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Biosolubilization Potential of Rock Phosphate and Phosphatase Production by some Phosphate Solubilizing Fungi

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Abstract: Biomining refers to the utilization of microorganisms to convert metals from rock phosphate ore (RPO) into soluble compounds, with the aim of extracting elements such as phosphorus. A total of fourteen fungal isolates were isolated, purified, and tested for their ability to dissolve RPO. The most active isolate was identified as *Aspergillus niger* (Assiut University Mycological Center (AUMC) 16252) after extensive testing. A variety of rock phosphate concentrations, ranging from 5 g/L to 400 g/L, were investigated. In response to gamma radiation exposure, many isolates, but especially *A. niger*, increased phosphatase activity. *A. niger* showed the highest activity, which increased even at high radiation gamma doses. *A. niger* possess the highest value of solubilized P under static condition (34.3 **% P_2O_5**). Flow culture studies have revealed that the capacity of *A. niger* to create phosphatases is regulated by growth rate, phosphate availability, and pH. When *A. niger* was cultured under static conditions at 28 °C, the specific activities of Acidic phosphatase and Alkaline phosphatase attained maximum values of 21 and 23.2 mg/mL, respectively. The pH of the substrate raised to 7 following the addition of rock phosphate and subsequently reduced to 5.02 after *A. niger* were infected and cultured for 3 days. These results imply that some fungus with less reactive RP can be utilized as natural fertilizers. This can help reduce the demand for synthetic fertilizers while also improving the environment and soil quality.

Keywords: Fungi, Biomining, Phosphatase, Rock phosphate, Aspergillus niger.

1 Introduction

Phosphorus (P) is an element necessary for plant growth and development. Phosphorus' low availability is owing to its proclivity to form insoluble complexes with cations such as aluminum and iron in acidic soils and calcium and magnesium in alkaline soils Meanwhile, insufficient phosphorus fertilizer recovery is caused by the fact that phosphorus given as fertilizer is predominantly absorbed by the soil and is not immediately available for plant uptake [1, 2]. Phosphate rock is a sedimentary rock that contains high concentrations of phosphorus-bearing minerals, primarily apatite. Phosphorus, which is naturally found in phosphate, is a crucial mineral for plants' growth and development. It has long been recognized to apply phosphate rock as fertilizer. Although phosphate rock releases soluble phosphorus at a slower rate than industrial phosphorus fertilizers, its low cost makes it an appealing phosphate fertilizer [3]. Around 217 million tons of phosphate were produced annually around the world in 2012, and there are 67 billion tons of reserves [4]. Researchers predict that current phosphate deposits may meet the world's phosphate needs for more than a century, even with population growth. Each year, Egypt produces more than 6 million tons of phosphate and holds roughly 4% of the global phosphate reserves, which amount to 2.78 billion tons [3]. Traditionally, RP is treated chemically by reacting it with phosphoric acid or sulfuric acid to create partially acidulated RP, but this method is expensive and environmentally harmful [5]. A more practical and affordable solution is the application of P-solubilizing microorganisms to depleted soil, which can make RP soluble in soils. These microbes have the ability to solubilize the phosphorus in rock phosphates, making it more available for plant uptake [6]. The utilization of fungi capable of solubilizing phosphate represents a promising

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biotechnological approach for effective phosphorus (P) fertilization management. This method allows for the utilization of rock phosphates (RP) or the recovery of phosphorus fixed in soil particles [7]. P solubilizing fungi (PSF) release various enzymes, including acid and alkaline phosphatases, phosphonates, phytases, and siderophores, that are crucial for solubilizing organic P in soil. Quantifying the activity of these enzymes can provide valuable information about their respective contributions to the P solubilization of RP-enriched composts [8]. This study aims to isolate and characterize phosphate-solubilizing fungi (PSF) from RP. It is also in the process of developing a novel method that utilizes these fungal isolates to dissolve medium- to low-grade phosphate rock, aiming to achieve the highest value of solubilized phosphate with a strong emphasis on beneficiation capability. This approach could increase phosphorus solubility in soil, leading to higher agricultural yields and reduced reliance on chemical fertilizers. By leveraging the natural ability of PSF to solubilize RP and advancing sustainable techniques, this research seeks to offer an eco-friendly and economically viable solution to the problem of low phosphorus availability in agricultural soils.

2 Materials and Methods

2.1 Materials

2.1.1 Rock Phosphate sample

The phosphate deposits in the Abu Tartur district account for approximately one-third of Egypt's total phosphate reserves, totaling over 1000 million tons. These deposits have a phosphorus pentoxide (P_2O_5) content with a medium-grade ores (17–25% P_2O_5) concentration [9].

It should be noted that we utilized the byproducts (wastes) of phosphate rock mining, which contain 12.4 g/kg of P_2O5.

2.1.2. Microbial strain

The isolated strain, *Aspergillus niger* (AUMC 16252), was chosen among 14 isolates and tested for the solubilization potential of rock phosphate following identification at the AUMC, Assiut, Egypt. This strain was then sequenced, and the recovered sequences were deposited in GenBank with the accession number OR856083. The sequence was compared to sequences in the Gen-Bank database using Basic Local Alignment Search Tool (BLAST) algorithms**.**

2.1.3. Media used

i. PVK medium [10]**:** ((NH4)2 SO4 at 0.5 g/L, MgSO4.7H2O at 0.1 g/L, NaCl at 0.02 g/L, KCl at 0.02 g/L, $FeSO_4$ at 0.003 g/L, $MnSO_4$ at 0.003 g/L, $Ca_3(PO_4)_2$ at 5 g/L, glucose at 10 g/L, yeast extract at 0.5 g/L, and agar at 15 g/L.

ii. Czapek-Dox's agar medium The medium used **[**11**]:** contained the following ingredients: 30.0 g/L sucrose, 2.0 g/L NaNO3, 1.0 g/L KH2PO4, 0.5 g/L MgSO4.7H2O, 0.5 g/L KCl, 0.001 g/L FeSO4.5H2O, 0.001 g/L CaCl2, 15.0 g/L Bacto-agar, and 0.065 g/L rose bengal (used as a bacteriostatic agent, obtained from Merck).

iii. Potato Dextrose Agar (PDA) [12] **g/l:** Potato slices, 250; Glucose, 20; Agar, 20.

2.2. Methods

2.2.1 Isolation of phosphate solubilizing fungi

Phosphate rock was sifted and ground into a fine powder. Powder was used as the separation source and different soil concentrations were prepared as 5g/l, 10g/l, 20g/l, 30 g/l, 40g/l. These concentrations were sterilized separately in sterile bottles using a UV lamp. Sterile rock phosphate mixed with prepared and autoclaved Potato dextrose agar (PDA) and Czapek-Dox**'s** agar media then immediately poured into sterile petri dishes. The media was inoculated with 50µm of soil suspension (1 gm of soil/ 10ml H_2O) and incubated at 30 °C.

2.2.2. Estimation of rock phosphate decomposing fungi

Fourteen fungal isolates were assessed for their ability to solubilize rock phosphate on PVK media. Unlike using Tricalcium phosphate as the phosphate source at the normal concentration of 5 g/l, this study utilized different concentrations of rock phosphate (ranging from 5 to 40 g/l). The aim was to investigate the isolates' capability to thrive and solubilize higher concentrations of rock phosphate.

2.2.3. Screening for most potent Phosphatase producers

Nine isolates were purified and assessed for phosphatase activity on PVK media with rock phosphate (50 g/l). The plates were incubated for varying durations: 24, 48, 72, 96 hours, and 7 days. The objective was to explore the isolates' ability to thrive at elevated rock phosphate concentrations and examine the impact of time on rock phosphate solubilization.

2.2.4. Irradiation of most potent rock phosphate solubilizing isolates

The irradiation source used in this study was a Cobalt-60, 220 Gamma cell located in the Cyclotron project of the Egyptian Atomic Energy Authority. At the time of the experiment, the radiation dose was 563.2 Gy/h. The tested

isolates were exposed to doses of 250, 500, 750, 1000, 1250, 1500, 1750 and 2000 Gy.

2.2.5. Microscopic examination and molecular identification of most effective fungus isolate

To perform morphological identification, the phosphate solubilizing isolate was cultivated on various agar media, including Czapek-Dox agar, PDA, and malt extract agar. The resulting growth and morphology of the strains on the different media were used to identify them ([12]; [13]; [14]; [15, 16]; and [17]). *A. niger* were inoculated on PDA media supplemented with RP (1000 g/l) and on PDA without RP to compare the morphological changes on the isolates and microscopic examination of *A. niger* on PDA supplemented with RP (1000 g/l) and without RP will be done. For molecular identification, the fungal sample was cultivated on Czapek's agar (CZA) medium and kept in an incubator at 28°C for 5 days [18]. DNA extraction procedures were conducted at the Molecular Biology Research Unit of Assiut University utilizing the Patho-gene-spin DNA/RNA extraction kit from Intron Biotechnology Company in Korea. Collaborating with SolGent Company in Daejeon, South Korea, polymerase chain reaction (PCR) and subsequent sequencing were performed. The internal transcribed spacer (ITS) regions of the ribosomal ribonucleic acid (rRNA) gene in each fungal isolate were amplified using universal primers, ITS1 (forward) and ITS4 (reverse), incorporated into the reaction mixture with the compositions ITS1 (5' - TCCGTAGGTGAACCTGCGG - 3') and ITS4 (5' - TCCTCCGCTTATTGATATGC - 3'). Sequencing of the purified PCR products (amplicons) employed the same primers, with ddNTPs incorporated in the reaction mixture following White et al.'s protocol [19]. The obtained sequences underwent analysis using the Basic Local Alignment Search Tool (BLAST) on the National Center of Biotechnology Information (NCBI) website. MegAlign software version 5.05 from DNA Star facilitated sequence analysis and phylogenetic tree construction. The sequences were deposited in GenBank with assigned accession numbers and are archived at the Mycological Center of Assiut University, accompanied by a deposition number (http://www.aun.edu.eg/aumc.htm).

2.2.6. Evaluation of the Solubilization Index on Solid Medium of A. niger at different time

The phosphate soluble activity of *A. niger* was evaluated in vitro on Pikovskaya agar medium according to the method described by Iman [20]. In the study, fungal isolates were inoculated in triplicate on agar plates under sterile conditions and incubated at 25-28°C for 3 and 8 days respectively. Uninoculated PKV agar plates served as controls. To evaluate the solubilizing activity of the isolates, centimeter-scale measurements of the diameters of clear zones and colonies were performed. The phosphate solubilization index was then calculated by equation (1) using the ratio of the overall diameter (colony + halo zone) to the colony diameter.

$$\text{Solubilizing index (SI)} = \frac{colony\ diameter + halo\ zone\ diameter}{colony\ diameter} \quad (1)$$

2.2.7. Determination of A. niger activity on different concentration of rock phosphate at different time

The solubilizing activity of *A. niger* was evaluated at different concentrations of rock phosphates between 50 to 400 g/L and at different times 48, 72, and 96 hrs. The aim was to determine the efficacy of *A. niger* in solubilizing phosphate rocks at different concentrations.

2.2.8. Improvement of fungal growth and RP solubilization

Further research was conducted on *A. niger* to determine the optimal conditions for phosphate solution on PVK agar media. The study evaluated the optimal pH, carbon source concentration, nitrogen source, and solubilization temperature. To investigate the impact of pH on the solubilizing index, Fungal isolates were grown at various pH levels, including 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. Different carbon sources, including dextrose, fructose, lactose, galactose, maltose, and sucrose, were added separately to the base medium (at a concentration of 10 g/L). The effects of various nitrogen sources, such as peptone, tryptone, yeast extract, beef extract, ammonium chloride, and sodium nitrate, were examined at a concentration of 0.5 g/L. The fermentation process was carried out for 7 days at different temperatures, including 20°C, 25°C, 30°C, 35°C, and 40°C to determine the optimal temperature for phosphate solubilization. The solubilization index was calculated to optimize the solubilization of rock phosphate as shown in equation (1).

2.2.9. P-Solubilization Efficiency of Selected Isolates in Liquid Media

A. niger, identified as the most efficient rock phosphate decomposer based on the solubilization index on solid medium, was further tested for its phosphatase activity under different conditions (acidic or alkaline, shaker or static).

Preparation of Inoculate. To prepare spore suspensions [21], a culture plate containing a 10-day-old fungal culture was covered with 20 mL of sterile water in aliquots, and the surface of the fungal colony was carefully scraped with a sterile glass rod. The resulting suspension was filtered through Whatman number 42 filter paper and transferred to a sterile glass vial. The spore concentration of the suspension was determined with a hemocytometer, and

sterile distilled water was added to adjust the spore concentration of the isolates to approximately 106 spores per milliliter.

Acid and alkaline phosphatase release in rock phosphate. The method described by Tabatabai [22], and Aseri [23] was utilized to assess the acidic and alkaline phosphatase activity of *A. niger*. In detail, *A. niger* supernatant collected after three and eight days was mixed with corresponding working buffer solution and p-nitrophenyl phosphate disodium salt hexahydrate substrate solution. The same procedure was used for a control sample. After incubation, a series of solutions were added, followed by vigorous shaking and filtration. The yellow color intensity in the samples was measured with a spectrophotometer at a wavelength of 410 nm. The working buffer for measuring alkaline phosphatase activity was prepared by combining a universal buffer stock solution, deionized water, and 0.1 M NaOH. Phosphatase activity was determined using a standard solution, and the results were expressed in mg/h/g/ml. Tabatabai's buffer stock solution included tris-(hydroxymethyl)-aminomethane, maleic acid, citric acid, and boric acid. A 1 M sodium hydroxide (NaOH) solution was prepared, and the final volume of the buffer stock solution was adjusted. This method allows for precise assessment of phosphatase activity and provides valuable insights into *A. niger's* enzymatic capabilities in nutrient cycling.

Quantitative estimation of phosphate solubilization. The Phosphate solubilization studies were performed in 100 mL of PVK liquid medium in Erlenmeyer flasks, which were supplemented with 5% (w/v) rock phosphate. The concentration of rock phosphate used in the study was equivalent to "P" 10,000 g/mL. The conditions used for the solubilization study were the same as those used for TCP solubilization [24]. The amount of Pi released was then estimated using the molybdate-ascorbic acid method [25]. Quantification of phosphate release was performed was conducted spectrophotometrically using the chlorostannous-reduced molybdophosphoric acid blue method, as described by Jackson [26] and Thakur [27]. The amount of P-solubilized was expressed as equivalent phosphate (µg/mL).

2.2.10. Phosphate Solubilization and pH in PVK Broth

Before sterilization, the initial pH of the medium was adjusted to 7.0. The spore suspensions for each fungal isolate were prepared using the method described by Elias [5]. In the study, each sterilized conical flask containing PVK broth was inoculated with 10 ml of spore suspension for the fungal culture (with a spore concentration of 10^7 spores/ml). The control was treated with 10 ml of sterile distilled water in sterilized PVK broth. Three replicates were prepared for each fungal isolate. The cultures were incubated for seven days at 28°C in a static environment on a rotary shaker (Sanco, India) at 130 rpm.

3 Result and Discussion

3.1 Isolation of most potent RP solubilizing fungi

About 34 fungal isolates with different concentrations of rock phosphate were isolated. Seven isolates with 5 g/l rock phosphate, six isolates with 10 g/l, eight isolates with 20 g/l, eight isolates with 30 g/l, and five isolates with 40 g/l. The isolates obtained in the study were purified on PDA agar media to obtain pure isolates for further analysis. A total of 14 isolates were obtained, purified, and screened for their ability to solubilize rock phosphate. The final count of purified isolates was as follows: Two isolates were obtained from a rock phosphate concentration of 5 g/L (R2 and R5), two isolates were obtained from a rock phosphate concentration of 10 g/L (R9 and R10), Four isolates were obtained from a rock phosphate concentration of 20 g/L (R3, R4, R8, and R13), Three isolates were obtained from a rock phosphate concentration of 30 g/L (R1, R7, and R12) and Three isolates were obtained from a rock phosphate concentration of 40 g/L (R6, R11, and R14)

3.2 Estimation of rock phosphate decomposing fungi

The solubilization capacity of rock phosphate was evaluated in 14 fungal isolates using PVK media with different rock phosphate concentrations (5, 10, 20, 30, and 40 g/l) to explore the isolates' ability to thrive at higher rock phosphate concentration. It was observed that the growth diameter of isolates R3, R4, R5, R10, and R12 decreased with increasing rock phosphate concentration as shown in the table. (1). The process of solubilizing rock phosphate can benefit plants because it releases phosphorus, an essential nutrient for plant growth. Screening fungal isolates for their ability to solubilize rock phosphate is important because it can aid in identifying potential candidates for enhancing plant growth in phosphorus-deficient soils. Using different rock phosphate concentrations in the PVK media during the screening process can help determine the optimal concentration by promoting the solubilization ability of fungal isolates. In this case, the growth diameter of isolates R3, R4, R5, R10, and R12 decreased with increasing phosphate concentration, indicating that these isolates may have a limited ability to solubilize higher rock phosphate concentrations. Further studies can be conducted to explore the solubilization potential of these isolates under different conditions and concentrations.

Table 1: Fungal growth on different concentration of rock phosphate (g/l). (-): to no growth appears on the agar plates supplemented with different RP concentration.

Isolate's code	Diameter of fungal growth on different rock phosphate concentration (cm)				
	5 g/l	10 g/l	20 g/l	30 g/l	40 g/l
R1	1.5	1	1	0.5	-
R2	2	1.5	1	1	1
R3	2	2	1	1	1
R4	0.3	0.3	0.2	-	-
R5	2	-	-	-	-
R6	5.5	5.5	5.5	3	3
R7	7	6.5	3	1	1
R8	6.5	5	5	3	2
R9	3	3	2.8	2	1
R10	0.5	0.5	-	-	-
R11	9	7	5.5	4	2
R12	-	-	-	-	-
R13	9	8	8	8	8
R14	9	9	9	8	8

3.3 Screening for most potent Phosphatase producers on PVK with rock phosphate

Nine fungal isolates demonstrated noteworthy phosphate-solubilizing activity up to a concentration of 40 g/L. The rock phosphate (RP) concentration was subsequently increased to a higher level, reaching 50 g/L to assess the ability of the isolates to continue their phosphate-solubilizing activity at an elevated level of rock phosphate, potentially providing insights into the fungi's adaptability and effectiveness in solubilizing phosphorus from more concentrated sources. The results indicated that R11 had the highest activity, which was visible after 24 hours of incubation and increased with time as in Table. (2). R2, R9, R13, and R14 followed R11 in their activity for rock phosphate solubilizing, with their activity becoming apparent after 48 hours of incubation. The solubilization activity of these fungi on rock phosphate is depicted in Figure 1. The results of this study suggest that R11 may be a promising candidate for solubilizing rock phosphate at higher concentrations, as it demonstrated the highest activity even at a concentration of 50 g/l. Additionally, the delayed activity observed in R2, R9, R13, and R14 may be due to differences in their metabolic pathways or the time required for them to adapt to the higher concentration of rock phosphate.

Table 2. Effect of different time on isolates growth on 50 g/l rock phosphate. (-) indicate that no growth appears, (+) indicate that the growth begins to appear, (++) indicate that there is a moderate growth.

Isolate's code	Diameter of fungal growth on PVK media with 50 g/l rock phosphate per Time				
	24 hrs	48 hrs	72 hrs	96 hrs	7 days
R1	-	-	+	++	(2cm)
R2	-	+	++	++	(5cm)
R6	-	-	+	++	(8cm)
R7	-	-	+	++	7 cm
R8	-	-	+	++	8 cm
R9	-	+	++	++	9cm
R11	+	++	+++ (1cm)	++++ (2.5cm)	9 cm
R13	-	+	+	+	9cm
R14	_	+	+	++	9cm

3.4. Effect of irradiation on the efficacy of fungal isolates to solubilized rock phosphate

Nine fungal isolates, which demonstrated high activity in solubilizing rock phosphate up to a concentration of 40 g/l RP, were exposed to different doses of gamma radiation up to 2000 Gy. Subsequently, the growth rate of these isolates was evaluated at a concentration of 50 g/l RP after 7 days. The results indicated that R11 had the highest activity, and its activity increased with radiation exposure, even at high doses. The exposure of fungal isolates to gamma radiation can induce mutations and alter their characteristics, including their ability to solubilize rock phosphate [28]. The results of this study suggest that R11 may have a higher potential for solubilizing rock phosphate after exposure to gamma radiation. This finding may have practical applications in enhancing plant growth and soil fertility in phosphorus-deficient soils. However, further studies are needed to investigate the mechanisms underlying the increased activity of R11 and to evaluate the effects of gamma radiation on other aspects of fungal physiology and ecology.

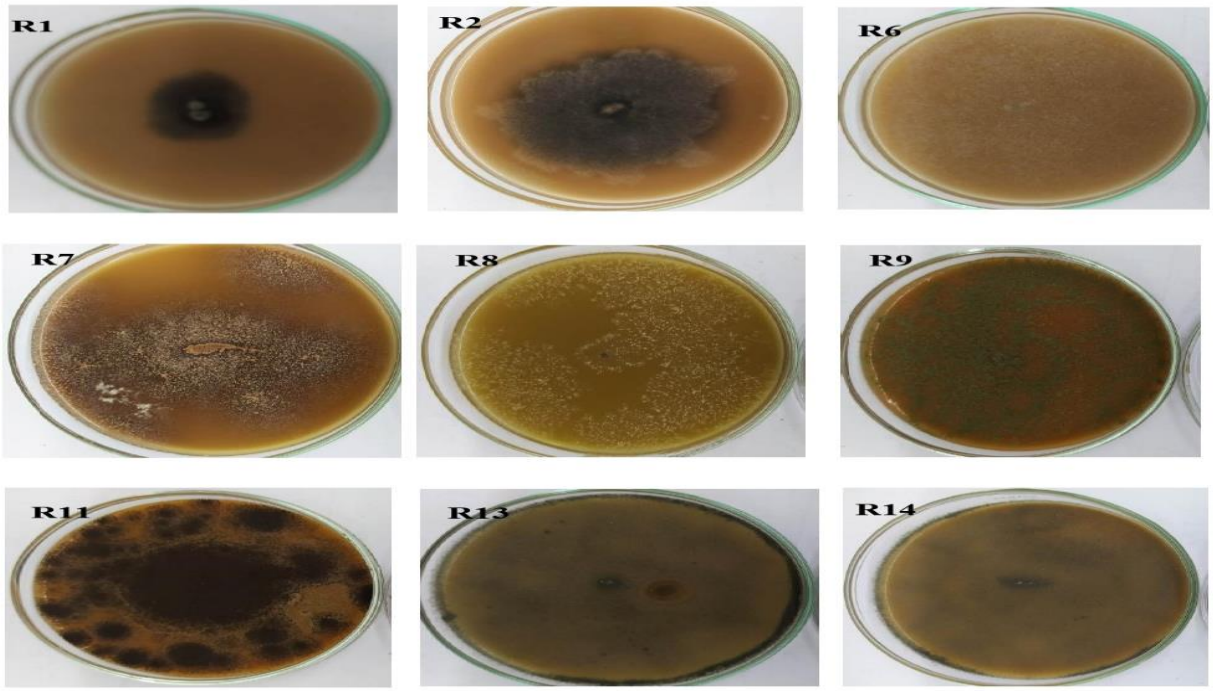

Fig. 1. Fungal isolates growth on RP (50 g/l).

3.5. Microscopic examination and molecular identification of most potent fungal isolate

The most effective and dominant isolate for solubilizing phosphate was R11, which was identified morphologically at the Mycological Center of Assiut University as *Aspergillus niger* Van Tieghem, following the classification method outlined by Moubasher [13]. The identification of R11 as *Aspergillus niger* Van Tieghem was based on morphological characteristics, which include the shape and size of the fungal structures such as the spores and hyphae. The classification method used to identify R11 follows the guidelines established by Moubasher [13], which is a widely used taxonomic system for fungi. *A. niger* exhibited a clear difference in its growth behavior, sporulation, and microscopic examination when grown on PDB media with or without rock phosphate. As shown in Figure 2, the addition of rock phosphate to the PDB media resulted in an increase in the rate of fungal growth and sporulation, and there was a clear difference in its microscopic examination.

These findings showed that when rock phosphate was added to the PDB media, A. niger exhibited a noticeable change in its growth behavior.

This suggests that the presence of rock phosphate positively influenced the fungus's overall development and reproductive processes. Microscopic examination of *A. niger* revealed clear differences between the two conditions. This indicates that the structural and morphological features of the fungus were influenced by the presence of rock phosphate and it is clear the high solubilization rate of RP in fig (2-b).

The microscopic examination likely provided insights into the cellular and structural changes associated with the interaction between *A. niger* and rock phosphate. The molecular identification of the isolated fungal strain relied on the sequence analysis of the internal transcribed spacer (ITS) sections of the nuclear-encoded rDNA. The obtained sequence exhibited 100% identity and98% - 100% coverage with various strains of *Aspergillus niger*, including the type material ATCC 16888T with GenBank accession no. AF138904; *P. chrysogenum* was used as the out-group strain. A. and P. denote *Aspergillus* and *Penicillium*, respectively. Genomic DNA extraction and PCR amplification of rDNA were performed for strain R11, and the resulting amplicons were sequenced. The phylogenetic tree (Figure. 3) constructed based on the alignment of the 5.8S region demonstrated a close evolutionary distance between isolate R11 and *Aspergillus niger*.

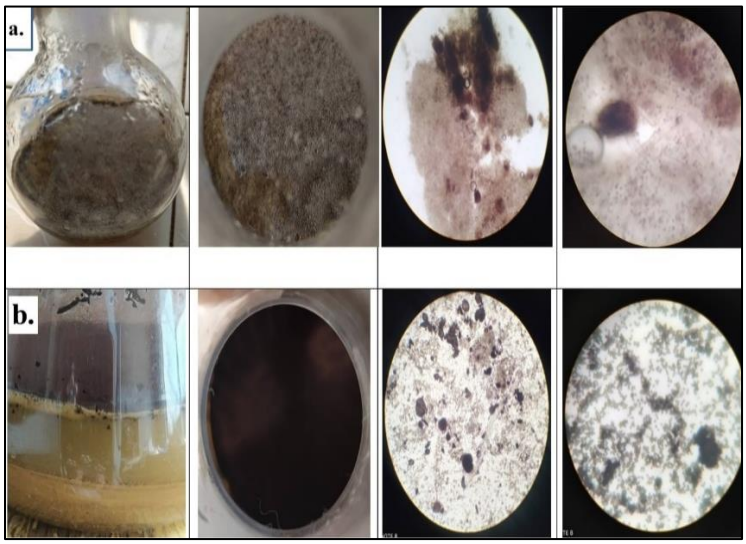

Fig.2: A. niger growth and microscopic examination on a: PDB without RP (control), b: PDB with RP 1000 g/l.

3.6 Determination of Solubilization Index on Solid Medium of A. niger

Among the isolates compared, *A. niger* was the only one that produced a clear zone on the inoculated plates after 48 h. of incubation, and the diameter of the zone increased with time as shown in Figure 4.

The solubility index (SI) of *A. niger* was found to be 1.26, and no significant changes in the SI were observed over time, according to data presented in Table 3. Comparing this result with another study by Elias [5], it was found that among the 167 phosphate-solubilizing fungal isolates screened, JUCaF38 (*Aspergillus* sp.) exhibited the most efficient phosphate solubilization on PV plates with an SI of 3.05, followed by JUHbF95 (*Aspergillus* sp.) with an SI of 2.87 and JUFbF59 (*Penicillium* sp.) with an SI of 2.39. The lowest SI of 1.10 was observed in the isolate JUHbPSF61 [5]. These findings suggest that the efficiency of phosphate solubilization by fungal isolates can vary widely, and factors such as the type of fungus and the

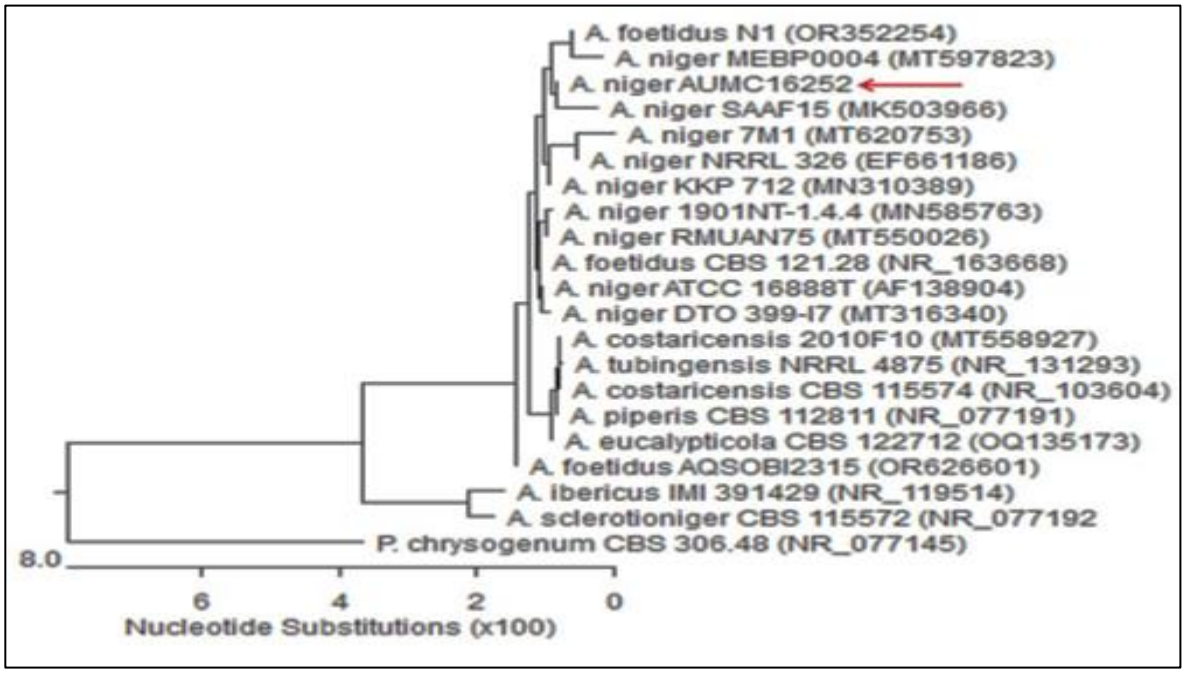

Fig. 3: Phylogenetic tree constructed using the ITS sequences of the rDNA of the fungal strain isolated in this wniger AUMC 16252) and closely similar sequences obtained from the GenBank

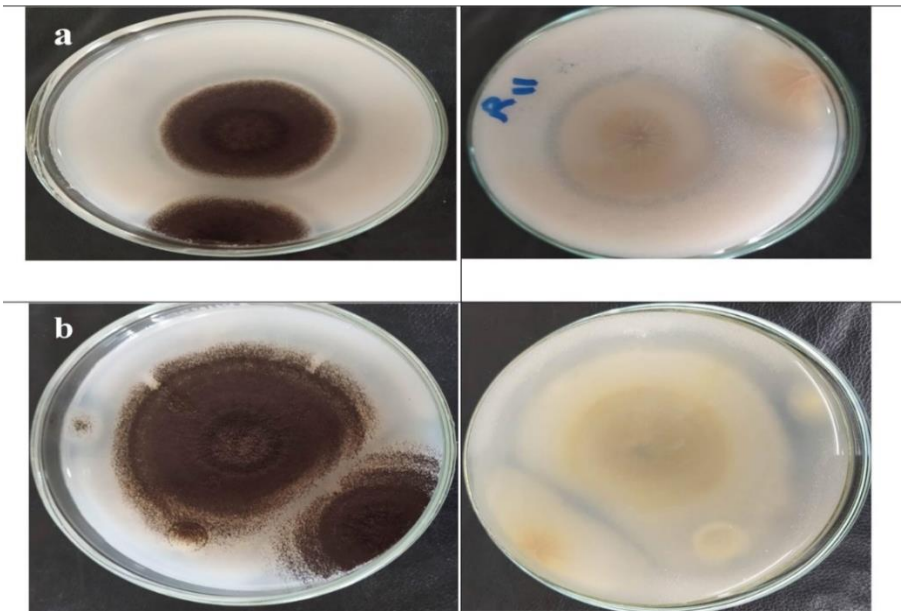

Fig. 4: A. niger growth on PVK Supplemented with Tricalcium phosphate as P source.

Table 3: solubility index of A. niger after 3 and 8 days.

SI	Colony diameter (cm)	Colony diameter+halozone diameter (cm)	Solubility Index (SI)
After 3 days	4	5	1.25
After 8 days	6.4	8.1	1.26

conditions of incubation can influence the solubilization capacity. Further studies can be conducted to explore the physiological and genetic mechanisms underlying phosphate solubilization by different fungal isolates, which can aid in the development of efficient and sustainable agricultural practices.

3.7 Determination of A. niger activity on different concentration of rock phosphate at different time

A. niger was the only isolate that produced a clear zone on the inoculated plates after 48 h. of incubation, and the diameter of the zone increased with time as shown in Figure 4. To thoroughly assess its effectiveness, *A. niger* underwent a comprehensive evaluation by being subjected to examination across a range of rock phosphate concentrations, varying from 50 to 400 g/L. This broad spectrum of concentrations allows for a more detailed analysis of how *A. niger* responds to and performs under different levels of rock phosphate, providing a more comprehensive understanding of its efficacy across a wide concentration range. Figure 5 clearly illustrates that the capability of A. niger to solubilize rock phosphate remained evident even at the highest concentration (400 g/L). Notably, *A. niger* showed its most robust growth and solubilization performance when exposed to 50 g/L of rock phosphate; however, sporulation was delayed at these concentrations, and only manifesting after 96 hours of incubation. Conversely, as the concentration of rock phosphate increased, sporulation was expedited, commencing after 72 hours of incubation. Figure 4 effectively depicts *A. niger's* various degrees of activity with growth evident after 48 hours and sporulation initiating after 72 hours, culminating at its zenith after 96 hours. The growth kinetics of *A. niger* across a spectrum of rock phosphate concentrations over time are elucidated in Figure 5. These findings highlight *A. niger's* ability to efficiently solubilize rock phosphate at greater concentrations, which increases as the incubation period proceeds.

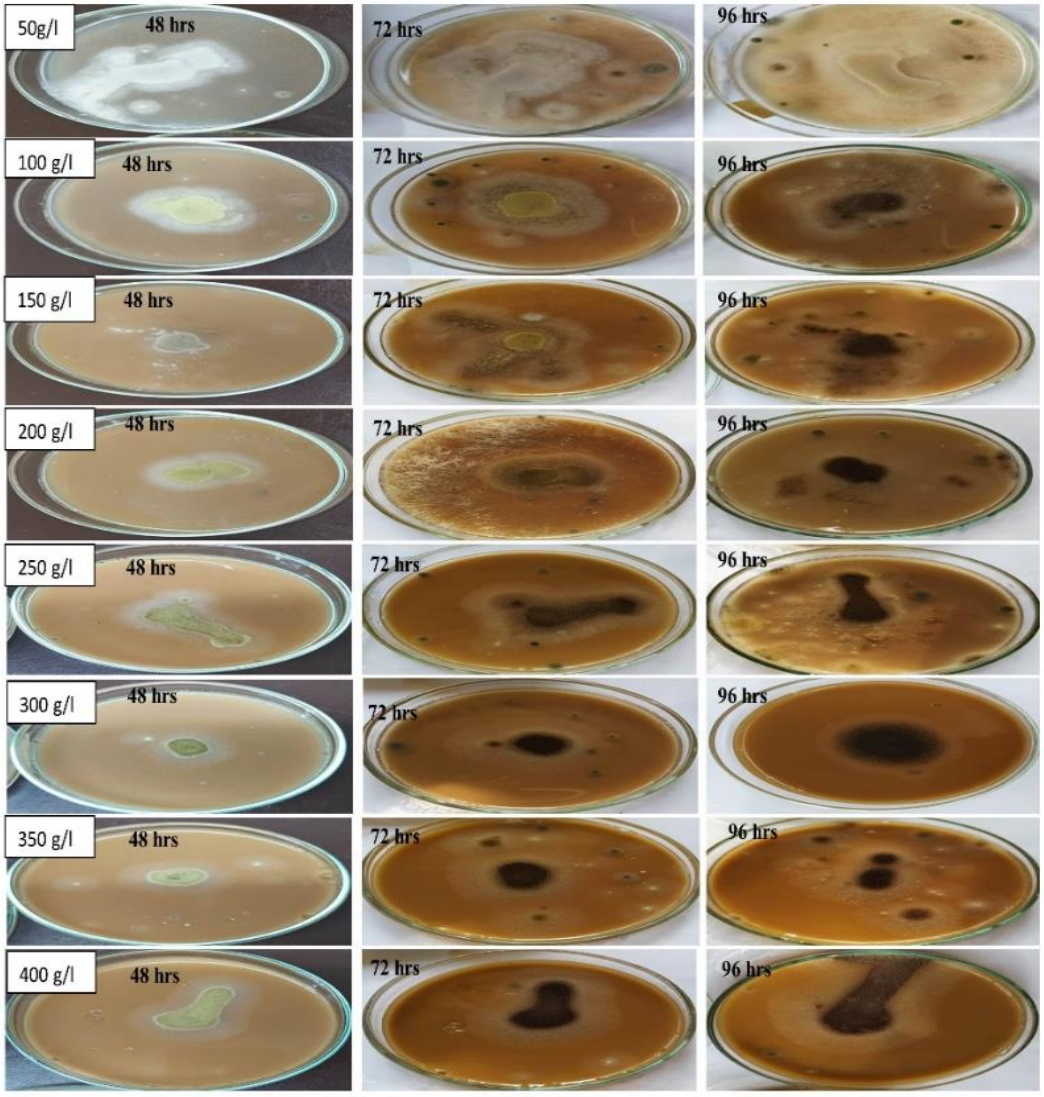

Fig. 5: A. niger growth on different concentration of RP from 5 to 400 g/l.

3.8 Improvement of rock phosphate solubilization

The optimal conditions for fungal isolates to solubilize phosphate were investigated by growing the isolates on PVK agar media under varying conditions until the maximum solubility index was simultaneously attained.

phosphate solubilization by different fungal isolates, which can aid in the development of efficient and sustainable agricultural practices.

The results are depicted in Figure 6. *A. niger* showed the highest solubility index when grown on PVK agar media adjusted to a pH of 6 (Fig. 6-a), supplemented with sucrose as a carbon source (Fig. 6-b), temperature of 30 °C (Fig. 6-c), and with yeast extract added as a nitrogen source (Fig. 6-d). The optimal conditions identified for *A. niger* can be used to increase its activity in solubilizing rock phosphate, which can contribute to improved soil fertility and plant growth in phosphorus-deficient soils.

3.9. P-Solubilization Efficiency of Selected Isolates in Liquid Media

3.9.1. Rate of RP solubilization

As reported in Table 4, fungal growth led to a high solubilization rate of both static and shaking conditions, considering RP wastes with 12.4% P2O5. The remarkable solubilization activity of *A. niger* is linked to its heightened enzyme activity. This aligns with De Oliveira Mendes ' findings, highlighting that *Aspergillus niger* demonstrates

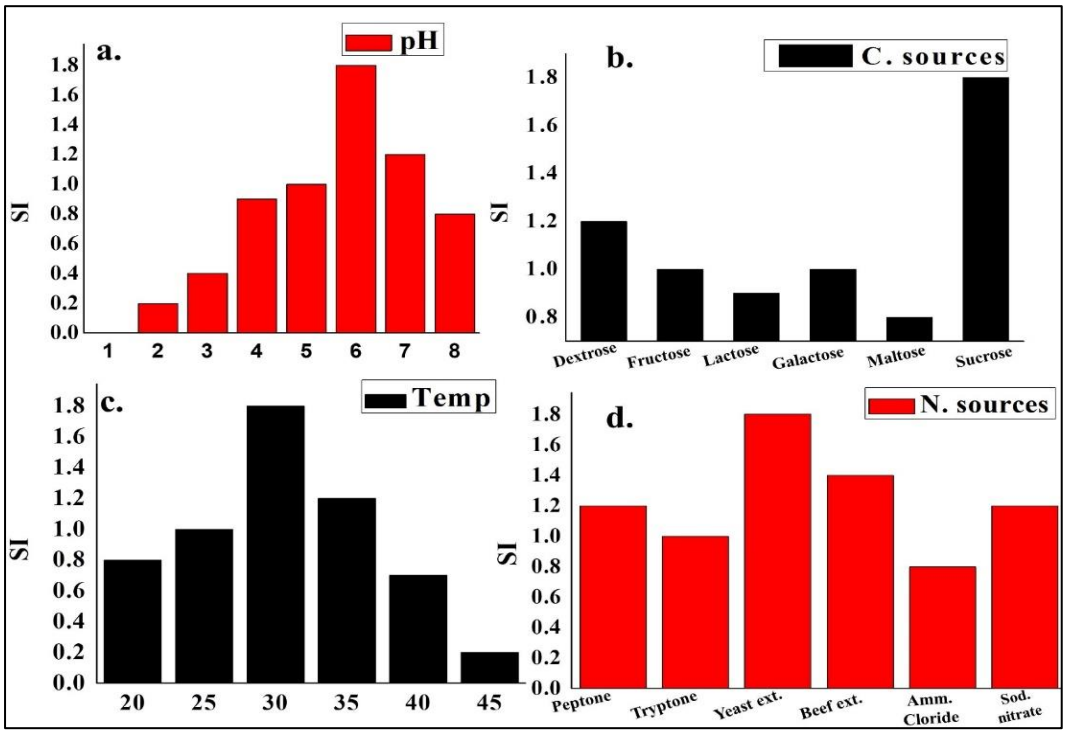

Fig. 6: Optimization of solubilizing index of A. niger.

Table 4: Phosphate concentration in RP waste before and after fungal growth.

Sample	*A. niger* growth			
	In filterate after fungal growth		In soil after Fungal growth	
	Static	Shaking	Static	Shaking
P2O5 (%)	32.07±0.01	21.67±0.01	2.00±0.08	2.00±0.09
P (%)	14.0	9.5	1.0	0.9
Solubilized P (g kg^{-1})	140.0	94.6	9.7	8.8
% of P_2O_5 under Static	34.3%			
% of P_2O_5 under Shaking	23.7%			

the highest phosphorus solubilization rate compared to all Penicillium species [29]. The obtained results reveal that *A. niger* has the capability to break down complex forms of phosphorus in the tested waste. This ability is attributed to the enzyme activity of *A. niger* and the positive changes observed during its growth (referred to as "beneficiation" in the rock phosphate and “solubilization” in the filterate). When analyzing the amount of rock phosphate (RP) solubilized by the fungal isolate, measured in terms of phosphorus oxide (P2O5), it was notably lower in the fungal filterate under shaking conditions (21.67 %) compared to static condition (32.07 %). he primary finding is that the highest concentration of soluble phosphate was detected in the liquid portion following fungal growth, with no significant difference in solubilized phosphate in the solid portion under both static and shaking conditions. Results show an increase in solubilized phosphate as P2O5, rising from 12.4% to 23.7% under shaking conditions and reaching 34.3% under static conditions after fungal growth in both tested portions. The fungal isolate demonstrated greater activity under static conditions compared to shaking conditions, with the highest value of solubilized phosphate concentrated in RP after fungal growth under static conditions **140.0 (g kg^{-1})**. The results indicate that the solubilization rate exceeded 100% under both static and shaking conditions. This phenomenon can be attributed to microbial activity, particularly phosphate-solubilizing microorganisms, which play a crucial role in releasing phosphate from rock phosphate. Several researchers have quantitatively studied the ability of phosphate-solubilizing microorganisms (PSM) to solubilize insoluble phosphate in pure liquid culture medium. The microbial solubilization of soil phosphorus in a liquid medium is often attributed to the excretion of organic acids [30]. These microorganisms can enhance the solubilization process, potentially leading to a solubilization rate greater than 100% There was a further increase in the value of solubilized phosphate, as reported by [29], for *A. niger* (1097 mg L^{-1}). These findings imply

that the solubilization activity of the fungal isolate is influenced by the growth conditions, with a suggestion that static conditions may be more favorable for its activity. similar findings was obtained by Elias, where all fungal isolates exhibited a significantly ($p < 0.05$) higher amount of phosphate solubilization compared to the uninoculated control [5]. In our prior research on streptomyces, it was observed that the phosphorus percentage (%P) in fine RP samples exhibited a rise of 12.3% for the room temperature sample and 20.87% for the 28 ºC sample after growth. Conversely, supernatants cultivated under all conditions maintained a consistent concentration of 0.344%P [31].

3.9.2. Acidic and alkaline Phosphatase activity

The assay of acid and alkaline phosphatases was carried out using the method described by Soumare., et al [32]. Table 5 shows the ability of the fungal isolate to produce acidic and alkaline phosphatase activity under static and shaker conditions after 3 and 8 days. The data presented in Table 5 demonstrate that *A. niger* exhibited acidic and alkaline phosphatase activities under all tested conditions. However, the isolate showed its highest ACase and ALase activity after 3 days of incubation under static conditions in fungal cells, with more alkaline phosphatase activity than acidic. Obviously, growth at room temperature for 3 days under static conditions contained the most acidic (21.00 µg p-nitrophenol g^{-1} ml^{-1}) and alkaline (23.21 µg p-nitrophenol g^{-1} ml^{-1}) activities, while the activity decreased significantly by 8 days. This is because the fungal phosphatase activity gives rise to a polyphosphate kinase accountable for the, undergoes hydrolysis, leading to the organic release of exopolyphosphates [33]. These findings are consistent with the results reported by Soumare, who indicated that the secretion of acid and alkaline phosphatase indicated that P18, BC10, and BC11 strains would be able to mineralize organic P sources from root exudates [32].

3.10. Relation between Phosphate Solubilization and pH in PVK Broth

The fungal isolate was observed to secrete phosphatase from the initial day of incubation, with levels increasing gradually until the third day. During this period, the pH of the *A. niger* medium decreased from an initial value of 7.01 and continued to decrease in the presence of RP in the PVK broth, reaching 5.02 on a shaker and 5.07 under static conditions by the third day, and varying levels of decrease were observed during the fifth and seventh days of incubation. In a study by Doilom., et al [34], it was found that all fungal strains released phosphate on the first day and showed an increasing trend as compared to the control. The fungal isolate KUMCC 18-0196 exhibited the most significant reduction in pH, from an initial value of 7.01 to 3.33 ± 0.01 on the seventh day of incubation, which was markedly different from the control and other fungal strains [34]. In liquid medium, microbial solubilization of soil phosphorus is frequently attributed to the excretion of organic acids by phosphate-solubilizing microorganisms (PSM). Organic acids produced by PSM, such as oxalic acid, lower pH, chelate cations, and compete with phosphate for adsorption sites in soil. Notably, *Aspergillus niger* ATCC 1015 produced the highest amount of oxalic acid, reaching 155 mM [35]

Table 5: Acidic and alkaline Phosphatase activity of *A. niger*.

Phosphatase activity (µg p-nitrophenol g^{-1} ml^{-1})							
Acidic Phosphatase				**Alkaline Phosphatase**			
After 3 days		**After 8 days**		**After 3 days**		**After 8 days**	
Shaking	**Static**	**Shaking**	**Static**	**Shaking**	**Static**	**Shaking**	**static**
2.34±0.11	21.00±1.3	1.18±0.0	4.77±0.6	2.53±0.0	23.21±0.22	1.46±0.01	5.83±0

4 Conclusion

In conclusion, fourteen isolates were tested for their capacity to solubilize rock phosphate (RP). Among these, *A. niger* demonstrated the highest activity across different

concentrations of RP, ranging from 5 to 400 g/L, providing valuable insights into the fungus's behavior and effectiveness. Additionally, when exposed to gamma growth, sporulation, and microscopic characteristics at different RP concentrations contribute to a comprehensive understanding of *A. niger's* adaptive capabilities.

prolonged accumulation of inorganic phosphate polymers specifically polyphosphate. This polyphosphate polymer The notable changes observed in fungal behavior indicate that *A. niger* may possess the ability to adapt and perform optimally under different environmental phosphorus conditions. The adeptness of *A. niger* in biobeneficiation is evident not only in its ability to yield the highest value of solubilized phosphorus but also in the clear formation of different layers during fungal growth on a concentration of 1000 g/L. This information is crucial for applications in agriculture or environmental management, where the solubilization of phosphates is of significance. Further studies could delve into the specific mechanisms underlying *A. niger's* response to varying RP concentrations, enhancing our understanding and potentially leading to practical applications in sustainable resource management.

Abbreviations

PVK: Pikovskaya medium

SI: solubilization index

PSF: Phosphate solubilizing fungi

P: phosphorus

RP: Rock phosphate

PDA: Potato Dextrose Agar

A. niger: *Aspergillus niger*

ACase: Acidic phosphatase

ALase: alkaline phosphatase

Funding: Not applicable.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests

Authors Contributions

The description given details each author's contribution to the research project. **Rofaida S. Abdelkader** carried out experimental methods, identified data, deposited it in GenBank, produced the first draft, and edited the text. **Susan E. Weesa** contributed to data representation and analysis. **Mohamed G.A** proposed the research topic, planned and designed the study. All authors read and approved the final manuscript.

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