EGYPTIAN ACADEMIC JOURNAL OF
BIOLOGICAL SCIENCES
MICROBIOLOGY
G

ISSN
2090-0872

WWW.EAJBS.EG.NET

Vol. 16 No. 2 (2024)

Citation: *Egypt. Acad. J. Biolog. Sci. (G. Microbiolog) Vol.16 (2) pp.49- 79 (2024)*
DOI: 10.21608/EAJBSG.2024.371575

Egypt. Acad. J. Biolog. Sci., 16(2):49-79 (2024)

Egyptian Academic Journal of Biological Sciences
G. Microbiology

ISSN: 2090-0872
https://eajbsg.journals.ekb.eg/

Biosolubilization of Rock Phosphate by *Streptomyces sp*. MMA-NRC isolated from Rhizospheric Soil and Assessment of Ability on Wheat Growth Promotion: Insights from Genetic Improvement Random Mutation Induction Approach

Nagwa M. Abd El-Aziz[1], Nora N. El-Gamal[2], Maysa E. Moharam[2], Sayeda A. Abdelhamid[3] and Nashwa M. El-Sheikh[4]

[1]Microbial Genetic Department Biotechnology Research Institute, National Research Centre, 33 El Buhouth ST, Postal code 12622, Dokki, Cairo, Egypt.
[2]Microbial Chemistry Department, Biotechnology Research Institute, National Research Centre, 33 El Buhouth ST, Postal code 12622, Dokki, Cairo, Egypt.
[3]Microbial Biotechnology Department, Biotechnology Research Institute, National Research Centre, 33 El Buhouth ST, Postal code 12622, Dokki, Cairo, Egypt.
[4]Soils, Water and Environment Research Institute, Agricultural Research Centre, Giza, Egypt.
*E.Mail: nagwamkh@gmail.com

ARTICLE INFO
Article History
Received:2/7/2024
Accepted:3/8//2024
Available:7/8/2024

***Keywords*:**
Phosphatase, *Streptomyces*, mutagenesis, RSM, biofertilizer.

ABSTRACT

In this study, we examined 50 bacterial strains collected from five different rhizospheres of cultivated wheat plants and assessed their phosphatase activity. The samples were cultured for a duration of 3 and 7 days in NBRIP medium, with rock phosphate (RP) being utilized as the source of phosphate. The best isolate exhibiting the most activity was identified as isolate no. 19, the best-exhibiting activity which recorded a value of 24.27 and 35.44 mg L^{-1} after 3 and 7 days of incubation, respectively. The strain was molecularly identified as *Streptomyces sp*. MMA using PCR and sequencing of the 16Sr DNA gene, which was entered under accession number OR770185 in the NCBI database. Sequential mutagenesis was performed using ethidium bromide (EtBr) and acridin orange (AO). Several mutants were generated, and the most efficient phosphate solubilizing mutant, designated as mutant *Streptomyces sp.* AO-31 exhibited phosphatase activity of 60.12 mg L^{-1}. Response Surface Methodology (RSM) was applied to *Streptomyces sp.* mutant AO-31. The highest phosphatase-specific activity of 76.55 mg L^{-1} was achieved under optimized culture conditions of temperature 37°C, pH 7, sucrose of 1.5%, yeast extract of 1.5%, 5 days of incubation, and a rock phosphate (RP) concentration of 7 g/L. Treatments of biofertilizer of rock phosphate with mutant strain *Streptomyces sp.* AO-31 showed an increase in dry matter yield of wheat plants, P content in wheat plants, P uptake of wheat plants and available soil phosphorus after harvesting, compared to the addition of rock phosphate with wild-type strain *Streptomyces sp.* MMA-NRC, and the addition of rock phosphate without the addition of biofertilizers.

Citation: *Egypt. Acad. J. Biolog. Sci. (G. Microbiolog) Vol.16 (2) pp.49- 79 (2024)*
DOI: 10.21608/EAJBSG.2024.371575

INTRODUCTION

Phosphorus (P) is a vital macronutrient that is crucial for the growth and development of plants. It plays a crucial role in fundamental plant metabolic activities such as cellular energy storage, photosynthesis, and respiration) Malhotra *et al.,* 2018). The principal growth-limiting nutritional component might vary depending on environmental and biological conditions.(Rajkumar and Kurinjimalar, 2021). Nevertheless, the concentration of soluble phosphorus in soil is often modest, ranging from 0.4 to 1.2 grams per kilogram. (Joe *et al.,* 2018). The majority of soil phosphorus (about 95–99%) exists in insoluble forms, rendering it inaccessible for plant utilization. (Mara *et al.,* 2014). Historically, farmers have utilized both organic and inorganic fertilizers to address nutrient deficits and ensure optimal nutrient levels in their fields. (Tiwari *et al.,* 2020). Soil P is distinguished by its limited movement in comparison to other crucial nutrients like nitrogen or potassium. Plant nutrition models have consistently demonstrated that the sluggish diffusion of inorganic phosphate (Pi) is a significant constraint on plants' ability to acquire P. (Barber, 1995). This implies that plants require supplementary mechanisms to obtain phosphorus (Pi) when there is a scarcity of phosphorus in the soil, as their roots can only access a small portion of the total phosphorus present in the soil. Despite the application of chemical fertilizers to soils, plants can only utilize limited quantities of phosphorus (P) fertilizer due to the intricate nature of P in promoting the growth of fragile soil structures. A significant amount of phosphorus (P) provided as chemical fertilizer is lost from the plant-soil system due to its interaction with calcium ions (Ca2+) in calcareous soils, and aluminum ions (Al3+) and iron ions (Fe3+) in acidic soils. (Izhar Shafi *et al.,* 2020), Hence, around 80% of the applied phosphorus (P) becomes inaccessible to the plant. (Salvagiotti *et al.,* 2017). However, the excessive and illogical application of nutrients in agroecosystems can lead to a detrimental environmental impact. This issue has gained the attention of stakeholders throughout the food value chain. (Choudhury and Kennedy, 2005). FAO reports that agriculture utilizes around 175.5 million tons of chemical fertilizers to achieve optimal agricultural productivity and satisfy the growing demands of habitats. The majority of phosphate fertilizers are produced by utilizing rock phosphate (RP) as the primary source of P2O5. Global rock phosphate supply is constrained despite rising demand. (Suleman *et al.,* 2018). The significant limitation in directly using RP as a soil amendment is its extremely low solubility. (Baig *et al.,* 2012). Nevertheless, P forms that cannot dissolve, such as tricalcium P [Ca3 $(PO_4)_2$], aluminum P ($AlPO_4$), and iron P ($FePO_4$), can be made soluble by organic acids, phosphatase enzymes, and complexing agents created by soil microorganisms that live in various soil habitats. (Vaishampayan *et al.,* 2001). Multiple studies have documented the utilization of microorganisms to dissolve insoluble phosphate compounds as an alternative approach to phosphate fertilizers. (Adnan *et al.,* 2020). Using these telluric competent strains is an agroecological method that improves agricultural fertility and reduces farmers' reliance on inorganic fertilizers, hence enhancing soil health. (Janati *et al.,* 2021). Over the course of many years, agricultural microbiologists have researched the capacity of specific bacteria to break down P fertilizers and P chelated in soil, in line with sustainable practices. With the intention of achieving this goal, (Behera *et al.,* 2017) It has been observed that the ability of PSB to dissolve P from soil or fertilization activities in agricultural fields is strongly influenced by their release of organic acids, including citric, formic, oxalic, lactic, acetic, and malic acids. Furthermore, PSBs have the ability to enhance the availability of phosphorus in plants by altering soil

activities in the rhizosphere. This results in the provision of vital nutrients to plants and the creation of growth regulators in different agricultural systems. (Adnan *et al.,* 2020; Islam *et al.,* 2021). Additionally, PSBs have the ability to generate growth-promoting hormones like Gibberellins and Indol Acetic Acid (IAA), which have a positive impact on plant growth. (Khan *et al.,* 2020). At present, the majority of bacteria categorized as PSBs are found within the *Pseudomonas*, *Serratia*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Bacillus*, *Rhizobia*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Acinetobacter*, and *Pantoea* genera (Seenivasagan and Babalola, 2021). Morocco possesses a substantial portion of the world's phosphorus reserves, accounting for 75% of the global supply. Additionally, it holds the top position as the largest exporter of phosphorus and its byproducts. The global market country's contribution exceeds 30% (Hakkou *et al.*, 2016). Morocco's strategic position in terms of commercial and technological aspects of phosphate extraction is not yet matched by a bio-industry that supports the biotechnology sector. This could potentially create opportunities for maximizing the utilization of soil phosphorus. While PSBs can offer agronomic advantages, their presence in the soil may not always be sufficient to outcompete other native bacteria (Acevedo *et al.,* 2014). Therefore, it is necessary to choose, describe, and identify these telluric bacteria in order to build sustainable cropping systems that both enhance crop productivity and promote economic-environmental sustainability.

The objective of this study was to isolate and genetically characterize previously unidentified bacterial strains that exhibit high efficiency in producing extracellular phosphatase. These cultures were capable of utilizing rock phosphate solid waste as the only carbon source in submerged fermentation conditions. Enhancement of the phosphate-producing chosen strain via appropriate chemical mutagenesis.

MATERIALS AND METHODS

Soil Sample Collection:

Soil samples were taken from five different rhizospheres of cultivated wheat plants from different localities in the Agricultural Research Centre (ARC), Giza, Egypt. Rhizospheric soil samples were carefully collected and placed in labeled sterile plastic bags and stored at –20°C before rhizobacteria isolation.

Culture Media, Isolation and Purification of Rock Phosphate Solubilizing Bacteria RPSB:

A soil suspension was created in order to extract RPSBs from a dilution cascade. To achieve this objective, 10 grams of soil were mixed with 100 milliliters of sterile phosphate-buffered saline (PBS) solution with a pH of 7. The mixture was then agitated at a speed of 190 revolutions per minute for a duration of 45 minutes. Afterwards, the solution was diluted in a series of steps, resulting in a final dilution of 10^{-6}. Approximately 100 µL of each dilution was applied onto National Botanical Research Institute Phosphate (NBRIP) agar plates, which consist of (g/L): glucose 10 g, $(NH_4)_2SO_4$ 0.1 g, $MgSO_4.7H_2O$ 0.25 g, KCl 0.2 g, and agar 15 g. The agar is supplemented with 5 g of rock phosphate (RP) and dissolved in 1000 ml of distilled water. The pH of the medium is adjusted to 7 for stability (Nautiyal, 1999). The NBRIP medium was then complemented with cycloheximide to inhibit fungal growth. Pikovskaya (PVK) agar plates, which consist of (g/100ml): 1g glucose, 0.5g rock phosphate (RP), 0.05g Yeast extract, 0.05g $(NH_4)_2SO_4$, 0.02g KCl, 0.01g $MgSO_4.7H_2O$, 0.02g NaCl, 0.0002g $FeSO_4$, 0.0002g $MnSO_4$, agar 15 g and the pH of the medium is adjusted to 7 for stability. NBRIP and PVK agar plates were incubated for 3 to 5 days at 37ºC (Shekhar Nautiyal, 1999). During this incubation period, the presence of obvious zones of solubilization around the colony facilitated the identification of PSBs. Each distinct

morphological colony type was isolated, purified, and subjected to five rounds of restriction from a single colony to ensure purification on NBRIP and PVK plates. These colonies were then identified as phosphate-solubilizing bacteria based on their growth on NBRIP media and morphological characteristics. The phosphate solubilizing bacterial strain obtained in this investigation was cultured on NBRIP agar slants for further analysis before cryopreservation in sterile glycerine (20%) at –80◦C. For actinomycetes preparations, Plate Count Agar medium (P.C) (Himedia, West Chester, Pennsylvania, USA) was utilized.

Quantitative Estimation of Phosphate Solubilizing Assessment Using Rock Phosphate:

The quantitative solubilization ability of RP by phosphate-solubilizing bacteria was tested in a liquid medium containing PVK and NBRIP. To do this, a preculture in plate count medium was prepared overnight and subsequently transferred to NBRIP and PVK broth medium. Following three and seven days of incubation at 37°C in an orbital shaker (180 rpm), 20 millilitres of the bacterial cultures were removed and centrifuged for fifteen minutes at 12,000 g. After being extracted, supernatants were measured for pH and their assimilable P content of the flasks was measured after the 3 and 7-day incubation periods. The control treatments were administered without inoculation in the same way. The available phosphorus was measured spectrophotometrically in accordance with Murphy and Riley, 1962, and extracted using 0.5 M $NaHCO_3$ at pH 8.5 (Olsen *et al.,* 1954). The ascorbic acid colorimetric method was used to determine the assimilable P concentration (Murphy and Riley, 1962). After a few minutes of incubation at room temperature, around 1 mL of supernatant was combined with 160 µL of a reaction solution, and the optical density (OD) was measured at 880 nm. The calculation of phosphate-accessible content was performed using a standard KH_2PO_4 P solution with concentrations ranging from 0 to 1 mg L^{-1}. A pH metre was used to measure the pH. The connection between the concentration of compound C (P[C]) and the pH level was consistently positive in all strains that exhibited high solubility of phosphorus (P). The correlation coefficients (R) for the relationship between P [C] and pH medium were higher than 0.84. Nevertheless, in several applications, the regression equations for the concentration of P [C] in relation to the pH of the medium were represented by linear models.

Morphological and Biochemical Characterization of Rock Phosphate Solubilizing Bacteria RPSB:

According to Bergey's Manual (Systematic Bacteriology, 2nd ed., vol. 5, eds., 2012), phosphate-solubilizing bacteria were characterised, and Gramme staining, cell morphology, and motility tests were carried out. Isolate was also tested for catalase, gelatinase, and urease activities. Experiments were repeated three times for isolation.

Molecular Identification of 16S rDNA Gene PCR Amplification of Bacterial Isolate:

We followed the instructions provided by QIAGEN's QIAamp DNA Mini Kit and the GeneJET Genomic DNA Purification Kit to extract the genomic DNA (Sambrook and Russell, 2001). Using forward primer 63f (5'- CAGGCCTAAC ACATGCAAGTC-3') and reverse primer 1387R (5'- GGGCGGWGTGTACAAGG C-3'), a polymerase chain reaction (PCR) was performed. applying the corresponding forward and reverse primers. For the amplification of a segment of the nuclear ribosomal gene cluster that contains the 16s rRNA gene (Singh and Saxena, 2010). Thirty cycles of denaturation at 95°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min/1 kbp were included in the PCR protocol. The final extension was conducted at 72°C for 5 min. On a 1% agarose gel, the PCR result was seen using a 100-bp ladder DNA marker (Invitrogen,

California, USA) (Altschul *et al.,* 1990). Subsequently, the PCR products that had been purified were subjected to sequencing, and using the basic local alignment search tool (BLAST) software obtained from the National Center for Biotechnology Information GenBank (www.ncbi.nlm. nih.gov/BLAST) the resulting sequence data were examined. All sequences were aligned with Clustal Omega that was obtained from: Clustal Omega < EMBL-EBI and Version 4 of the MEGA X programme was utilized to create phylogenetic trees (Tamura *et al.,* 2007; Rozewicki *et al.,* 2019).

Acridin Orange (AO) and Ethidium Bromide (Eth.Br) Mutagenesis:

For a period of three days, the wild-type *Streptomyces sp.* was cultivated on plate count broth medium at 37°C. After that, 10 millilitres of the culture were centrifuged at 9000 *xg* for ten minutes at 4°C to separate the cell biomass. Then, 10 millilitres of sterile saline solution (0.9%) were used to reconstitute the pellet of cellular biomass (Sanaa, 2018). Regarding acridin orange (AO) and ethidium bromide (EthBr). (Al-Awadi *et al.,* 2019; Akbar *et al.,* 2015; Akbar *et al.,* 2013; Kamalambigeswari *et al.,* 2018) added AO to the suspension to reach a final concentration of 60 mg and incubated at 37°C for 60 minutes in order to induce mutagenesis on the individual plates using 10 mg/ml EthBr. After every mutagenesis treatment, the specimen was cleaned with sterile saline solution, placed onto agar plates containing plate count media, and cells were collected by centrifugation at 2800 xg for 15 min. These plates were then incubated for three days at 37ºC. Then, in order to find mutants that manufacture phosphatase with high efficiency, surviving colonies were examined for phosphatase-specific activity (Duarte *et al.,* 2011).

Determination of the Optimal Conditions of Rock Phosphate Solubilizing Bacteria RPSB for Phosphatase Production:

The optimization of growing conditions for phosphatase production was carried out through two phases of statistical design research. The statistics software Design-Expert® 6.0.8. The experimental design and analysis software was developed by Stat-Ease, a company based in Minneapolis, MN, USA. Initially, we determined the most suitable carbon sources. (glucose, fructose, sucrose, arabinose, xylose, rafinose, lactose, galactose, mannitol and casein), optimal nitrogen sources (tryptone, peptone, yeast extract, malt extract and beef extract), the activity of this enzyme in 0.5% NBRIP medium was measured for phosphatase production, and it was incubated for three days at 37°C on a shaker (Parhamfar *et al.,* 2016). The second stage was to assess each independent variable using response surface methodology (RSM). These variables were temperature, pH, incubation duration, carbon source, and nitrogen source and rock phosphate concentration. A Box Behenken design was used to evaluate these aspects at two levels. presented in Table 1. The response of phosphatase activity was evaluated using 86 experimental designs (Gupta and Singh, 2013; Singh and Saxena, 2010). The equation $Y = \beta o+\beta 1X1+\beta 2X2+\beta 3X3+ \beta 11X12 + \beta 22X22 + \beta 33X32 + \beta 12X1X2+ \beta 13X1X3+ \beta 23X2X3$ was used to quantify phosphatase activity. Analyzing the variance and Fisher's F test ($p < 0.05$) proved the significance of each coefficient. We utilized three-dimensional (3D) contour plots to visually represent the quadratic models. Additionally, we conducted an analysis of variance (ANOVA) on the response surface methodology (RSM) data collected for phosphatase production. The experiments were conducted thrice. (Tiwary and Gupta, 2010).

Table 1: Experimental factors and level of minimum and maximum range for statistical screening using Box Behenken design.

Factors	Independent Factor	Unit	Type	Range Level		
				Minimum (−1)	Medium (0)	Maximum (+1)
X1	**Incubation time**	Days	Factor	3	5	7
X2	**pH**	+H	Factor	5	7	9
X3	**Sucrose (carbon source)**	% (w/v)	Factor	0.5	1.5	2.5
X4	**yeast extract (nitrogen source)**	% (w/v)	Factor	0.5	1.5	2.5
X5	**Rock phosphate concentration**	g/L	Factor	3	5	7
X6	**Temperature**	°C	Factor	30	37	44
X7	**phosphatase activity**	mg L^{-1}	Response			

Pot Experiments:

An experimental study was conducted in a controlled environment at the Agricultural Research Center experimental Station in Giza Governorate, Egypt, during the winter season of 2023/22024. The study involved the usage of pots. This study aims to assess the efficacy of bio-enriched rock phosphate as a fertilizer for direct application to clay soil, and its impact on the growth and nutrient absorption of wheat. The soil utilized in this experiment was obtained from the topmost layer (0-20 cm) of an agricultural experimental farm at the Agricultural Research Center, Giza. Table 2, contains several physicochemical parameters of soil. The pot experiment was conducted using plastic pots that were 40 cm tall and had a diameter of 30 cm. The pots were designed with a drainage aperture at the bottom. The experiment followed a completely randomized design (CRD). Every pot included 8 kilograms of dirt. The study utilized rock phosphate that was enriched with distinct microbial strains (mutant AO-31 or wild type MMA-NRC) at two levels: 1.2-ton ha-1 (equivalent to 6 g pot-1) and 2.4 ton ha-1 (equivalent to 12 g pot-1). The specific treatments applied in this investigation are listed in Table 2. Each pot was initially planted with 10 seeds of wheat (*Triticum aestivum L., Gemmeiza* 10 variety). After all the seeds had sprouted, the number of plants in each pot was reduced to 4. Following the application of all treatments, all pots were promptly irrigated. The control treatment involved the application of superphosphate (15.5% P_2O_5) at a rate of 476.19 kgha-1 (2.4 g pot-1) without the use of rock phosphate. Furthermore, two doses of ammonium nitrate (33.5% N) were administered at a rate of 866.32 kg ha-1 (4.3 g pot-1), along with potassium sulphate (48% K_2O) at a rate of 119 kg ha-1 (0.6 g pot-1). The initial dose was administered two weeks after planting, followed by a subsequent dose five weeks following planting. After a period of 120 days following planting, the plants were collected and subjected to drying in an oven at a temperature of 70°C for a duration of 72 hours. The dry matter of grains and straw (measured in grams per pot) was recorded, then finely pulverized and stored for chemical analysis of their nutrient content, which was subsequently computed as a percentage. In addition, the weights of 1000 grains (g) were documented. Furthermore, following the harvest, soil samples were obtained from each pot, dried in the air, and then crushed. The material was processed using a hardwood roller, filtered through a 2 mm screen, and then stored for further investigation.

Table 2: The chemical composition analysis of rock phosphate (RP).

Chemical composition	Rock phosphate (RP)
P_2O_5	28.6 %
SiO_2	7.4 %
MgO	0.59 %
CaO	44.8 %
$CaCO_3$	7.98 %
SO_3	0.58 %
pH	8.67
EC	3.47 dS m

Soil Analysis:

The pipette technique was employed to ascertain the particle-size distribution. The textural class was determined using the USDA textural class triangle. The soil organic carbon content was assessed using the modified Walkley and Black technique. The Collins calcimeter was used to quantify the percentage of inorganic carbonate concentration (% $CaCO_3$). The pH of the soil was determined by measuring the soil-to-water suspension in a 1:2.5 ratio using a digital pH meter. An electrical conductivity meter was used to evaluate the electrical conductivity (EC) of the soil-to-water extract at a 1:5 ratio. The microkjeldahl method was employed to determine the total soil nitrogen content. The available phosphorus was extracted by 0.5 M $NaHCO_3$ at pH 8.5 and spectrophotometrically determined. The available potassium was extracted with 1 N ammonium acetate at pH 7.0 and determined using the flame photometer. The experimental soil's physical and chemical properties were determined according to Klute (1986) and Page *et al.* (1982), as displayed in Table 3.

Table 3: Physical and chemical properties of soil under study.

Properties	Value	Properties	Value
Sand (%)	27.48	Available micronutrients (mg kg^{-1})	
Silt (%)	34.22	Fe	6.44
Clay (%)	38.30	Mn	5.59
Texture	Clay loamy	Zn	4.25
$CaCO_3$ gkg^{-1}	41.6	Soluble ions(meq/L)	
EC (dS m^{-1})	2.66	Ca^{++}	11.8
pH (1:2.5) susp.	7.82	Mg^{++}	9.2
Organic Matter (%)	2.11	Na^{+}	4.6
Available macronutrients (mg kg^{-1})		K^{+}	0.57
N	39.30	HCO_3^{-}	5.5
P	6.50	Cl^{-}	11.0
K	370	$SO^{—}_4$	9.67

Plant Analysis:

A 0.5 g sample of the dehydrated plant material was subjected to digestion using a mixture of sulfuric acid and hydrogen peroxide in a ratio of 20:5. The resulting digests were then analyzed for phosphorus using the same methods described for soil analysis.

Statistical Analysis:

The collected data underwent statistical analysis of variance (ANOVA) utilizing the least significant difference (L.S.D.) at a significance level of 0.05. according to Snedecor and Cochran (1980).

RESULTS AND DISCUSSION

Selection and Purification of Rock Phosphate Solubilizing Bacterial Isolates RPSB:

Soil samples were taken from five different rhizospheres of cultivated wheat plants from different localities in the Agricultural Research Centre (ARC), Giza, Egypt. The RPSB bacteria were isolated by selecting isolates that exhibited distinct morphological differences in colony appearance due to RP solubilization on NBRIP agar media. Colonies that were deliberately isolated to assess their ability to dissolve substances. At first, a total of 50 isolates were chosen. These isolates were then transferred onto the NBRIP medium three times to ensure their effectiveness and consistency. The phosphate solubilizing bacterial strain developed in this study was cultivated on NBRIP agar medium and identified as a rock phosphate solubilizing bacteria based on its growth on NBRIP media and morphological characteristics. The bacteria were subsequently subcultured on NBRIP agar slants for additional examination.

Quantitative Assay of Rock Phosphate Solubilizing Bacterial Isolates RPSB:

All of the 50 bacterial isolates were isolated, purified, and evaluated for their production of available phosphorus to solubilizing rock phosphate RP by the plate assay method in the phosphatase NBRIP and PVK agar medium with and without glucose addition, containing 0.5% rock phosphate (RP) after 3- and 7-days incubation at 37°C, as shown in Table 4. The findings revealed a wide range of phosphatase-specific activity. Production of the available phosphor of high efficiency isolates No. 19 in NBRIP medium without glucose addition at seven days incubation (35.44 mg L^{-1}) over 3 days incubation (24.27 mg L^{-1}), as shown in Figure 1. Production of available phosphor in NBRIP medium with glucose addition at seven days incubation (30.57 mg L^{-1}) over 3 days incubation (19.36 mg L^{-1}). Production of available phosphor in PVK medium without glucose addition at seven days incubation (31.49 mg L^{-1}) over 3 days incubation (20.73mg L^{-1}). Production of available phosphor in PVK medium with glucose addition at seven days incubation (27.18 mg L^{-1}) over 3 days incubation (18.58 mg L^{-1}). Results indicated that NBRIP medium without glucose addition was the high-efficiency phosphatase production medium. Then; the highest ability to fully break down 0.5% rock phosphate (RP) within 7 days incubation, which recorded 35.44 mg L^{-1} in NBRIP agar medium without glucose addition, for isolate No.19, as shown in Table 4. Besides, NBRIP medium pH inoculated with selected isolates strongly decreased from 7.00 to 3.82 (RP) after 7 days of incubation.

Table 4: Phosphatase activity mg/L^{-1} of isolate No.19 in NBRIP and PVK medium with and without glucose addition after 3 and 7 days incubation

Media	Phosphatase activity (mg L^{-1})	
	3 days incubation	7 days incubation
NBRIP medium with glucose	19.36	30.57
NBRIP medium without glucose	**24.27**	**35.44**
PVK medium with glucose	18.58	27.18
PVK medium without glucose	20.73	31.49

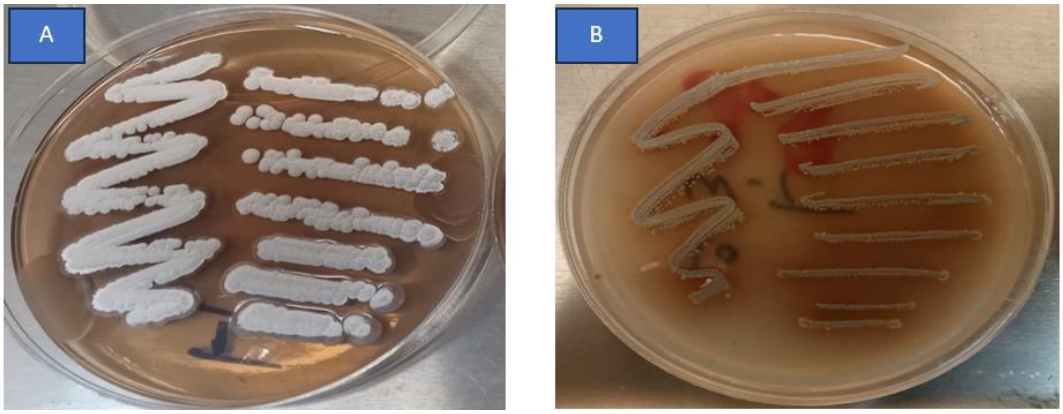


Fig. 1: Colony morphology of *Streptomyces sp.* MMA-NRC strain on **A;** plate count agar plate and **B;** NBRIP rock phosphate agar plate without glucose addition after 3 days incubation

Physiological and Biochemical Characterization Of Selected Rock Phosphate Solubilizing Bacterial Isolate RPSB:

The physiological and biochemical characteristics of isolate No. 19 were explained in Table 5, the results showed that isolate No.19 was able to hydrolyze gelatin and casein, wasn't able to hydrolyze starch, positive to catalase test and positive to citrate utilization, and couldn't grow at low temperature (4^{o}C) and could grow at pH 4. Isolate No.19 was negative to Voges proskauer test. Positive to nitrate reduction and urease production. The gelatin hydrolysis test indicates that isolate No.19 can produce proteolytic enzymes capable of breaking down proteins in a gelatinous substance. The results presented in Table 5, demonstrate the utilization of various carbon sources by isolate No.19. Isolate No.19 exhibited the capability to modify and adapt to various carbon sources, as well as the ability to modify and adapt to all nitrogen sources.

Table 5: Physiological, biochemical properties and utilization of carbon, and nitrogen sources of highly selected isolate No.19

Assay Parameters	**Isolate No.19**
Gram staining	+
Catalase test	+
Oxidase Test	+
Oxidative/Fermentative	Fermentative
Gelatin hydrolysis	+
Starch hydrolysis	-
Levan form	-
Voges Proskauer test	-
Arginine Dehydrolase	-
Growth on:	
NaCl 1.0%	+
NaCl 2.0%	+
NaCl 4.0%	+
NaCl 6.0%	+
NaCl 8.0%	+
NaCl 10.0%	+
NaCl 12.0%	+
Temperature of 4ºC	-
Temperature of 15ºC	+
Temperature of 26ºC	+
Temperature of 30ºC	+
Temperature of 40ºC	-
pH4	+
pH5.5	+
pH7	+
pH8.5	+
pH10	+
Growth on MacConkey agar	-
Casein hydrolysis	+
Citrate utilization	+
H_2S production	-
Indole production	-
Nitrate reduction	+
Methyl red test	-
Urease production	+
Carbon sources utilization and remodel:	
Glucose	+
Fructose	+
Sucrose	+
Arabinose	+
Xylose	+
Rafinose	+
Lactose	+
Galactose	+
Mannitol	+
Casein	+
Citrate Utilization	+
Nitrogen sources utilization and remodel:	
Peptone	+
Tryptone	+
Yeast extract	+
Malt extract	+
Beef extract	+

Note: -: negative reaction, +: positive reaction

Molecular Identification of Bacterial Isolates No.19 Using 16S rDNA Gene Alignment in Genbank (Blast) and Phylogenetic Tree Analysis:

The nucleotide sequence of the No.19 isolate's 16S rDNA gene has been recorded in GenBank with the accession number OR770185. To determine the likely identity of the strain, the obtained sequence was evaluated using BLAST searches with the "blastn" program on the NCBI website (www.ncbi.nlm.nih.gov/BLAST). The BLAST analysis indicated that this nucleotide sequence showed a 99% identity with *Streptomyces sp.*, based on sequence homology. The isolates were identified as *Streptomyces sp.* MMA-NRC strain, as shown in Figure 2.

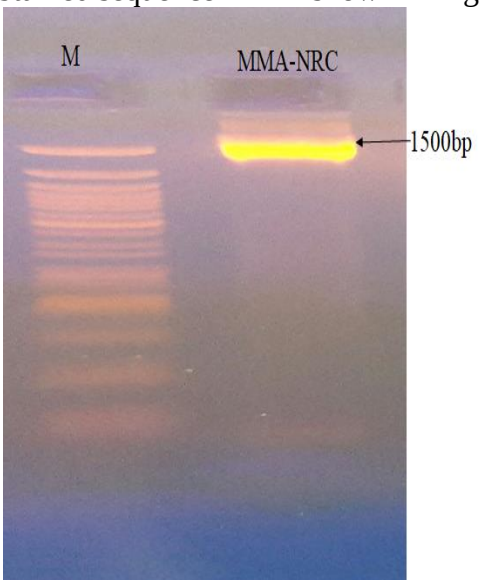


Fig. 2: Agarose gel electrophoresis of **lan1**: (M) marker 100bp; and **Lan 2**: amplified PCR *16s rDNA* (1500 bp) of *Streptomyces sp. MMA-NRC* .

The phylogenetic tree, based on the 16S rDNA gene sequence, revealed that isolate No.19 belongs to the same group and is closely related to *Streptomyces sp.* then named *Streptomyces sp.* MMA-NRC, as depicted in Figure 3.

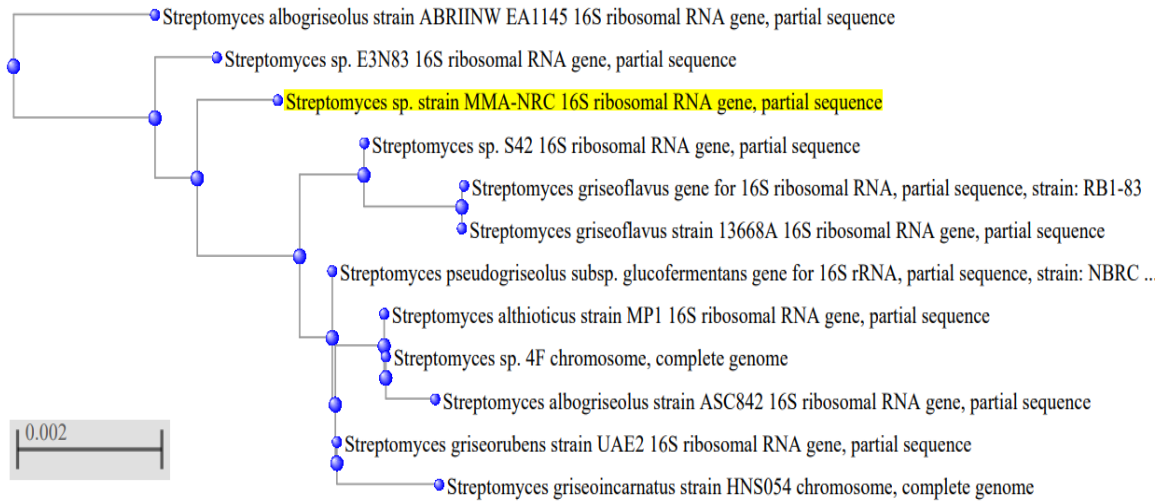


Fig. 3: Phylogenetic tree constructed based on 16S rRNA gene sequences of *Streptomyces sp.* MMA-NRC with other *Streptomyces* species obtained from GenBank database.

In 2022, Maharana and Dhal the top RP solubilizers among the 13 bacterial isolates were determined to be *Bacillus cereus* S0B4, *Solibacillus isronensis* S0B8, and *Bacillus amyloliquefaciens* S0B17 strains. The capacity of *S. isronensis* S0B8 to solubilize RP is reported for the first time in this paper. On the seventh day, the highly powerful strain *B. cereus* S0B4 had the highest level of soluble P (338.5 mg/L). Mayadunna *et al.*, 2023, Phosphate solubilization efficiency of several discovered isolates was examined in Pikovskava's (PVK) broth using TCP (5 g L^{-1}) and ERP (5 g L^{-1}) as P sources. To produce the biofertilizer, twelve microbial isolates with improved phosphate solubilization efficiency were chosen. A considerable quantity of accessible phosphorus (896.98 ± 10.41) mg L^{-1} was found in isolate F10 in ERP broth, while F5 (*Aspergillus sp*.) in TCP broth had the second-highest level at 991.43 ± 1.37 mg L^{-1}. According to Janati *et al.* (2022), 12 of the 64 isolated strains were deemed to be of potential biotechnological interest due to their P solubilization and strong tolerance to a variety of extreme environments. Additionally, with a PSI of 4.1, strain WJEF15 demonstrated the highest P solubility efficiency in NBRIP solid media; in contrast, strain WJEF61 was found to be the most effective strain in NBRIP-TCP liquid medium, releasing 147.62 mg.l^{-1} of soluble P. On the other hand, strain WJEF15 demonstrated maximum solubilization with 25.16 mg.l^{-1} in the NBRIP-RP medium. Ates, 2023, using TCP and RP as phosphate sources, 118 bacterial isolates with the ability to solubilize phosphate were cultured for 72 hours in the National Botanical Research Institute phosphate (NBRIP) medium. The medium's pH and inorganic phosphorus (Pi) content were measured at the conclusion of the incubation. After the bacteria were incubated, an average of 57.87 mg L^{-1} Pi was observed in the rock phosphate medium; in the TCP medium, this rate was determined to be 421 mg L^{-1}. The pH of the NBRIP media was found to vary between 3.60 and 5.05 after 72 hours of incubation, and TCP dissolved seven times more to rock phosphate.

***Streptomyces sp.* MMA-NRC Multistep Mutation Induction for Phosphatase Production Improvement**:

At the beginning of the mutation process, the wild-type *Streptomyces sp.* MMA-NRC strain was exposed to ethidium bromide (Eth.Br) at a concentration of 10 mg/ml for a period of 60 minutes. Subsequently, a grand total of fifty colonies that managed to survive were separated and examined for their phosphatase-specific activity. Out of all these colonies, only five mutants exhibited a significant level of efficacy in terms of their specific phosphatase activity. The findings indicated that the mutant E-19 exhibited the greatest level of activity, measuring 41.36 mg L^{-1} compared to the wild-type *Streptomyces sp*. MMA-NRC exhibited a phosphatase-specific activity of 24.27 mg/L after three days of incubation and pH decreased from 7 to 5.26.

During the initial stage of the mutation process, the mutant E-19 strain was subjected to acridin orange (AO) at a concentration of 60 mg for a duration of 60 minutes. Afterwards, a total of fifty colonies that successfully survived were isolated and analyzed for their phosphatase-specific activity. Among all of these colonies, only nine mutants demonstrated a notable level of effectiveness in relation to their unique phosphatase activity. The activity demonstrated mutant AO-31 a precise value of 60.12 mg L^{-1}, while the E-19 wild-type mutant displayed a specific activity of 41.36 mg L^{-1} and a reduction in pH from 5.26 to 3.67., as shown in Table 6 and Figure 4.

Table 6: Estimation of phosphatase-specific activity produced by strain *Streptomyces sp.* MMA-NRC and its mutants after 3 days incubation at 37°C.

Strain	phosphatase specific activity (mg L^{-1})	pH
First step mutation with ethidium bromide (Eth.Br)* mutagenesis		
Parent *Streptomyces sp.* MMA-NRC	**24.27**	**7.00**
Eth.Br mutants		
E-14	33.90	6.57
E-19	**41.36**	**5.26**
E-26	36.31	6.12
E-37	34.77	6.36
E-43	39.34	5.89
Second step mutation with acridin orange (AO) mutagenesis**		
Parent E-19	**41.36**	**5.26**
AO mutants		
AO-8	51.55	4.82
AO-12	53.73	4.65
AO-27	54.27	4.45
AO-31	**60.12**	**3.67**
AO-33	57.03	3.94
AO-39	51.27	4.84
AO-42	52.22	4.71
AO-45	52.60	4.70
AO-49	53.46	4.69

* EthBr 10 mg/ml concentration ** AO of 60 mg concentration.

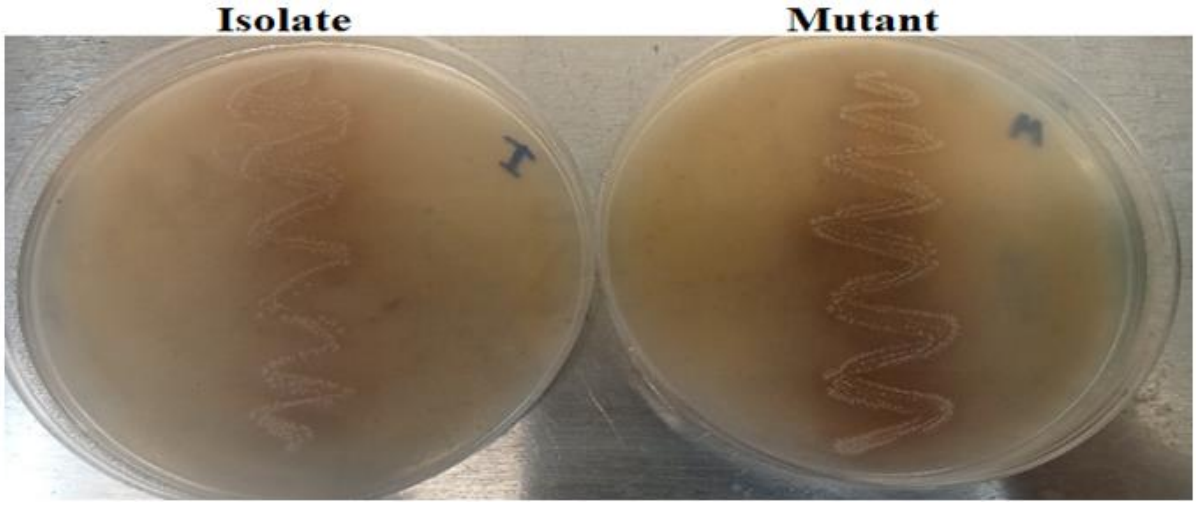


Fig. 4: Colony zone of strain *Streptomyces sp.* MMA-NRC and mutant *Streptomyces sp.* AO-31 on NBRIP rock phosphate agar plate without glucose addition after 3 days incubation

(Abd El-Aziz *et al.*, 2023a); *Streptomyces werraensis* strain KN23 was treated using a method called sequential mutagenesis. This involved exposing it to UV light, hydrogen peroxide (H_2O_2), and sodium azid (SA), which resulted in the production of several mutants. The SA-27 mutant, whose keratinase activity was 106.92 U/mL, was the most effective in terms of keratin breakdown. According to Khalil *et al.* (2022), a multistep mutation-induction procedure was used to create super-keratinase-producing mutants in the yeast *Pichia kudriavzevii* YK46 through mutagenesis induction using ethidium bromide (Eth.Br), UV light, and ethyl methane sulfonate (EMS). Based on mutagenesis results, EMS-37, a mutant with an activity of 211.90 U/ml, was identified as the best keratinolytic

efficiency mutant. Abd El-Aziz *et al.*, 2023c, *Rhodotorula mucilaginosa* PY18 was further increased by successive mutagenesis using UV, ethidium bromide (Eth.Br), and hydrogen peroxide (H_2O_2). This resulted in a mutant known as *Rhodotorula mucilaginosa* E54, which had a specific activity of 114.2 U/mg over the wild type 46.35 U/mg.

Screening of Significant Carbon and Nitrogen Variables:

In order to determine the optimal carbon and nitrogen sources for synthesizing phosphatase in a mutant strain called *Streptomyces sp*. AO-31, different carbon and nitrogen sources were examined. The findings revealed that the medium having a concentration of 24.65 mg L^{-1} of sucrose and 16.72 mg L^{-1} of yeast extract displayed the highest levels of phosphatase activity, as presented in Figure 5a and 5b**.**

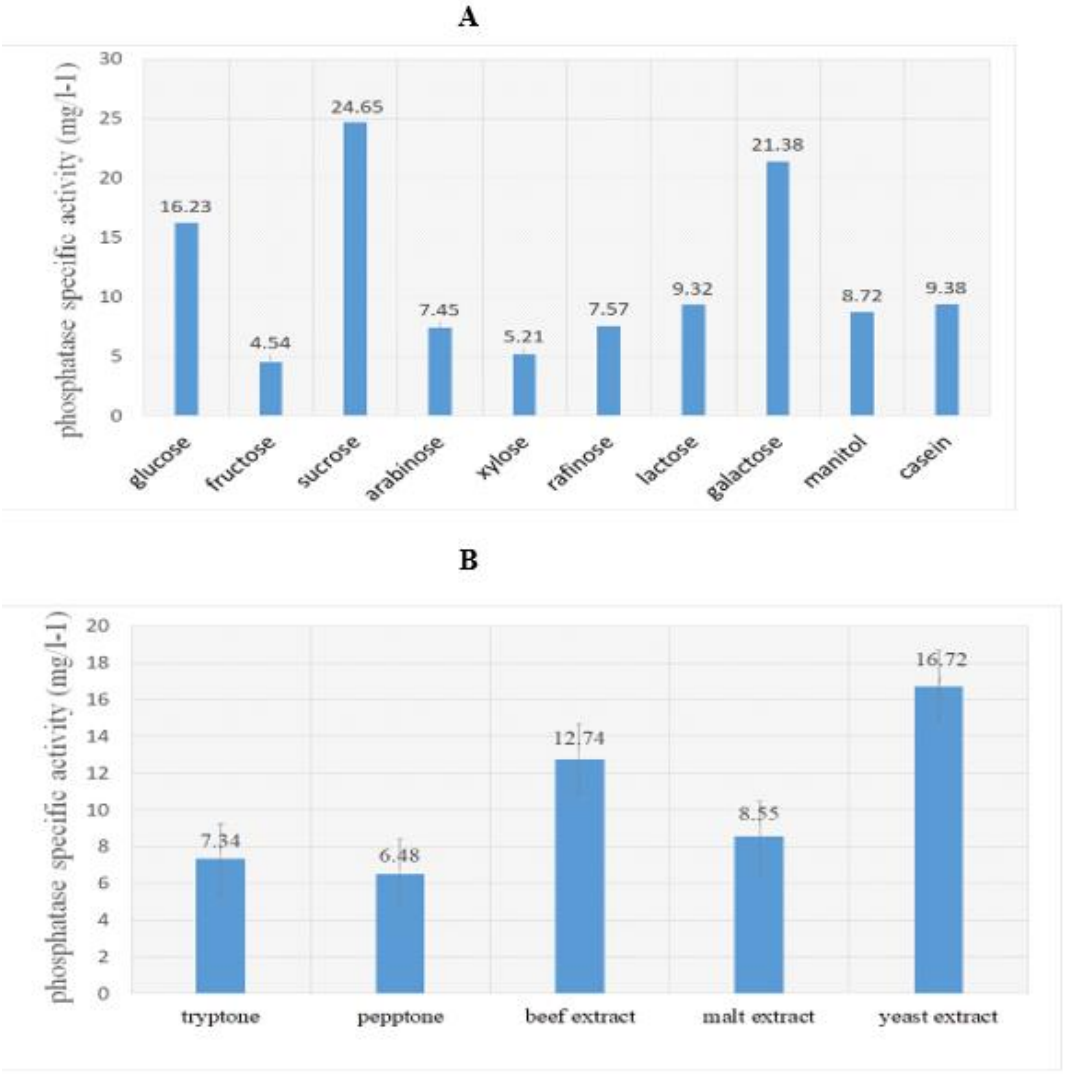


Fig. 5: Medium optimization conditions by supplementing different **A**; carbon sources. **B**; nitrogen sources of mutant *Streptomyces sp*. AO-31.

Optimizing Phosphatase Activity Through Response Surface Methodology (RSM):

To improve and maximize the production of phosphatase by a mutant strain *Streptomyces sp.* AO-31, it is crucial to analyze the complex data using response surface methodology (RSM). The work employed a central composite model to produce phosphatase by conducting an 86-run experimental design on a mutant strain of *Streptomyces sp.* AO-31. The matrix comprised six components, each with three levels (-1, 0, and +1), and there were three

replicas at the central point. Tables 7, 8, 9 and 10 showcase the self-governing components by employing a coded matrix and include the corresponding solutions, as well as the actual and predicted values for phosphatase activity. The enzyme activity exhibited variability over the 86 iterations of the experiment as a result of employing various settings in each iteration, underscoring the need to statistically optimize fermentation conditions over traditional methods. The culture conditions were tuned to maximize phosphatase activity by increasing the temperature to 37°C, pH to 7, sucrose concentration to 1.5% (as the carbon source), yeast extract concentration to 1.5% (as the nitrogen source), incubating for 5 days, and using a rock phosphate (RP) concentration of 7 g/L. The outcome was an activity of 76.55 mg L^{-1} (Run 56). The coefficient of determination (R^2) exhibited a high level of accuracy in the model, with a value of 0.9404.

The found correlation is statistically substantial, indicating that the current model for phosphatase synthesis is dependable. The equation expressing the final outcome, considering second-order components, can be stated as follows: The equation is Y = -47.90 + 3.47X1 + 9.03X2 + 7.90X3 + 8.60X4 - 1.77X1X2 - 2.07X1X3 - 1.27X1X4 - 3.37X2X3 - 3.30X2X4 - 1.73X3X4 - 2.63X1 squared - 4.23X2 squared - 4.57X3 squared - 4.37X4 squared. The variable Y indicates the reaction or phosphatase yield, while X1, X2, X3, and X4 correspond to pH, incubation time, xylose %, and malt extract percentage, respectively.

Table 7: The study focuses on developing various experiments using the response surface methodology to investigate the relationship between independent variables and responses in the mutant *Streptomyces sp* AO-31.

Run	Factor A: temp.	Factor B: pH	Factor C: sucrose	Factor D: yeast extract	Factor E: incu. time	Factor F: RP conc.	Actual Value phosphatase specific activity (mg L^{-1})	Predicted Value phosphatase specific activity (mg L^{-1})
1	44	9	0.5	0.5	7	7	64.24	65.23
2	37	7	1.5	1.5	7	5	73.36	74.21
3	37	7	1.5	1.5	5	5	75.22	74.65
4	44	9	0.5	2.5	7	7	65.54	65.48
5	44	9	2.5	2.5	7	3	66.03	65.28
6	44	5	0.5	0.5	7	3	67.37	67.83
7	30	5	2.5	2.5	7	7	67.83	68.83
8	30	9	0.5	0.5	3	3	65.11	64.86
9	30	9	2.5	0.5	7	7	65.95	65.45
10	30	9	2.5	2.5	3	3	64.15	64.37
11	37	7	1.5	1.5	5	5	74.25	74.65
12	30	5	2.5	2.5	3	3	68.85	67.85
13	44	9	0.5	2.5	7	3	66.47	66.27
14	44	5	2.5	2.5	3	7	66.67	67.61
15	37	7	1.5	1.5	5	5	75.28	74.65
16	30	5	2.5	0.5	3	3	68.66	68.11
17	44	9	0.5	0.5	3	7	66.32	64.73
18	44	9	2.5	0.5	3	3	64.94	64.89
19	30	9	2.5	0.5	3	7	64.64	64.40
20	37	7	1.5	1.5	5	5	72.24	74.65
21	44	7	1.5	1.5	5	5	74.88	74.44
22	44	9	2.5	0.5	7	3	65.52	65.71
23	30	5	0.5	0.5	7	3	67.41	67.96
24	44	5	0.5	0.5	7	7	67.49	67.67
25	37	7	1.5	1.5	5	5	75.32	74.65
26	30	9	2.5	2.5	7	7	66.29	65.27
27	44	5	2.5	0.5	7	3	69.45	68.88
28	30	5	2.5	2.5	7	3	68.48	68.90
29	44	9	0.5	0.5	7	3	66.26	65.80

30	30	5	0.5	2.5	3	3	68.46	67.93
31	37	7	1.5	1.5	5	5	73.35	74.65
32	44	5	2.5	2.5	7	7	69.99	68.16
33	30	7	1.5	1.5	5	5	74.33	74.62
34	44	9	2.5	0.5	7	7	63.26	65.32
35	37	7	1.5	1.5	5	5	75.41	74.65
36	44	9	0.5	2.5	3	3	64.56	65.44
37	44	5	2.5	0.5	3	3	68.73	68.38
38	30	5	2.5	2.5	3	7	67.24	67.92
39	30	9	2.5	2.5	3	7	64.96	64.02
40	37	7	0.5	1.5	5	5	74.65	74.41
41	37	7	1.5	1.5	5	5	73.27	74.65
42	30	5	0.5	0.5	3	7	66.66	67.39
43	44	5	2.5	2.5	7	3	67.48	68.35
44	44	9	2.5	0.5	3	7	64.04	64.63
45	37	5	1.5	1.5	5	5	69.48	68.83
46	30	9	2.5	0.5	3	3	63.27	64.54
47	44	9	2.5	2.5	3	3	65.39	64.26
48	30	9	2.5	2.5	7	3	65.67	65.75
49	44	9	2.5	2.5	7	7	64.35	64.67
50	37	7	2.5	1.5	5	5	74.32	74.41
51	30	5	0.5	2.5	3	7	69.69	67.81
52	37	9	1.5	1.5	5	5	65.37	65.87
53	44	5	0.5	2.5	7	7	67.33	67.83
54	44	5	0.5	2.5	3	3	67.12	67.70
55	44	9	0.5	2.5	3	7	66.03	64.79
56	37	7	1.5	1.5	5	7	76.55	75.43
57	44	9	2.5	2.5	3	7	63.23	63.79
58	37	7	1.5	0.5	5	5	75.29	74.24
59	44	5	0.5	2.5	7	3	69.66	68.20
60	30	9	2.5	0.5	7	3	66.73	65.73
61	37	7	1.5	1.5	5	5	75.93	74.65
62	30	5	2.5	0.5	3	7	67.74	68.39
63	30	5	0.5	2.5	7	3	69.88	68.79
64	37	7	1.5	1.5	5	3	74.72	75.69
65	44	5	0.5	0.5	3	3	66.69	67.52
66	30	9	0.5	0.5	7	7	66.67	65.40
67	30	5	2.5	0.5	7	7	69.16	69.11
68	30	5	0.5	0.5	3	3	67.43	67.29
69	30	5	0.5	0.5	7	7	68.21	67.92
70	30	9	0.5	2.5	3	7	63.26	65.06
71	44	5	0.5	2.5	3	7	66.77	67.46
72	30	5	0.5	2.5	7	7	67.64	68.54
73	30	9	0.5	0.5	3	7	63.38	64.54
74	44	5	2.5	2.5	3	3	66.62	67.66
75	37	7	1.5	1.5	3	5	74.44	73.44
76	37	7	1.5	1.5	5	5	75.48	74.65
77	44	5	2.5	0.5	3	7	69.82	68.54
78	30	9	0.5	2.5	7	7	66.47	66.11
79	37	7	1.5	2.5	5	5	73.33	74.23
80	30	9	0.5	2.5	7	3	65.04	66.79
81	44	5	0.5	0.5	3	7	67.11	67.50
82	44	9	0.5	0.5	3	3	65.34	65.16
83	30	9	0.5	0.5	7	3	66.35	65.86
84	44	5	2.5	0.5	7	7	69.39	68.90
85	30	5	2.5	0.5	7	3	68.21	68.97
86	30	9	0.5	2.5	3	3	66.24	65.59

Table 8: Fit summary

Source	Sequential p-value	Lack of Fit p-value	Adjusted R^2	Predicted R^2	
Linear	0.0820	0.0005	0.0632	0.0470	
2FI	1.0000	0.0002	-0.1434	-0.1755	
Quadratic	< 0.0001	0.7227	0.9127	0.8691	Suggested
Cubic	0.3489	0.7844	0.9183	0.7948	Aliased

Table 9: Sequential model sum of squares (Type I)

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Mean vs Total	4.036E+05	1	4.036E+05			
Linear vs Mean	156.03	6	26.00	1.96	0.0820	
2FI vs Linear	11.74	15	0.7829	0.0483	1.0000	
Quadratic vs 2FI	966.56	6	161.09	130.07	< 0.0001	Suggested
Cubic vs Quadratic	34.72	26	1.34	1.15	0.3489	Aliased
Residual	37.11	32	1.16			
Total	4.048E+05	86	4706.93			

Choose the polynomial with the highest degree that has substantial additional terms and is not aliased.

Table 10: Model summary statistics

Source	Std. Dev.	R^2	Adjusted R^2	Predicted R^2	PRESS	
Linear	3.65	0.1294	0.0632	0.0470	1149.52	
2FI	4.03	0.1391	-0.1434	-0.1755	1417.88	
Quadratic	1.11	0.9404	0.9127	0.8691	157.83	Suggested
Cubic	1.08	0.9692	0.9183	0.7948	247.54	Aliased

Focus on the model maximizing the **Adjusted R^2** and the **Predicted R^2**.

The Model Validation:

The suggested model's validity was assessed by calculating the phosphatase production of the mutant *Streptomyces sp.* AO-31 for each trial in the matrix. The data obtained from the experiment, as shown in Table 7, indicate that the highest phosphatase output recorded was 76.55 mg L−1, which nearly corresponds to the expected value of 75.43 in run 56. The following section, specifically Tables 11, 12, and 13, provides a detailed statistical analysis of the variance data related to the production of phosphatase by the mutant AO-31. The model had a robust statistical significance, as shown by an F value of 33.92. The confirmation was reinforced by Fisher's F test, which produced a probability value (P model > F) of 0.01. The studies demonstrated improved accuracy and reliability, as evidenced by 'Prob>F' values below 0.05 and a significantly reduced coefficient of variation (5.88%). The findings demonstrated a substantial correlation between the observed and projected values, with all variables exerting a substantial influence on the phosphatase production statistics. The statistical optimization conducted in this investigation resulted in an improvement in phosphatase biosynthesis compared to the original medium, reaching a level of 76.55 mg L^{-1}. Figure 6, demonstrates the influence of multiple factors and their interconnectedness on phosphatase production. Response surface curves were generated to depict the interaction among multiple factors and determine the best level of each variable that yields the maximum response. Each picture illustrates the effect of two variables while holding all other factors constant at zero values. The highest recorded reaction value occurred at a temperature of 37°C, a pH level of 7, a sucrose concentration of 1.5% (as the carbon source), a yeast extract concentration of 1.5% (as the nitrogen source), an incubation period of 5 days, and a concentration of 7 g/L for rock phosphate (RP).

Table 11: Lack of fit tests

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Linear	1036.55	70	14.81	9.81	0.0005	
2FI	1024.81	55	18.63	12.35	0.0002	
Quadratic	58.25	49	1.19	0.7878	0.7227	Suggested
Cubic	23.53	23	1.02	0.6779	0.7844	Aliased
Pure Error	13.58	9	1.51			

The selected model should have an insignificant lack of fit.

Table 12: Analysis of variance (ANOVA) for Response Surface Quadratic Model CCD) by mutant *streptomyces* AO-31

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1134.33	27	42.01	33.92	< 0.0001	significant
A-temp	0.5400	1	0.5400	0.4360	0.5117	
B-pH	144.48	1	144.48	116.65	< 0.0001	
C-C. Source sucrose	0.0007	1	0.0007	0.0005	0.9815	
D-N. Source yeast extract	0.0002	1	0.0002	0.0002	0.9895	
E-incubation time	9.95	1	9.95	8.03	0.0063	
F-rock phosphate	1.06	1	1.06	0.8571	0.3584	
AB	0.0225	1	0.0225	0.0182	0.8932	
AC	0.0072	1	0.0072	0.0058	0.9394	
AD	0.8327	1	0.8327	0.6723	0.4156	
AE	0.5256	1	0.5256	0.4244	0.5173	
AF	0.0564	1	0.0564	0.0455	0.8318	
BC	5.21	1	5.21	4.21	0.0448	
BD	0.0342	1	0.0342	0.0276	0.8686	
BE	0.4323	1	0.4323	0.3491	0.5569	
BF	0.6972	1	0.6972	0.5630	0.4561	
CD	3.24	1	3.24	2.62	0.1112	
CE	0.1388	1	0.1388	0.1120	0.7390	
CF	0.1369	1	0.1369	0.1105	0.7407	
DE	0.1521	1	0.1521	0.1228	0.7273	
DF	0.1828	1	0.1828	0.1476	0.7023	
EF	0.0756	1	0.0756	0.0611	0.8057	
A^2	0.0363	1	0.0363	0.0293	0.8647	
B^2	127.39	1	127.39	102.86	< 0.0001	
C^2	0.1413	1	0.1413	0.1141	0.7367	
D^2	0.4178	1	0.4178	0.3374	0.5636	
E^2	1.64	1	1.64	1.32	0.2548	
F^2	1.96	1	1.96	1.59	0.2130	
Residual	71.83	58	1.24			
Lack of Fit	58.25	49	1.19	0.7878	0.7227	not significant
Pure Error	13.58	9	1.51			
Cor Total	1206.16	85				

Factor Coding Is Coded:

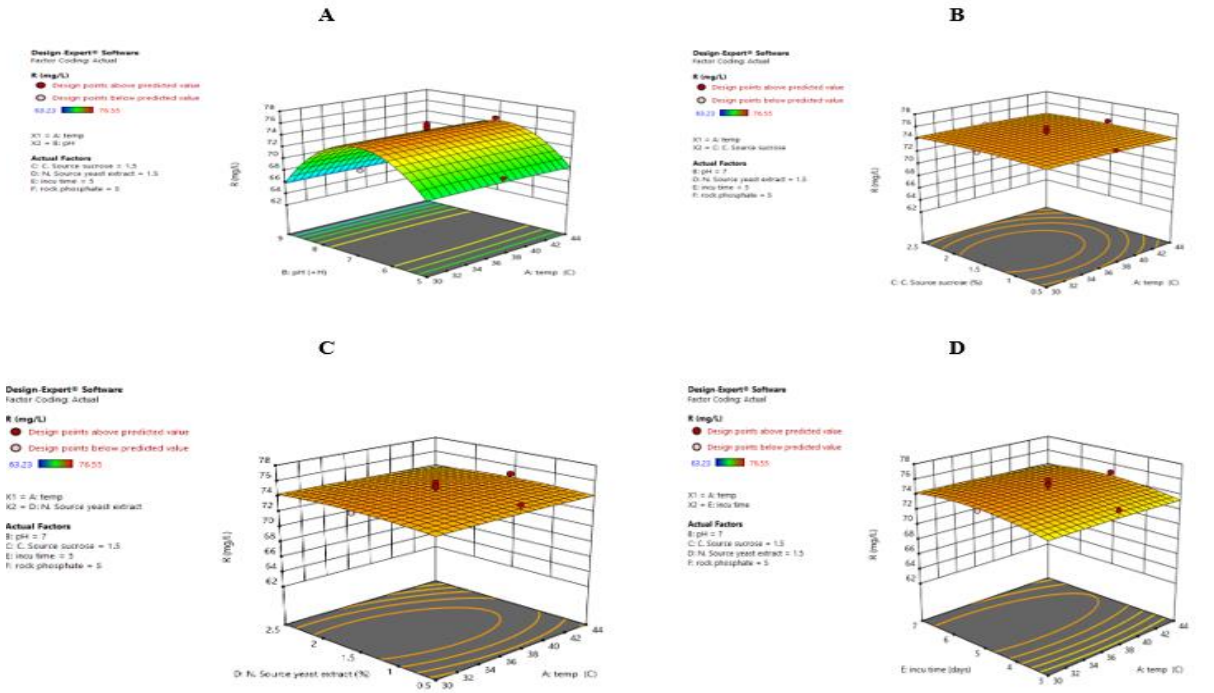
The sum of squares is Type III – Partial**.** The Model F-value of 33.92 indicates that the model is statistically significant. The probability of an F-value of this magnitude occurring only due to noise is extremely low, at 0.01%. P-values below 0.0500 imply that the model terms are statistically significant. Significant model terms in this scenario include B, E, BC, and B^2. Values beyond 0.1000 suggest that the model terms lack significance. If your model has numerous insignificant model terms (except those necessary to support

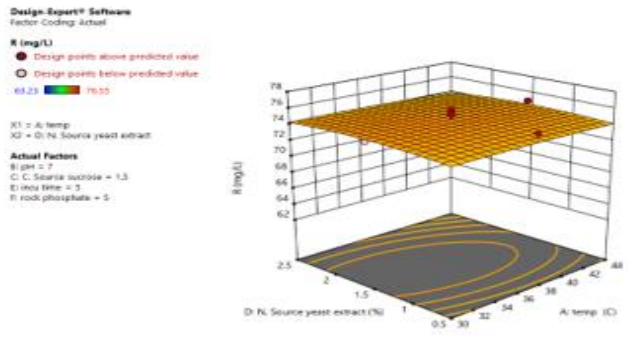
hierarchy), reducing the model may enhance its performance**.** The Lack of Fit F-value of 0.79 indicates that the Lack of Fit is not statistically significant compared to the pure error. The probability that a Lack of Fit F-value of this magnitude could arise solely from noise is 72.27%. The absence of a substantial lack of fit is desirable as it indicates that the model is fitting well.

Table 13: Fit statistics.

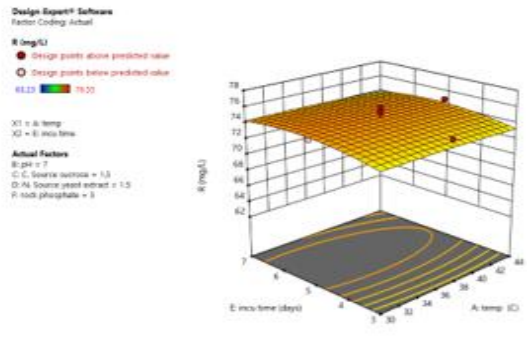
Std. Dev.	1.11	**R^2**	0.9404
Mean	68.50	**Adjusted R^2**	0.9127
C.V. %	1.62	**Predicted R^2**	0.8691
		Adeq Precision	18.7279

The Predicted R^2 value of 0.8691 is reasonably consistent with the Adjusted R^2 value of 0.9127, indicating that the discrepancy between the two values is less than 0.2. Adeq Precision measures the signal-to-noise ratio. A ratio greater than 4 is desirable. Your ratio of 18.728 indicates an adequate signal. This model can be used to navigate the design space.

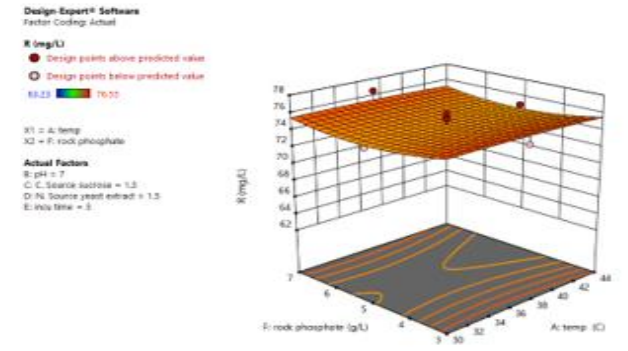



Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = A: temp
X2 = D: N. Source yeast extract
Actual Factors
B: pH = 7
C: C. Source sucrose = 1.5
E: incu time = 5
F: rock phosphate = 5
R (mg/L)
D: N. Source yeast extract (%)
A: temp (C)

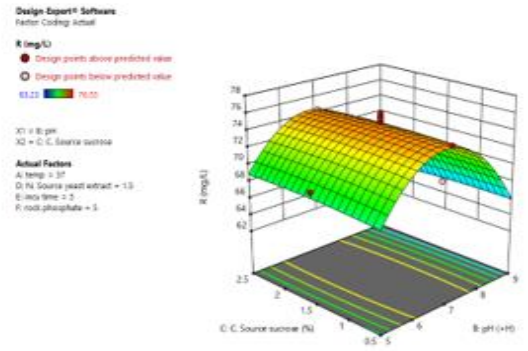
E


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = A: temp
X2 = E: incu time
Actual Factors
B: pH = 7
C: C. Source sucrose = 1.5
D: N. Source yeast extract = 1.5
F: rock phosphate = 5
R (mg/L)
E: incu time (days)
A: temp (C)

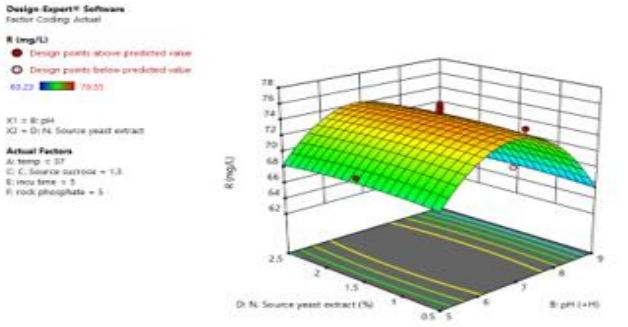
F


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = A: temp
X2 = F: rock phosphate
Actual Factors
B: pH = 7
C: C. Source sucrose = 1.5
D: N. Source yeast extract = 1.5
E: incu time = 5
R (mg/L)
F: rock phosphate (g/L)
A: temp (C)

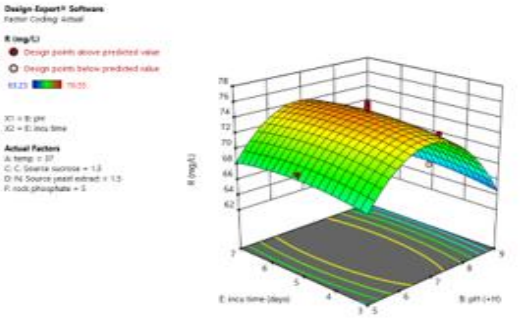
G


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = B: pH
X2 = C: C. Source sucrose
Actual Factors
A: temp = 37
D: N. Source yeast extract = 1.5
E: incu time = 5
F: rock phosphate = 5
R (mg/L)
C: C. Source sucrose (%)
B: pH (+H)

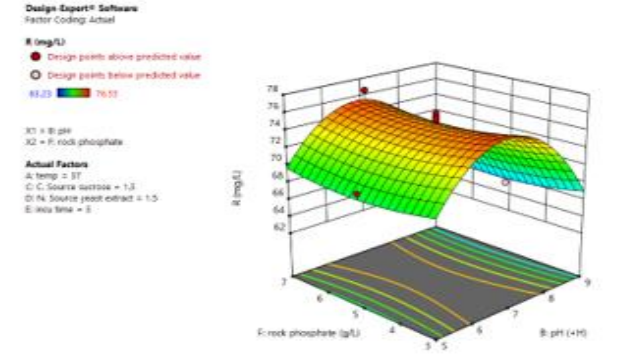
H


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = B: pH
X2 = D: N. Source yeast extract
Actual Factors
A: temp = 37
C: C. Source sucrose = 1.5
E: incu time = 5
F: rock phosphate = 5
R (mg/L)
D: N. Source yeast extract (%)
B: pH (+H)

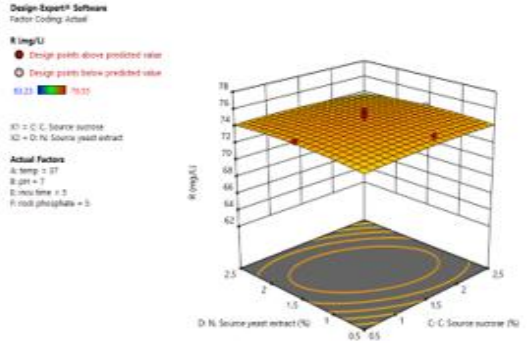
I


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = B: pH
X2 = E: incu time
Actual Factors
A: temp = 37
C: C. Source sucrose = 1.5
D: N. Source yeast extract = 1.5
F: rock phosphate = 5
R (mg/L)
E: incu time (days)
B: pH (+H)

J


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = B: pH
X2 = F: rock phosphate
Actual Factors
A: temp = 37
C: C. Source sucrose = 1.5
D: N. Source yeast extract = 1.5
E: incu time = 5
R (mg/L)
F: rock phosphate (g/L)
B: pH (+H)

K


Design-Expert® Software
Factor Coding: Actual
R (mg/L)
Design points above predicted value
Design points below predicted value
63.23
79.55
X1 = C: C. Source sucrose
X2 = D: N. Source yeast extract
Actual Factors
A: temp = 37
B: pH = 7
E: incu time = 5
F: rock phosphate = 5
R (mg/L)
D: N. Source yeast extract (%)
C: C. Source sucrose (%)

L

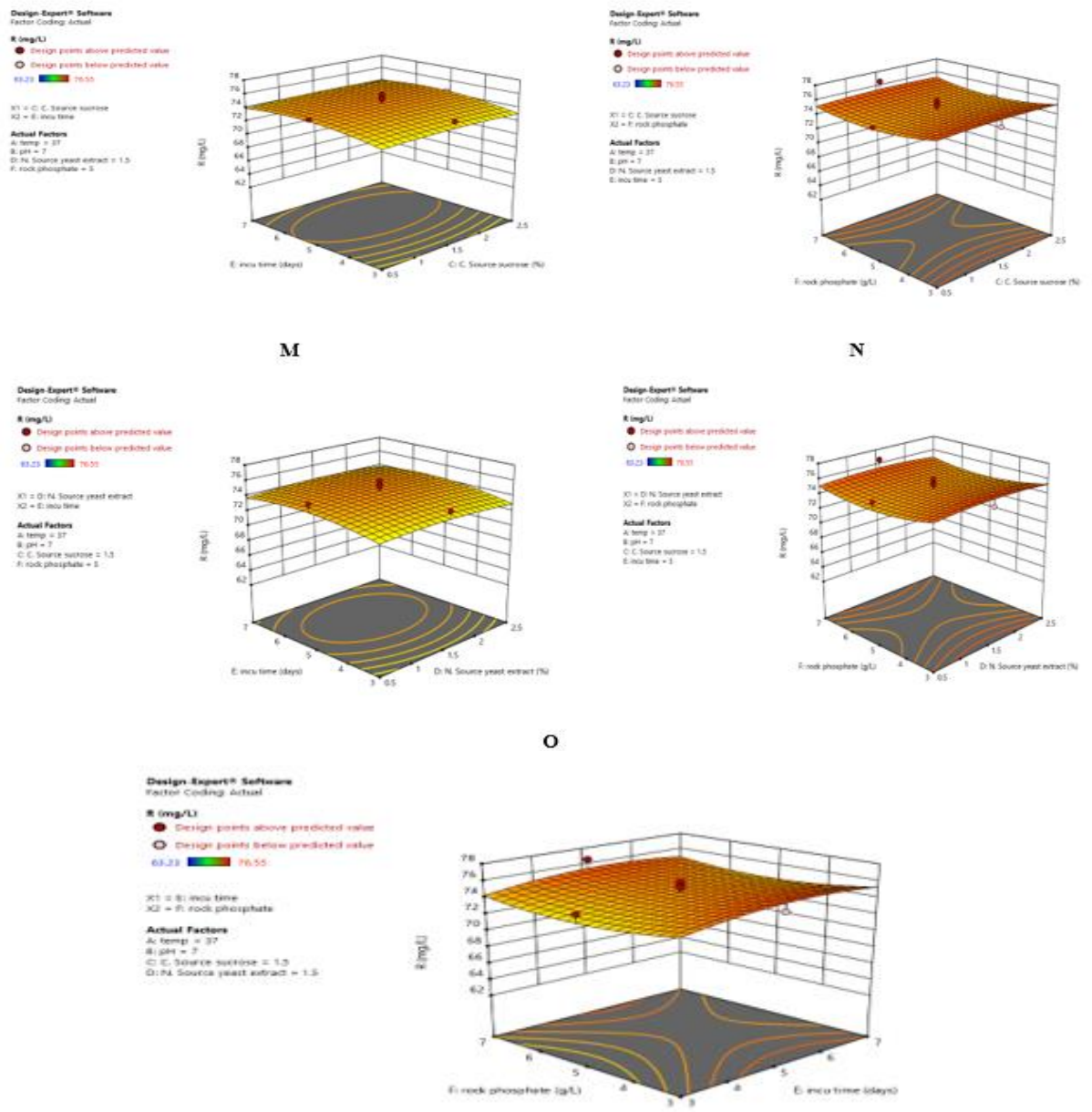


Fig. 6: Contour plots of keratinase activity as a function of the interactions of six variables by keeping the other at the centre level. **(a)** pH, temp. **(b)** sucrose, temp. **(c)** yeast extract, temp. **(d)** incub.time, temp. **(e)** rock phosphate, temp. **(f)** sucrose, pH **(g)** yeast extract, pH **(h)** incub.time, pH **(i)** rock phosphate, pH **(j)** sucrose, yeast extract **(k)** incub.time, sucrose **(l)** rock phosphate, sucrose **(m)** incub.time, yeast extract **(n)** rock phosphate, yeast extract **(o)** rock phosphate, incub. time. on phosphatase production by mutant *Streptomyces sp.* AO-31.

The main aim of this research was to identify the optimal conditions for the production of phosphatase by evaluating the effects of various enhancing factors. By employing this technique, we effectively enhanced the environmental conditions necessary for the optimal production of enzymes. We employed response surface methodology (RSM) to simultaneously investigate the primary and interaction effects of several environmental factors on the synthesis of phosphatase. These results support previous research that has also utilized RSM to enhance enzyme

production under various growth conditions. (Abd El-Aziz *et al.,* 2023a), The optimization of keratinase expression in the *Streptomyces werraensis* KN23 mutant SA-27 was achieved through the utilization of Response Surface Methodology, which involved the incorporation of many parameters. The culture conditions were refined to achieve a maximum specific activity of keratinase, which reached 129.60 U/mL. The results were achieved by maintaining a pH of 7, incubating for a period of 72 hours, and utilizing a yeast extract concentration of 5% and a sugar concentration of 1.5%. For instance, (Khalil, *et al.,* 2022), The highly productive mutant EMS-37 was cultivated under ideal conditions utilizing Response Surface Methodology (RSM). The maximum level of keratinase activity was attained by adjusting the culture conditions to pH 5, incubating for 72 hours, and using 2.5% glucose and 2.5% beef extract. whilst (Ire *et al.,* 2018) Applied the identical methodology of Response Surface Methodology (RSM) to enhance the efficiency of producing an acidic protease by *Penicillium bilaiae*. The utilization of Response Surface Methodology (RSM) is gaining popularity due to its ability to effectively integrate optimal conditions for activities that involve several factors. **(**Haile *et al.,* 2022) Investigated the impact of incubation duration, acidity level, heat, and concentration of substrate on the enzymatic activity of rock phosphatase in the R5 isolate of *Serratia marcescens*. The optimal parameters for the experiment were a 72-hour incubation period, a pH of 8, a temperature of 30°C, and a substrate concentration of 1%.

Effect of Phosphate Rocks Bio-Enriched on The Growth of The Wheat Plant:

Dry Matter Yield of Wheat Plants:

The data from Table 14, shows that the treatments involving the application of rock phosphate bio-enriched resulted in a significant increase in dry matter compared to treatments without inoculation of rock phosphate. The highest dry matter was observed when rock phosphate was bio-enriched by mutant *Streptomyces sp*. AO-31 at a rate of 1.2 ton ha-1. This treatment recorded values of 3.77 g pot-1 and 13.17 g pot-1 for dry weight of grain and straw, respectively. Additionally, the treatment resulted in a weight of 51.88 g for 1000 grain, a biological yield of 16.95 g pot-1, and a harvest index (HI) of 22.3. The application of rock phosphate bio-enriched at a rate of 2.4-ton ha-1 resulted in a higher plant dry matter yield for wheat plants compared to the same biological treatments at a rate of 1.2-ton ha-1. This phenomenon can be ascribed to specific microbial strains, such as acidogenic bacteria, that directly facilitate plant growth by generating chemicals that stimulate plant development and improve the mobilisation of nutrients. Additionally, these bacteria contribute to the process of making insoluble and unavailable nutrient components soluble, resulting in a higher availability of nutrients in the soil. Furthermore, they augment the chemical and biological characteristics of soil by dissolving calcium carbonate, reducing soil pH, and allowing the removal of soluble salts through irrigation water. This process facilitates the improvement of plant growth and is evident in the reported characteristics of plant growth, as depicted in Figure 7. Comparable findings were discovered by (Farrag and Bakr 2021). According to the results in Table 14, we observed that the application of rock phosphate bio-enriched with bacteria resulted in a significant improvement in the dry matter yield of wheat plants compared to other treatments.

Our findings are consistent with the conclusions of Fiorentino *et al.* (2018) *Trichoderma* significantly outperforms the control group in many plant growth metrics, including plant height, biomass production, root length, and biological yield. Several direct and indirect actions on plants are responsible for *Trichoderma's* positive influence on plant growth enhancement. One of these impacts is the release of

molecules like tiny peptides and volatile organic compounds. These substances help the root system grow by improving its architecture via auxin action, which affects things like root length, branching, and density. The presence of indole-3-ethanol, indole-3-acetaldehyde, and indole-3-carboxaldehyde promotes better plant growth. In an independent probe, Bononi *et al*. (2020) *Trichoderma* strains were shown to enhance the effectiveness of phosphorus uptake by as much as 141% and promote soybean growth by a range of 2.1% to 41.1%. Additionally, Lajim *et al*. (2021) the study revealed that applying *Trichoderma spp*. to the soil had a beneficial impact on plant height, germination, root length, and root mass when compared to the control group. Furthermore, *Trichoderma* and plants collaborate to enhance growth by acidifying the medium through P solubilization. This is achieved by *Trichoderma* producing organic acids, which in turn inhibits the primary root and leads to the development of secondary roots. *Trichoderma* boosts the growth of lateral roots instead of the production of new roots, which helps to counteract the acidity of the medium and promotes early root development.

Fig. 7: Effect of phosphate rocks bio-enriched on the growth of the wheat plant

The dry weight of straw and grains inoculated with mutant *Streptomyces sp*. AO-31 showed relative increases of 9.89%, 17.36%, 9.01%, and 15.21% compared to the wild-type *Streptomyces sp*. MMA-NRC and the control group without microbial strains. These results were observed at a rock phosphate level of 12 tons ha-1. The application of rock phosphate at a rate of 24 tons ha-1 resulted in a relative increase of 5.16%, 13.14%, 5.57%, and 14.59% in the dry weight of straw and grains when infected with mutant *Streptomyces sp*. AO-31 compared to wild-type *Streptomyces sp*. MMA-NRC and without any microbial strain inoculation. The application of rock phosphate resulted in a relative increase of 6.47% and 7.12% in straw and grain production, respectively, at a rate of 2.4 tons per hectare compared to a rate of 1.2 tons per hectare. This information may be found in Table 14.

Table 14: The study investigates the impact of the application rate of rock phosphate, in combination with bio-enriched substances, on the yield and characteristics that contribute to the yield of wheat plants.

Treatments	Straw (g/pot)	Grains (g/pot)	Weight of 1000 grains(g)	Biological yield	HI
Rock phosphate (6 g/pot)	10.52	3.01	51.45	13.53	22.5
Rock phosphate (6 g/pot) +Wild type MMA	11.47	3.23	51.46	14.70	21.9
Rock phosphate (6 g/pot)+ Mutant AO-31	12.73	3.55	51.88	16.28	21.8
Rock phosphate (12 g/pot)	11.44	3.22	52.51	14.66	21.9
Rock phosphate (12 g/pot) +Wild type MMA	12.49	3.56	51.85	16.06	22.3
Rock phosphate (12 g/pot) + Mutant AO-31	13.17	3.77	51.88	16.45	22.3
Superphosphate (control)	15.43	4.24	52.52	19.67	21.5
L.S.D. 0.05					
Rock phosphate (RP)	n.s	n.s	n.s	n.s	n.s
Biofertilizers (Bio.)	n.s	0.61	1.32	1.46	4.39
RP*Bio.	n.s	n.s	n.s	n.s	n.s

P Content in The Wheat Plant:

The data in Table 15 demonstrated that the levels of phosphorus in the straw and grains of wheat plants were affected by the inoculation of the chosen microorganisms *Streptomyces sp.* AO-31 and *Streptomyces sp.* MMA-NRC, as well as the varying amounts of rock phosphate application. There was a notable rise in the percentage of phosphorus (P) in wheat plants when rock phosphate bio-enriched was applied at a rate of 2.4 tons per hectare, compared to the treatment without inoculation. Additionally, applying rock phosphate bio-enriched at 2.4 tons per hectare led to a significant increase in the phosphorus concentrations in both the straw and grains of the wheat plants, compared to the same biological treatments at a rate of 1.2 tons per hectare.

P Uptake of Wheat Plants:

The uptake of phosphorus (P) by wheat plants grown in soil treated with various substances during the growing season is seen as an indication of how the treatments affect the plant's ability to utilize the nutrients present in the soil. The data in Table 15 showed that the highest uptake values of phosphorus (P) by straw and grains of wheat plants were observed for treatments where rock phosphate was applied at a rate of 2.4-ton ha-1 and enriched with mutant *Streptomyces sp*. AO-31. The mutant strain displayed uptake values of 23.71 mg pot-1 P by straw and 15.99 mg pot-1 P by grains of wheat plants. Similarly, rock phosphate enriched with wild-type *Streptomyces sp.* MMA-NRC at the same rate exhibited uptake values of 18.77 mg pot-1 P by straw and 13.51 mg pot-1 P by grains of wheat plants. However, based on the data in Table 15, we noticed that there was a significant increase in the uptake of phosphorus by wheat plants when rock phosphate bio-enriched at a rate of 2.4 ton ha-1 was applied, compared to the same biological treatments at a rate of 1.2 ton ha-1. Furthermore, the application of rock phosphate bio-enriched resulted in a substantial enhancement in the uptake of phosphorus by wheat plants, compared to rock phosphate without inoculation.

Table 15: Concentrations and uptake of phosphorus in straw and grains of wheat plants as well as available phosphorus in soil after harvesting.

Treatments	P (%) straw	P uptake (mg/pot) straw	P(%) grains	P uptake (mg/pot) grains	P(mg/kg)in soil
Rock phosphate (6 g/pot)	0.09	10.08	0.24	7.34	16.3
Rock phosphate (6 g/pot) +Wild type MMA	0.12	13.63	0.30	9.84	17.0
Rock phosphate (6 g/pot)+ Mutant AO-31	0.14	17.96	0.36	12.84	21.7
Rock phosphate (12 g/pot)	0.13	15.23	0.32	10.41	22.3
Rock phosphate (12 g/pot) +Wild type MMA	0.15	18.79	0.37	13.51	22.5
Rock phosphate (12 g/pot) + Mutant AO-31	0.18	23.71	0.42	15.99	29.3
Superphosphate (control)	0.21	33.30	0.50	21.61	36.7
L.S.D. 0.05					
Rock phosphate (RP)	0.008	3.02	0.003	1.05	1.39
Biofertilizers (Bio.)	0.002	5.20	0.006	1.81	2.42
RP*Bio.	n.s	n.s	n.s	n.s	n.s

The utilization of rock phosphate in conjunction with phosphate-solubilizing bacteria, such as Bacillus, resulted in an enhanced absorption of nutrients by plants. This can be attributed to some strains of phosphate-solubilizing bacteria, like Bacillus, which have the ability to create organic acids and CO_2. These chemicals facilitate the conversion of some minerals, such as phosphorus (P), from insoluble forms to soluble ones. As a result, the concentration of nutrients near plant roots increases, making them more readily available in the soil solution. Additionally, these molecules decrease the likelihood of nutrient fixation by soil factors. Moreover, these strains possess the capacity to synthesize certain chemicals and enzymes that facilitate the growth and elongation of plant roots. Consequently, this enhances the roots' capability to assimilate nutrients from the soil. These results are in agreement with those of (Farrag and Bakr 2021) furthermore, observed that there was a significant increase in the uptake of phosphorus by wheat plants when treated with rock phosphate that was supplemented with *Bacillus* bacteria. Li *et al.*, 2015, the study confirmed that the application of bacteria resulted in a significant boost in plant development and improved the absorption of nutrients by plants, ultimately leading to the promotion of plant growth. However, the complex nature of bacteria-plant interactions prevents us from definitively attributing the promotion of plant growth solely to mineral solubilization by bacteria. Furthermore, the positive effects on the root system, such as increased root length, volume, and number of root tips, may have facilitated improved contact between the roots and the minerals studied. These findings are particularly important for nutrient uptake in conditions of limited nutrient availability. It is a mechanism via which microorganisms influence plant growth.

The percentages of relative increase were 14.28, 35.71, 16.67 and 33.34 % for concentrations of phosphorus in straw and grains of wheat plants inoculated with mutant *Streptomyces sp.* AO-31 compared to wild-type *Streptomyces sp.* MMA-NRC and without inoculation by microbial strains at the level 1.2 of ton ha^{-1} rock phosphate. The uptake of phosphorus in straw and grains was 24.15, 43.87, 22.36 and 42.83 %, respectively. The relative increase at the level 2.4 ton ha^{-1} of application rock phosphate were 16.67, 27.78, 11.90 and 23.81 % for concentrations of phosphorus in straw and grains inoculated with mutant *Streptomyces sp.* AO-31 compared to wild-type *Streptomyces sp.* MMA-NRC and without inoculation by microbial strains. The

uptake of phosphorus in straw and grains were 20.75, 35.76, 15.51 and 34.89 %, respectively. As for the effect of the application of rock phosphate, the relative increase of concentrations of phosphorus in straw and grains were 13.34 and 18.92 %, while the uptake of phosphorus in straw and grains was 13.34 and 18.92 %, at the level 2.4-ton ha^{-1} compared to level 1.2-ton ha^{-1}.

Available Soil Phosphorus After Harvesting:

The data in Table 15 indicate that the phosphate rock treatments enriched with Streptomyces sp. MMA-NRC resulted in the greatest amounts of accessible soil phosphorus, measuring 29.3 mg kg-1. These values were seen after applying the treatment at a rate of 2.4 tons ha-1. However, the rock phosphate application without inoculation resulted in the lowest soil phosphorus concentrations of 16.3 and 22.3 mg kg-1 at application rates of 1.2 and 2.4 tons ha-1, respectively. Furthermore, based on the data in Table 15, we observed a significant increase in soil phosphorus levels when superphosphate (15.0% P2O5) was applied, compared to all treatments involving the application of rock phosphate bio-enriched, which resulted in a soil phosphorus level of 36.7 mg kg-1. This increase in soil phosphorus contributes to a decrease in soil pH, and these products aid in the conversion of insoluble forms of phosphorus into soluble forms. All of these factors have a substantial impact on enhancing the availability of phosphorus in the soil. Comparable outcomes were achieved by Farrag and Bakr 2021. Adnan *et al.* (2017), scientists have shown that acid-producing bacteria like *Bacillus* may change the nutritional makeup of phosphorus (P) and improve its solubility in soil in a number of ways. To make phosphorus more soluble in soil, for example, they may reduce the soil's pH by producing organic acid and mineral acids. To make phosphorus available to plants, it is essential that *Trichoderma* strains be able to dissolve phosphate while simultaneously creating a variety of organic acids. This is due to the fact that these organic acids may change soil phosphate into plant-soluble di- or monobasic phosphates.

Furthermore, it was observed that the application of rock phosphate bio-enriched at a level of 1.2 ton ha-1 did not result in a significant increase in the available phosphorus content of the soil, compared to the same biological treatments at a level of 2.4 ton ha-1. This lack of significant increase may be attributed to the high concentration of calcium oxide and calcium carbonate in the rock phosphate used, as indicated in Table 15. When the pH level is high and the concentration of soluble calcium increases, a series of fixation reactions occur that gradually decrease the solubility of phosphorus. Phosphorus is thus reduced in availability due to its conversion into less soluble molecules such as octa calcium phosphate or dicalcium phosphate dihydrate. This component may be responsible for the decrease in soluble phosphorus content, which is amplified with increasing rock phosphate application rates.

The percentages of relative increase were 21.66 and 24.88 % of available phosphorus in soil after harvesting inoculation with mutant *Streptomyces sp.* AO-31 compared to wild-type *Streptomyces sp.* MMA-NRC and without inoculation by microbial strains at the level 1.2 of ton ha^{-1} rock phosphate. While the relative increase at the level 2.4 ton ha^{-1} of application rock phosphate was 23.21 and 23.89 %. As for the effect of the application of rock phosphate, the relative increase of available phosphorus in soil was 25.79 % at the level 2.4 ton ha^{-1} compared to level 1.2 ton ha^{-1}. Application of superphosphate (control) was superior compared to all treatments in the experiments in increasing dry matter yield of wheat plants, P content in wheat plants, P uptake of wheat plants and available soil phosphorus after harvesting

Conclusion

Several bacterial strains were tested for their ability to produce phosphatase in

this investigation. The phosphatase enzyme production was maximum in *Streptomyces sp*. NM-NRC. *Streptomyces sp*. NM-NRC NM-NRC has its phosphatase expression increased using chemical mutagenesis using acridin orange (AO) and ethidium bromide (EtBr). *Streptomyces sp*. AO-31, a mutant strain, showed more phosphatase activity than its wild-type counterpart, *Streptomyces sp*. MMA-NRC.Through the use of response surface approaches, a synergistic combination of effective parameter interactions was utilized to optimize microbial phosphatase enzyme production by mutant AO-31. It was determined that 37 degrees Celsius with a pH of 7 provided the best environment for the production of phosphatase enzyme, sucrose (carbon source) of 1.5%, yeast extract (nitrogen source) of 1.5%, 5 days of incubation, and a rock phosphate RP concentration of 7 g/L. These circumstances resulted in a high level of phosphatase activity. Treatments of biofertilizer of rock phosphate with mutant strain *Streptomyces sp.* AO-31 showed an increase in dry matter yield of wheat plants, P content in wheat plants, P uptake of wheat plants and available soil phosphorus after harvesting, compared to the addition of rock phosphate with wild-type strain *Streptomyces sp.* MMA-NRC, and the addition of rock phosphate without the addition of biofertilizers. These results indicate that mutant strain *Streptomyces sp.* AO-31 was a promising strain in rock phosphate solubilization and has the ability to wheat plant growth promotion.

Declarations:

Ethical Approval: Not applicable.

Conflicts of Interest: The authors declare that they have no competing interests.

Authors Contributions: Each author contributed equally to the development of the research technique and the execution of the experiments. Edited, revised, and contributed to the essay while also helping with data analysis and illustrations. I evaluated these portions of the article and also prepared the figures.

Consent to publication: Not applicable.

Funding: National Research Centre**,** Egypt, Grant ID 13020121

Availability of Data and Materials: *Streptomyces sp.* MMA-NRC was sequenced and added to the NCBI database with the accession number OR770185. You may discover all the additional data that backs up the study's conclusions in the paper itself.

Acknowledgements: We express our gratitude to the National Research Centre in Cairo, Egypt, for their ongoing assistance.

REFERENCES

Abd El-Aziz, N.M., Khalil, B.E., Ibrahim, H.F. (2023a). Enhancement of feather degrading keratinase of *Streptomyces swerraensis* KN23, applying mutagenesis and statistical optimization to improve keratinase activity. *BMC microbiology*, 23(1):158.

Abd El-Aziz, N, M., Moharam, M.E., El-Gamal, N.N., Khalil, B.E. (2023c). Enhancement of novel *Endo-polygalacturonase* expression in *Rhodotorula mucilaginosa* PY18: insights from mutagenesis and molecular docking. *Microbial Cell Factories*, 22(1): 1-23.

Acevedo, E., Galindo-Castañeda, T., Prada, F., Navia, M., and Romero, H. M. (2014). Phosphate-solubilizing microorganisms associated with the rhizosphere of oil palm (Elaeis guineensis Jacq.) in Colombia. *Applied Soil Ecology*, 80:26–33.

Adnan, M., Fahad, S., Zamin, M., Shah, S., Mian, I. A., Danish, S. (2020). Coupling phosphate-solubilizing bacteria with phosphorus supplements improve maize phosphorus acquisition and growth under lime induced salinity stress. *Plants*, 9:900.

Adnan, M., Shah, Z., Fahad, S., Arif, M., Alam, M., Khan, I. A., Mian, I.A., Basir, A., Ullah, H., Arshad, M., Rahman, I., Saud, S., Ihsan, M.Z., Jamal, Y., Amanullah, Hammad, H.

M. and Nasim, W. (2017). Phosphate-Solubilizing Bacteria Nullify the Antagonistic Effect of Soil Calcification on Bioavailability of Phosphorus in Alkaline soils. *Scientific Reports*, 7: 16131.

Akbar, S., Prasuna, R., Khanam, R. (2015). Strain improvement by induction of mutagenesis for hyper production of pectinase using *Aspergillus tamarii*. *Journal of Scientific and Industrial Research*, 74(3):160-164

Akbar, S., Prasuna, R.G., Khanam, R. (2013). Multistep mutagenic strain improvement in *Aspergillus Carbonarius* to enhance pectinase production potential. *International Journal of Applied Biology and Pharmaceutical Technology*, 4(2): 92-98

Al-Awadi, Salwa and Mohammed, Nebras. (2019). Mutagenicity Acridine Orange Mutagen On The Biological Activity of *S.aureus* Isolated From Tonsillitis. *International Journal of Drug Delivery Technology*, 9. 693-696.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J. (1990). Basic local alignment search tool. *Journal of molecular biology*, 215(3), 403-410.

Ateş, Ö. (2023). Phosphate Solubilizing Bacteria Isolation Medium: Rock Phosphate or Tricalcium Phosphate. *Geomicrobiology Journal*, 40(8–10): 751–755.

Baig, K. S., Arshad, M., Shaharoona, B., Khalid, A., and Ahmed, I. (2012). Comparative effectiveness of *Bacillus spp*. Possessing either dual or single growth promoting traits for improving phosphorus uptake, growth and yield of wheat (*Triticum aestivum L*.). *Annals of Microbiology*, 62: 1109–1119

Barber, S. A. (1995). Soil nutrient bioavailability: A mechanistic approach. Hoboken, NJ: John Wiley and Sons

Behera, B. C., Yadav, H., Singh, S. K., Mishra, R. R., Sethi, B. K., Dutta, S. K. (2017). Phosphate solubilization and acid phosphatase activity of *Serratia sp*. Isolated from mangrove soil of Mahanadi River delta, Odisha, India. *Journal of Genetic Engineering and Biotechnology*, 15(1): 169–178.

Bononi, L., Chiaramonte, J. B., Chiaramonte, C. C., Moitinho M. A. and Melo I. S. (2020). Phosphorus solubilizing *Trichoderma spp*. from Amazon soils improve soybean plant growth. *Scientific Reports*, 10(1): 1–13.

Choudhury, A., and Kennedy, I. (2005). Nitrogen fertilizer losses from rice soils and control of environmental pollution problems. *Communications in Soil Science and Plant Analysis*, 36: 1625–1639.

Duarte, T., Oliveira, S., Macrae, A., Cedrola, S., Mazotto, A., Souza, E., Melo, A., Vermelho, A. (2011). Increased expression of keratinase and other peptidases by *Candida parapsilosis* mutants. *Brazilian Journal of medical and biological research*, 44:212-216.

Farrag, H. M. and Bakr, A.A. A. (2021). Biological reclamation of a calcareous sandy soil with improving wheat growth using farmyard manure, acid producing bacteria and yeast. *SVU-International Journal of Agricultural Sciences*, 3(1):53-71.

Fiorentino, N., Ventorino, V., Woo, S. L., Pepe, O., Rosa, A. D., Gioia, L., Romano, I., ombardi , N., Napolitano, M., Colla, G. and Rouphael, Y. (2018). *Trichoderma* -based biostimulants modulate rhizosphere microbial populations and improve n uptake efficiency, yield, and nutritional quality of leafy vegetables. *Frontiers in Plant Science*, 9:1–15.

Gupta, S., Singh, R. (2013). Statistical modeling and optimization of keratinase production from newly isolated *Bacillus subtilis* RSE163. *International Journal of Advanced Biotechnology and Research*, 4(1): 167-174.

Haile S, Masi C, Tafesse M. 2022. Isolation and characterization of rock phosphatease-producing bacteria (*Serratia marcescens*) from avocado peel waste for juice clarification. *BMC Microbiology*, 24:22(1):145.

Hakkou, R., Benzaazoua, M., and Bussière, B. (2016). Valorization of phosphate waste rocks and sludge from the moroccan phosphate mines: Challenges and perspectives. *Procedia Engineering*, 138: 110–118.

Ire, F.S., Aguguo, V.J., Ezebuiro, V. (2018). Optimization of invertase from *Aspergillus niger* grown on low-cost agricultural wastes by response surface methodology (RSM). *Journal of Advances in Microbiology*, 13(4): 1-15.

Islam, M. S., Fahad, S., Hossain, A., Chowdhury, M., Iqbal, M. A., Dubey, D.-A. (2021). "Legumes under drought stress: Plant responses, adaptive mechanisms and management strategies in relation to nitrogen fixation," in Engineering tolerance in crop plants against abiotic stress, eds S. Fahad, *et al.*, (Boca Raton: CRC Press), 336.

Izhar Shafi, M., Adnan, M., Fahad, S., Wahid, F., Khan, A., Yue, Z., et al. (2020). Application of single superphosphate with humic acid improves the growth, yield and phosphorus uptake of wheat (*Triticum aestivum L*.) in calcareous soil. *Agronomy*, 10:1224.

Janati W, Mikou K, El Ghadraoui L, Errachidi F. (2022). Isolation and characterization of phosphate solubilizing bacteria naturally colonizing legumes rhizosphere in Morocco. *Front Microbiology*, 26:13:958300.

Janati, W., Benmrid, B., Elhaissoufi, W., Youssef, Z., Nasielski, J., and Adnane, B. (2021). Will P bio-solubilization stimulate biological N2 fixation in grain legumes?. *Frontiers in Agronomy*, 3:637196.

Joe, M. M., Deivaraj, S., Benson, A., Henry, A. J., and Narendrakumar, G. (2018). Soil extract calcium phosphate media for screening of phosphate-solubilizing bacteria. *Agriculture and Natural Resources*, 52(3): 305–308.

Kamalambigeswari, R., Alagar, S., Sivvaswamy, N. (2018). Strain improvement through mutation to enhance pectinase yield from *Aspergillus niger* and molecular characterization of *polygalactouronase* gene. *Journal of Pharmaceutical sciences and research*, 10(5): 989-994.

Khalil, B. E., Ibrahim, H. F. & Abd El-Aziz, N. M. (2022). Improvement of *Pichia kudriavzevii* Egyptian isolate for keratinase production. *Egyptian Pharmaceutical Journal*, 21(2): 192–206.

Khan, M. A., Asaf, S., Khan, A. L., Jan, R., Kang, S.-M., Kim, K.-M. (2020). Thermotolerance effect of plant growth-promoting *Bacillus cereus* SA1 on soybean during heat stress. *BMC Microbiology*, 22; 20(1):175.

Klute, A. (1986). Methods of Soil Analysis. Part-1: Physical and Mineralogical Methods (2nd). Amer. Soc. Agron. Madison. Wisconsin. U. S. A.

Lajim, S. N. H. M., Badaluddin, N. A., Khandaker, M. M., Sajili, M. H., Abu Bakar, M. F. and Lokman, M. N. (2021). Potential of *Trichoderma spp*. as plant growth promoter on Okra (Abelmoschus esculentus). *Bioscience Research Journal*,18: 388-394.

Li, R.X., Cai, F., Pang, G., Shen, Q.R., Li, R., Chen, W. (2015). Solubilization of phosphate and micronutrients by *Trichoderma harzianum* and its relationship with the promotion of tomato plant growth. *PLoS One*, 10 (6): e0130081.

Maharana, R., Dhal, N.K. 2022. Solubilization of rock phosphate by phosphate solubilizing bacteria isolated from effluent treatment plant sludge of a fertilizer plant. *Folia Microbiologica*, 67: 605–615.

Malhotra, H., Vandana, Sharma, S., and Pandey, R. (2018). "Phosphorus nutrition: Plant growth in response to deficiency and excess," in Plant nutrients and abiotic stress tolerance, eds M. Hasanuzzaman, M. Fujita, H. Oku, K. Nahar, and B. Hawrylak-Nowak (Singapore: Springer), 171–190.

Mara, P. R., Isabel, C. M. C. J., Luiz, C. R., dos, S., Marcos, A. S., Flvia, D. P. (2014). Phosphate solubilization and phytohormone production by endophytic and rhizosphere *Trichoderma* isolates of guanandi (*Calophyllum brasiliense Cambess*). *African Journal of Microbiology Research*, 8(27): 2616–2623.

Parhamfar, Maryam; Badoei Dalfard, Arastoo; Parhamfar, Milad and Fahimirad, Shohreh. (2016). Purification and Characterization of an Extracellular Phosphatase Enzyme from *Bacillus spp*. *Journal of Cell and Molecular Research.* 8:(2): 90-97.

Mayadunna, N.; Karunarathna, S.C.; Asad, S.; Stephenson, S.L.; Elgorban, A.M.; Al-Rejaie, S.; Kumla, J.; Yapa, N.; Suwannarach, N. (2023). Isolation of Phosphate-Solubilizing Microorganisms and the Formulation of Biofertilizer for Sustainable Processing of Phosphate Rock. *Life*, *13*, 782.

Murphy, J. & Riley. J. R. (1962). A modified single solution method for the determination of phosphorus in natural waters. *Analytica Chimica Acta*, 27:31-36.

Olsen, S. R., Cole, C. V., Watanabe, F. S. & Dean. L. A. (1954). Estimation of available phosphorus in soils by extraction with NaHCO3, USDA Cir.939. U.S. Washington.

Page, A.L.; R.H. Miller and D.R. Keeney (1982). Methods of Soil Analysis. Part-2: Chemical and Microbiological Properties. (2nded.) American Society of Agronomy, Madison, Wisconsin. USA.

Rajkumar, R., and Kurinjimalar, C. (2021). "Microbes and plant mineral nutrition," in Microbiological activity for soil and plant health management, eds R. Soni, D. C. Suyal, P. Bhargava, and R. Goel (Berlin: Springer), 111–132.

Rozewicki, J., Li ,S., Amada, K.M., Standley, D.M., Katoh, K. (2019). MAFFT-DASH: integrated protein sequence and structural alignment. *Nucleic acids research*, 47(W1): W5-W10.

Salvagiotti, F., Prystupa, P., Ferraris, G., Couretot, L., Magnano, L., Dignani, D. (2017). N:P:S stoichiometry in grains and physiological attributes associated with grain yield in maize as affected by phosphorus and sulfur nutrition. *Field Crops Research*, 203: 128–138.

Sambrook J. and Russell, D. (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York.

Sanaa, T. (2018). High Keratinase Production and Keratin Degradation By A Mutant Strain Kr II, Derived From *Streptomyces radiopugnans* Kr I2. *Journal of Applied Biological Sciences*, 12(2): 1-7.

Seenivasagan, R., and Babalola, O. O. (2021). Utilization of microbial

consortia as biofertilizers and biopesticides for the production of feasible agricultural product. *Biology*, 10:1111.

Shekhar Nautiyal C. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*, 170(1): 265-270.

Singh, B., Saxena, A. (2010). Surrogate markers of insulin resistance :A review. *World journal of diabetes*, 1(2): 36.

Snedecor,G.W. and W.G.Cochran. 1980. " Statically methods" 7 th ed., Iowa Stat. Univ.

Suleman, M., Yasmin, S., Rasul, M., Yahya, M., Atta, B. M., and Mirza, M. S. (2018). Phosphate solubilizing bacteria with glucose dehydrogenase gene for phosphorus uptake and beneficial effects on wheat. *PLoS One*, 13:e0204408.

Tamura K., Dudley J., Nei M. and Kumar S. (2007). MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*, 24: 1596–1599.

Tiwari, S., Patel, A., Pandey, N., Raju, A., Singh, M., and Prasad, S. M. (2020). "Deficiency of essential elements in crop plants," in Sustainable solutions for elemental deficiency and excess in crop plants, eds K. Mishra, P. K. Tandon, and S. Srivastava (Berlin: Springer), 19–52.

Tiwary, E., Gupta, R. (2010). Medium optimization for a novel 58 kDa dimeric keratinase from Bacillus licheniformis ER-15: biochemical characterization and application in feather degradation and dehairing of hides. *Bioresource technology*, 101(15): 6103-6110.

Vaishampayan, A., Sinha, R. P., Häder, D.-P., Dey, T., Gupta, A. K., Bhan, U. (2001). Cyanobacterial biofertilizers in rice agriculture. *The Botanical Review*, 67(4):453-516.