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Original Article

Isolation and Identification of *Penicillium rubens* from the Local Strain in Mosul, Iraq, and Investigation of Potassium Phosphate Effect on its Growth

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Abstract

Penicillium species is one of the most common microscopic filamentous fungi that have been isolated from widespread substrates. In this study, soil samples from different areas of Mosul, Iraq, were examined by morphological and molecular methods. Fungi were isolated and grown in diagnostic culture media, including Czapek Yeast Extract Agar, Malt Extract Agar, and 25% Glycerol nitrate agar at different temperatures of 15, 20, 25, and 30˚C. The growth rate of the fungus was also evaluated in the culture containing K_2HPO_4. In the molecular method, the obtained fragment was sequenced using the Internal Transcribed Spacer region primers after replication. Morphological evaluation of both macroscopic and microscopic features revealed that strains were *Penicillium rubens*, and molecular methods used have confirmed the isolated strain. The results of this study showed a decrease in the diameters of the colonies at different growth temperatures when doubling the amount of K_2HPO_4. In addition, the findings revealed that compared to growth inhibition in the control groups, the maximum growth inhibition was 16 and 29 mm on Czapek Dox Agar (modified) medium at incubation temperatures of 20˚C and 25˚C, respectively. As a result, K_2HPO_4 has an important role in inhibiting growth; therefore, it can be used as Fungicide.

Keywords: ITS regions, K_2HPO_4, *Penicillium rubens*, Morphological characters

1. Introduction

Penicillium species (spp.) are the most common fungi that live in environments with different temperatures, humidity ratios, and PH levels. They also compete with other organisms, such as bacteria (1). This class of fungi is one of the species of the *Aspergillus* family belonging to the order *Eurotiales* (2). *Aspergillus* can grow in soil, air, and food with various physiological properties. Some species tolerate high or low temperatures, some tolerate high salt or sugar concentrations, others can live in low-oxygen environments, and some of them have important effects on human life (3), (4). *Penicillium spp.* can cause problems in immunosuppressed patients. *Penicillium rubens* (*P. rubens*) sometimes causes local infections in humans, such as corneal inflammation and allergic lung disease. The primary difficulty of identifying the infections caused by *Penicillium spp*. was the variety of clinical symptoms (5, 6). *Penicillium* plays an important role in assisting plants to adapt to environmental conditions due to its presence in the rhizosphere through various biological processes (7). Some species of *Penicillium* produce dissolved siderophore, phosphorus, and phytohormones, such as gibberellin A3 and indole acetic acid, which are necessary for fungi (8). Primary media, especially

potato dextrose agar (PDA) is the widely well-known medium for growing *Penicillium* (9). The morphological examination is important to determine the colony shape and dimensions, which depend on using standard media for *Penicillium*, such as Malt Extract Agar (MEA) and Czapek Yeast Agar (CYA) (10). *Penicillium* colonies can be distinguished on the plate under the microscope by their green or white color; many numbers of conidiophores and chains of unicellular conidia appear from a specialized cell that is called phialide (11). *P. rubens* has key applications in Biotechnology, especially in the treatment of some plant and animal diseases since it is considered an important biological agent in bioremediation (12). Furthermore, it is regarded as a major factor in the production of pharmaceutical intermediates, as well as amino acids, and it has an essential role in resisting bacterial infections due to its production of β-lactam antibiotics (13). The growth and production of metabolites in fungi are mainly affected by different concentrations of culture medium components; in particular, Phosphate and Potassium significantly affect the formation of the cell wall in fungi (14). The presence of Potassium in addition to Magnesium, Iron, and sugars in the culture medium is fundamental for the isolation, growth, and diagnosis of fungi (15). Phosphate is a mediator for the interactions between fungi and other organisms, and it is considered a critical macronutrient for reproduction, as well as an important balancing factor between nutritional needs and environmental conditions (16). Molecular diagnosis by DNA sequencing is one of the most modern and successful methods for identifying strains of microorganisms since biochemical experiments and morphological features cannot be relied on to identify fungal species (17, 18). Preliminary identification of fungal genera was performed by the amplification of 16srRNA fragment, Internal Transcribed Spacers (ITS) regions, calmodulin, and b-tubulin genes by basic local alignment search tool (BLAST) algorithm [19], based on GenBank National Center for Biotechnology Information (19-21).

In this study, after the isolation and identification of *P. rubens* from the soil of local strain in Mosul, Iraq, an evaluation was conducted to investigate the effect of Potassium phosphate on the growth of *P. rubens*.

2. Materials and Methods

2.1. Collection and Isolation of Samples from Soil

In total, 40 soil samples were collected from different areas of Mosul, Iraq, in order to investigate the presence of *P. rubens*, and the dilution method was used for its isolation from soil samples (22). Totally, 10 g of each sample was weighed, added to 90 ml of sterile distilled water, mixed well for 10 min, and finally, a series of dilutions were made (10^{-1}-10^{-4}). One ml of each soil sample suspension was transferred to an empty petri dish. Afterward, cooled PDA was added with three replicates for each sample, and three Petri dishes were left without inoculation as the control group (23). The samples were incubated at 25˚C for five to seven days for the growth of the existing fungi.

2.2. Morphological Identification of *Penicillium rubens*

Penicillium spp. were morphologically diagnosed based on their growth on the three diagnostic media, including CYA, MEA, and 25% Glycerol nitrate agar (G25N) at different temperatures of 5˚C, 25˚C, and 37˚C (24, 25). To reactivate, *Penicillium spp*. isolates were grown in the PDA medium and incubated at 25±2˚C for seven days. The spore suspension method was used to inoculate the culture media. They were inoculated evenly with the spore suspension placed in 2.5 ml tubes, and a semi-solid medium containing 7 g of agar, as well as 10 g of sucrose in 1,000 ml of distilled water was used at temperatures of 5˚C, 25˚C, and 37˚C (26). The diameters of the growing colonies were measured by the ruler at the back of the dish, the results of which were recorded and compared to the taxonomic keys of the genus *Penicillium* (27).

2.3. Molecular Identification of *Penicillium rubens*

2.3.1. Extraction of DNA

A total of 50 mg of *P. rubens* was used for the extraction of genomic DNA by using an extraction kit (Zymo

Research environmental purification, Quick-DNA™ Miniprep Plus Kit) according to the Kit's instruction**.**

2.3.2. Polymerase Chain Reaction Amplification and Gel Electrophoresis

Polymerase chain reaction (PCR) was performed for duplicating one segment of about 330 nucleotides of the ITS region. DNA template 4 µL (100 ng) and 1 µL of each primer (Table 1) were added to the master mix constituents. The temperature program and cycling protocol for the amplification of the gene were performed according to table 2.

Table 1. Sequence of primers of the Internal Transcribed Spacer region

Primer	Sequence
Forward	TGAATCATCGACTCTTTGAACGC
Revers	TTTCTTTTCCTCCGCTTATTGATAT

Table 2. Temperature program and cycling protocol for the amplification of Internal Transcribed Spacer gene

No.	Stage	Tempreture ˚C	Time	No. of Cycles
1	Initual Denaturation	95	5 min.	1
2	Denaturation	95	45 sec.	
3	Annealing	55	1 min.	35
4	Extention	72	1 min.	
5	Final Extention	72	7 min.	1

Amplified PCR products were electrophoresed by 2% agarose gel, and the stained gel was analyzed under the UV transilluminator (28).

2.3.3. DNA Sequencing for Nucleotide Sequences

The PCR product, along with the designed primers, was sent to Hitachi company (Japan) for the sequencing of the ITS gene and determining the sequences of the product using the primers.

2.4. Potassium Phosphate Modification Study

To investigate the effect of Potassium on the growth of *P. rubens* in Czapek Dox Agar (CDA) and CYA at different temperatures of 15˚C, 20˚C, 25˚C, and 30˚C, 2 g of K_2HPO_4 was added to the culture media, and all samples were incubated for 5 to 7 days to compare growth and measure the diameter of the colonies.

3. Results

3.1. Cultural and Microscopical Identification

All *Penicillium spp.* isolates were identified to the genus by means of their morphological characteristics. *P. rubens* was identified morphologically by the three culture media previously mentioned (i.e., CYA, MEA, G25N) as shown in figure 1B. Additionally, *P. rubens* was determined under a light microscope as illustrated in figure 1A.

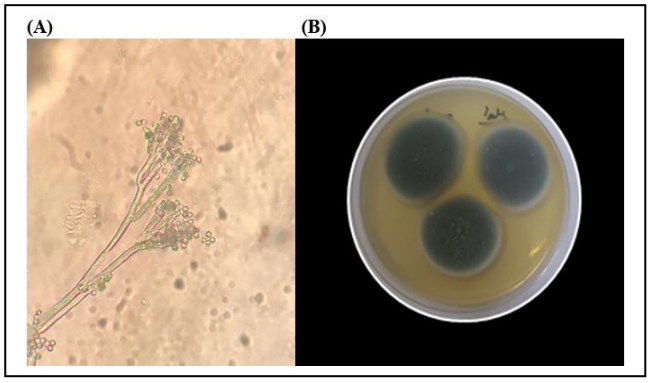

Figure 1. A. *Penicillium rubens* under 40× light Microscope. **B.** *Penicillium rubens* cultured in Malt Extract Agar

3.2. Results of Polymerase Chain Reaction and DNA Sequencing

As depicted in figure 2A, the DNA of *P. rubens* was extracted. The PCR amplification of the ITS gene in a reaction size of 330 bp is shown in figure 2B. After obtaining the nucleotide sequences of the genes, they were compared to the GenBank database using the BLAST tool (available on http://www.ncbi.nlm.nih.gov/blast/). The ITS sequences belonging to *P. rubens* were retrieved from the GenBank (MN413162.1) (Figure 3).

3.3. K_2HPO_4 and Temperature Records

After the end of the fungi incubation period in different culture media, the average colony diameters were measured in the control groups media (i.e., CDA and CYA) shown in table 3. They were also measured in the groups with doubled K_2HPO_4 (i.e., CDA-modified and CYA-modified), in which the variations in growth were observed according to different incubation temperatures, as depicted in figure 4. The highest and lowest growth of colony diameters were reported at 25˚C and 15˚C in the CDA medium, respectively.

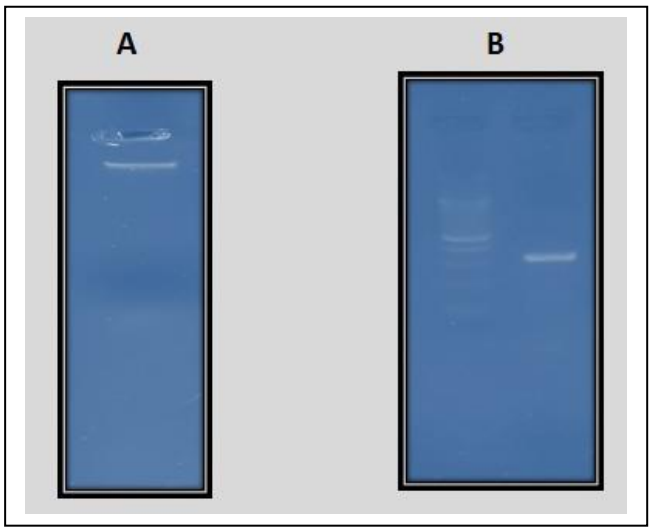

Figure 2. A. DNA extraction of the sample. **B.** PCR reaction for the Internal Transcribed Spacer in a reaction size of 330bp

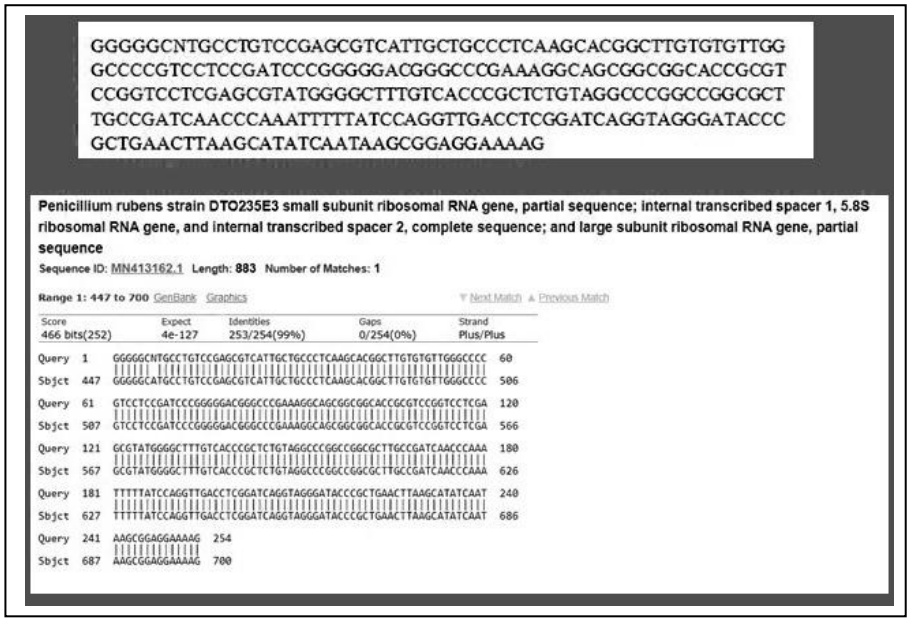

Figure 3. Nucleotides sequences of 16SrRNA for *Penicillium rubens*

Table 3. Average diameters of *Penicillium rubens* colonies at different temperatures

Temp. (ºC)	Growth diameter (mm) / CDA medium	Growth diameter (mm) / CDA -modified medium	Growth diameter (mm) / CYA medium	Growth diameter (mm) / CYA- modified medium
15	8	6	8	8
20	22	16	26	24
25	38	29	22	20
30	30	23	25	10

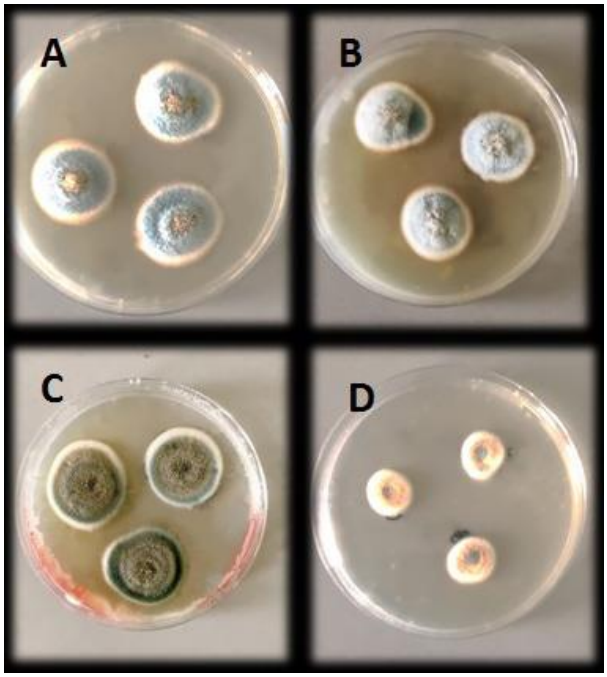

Figure 4. A. *Penicillium rubens* isolated in CDA* medium at 20˚C. **B.** *Penicillium rubens* isolated in CDA-modified medium at 20˚C. **4C.** *Penicillium rubens* isolated in CYA* medium at 30˚C. **4D.** *Penicillium rubens* isolated in CYA-modified medium at 30˚C
*Czapek Dox Agar
* Czapek Yeast Agar

4. Discussion

Identification of *Penicillium spp*. is based on morphological criteria, such as macroscopic and microscopic features, as well as classical cultivation methods. These methods are time-consuming, prone to contamination, as well as inaccurate classification, and might lead to identification problems. Currently, molecular methods focusing on genotypic characteristics are used to confirm the classical method and accelerate detection. In the present study, *P. rubens* showed a great ability to grow and sporulate on CYA and MEA media. The color of the colonies was pale yellow, as well as light green on the CYA medium, and the diameter of the colony was about 28 mm, which is compatible with the findings of a previous study conducted by Sawant, Vankudoth (29). According to the results, conidiophores of *P. rubens* belong to Terverticillate, which possess branches varying from one to three between the metulae and the stipe (30). However, the morphological identification of the fungus is not considered enough and cannot ascertain the type of fungus. Therefore, the diagnosis was completed by PCR reaction and ITS regions. Subsequently, *P. rubens* was diagnosed after sequencing and matching with the NCBI (Figure 3). Identification based on the replication of the ITS was used in previous studies, such as Guevara-Suarez, Sutton (31), (32). Internal Transcribed Spacer gene is an accurate and rapid identification test, which is considered important in the exploration and easy detection of fungal species in the environment. Results of this study showed a clear decrease in the diameters of the colonies at different growth temperatures when doubling the amount of K_2HPO_4. Furthermore, compared to the control groups, the maximum inhibition of the growth was 16 and 29 mm on CDA-modified medium at the incubation temperatures of 20˚C and 25 ˚C, respectively, in comparison with CDA medium at the same temperatures. However, the most prominent result was at 30˚C incubation temperature on CYA-modified medium, with the rate of growth inhibition equal to 10 mm, which is 15 mm less than that in the control group (i.e., CYA medium) at the same temperature, as depicted in figure 4D. It can also be observed that the growth diameters of the colonies were stable at 15˚C, where they were 8 mm in all groups, except for a slight decrease by 2 mm on CDA-modified medium. This range of temperatures was chosen since *P. rubens* is a mesophilic mold, which can grow between 5˚C and 35˚C, with its optimum temperature being at 25˚C-30˚C (33, 34). This result is consistent with the findings of a previous study, which used 10 types of fungi concluding that K_2HPO_4 and K_2HPO_4 have an inhibitory effect on growth through preventing spores formation by 100%, in addition to inhibiting the mycelial growth between 50% and 100% (35).

As morphological diagnosis in fungi is of significant importance in facilitating the identification of *Penicillium spp*., molecular identification is most

critical in determining the type of organism. In the future, using more differential media and identifying secondary metabolites are suggested as another method for the identification of fungi. Internal Transcribed Spacer technique is an important modern technique in molecular biology for the easy and rapid identification of microorganisms. The use of specific primers for a specific gene or whole-genome analysis results in more accurate results; however, K_2HPO_4 has an important role in inhibiting growth. Therefore, it can be used as Fungicides. It is possible to examine other medium-chemical components at different concentrations to show their effectiveness. Further studies are needed in utilizing more detailed growth temperatures with wider ranges to measure differences in growth for more specific results.

Authors' Contribution

Study concept and design: M. Y. A.
Acquisition of data: M. Y. A.
Analysis and interpretation of data: M. Y. A.
Drafting of the manuscript: R. M. H.
Critical revision of the manuscript for important intellectual content: B. S. A.
Statistical analysis: M. Y. A.
Administrative, technical, and material support: M. Y. A.

Conflict of Interest

The authors declare that they have no conflict of interest.

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