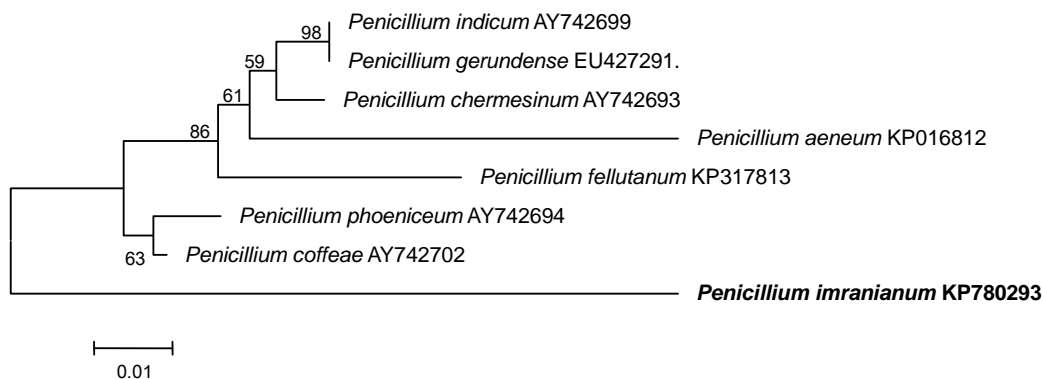


Fig. 3. Phylogenetic analysis of *P. imranianum* (in bold); Species (Italicized) are followed by their NCBI accession numbers.

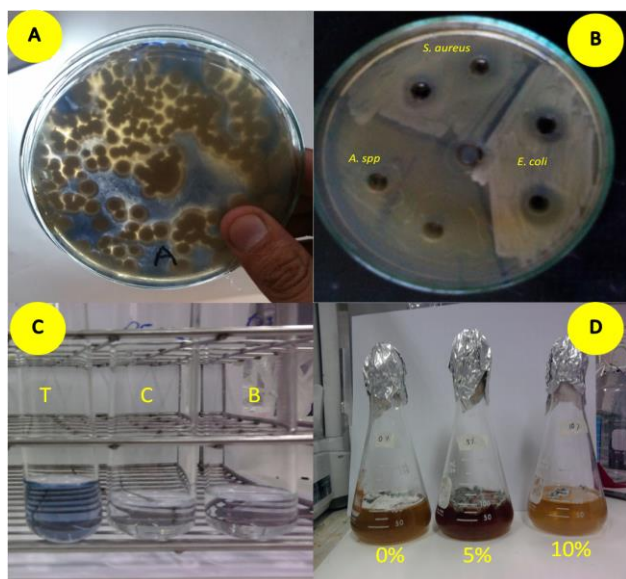


Fig. 4. Biotechnological screenings of *P. imranianum*; A: Plate screening of amylase; B: Antibacterial screenings of crude filtrates against *S. aureus*, *E. coli* & *A. spp*; C: TPC screening of antioxidant potential where T=test (having filtrate), C=control (having no filtrate) & B=blank (having only distilled water); D= salt (NaCl) induced red pigments shown at 0%, 5% & 10%.

Conclusions and Future Prospects: Currently, *P. imranianum* is present in active (agar plated and broth cultures) and stored (freeze dried and in 20% glycerol) forms. The stored forms of *P. imranianum* are available for public access with code EMCB-HF08, in Extremophilic Microbial Culture Bank (EMCB) of Research Unit for Biotechnology from Extremophilic Microorganisms (RUBEM), Institute of Biochemistry, University of Balochistan, Pakistan. Its stored and active forms are also available in Plant Biomass Utilization Research Unit (PBURU), Botany Department, Chulalongkorn University, Thailand and also submitted to Thailand Institute for Scientific and Industrial Research (TISTR), Thailand for public access having code 3655.

The discovery of *P. imranianum* in this climate change era can be a blessing providing metabolites available at high salt concentrations. These metabolites can be used in post global warming effects due to their extreme properties. Our research group is working on detailed characteristic studies of these metabolites which will be published soon or patented.

Acknowledgements

This research was funded by the Ratchadapisek Sompoch Endowment Fund (2016), Chulalongkorn University (CU59-049-EN).

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(Received for publication 25 March 2017)