Novel Research in Microbiology Journal (2022), 6(3): 1557-1582
Research Article

(Print) (ISSN 2537-0286)
(Online) (ISSN 2537-0294)
https://nrmj.journals.ekb.eg/
DOI: 10.21608/nrmj.2022.234592

Soil-borne alkaline phosphatase-producing *Bacillus* and *Penicillium* species as growth promoters of the *Corchorus olitorius* and *Amaranthus hybridus* plants

Olubusola A. Odeniyi*; Ladi Turaki

Department of Microbiology, University of Ibadan, Ibadan, Nigeria

*Corresponding author E-mail: busolaodeniyi@gmail.com

Received: 1 April, 2022; Accepted: 2 May, 2022; Published online: 3 May, 2022

Abstract

Phosphorus; is an essential element for plant growth, which may be abundant in the soil but unavailable, because it is poorly soluble and forms complexes with the metals. Microorganisms can solubilize and mineralize the insoluble phosphate into bioavailable forms. This study aimed to isolate and identify the phosphate-solubilizing microorganisms (PSMs), produce and characterize alkaline phosphatase, and determine their plant growth promoting abilities. About forty-one bacterial and twelve fungal isolates were isolated from the soil and water samples, and then screened for their phosphate solubilizing potentials on Pikovskaya (PVK), and the National Botanical Research Institute's Phosphate (NBRIP) growth media. The isolates of *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29) produced the highest alkaline phosphatase at pH 8, 42^{o}C on the 2nd and 3rd d of incubation; and they solubilized concentrations of 937.78 and 848.89 µg/ ml of phosphates, respectively. The optimum temperature and pH activity of the alkaline phosphatase produced by *Penicillium* sp. (PSF-8) were recorded at 50°C (1.145 U/ ml) and pH 9 (1.147 U/ ml), respectively. On the other hand, *Bacillus* sp. (PSB-29) expressed maximum activity at 40°C (1.232 U/ ml) and pH 8 (1.39 U/ ml), respectively. The Michaelis constant (K_m) and maximum velocity (V_{max}) for *Penicillium* sp. (PSF-8) were 23.596 mmol/ l and 2.940 µmol/ l/ min., whereas those for *Bacillus* sp. (PSB-29) were 11.889 mmol/ l and 0.0894 µmol/ l/ min., respectively. *Bacillus* sp. (PSB-29) enhanced the growth of both *Amaranthus hybridus* and *Corchorus olitorius*; by increasing the plant shoot and root length, biomass and phosphorus content, while *Penicillium* sp. (PSF-8) did not support *A. hybridus* growth. Finally, *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) were observed as potent bioagents for phosphate-solubilization during the farming activities.

Keywords: Phosphorus, *Penicillium* sp., *Bacillus* sp., Phosphate solubilization, Plant growth promotion

1. Introduction

Phosphorous (P); along with nitrogen (N) and potassium (K), are elements essential for the development of plants (Kalayu, 2019; Li *et al*., 2019). P is abundantly present in the earth's crust in an organic and inorganic forms, and makes up about 0.2% - 0.8% of the plants' dry weight (Obidi *et al*., 2018). The organic soil P mainly occurs as inositol phosphate/ phytate (myoinositol-1, 2, 3, 4, 5, 6-hexakisphosphates), while the inorganic forms exist in the deposits such as apatite; hydroxyapatite and oxyapatite (Rodriguez and Fraga, 1999; Kalayu, 2019). Although P may be abundant in the soil; however, only 0.01 % of the total P is available to the plants, because it is poorly soluble and forms complexes with the other soil metals; thus become unavailable to the plants (Li *et al*., 2019). The challenge of the unavailability of soluble P is being solved conventionally through the application of P chemical fertilizers (Zhang *et al*., 2017). However, this has caused environmental disadvantages such as eutrophication; algal blooms and groundwater contamination (Oteino *et al*., 2015; Ingle and Padole, 2017; EPA. 2021).

Microorganisms are essential soil constituents involved in several soil processes such as storage; nutrient mobilization, mineralization and release, decomposition, nitrogen fixation and de-nitrification (Alori *et al*., 2017). The PSMs mineralize the insoluble organic phosphate, and thereby increase the availability of nutrients to the plants (Sanjotha and Manawadi, 2016; Li *et al*., 2019; Boubekri *et al*., 2021). The bacteria (Dandessa and Bacha, 2018), yeasts, molds and algae; exhibit phosphate-solubilization and mineralize the poorly available P in the soil (Alori *et al*., 2017; Ingle and Padole, 2017; Zhang *et al*., 2017). These microorganisms produce organic acids and phosphomonoesterases while solubilizing the insoluble phosphate (Kalayu, 2019). The non-substrate specific phosphomonoesterases are termed as acid or alkaline phosphatases (ALPs) based on their pH optima. Among the microorganisms, the bacterial ALP are reported in several biological processes involving phosphate acquisition and transport system in the cell; microbial virulence, bacterial taxonomy and identification (Sharma *et al*., 2018), plant growth promotion (Behera *et al*., 2017) and synthesis of hydroxyapatite (Kalayu, 2019). Therefore, these bacterial spp. are applied as biofertilizers to promote the plant growth (Boubekri *et al*., 2021; Chen *et al*., 2021). Several previous studies have inoculated the soil and\or the crop with phosphate solubilizing/mineralizing microorganisms; as an approach to enhance the plant absorption of P, enhance the growth of plants, increase the harvest yield, and reduce the use of chemical fertilizers that negatively affect the environment (Alori *et al*., 2017; Li *et al*., 2017; Obidi *et al*., 2018; Kalayu, 2019).

Plant growth can be enhanced by the PSMs through stimulation of an efficient biological nitrogen fixation; the production of plant hormones, enzymes (alkaline and acid phosphatases), volatile organic compounds, and improve the availability of some trace elements (Boubekri *et al*., 2021; Chen *et al*., 2021). These microorganisms do not only solubilize the essential elements for plant growth promotion; however, they indirectly promote the plant growth by acting as biocontrol agents, which are active against some microbial plant pathogens. The PSMs secrete metabolic compounds (i.e. phenolics and flavonoids), siderophores, hydrogen cyanide and lytic enzymes, which prevent growth of the plant pathogens (Elias *et al*., 2016; Alori *et al*., 2017; Li *et al*., 2019).The positive characteristics of the PSMs in improving the soil fertility and plant yield make their continual study as a goal by many microbial researchers. Hence, the objective of this work was to investigate the phosphate solubilizing potentials of *Bacillus* and *Penicillium* species isolated from garden soil.

2. Materials and methods

2.1. Soil samples collection, and isolation of the soil microorganisms

Five soil samples of garden and garbage dumpsite, and sediments from fish ponds were collected from the Ibadan metropolis, Nigeria; to isolate the PSMs, according to Li *et al*., (2017). The fish pond water samples were collected into sterile 20 ml bottles, and transferred immediately to the Microbiology laboratory for further analysis (Ebrahimian and Motamedi, 2019). Microorganisms were isolated from the soil samples using the pour plate assay (Li *et al*., 2017). Briefly, 1 ml aliquots of serially diluted soil samples (10^{-2} to 10^{-10}) were inoculated into sterile 90 mm Petri plates. Approximately 15 ml of the corresponding media; Nutrient agar (NA) for bacteria and Potato dextrose agar (PDA) for the fungi; respectively, were poured into the seeded plates and then swirled gently to mix the media thoroughly with the serially diluted soil samples. The inoculated NA plates were incubated at 30^{o}C for 48 h, while the PDA plates were incubated at 27^{o}C for 120 h.

2.2. Screening of the microbial isolates for phosphate-solubilization activity

The distinct recovered microbial colonies were screened for phosphate solubilization by inoculation into 2 solid media, according to the method adopted by Nautiyal, (1999). The two used phosphate rich media were; Pikovskaya (PVK) medium composed of (g\ l); Glucose 1.0 g; $Ca_3(PO_4)_2$ 0.5 g; KCl 0.02 g; $MgSO_4$ 0.01 g; NaCl 0.02 g; yeast extract 0.05 g; $(NH4)_2SO_4$ 0.05 g; $MnSO_4$ 0.2 mg; $FeSO_4$ 0.2 mg, and agar 1.5 g (Pikovskaya, 1948), and the National Botanical Research Institute's Phosphate growth medium (NBRIP) composed of: Glucose 10 g: $Ca_3(PO_4)_2$ 5 g; $MgCl_2$ 5 g; $MgSO_4$ 0.25 g; KCl 0.2 g, $(NH_4)_2SO_4$ 0.1 g and agar 15 g (g\ l) (Nautiyal, 1999).

The pH of the media was adjusted to 7.0, while the salt components were autoclaved separately from the agar at 121^{o}C for 15 min.; allowed to cool to 50^{o}C, and then added aseptically to the sterile agar media before pour plating. After solidification, approximately 7 mm discs from actively growing fungal cultures were made using a sterile cork borer, and then placed at the center of each PVK and NBRIP Petri plates (using bromocresol purple as an indicator). The seeded plates were incubated at 25^{o}C for 7 d. On the other hand, each 24 h old bacterial isolate was point inoculated using a sterile straight inoculating needle on the surface of PVK and NBRIP (with bromocresol purple as an indicator) plates, and then incubated at 30^{o}C for 24-48 h. Phosphate-solubilization was detected visually through the formation of transparent halos around the fungal/ bacterial colonies (Nautiyal, 1999). Phosphate solubilization index (PSI) was determined by measuring the colony diameters and the clear zones of positive ones at the 7th day of incubation (Odeniyi and Itaba, 2020). The PSI was evaluated by using the formula of Elias *et al*., (2016):

Phosphate Solubilization Index (PSI) = (colony diameter + halo zone diameter)/ (colony diameter)

2.3. Quantitative determination of the phosphate solubilization potential

The bacterial and fungal isolates with a PSI greater than 3.0 were selected, and further characterized for their quantitative efficiencies to solubilize the phosphates in NBRIP broth medium. About 20 ml of the broth medium in 50 ml Erlenmeyer flasks was inoculated individually with 1 ml of 0.5 McFarland standard of each bacterial suspension, or\ and 7 mm agar plugs of the actively growing fungal isolates, and then incubated with shaking at 180 rpm for 5 and 7 d at 30 and 25^{o}C, respectively. After incubation, the final pH was recorded using a pH meter (Li *et al*., 2017), and the supernatants were obtained by centrifugation of the broth cultures at 4,000 rpm for 10 min. The concentration of the soluble phosphate was analyzed based on the method of Murphy and Riley, (1962); using increasing concentrations of a standard stock solution of KH_2PO_4. The quantity of the solubilized phosphate was determined by measuring the absorbance at 880 nm using a

spectrophotometer (Jenway Scientific Equipment, England, United Kingdom) against the standard curve of KH_2PO_4.

2.4. Phenotypic and biochemical identification of the selected phosphate-solubilizing isolates

The bacterial colonies that showed high PSI-producing activities were grown on NA plates, and examined for colony and microscopic morphological characteristics using the Gram and spore stains. These bacterial isolates were then subjected to several biochemical assays including; carbohydrate utilization, citrate utilization test, catalase test, oxidase test and indole test (Holt *et al*., 1994). The phosphate-solubilizing fungi (PSF) were identified by macroscopic and microscopic morphological observations after growing on PDA plates at 25°C for 3-5 d, in reference to Cheesbrough, (2005).

2.5. Microbial solubilization of the different phosphate sources

The ability of the selected phosphate solubilizing bacterial and fungal isolates to solubilize different phosphate sources was determined by growing the isolates on NBRIP broth medium, and replacing $Ca_3(PO_4)_2$ individually with $AlPO_4$, $FePO_4$, $(NH_4)_3PO_4$, NaH_2PO_4 and KH_2PO_4 as various sources of inorganic phosphate. Quantification of the released soluble P was determined as earlier described (Li *et al*., 2017).

2.6. Detection of Indole acetic acid (IAA) production

The selected microorganisms were grown individually in a 50 ml conical flask containing 20 ml of King B broth medium; composed of [peptone 20 g; K_2HPO_4: 1.15 g; $MgSO_4$: 1.5 g; glycerol: 10 g; H_2O: 1 l; 0.1% (w/v) Tryptophan] at pH 7.2 for 3 d at 30°C (Li *et al*., 2017). After incubation, the medium was centrifuged at 4000 rpm for 30 min., and 1 ml of the clear supernatant was mixed with 2 ml of Salkowski reagent in a test tube, and then incubated in the dark for 30 min. at 30°C. The development of a pink color indicated IAA production, and the optical density of the reaction was measured at 530 nm using a spectrophotometer (Jenway Scientific Equipment, England, United Kingdom). The concentration of IAA was estimated against a prepared IAA standard curve (Mohamed *et al*., 2019).

2.7. Production of crude alkaline phosphatase

About 1 ml of the selected bacterial suspension (0.5 McFarland standard) was inoculated into a 50 ml Erlenmeyer flask containing 20 ml NBRIP broth medium, and incubated at 180 rpm for 7 d at 30°C. One 7 mm agar plug of actively growing fungus was similarly inoculated into NBRIP broth medium and incubated at 25°C for 7 d. After incubation, the fungal and bacterial broth cultures were centrifuged at 4000 rpm for 15 min. to obtain a clear crude enzyme, which was used to estimate the alkaline phosphatase activity (Behera *et al*., 2017).

2.8. Alkaline phosphatase assay

The ALP enzyme potentials contained in the crude cell-free supernatants were assayed using the modified method of Sharma *et al*., (2018). ALP was assayed using *p*-nitrophenyl phosphate (*p*NPP), which is a colorless substrate that yields a yellow colorimetric end-product *p*-nitrophenol (*p*NP). Approximately 30 µl of the crude enzyme was mixed with 40 µl of 5 mM *p*NPP in 40 µl of 0.5 M Tris-HCl buffer (pH 10.0), and incubated at 30°C for 30 min. After incubation, the reaction was terminated by adding 120 µl of 20 mM NaOH solution. Concentration of the released *p*-nitrophenol was determined by measuring the absorbance at 405 nm using a microplate photometer (Multiscan FC, Thermo Fisher Scientific K.K., Tokyo, Japan), and then comparing the difference in absorbance between the test sample and the control. One unit of the alkaline phosphatase activity was defined as the quantity of enzyme required to release 1.0 µM of *p*-nitrophenol from *p*NPP in 1 min.

2.9. Effect of some growth parameters on ALP production by the selected isolates

2.9.1. Incubation time

About 5 ml of each bacterial suspension (0.5 McFarland standard), and 5 agar plugs (7 mm) of each actively growing fungal culture were inoculated individually into 100 ml of NBRIP broth. The inoculated media were incubated at 30°C and 25°C; respectively, with shaking at 180 rpm for 5 d, 7 d, respectively An aliquot of broth was aseptically withdrawn at 12 h intervals over the period of incubation, centrifuged, and then filter sterilized using a 0.22 µm membrane filter (Jathoth *et al*., 2015; Behera *et al*., 2017). The resulting cell-free broth was used to determine the amount of produced ALP with increasing the incubation time, as earlier described.

2.9.2. pH

The NBRIP broth was prepared as earlier described and the pH of each broth was adjusted using buffers to values of; 3, 4, 5, 6, 7, 8, 9, 10 and 11 (Chu *et al*., 2019). After autoclaving and cooling, 5 ml bacterial suspension of 0.5 McFarland standard and five 7 mm agar plugs of actively growing fungi were inoculated individually into each of 100 ml of the prepared NBRIP broths of varying pH values, and then the samples were processed as earlier described.

2.9.3. Temperature

The inoculated NBRIP media were incubated individually at varying temperatures of; 28°C, 37°C and 45°C with shaking at 180 rpm. After incubation, 10 ml of each broth was aseptically withdrawn periodically at 12 h intervals, centrifuged and then filtered using a 0.22µm membrane filter (Jathoth *et al*., 2015; Behera *et al*., 2017). The resulting culture filtrates were used to determine the quantities of the produced ALP as earlier described.

2.9.4. Carbon and nitrogen sources

Erlenmeyer flasks containing 100 ml each of NBRIP broth modified with different carbon sources such as; glucose, sucrose, maltose, starch and fructose, were sterilized and then inoculated as described above. For the nitrogen sources including; casein, gelatin, ammonium chloride, yeast extract and urea were used. Representative samples were aseptically withdrawn at 24 h intervals and processed as earlier described, to determine the amount ALP produced, in reference to Jathoth *et al*., (2015); Behera *et al*., (2017).

2.10. Production and partial purification of the ALP

The selected phosphate solubilizing bacterium and fungus were cultivated in the optimized NBRIP production broth medium. After incubation, the broth media were centrifuged at 4000 rpm for 15 min., to obtain the crude enzymes. These enzymes were partially purified through the acetone precipitation, dialyzed and then eluted through a Sephadex gel G-100 column chromatograph, at a flow rate of 0.5 ml/ min. (Niu *et al*., 2018).

2.11. Characterization of the partially purified ALP

2.11.1. Effect of pH on the ALP activity and stability

The effect of pH on enzyme activity was determined by using the citrate buffer (pH 3.0-5.0), acetate buffer (pH 6.0-7.0), Tris-HCl buffer (pH 8.0-9.0), and sodium carbonate/ sodium bicarbonate buffer (pH 10.0-11.0), in reference to Sharma *et al*., (2018); Chu *et al*., (2019). The reaction mixture of ALP individually contained 30 µl enzyme and 30 µl of each buffer of varying pH. The reaction mixtures were incubated for 30 min. at 30°C, after which the enzyme activity was assayed as previously described. For pH stability, the reaction mixtures contained equal volumes of the enzyme and the buffer of varying pH, which were incubated over 60 min. at 30°C. At 15, 30, 45 and 60 min. intervals, about 30 µl of *p*NPP was added to 30 µl of the enzyme mixture, and then assayed for the residual enzyme activity as previously described.

2.11.2. Effect of temperature on the enzyme activity and stability

To determine the effect of temperature on the ALP enzyme activity, the enzyme samples were assayed at different temperatures ranging from 27, 37, 40, 50, 60, 70, 80, 90 and 100^{o}C for 30 min. For the thermal stability, the enzyme was initially incubated at different temperatures ranging from 27^{o}C to 100^{o}C over a period of 60 min. (Sharma *et al*., 2018, Chu *et al*., 2019). At 15, 30, 45 and 60 min. intervals, an aliquot was withdrawn and assayed for enzyme activity as previously described.

2.11.3. Effect of different metal ions and surfactants concentration on ALP activity

Different metal ions such as; Mg^{2+}, Na^{+}, NH^{4+} and Ca^{2+}, and inhibitors including; urea, Tween-20, Triton-X100 and sodium dodecyl sulphate at various concentrations of 1; 2.5, 5.0 and 7.5 mM, were tested for their effect on the enzyme activity (Chu *et al*., 2019). Equal volume of the enzyme with varying concentrations of the metal ions and inhibitors was incubated individually at 30^{o}C for 30 min., and then the enzyme activity was assayed as previously described.

2.11.4. Effect of increasing the substrate concentration on the enzyme activity

Different concentrations of *p*-NPP (1-10 mM) were used to determine the kinetics of the alkaline phosphatases. The reaction mixture containing equal volumes of the enzyme and substrate was incubated at 30^{o}C for 30 min., and then assayed. The K_m and V_{max} values were determined using Lineweaver–Burk plots, according to Chu *et al*., (2019).

2.12. Plant growth promotion efficacy of the selected PSMs

A greenhouse experiment involving pots containing 1 Kg of twice-autoclaved farmyard soil mixed with 0.3 % $Ca_3(PO_4)_2$, was used to evaluate the effects of the alkaline phosphatase-producing microorganisms on the growth and development of *A. hybridus* and *C. olitorius* plants (Yadav *et al*., 2011; Adnan *et al*., 2020), commonly referred to as spinach and jute, respectively. Briefly, individual broths of the microbial cells were prepared, incubated, centrifuged, washed, and then the cell suspensions were adjusted to 10^8 cfu\ ml. Seeds of *C. olitorius* (variety; Oniyaya) and *A. hybridus* (variety; Local Green) were acquired from the University of Ibadan Teaching and Research Farm. The seeds were surface-sterilized with 3 % NaOCl for 30 min., and rinsed with sterile dist. water. The seeds were mixed individually with each microbial suspension and\or with co-inoculants of both microbial cultures, and the set-up was maintained under shaking (100 rpm) for12-18 h at 25°C. The treated seeds were then aseptically air-dried and used throughout the studies. Spinach and jute seeds treated with sterile dist. water served as controls (Adnan *et al*., 2020). The treated seeds (10 seeds\ pot) were sown at 3 cm depth, and each pot was inoculated individually with 1 ml of the microbial inoculants (Mohamed *et al*., 2019; Adnan *et al*., 2020). For co-inoculation, an equal volume of each microbial species [*Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8)] was mixed, and then 1 ml of this co-inoculant was used in inoculating the seeds of *A. hybridus* and *C. olitorius*. The experimental design consisted of 8 treatments in triplicates involving dist. water treated spinach seeds (DWTS), dist. water treated jute seeds (DWTJ), fungi-treated spinach seeds (FTS), fungi-treated jute seeds (FTJ), fungi-treated and bacterium-treated spinach seeds (FBTS), fungi-treated and bacterium-treated jute seed (FBTJ), bacterium-treated spinach seeds (BTS) and bacterium-treated treated jute seeds (BTJ). The assay was set up in the greenhouse and watered every 2 d with 100 ml of sterile dist. water. After 30 d, the growing plants were harvested to evaluate the growth and yield parameters including; the shoot height, root length, number of leaves, number of capsules (jute), and fresh and dry weights of the shoot and the root. The plant samples were oven-dried at 65°C for 48 h to a constant weight to analyze the plant dry weight, as described by the methods adopted by Mohamed *et al*.,

(2019); Naziya *et al*., (2019); Adnan *et al*., (2020). The P concentration in the plant samples was determined using the ashed plant materials, in reference to Pilar-Izquierdo *et al*., (2012); Adnan *et al*., (2020). The *C. olitorius* plant was kept growing for up to 60 d to determine the number of capsules that it produces.

2.13. Statistical analysis

The descriptive statistics was used to represent the mean values of duplicate data involving the number of microbial colonies, studies of ALP production and its residual activities. The plant growth studies were carried out in triplicates. The standard deviations (SD) were reported, and results were analyzed using Graph-Pad prism 8.

3. Results

3.1. Isolation of bacteria and fungi from the soil and water samples

Results presented in Table (1) show the count of the isolated bacteria and fungi from the soil and water samples on NA and PDA, respectively. The dumpsite soil sample produced the highest number of bacterial colonies (1.57×10^7 cfu\ g), while the lowest count was obtained from the concrete fish pond soil sample (6.0×10^6 cfu\ g). For the fungi, the garden soil sample recorded the highest number of fungal colonies (3.9×10^8 cfu\ g), whereas the concrete fish pond water sample expressed the lowest fungal count (1.3×10^8 cfu\ g). A total of 41 distinct bacterial and 12 fungal isolates were recovered from the soil and water samples.

Table 1: Total number of the bacterial and fungal colonies obtained from the soil and water samples

Source of microorganism	Bacteria	Fungi
	cfu\ (ml or g)	cfu\ (ml or g)
Garden soil	1.02×10^7	3.9×10^8
Dumpsite soil	1.57×10^7	3.4×10^8
Concrete fishpond soil	6.0×10^6	1.6×10^8
Earthen fishpond soil	9.4×10^6	2.4×10^8
Concrete fish pond water	8.0×10^6	1.3×10^8
Earthen fish pond water	1.21×10^7	3.8×10^8

3.2. Screening of the isolates for phosphate-solubilization potential

The microbial isolates exhibited variations in the phosphate solubilization index (PSI) ranging from 2.08 to 3.80 (Table 2). For fungi, isolate PSF-8 produced the maximum halo zone diameter of 55 mm, while isolates PSB-29 and PSB-18 recorded the maximum halo zone diameter of 15 mm for bacteria. The highest recorded PSI's were 3.03 and 3.80 for PSF-8 and PSB-29, respectively. A color change in the medium from purple to yellow around some of the isolates was indicative of the production of organic acids, which ultimately caused a pH drop in the medium. Only 27 bacterial and 6 fungal isolates were able to solubilize the phosphate, observed through the formation of halo zones on both PVK and NBRIP media. These isolates were able to solubilize Tricalcium phosphate on NBRIP medium more efficiently than PVK, as the clear halo zones were more pronounced and extensive. Thus, NBRIP medium was selected for continued microbial cultivation.

3.3. Quantitative analysis of phosphate-solubilization, and identification of the selected isolates

The 9 bacterial and a single fungus with PSI greater than 3.0 that were screened for phosphate-solubilizing ability on NBRIP broth medium, recorded solubilization of $Ca_3(PO_4)_2$; accompanied with a decrease in the medium pH from an initial pH 7.0 to a pH range of 4.2 to 5.54. Meanwhile, the concentration of the released soluble P by the bacterial isolates ranged from 216.67 µg/ ml (PSB-19) - 848.89 µg/ ml (PSB-29); however, the highest overall P concentration recorded by the fungal isolate PSF-8 was 937.78 µg/ ml. Accordingly, the isolates PSB-29 and PSF-8 were selected as the most efficient phosphate- solubilizing isolates, and were identified microscopically and phenotypically. PSF-8 was identified as a *Penicillium* sp., which was initially white; but with increasing the incubation time, it became dark blue/ green. The reverse of this isolate was cream colored, and it had globose conidia with septate hyphae under the light microscope. On the other hand, isolate PSB-29 was identified as *Bacillus* sp. This isolate was short rod and a spore former. It fermented and produced acid from glucose; sucrose, lactose maltose and sorbitol. It was indole and catalase positive, but showed negative results toward oxidase and citrate utilization. The two isolates were thus selected for further studies.

3.4. Solubilization of different phosphate sources by the selected *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29), and IAA production

Quantitative analysis of phosphate solubilization using different phosphate sources by the *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29) showed that both selected isolates were able to solubilize P from the other phosphate sources but with lower quantities, compared to that obtained on using $Ca(PO_4)_3$. The highest microbial solubilization of P was realized from $(NH_4)_3PO_4$ followed by NaH_2PO_4; recording 376.24 and 374.13 µg / ml for *Penicillium* sp. (PSF-8), and 219.87 and 213.87 µg/ ml, for *Bacillus* sp. (PSB-29), respectively. All phosphate solubilization activities were accompanied with a decrease in the pH of the broth media ranging from 3.46 - 4.95 for the *Penicillium* sp., and 4.54 -5.74 for the *Bacillus* sp. Moreover, it was observed that *Penicillium* sp. released more P than the *Bacillus* sp. The amount of indole acetic acid (IAA) produced by *Penicillium* sp. (8.97 mg/ l) was lower than that produced by *Bacillus* sp. (20.30 mg/ l).

3.5. Optimization of the growth parameters for maximum production of ALP by *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29)

Table 2: Phosphate-solubilization potency of the isolated microorganisms

Bacterial/ fungal code	Isolation source	Diameter (mm)		PSI	Medium pH	Soluble phosphate (µg/ ml)
		Colony	Halo			
PSB-1	Pond water	6.0	9.0	2.50	*ND	ND
PSB-2	Pond water	6.0	10.0	2.66	ND	ND
PSB-7	Pond water	9.0	11.0	2.22	ND	ND
PSB-8	Garden soil	7.0	9.0	2.28	ND	ND
PSB-11	Garden soil	6.0	13.0	3.10	4.22	267.78
PSB-12	Dump soil	8.0	12.0	2.50	ND	ND
PSB-15	Pond water	10.0	12.0	2.22	ND	ND
PSB-16	Garden soil	5.0	11.0	3.20	4.21	393.33
PSB-17	Pond soil	9.0	11.0	2.22	ND	ND
PSB-21	Pond soil	6.0	9.0	2.50	ND	ND
PSB-22	Garden soil	7.0	9.0	2.28	ND	ND
PSB-18	Garden soil	6.0	15.0	3.50	4.74	482.22
PSB-19	Dump soil	6.0	14.0	3.30	5.23	216.67
PSB-20	Pond soil	4.0	10.0	3.50	5.38	223.33
PSB-24	Pond water	4.0	7.0	2.75	ND	ND
PSB-25	Pond water	5.0	7.0	2.40	ND	ND
PSB-28	Garden soil	9.0	12.0	2.50	ND	ND
PSB-29	Garden soil	5.0	15.0	3.80	5.54	848.89
PSB-30	Garden soil	4.0	11.0	3.75	4.36	337.78
PSB-31	Garden soil	7.0	9.0	2.28	ND	ND
PSB-32	Pond soil	5.0	12.0	3.40	5.40	747.78
PSB-33	Pond soil	5.0	7.0	2.40	ND	ND
PSB-34	Dump soil	7.0	9.0	2.28	ND	ND
PSB-35	Garden soil	5.0	11.0	3.20	4.21	398.89
PSB-36	Garden soil	5.0	7.0	2.40	ND	ND
PSB-37	Garden soil	6.0	11.0	2.80	ND	ND
PSB-38	Pond soil	7.0	13.0	2.85	ND	ND
PSF-3	Garden soil	22.0	31.0	2.41	ND	ND
PSF-4	Pond soil	13.0	19.0	2.46	ND	ND
PSF-6	Garden soil	15.0	20.0	2.33	ND	ND
PSF-8	Garden soil	27.0	55.0	3.03	3.96	937.78
PSF-9	Garden soil	31.0	54.0	2.74	ND	ND
PSF-11	Pond soil	25.0	27.0	2.08	ND	ND

Where; ND: not determined, recorded results are means of duplicate readings

Maximum production of ALP enzyme by *Bacillus* sp. (PSB-29) (1.3 U/ ml) was recorded after incubation for 2 d (Fig. 1), and decreased with increasing the period of incubation. On the other hand, maximum enzyme production by *Penicillium* sp. (PSF-8) (1.6 U/ ml) was recorded after 3 and 4 d of incubation, and also decreased upon further incubation. The medium containing glucose supported the maximum ALP production by *Bacillus* sp. (PSB-29) (1.58 U/ ml) after 2 d of incubation (Fig. 2a), followed by fructose, sucrose, maltose, lactose and starch.

Glucose also supported maximum ALP production by *Penicillium* sp. (PSF-8) (1.92 U/ ml) after 3 d of incubation (Fig. 2b), followed by starch, fructose, sucrose, lactose and maltose. The highest production level of ALP (1.44 U/ ml) by *Bacillus* sp. (PSB-29) was observed in the broth medium containing ammonium sulphate on the 2nd d of incubation (Fig. 3a). The same medium supported ALP production by *Penicillium* sp. (PSF-8) (1.48 U/ ml), however, yeast extract yielded the maximum ALP (1.62 U/ ml) (Fig. 3b). A gradual increase in APL enzyme production was observed as the temperature increased resulting in maximum production by *Bacillus* sp. (PSB-29) (1.51 U/ ml) at 42^{o}C on 2nd and 3rd d (Fig. 4a). *Penicillium* sp. (PSF-8) produced the highest level of ALP (1.8 U/ ml) on the 3rd d at 37^{o}C. Meanwhile, on the 4th and 5th d at 42^{o}C (Fig. 4b), the ALP yield was 1.8 U/ ml. The broth medium pH did not affect the production of ALP by *Bacillus* sp. (PSB-29) (Fig. 5a); however, a gradual increase in ALP level that peaked at pH 8.0 to 9.0 on the 3rd and 4th d of incubation was observed in *Penicillium* sp. (PSF-8), which decreased thereafter (Fig. 5b).

3.6. Characterization of the partially purified ALP

Results demonstrated in Fig. (6) show characteristics of the stability and activity of the microbial ALP in response to different treatments. The activities of the ALPs of *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) were highest at pH 8 and pH 9 recording 1.349 U/ ml; 1.147 U/ ml; respectively, as shown in Fig. (6a). The optimum temperature for the ALP activity of *Bacillus* sp. (PSB-29) (Fig. 6b) was recorded at 40°C (1.232 U/ ml), while that for *Penicillium* sp. (PSF-8) was observed at 50°C (1.145 U/ ml). The lowest activity was recorded at 90°C and 100°C. The ALPs were stable at alkaline pH (Fig. 6e, f), and between 40-70°C for 60 min. (Fig. 6g, h), for *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8), respectively. Addition of 5.0 mM Mg^{2+} expressed the highest level of activity for both ALPs produced by *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8). Tween 20 (5 mM) caused an increased activity of 7 % in *Bacillus* sp. (PSB-29) (Fig 6j).

3.7. Effect of increasing the *p*-NPP substrate concentration on the ALP enzyme activity

The Lineweaver–Burk plot for the ALPs of *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) recorded with increasing the concentration of *p*-NPP as a substrate showed that the K_m and V_{max} for *Bacillus* sp. (PSB-29) were 11.889 mmol/ l and 0.0894 µmol/ l/ min.; respectively, while these for *Penicillium* sp. (PSF-8) were 23.596 mmol/ l and 2.940 µmol/ l/ min., respectively. It was observed that the substrate concentrations were directly proportional to ALP activity, where the highest recorded enzyme activities for *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) were 15.294 U/ ml and 12.43 U/ ml, respectively.

3.8. The selected phosphate solubilizing isolates as effective plant growth promoters

The effects of *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) on promoting the growth of *A. hybridus* and *C. olitorius* plants are demonstrated in Table (3).

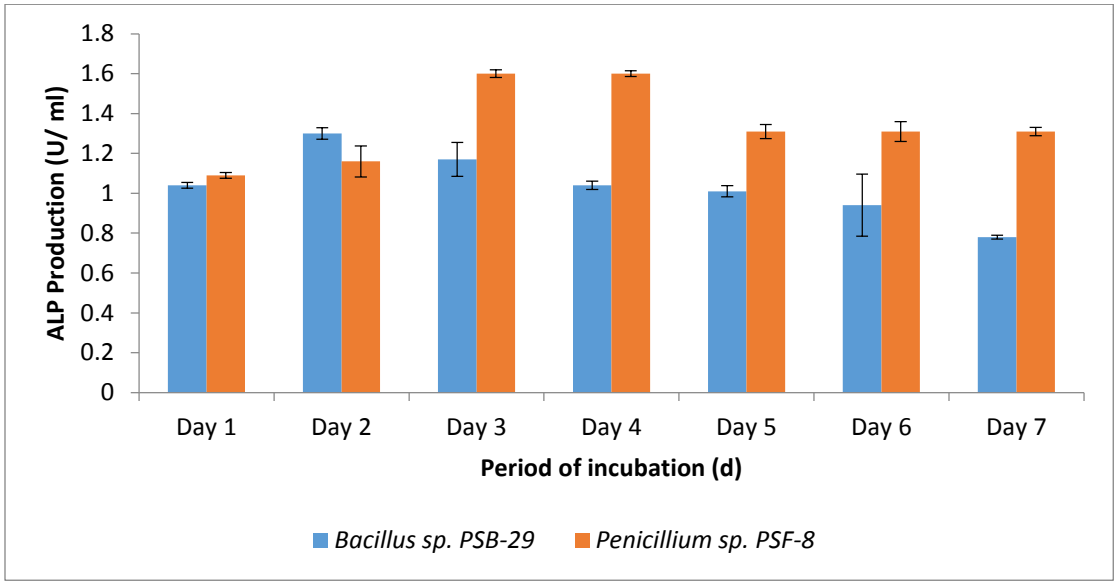
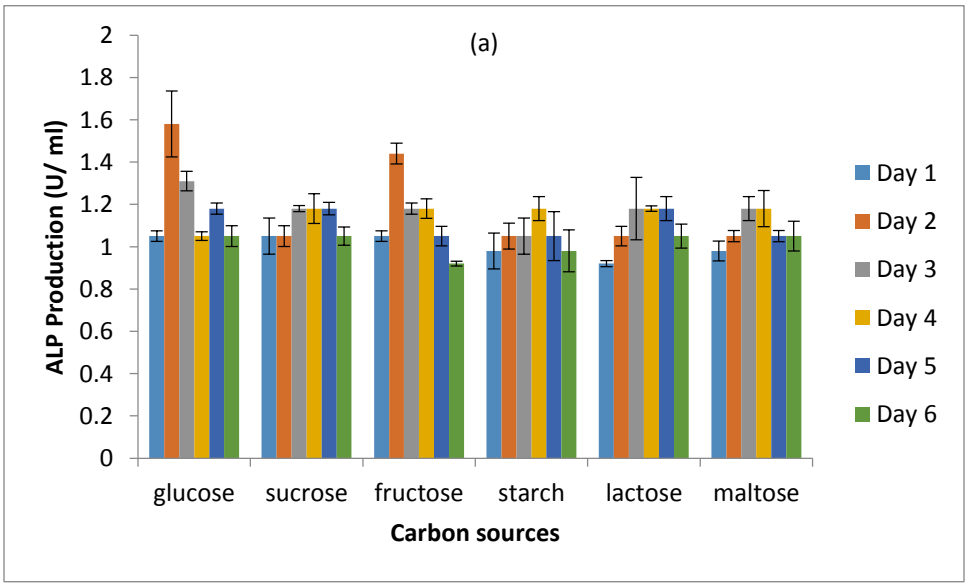


Fig. 1: Effect of incubation time on ALP production by *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8)



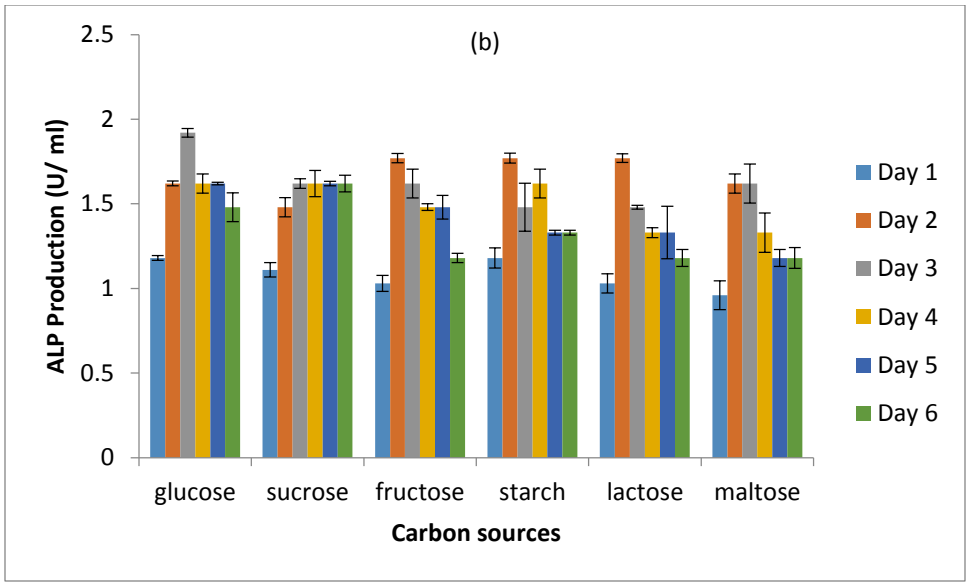
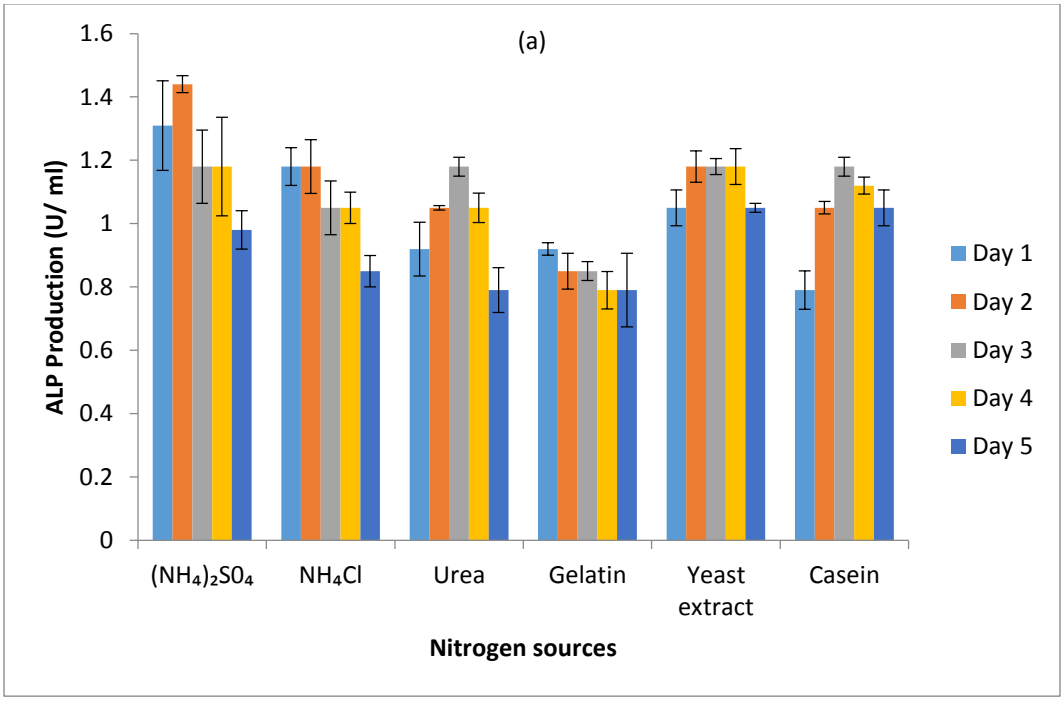


Fig. 2: Effect of different carbon sources on the production of ALP by; (a) *Bacillus* sp. (PSB-29) and (b) *Penicillium* sp. (PSF-8)



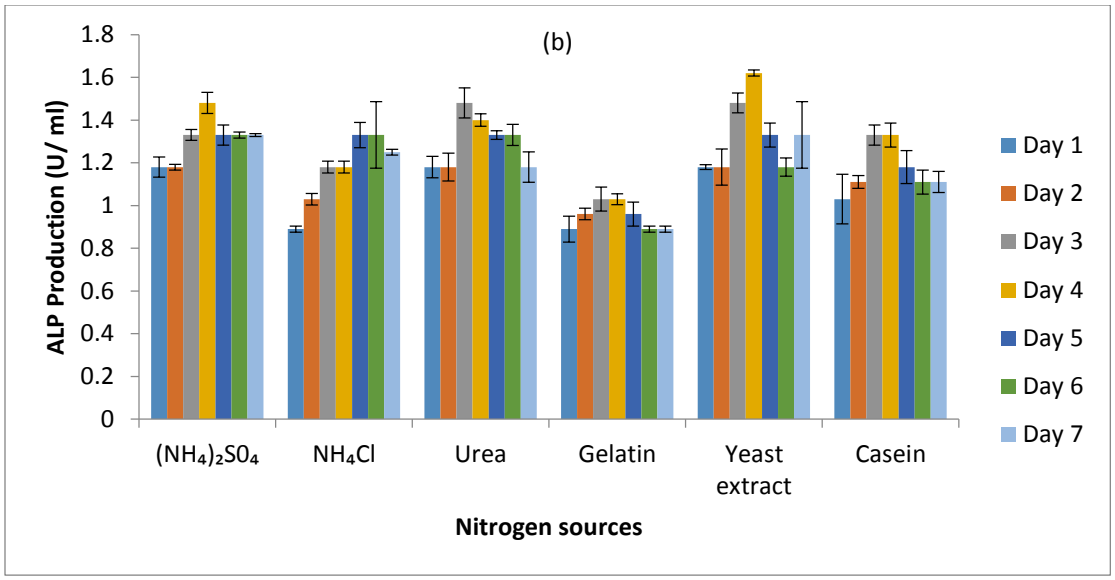
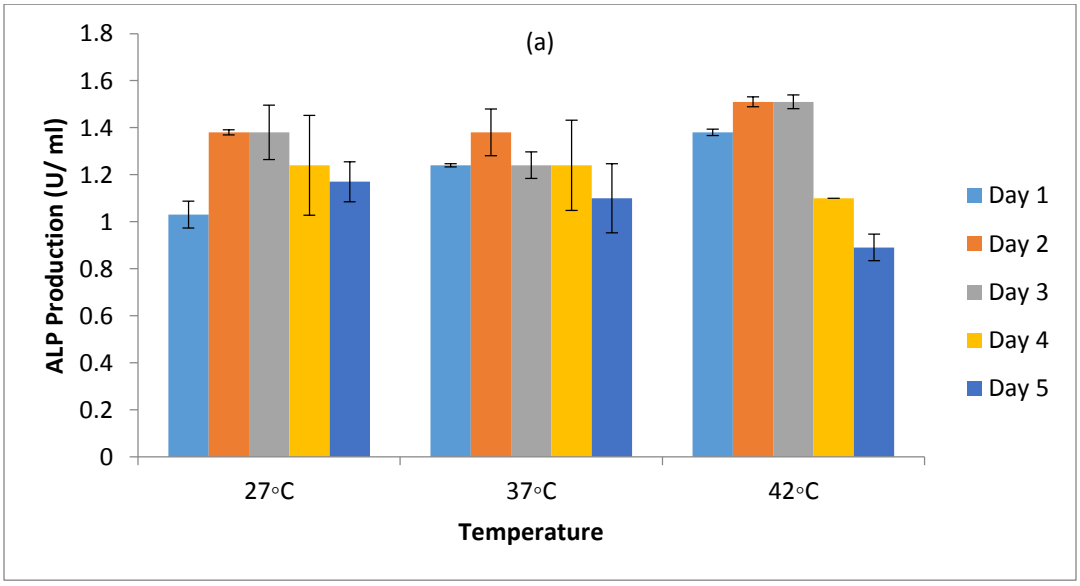


Fig. 3: Effect of different nitrogen sources on production of ALP by (a) *Bacillus* sp. (PSB-29) and (b) *Penicillium* sp. (PSF-8)



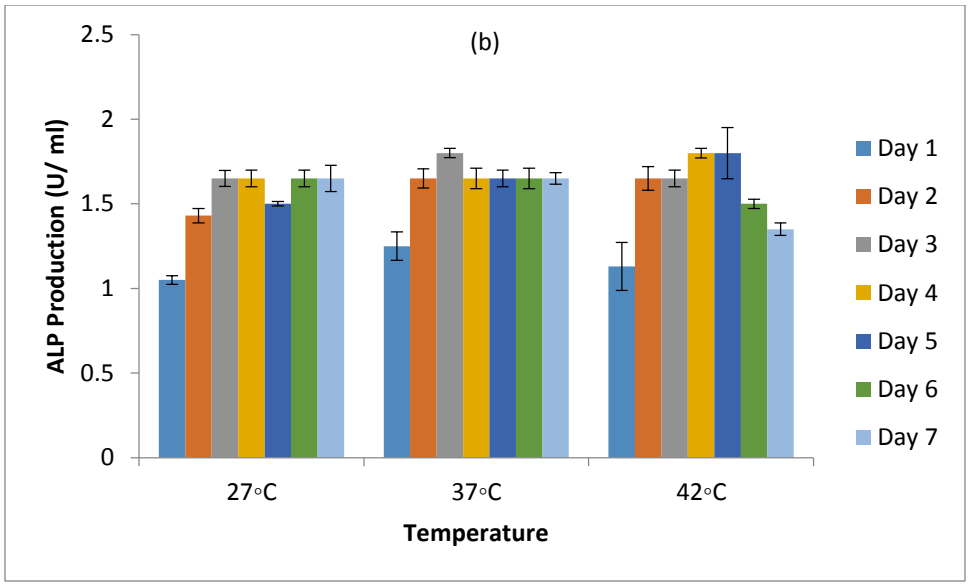
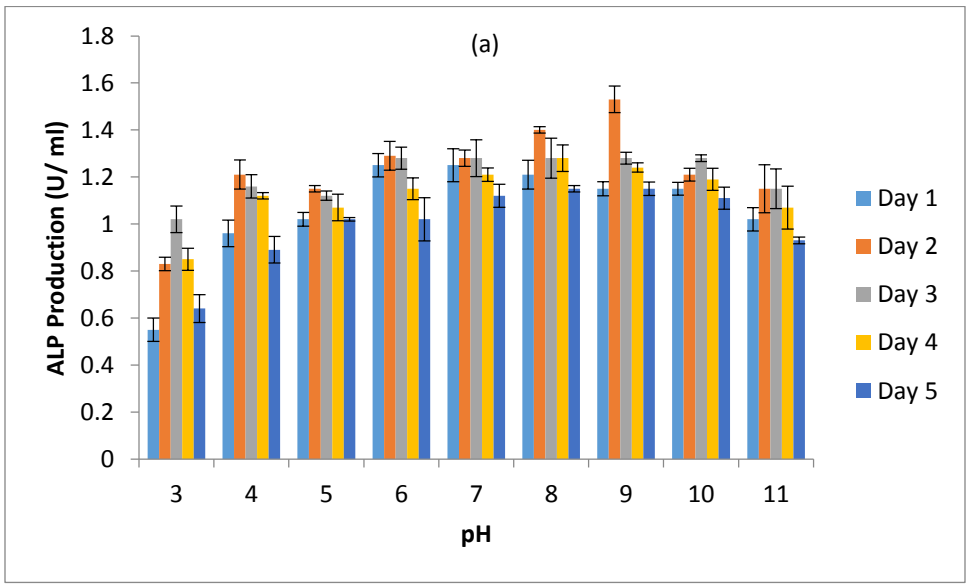


Fig. 4: Effect of temperature on production of ALP by (a) *Bacillus* sp. (PSB-29) and (b) *Penicillium* sp. (PSF-8)



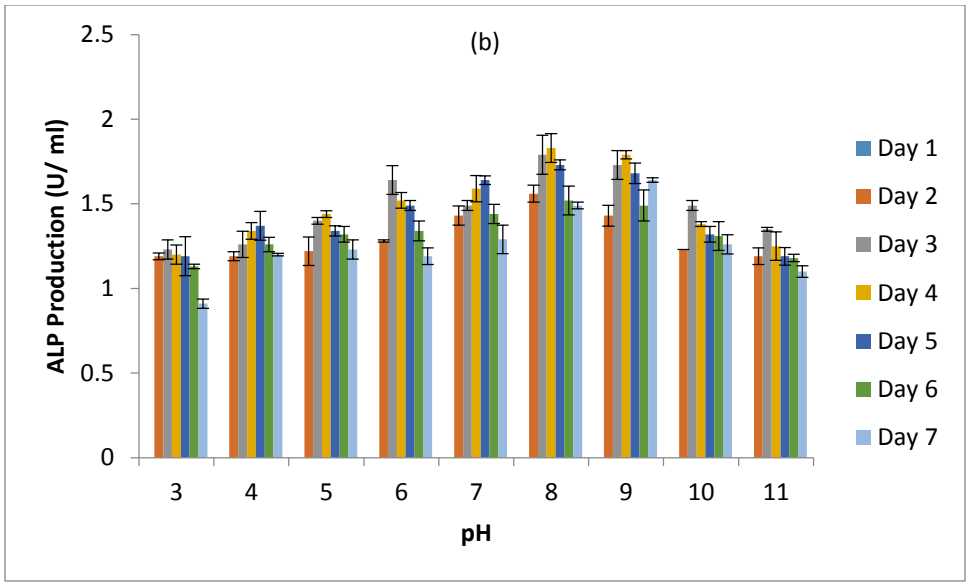
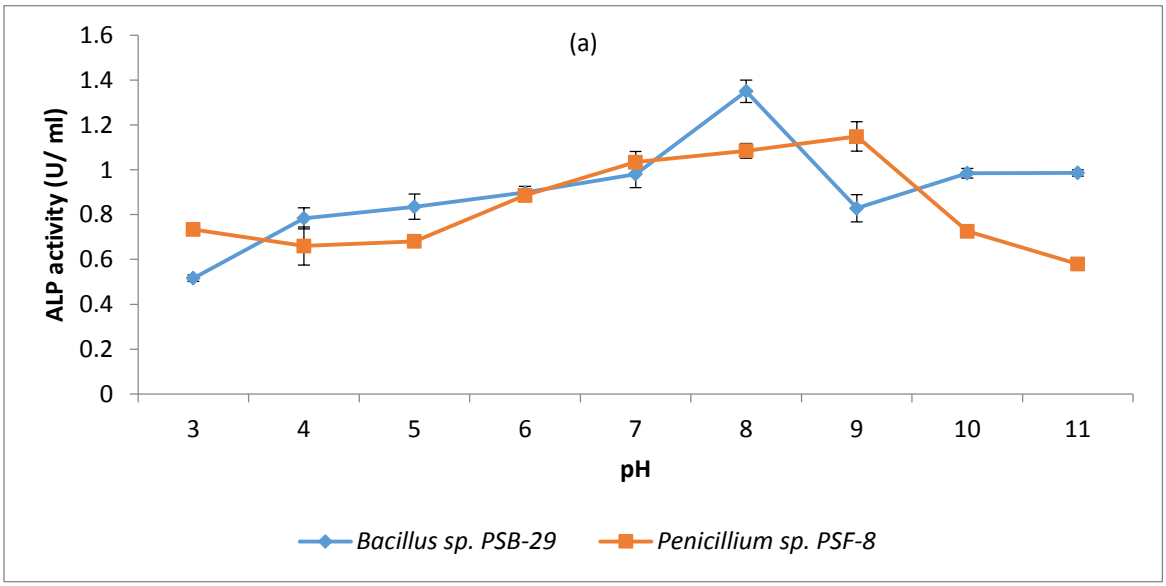
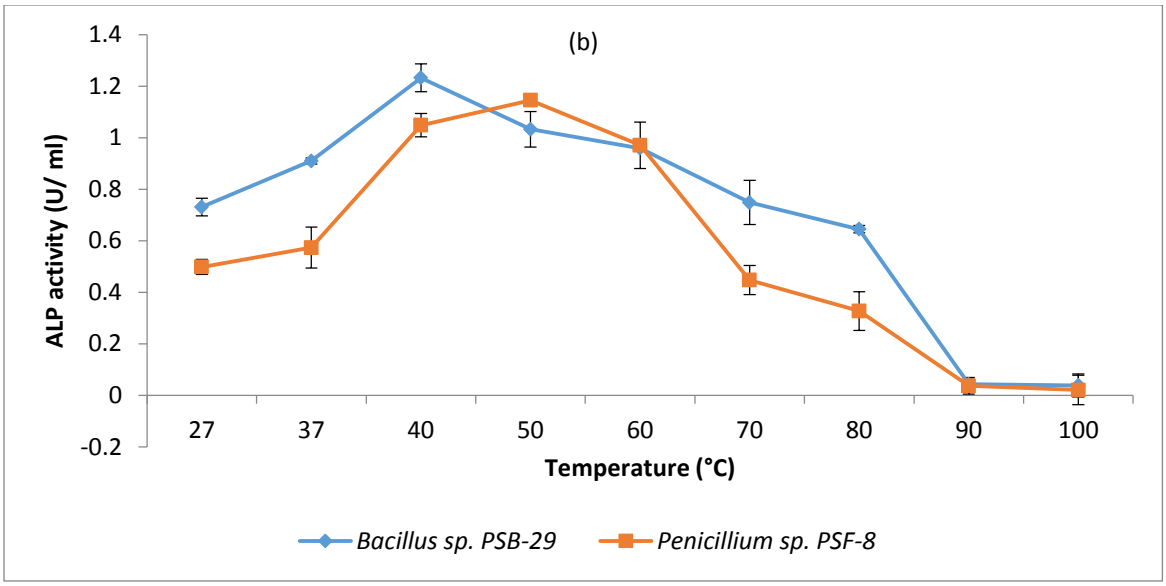
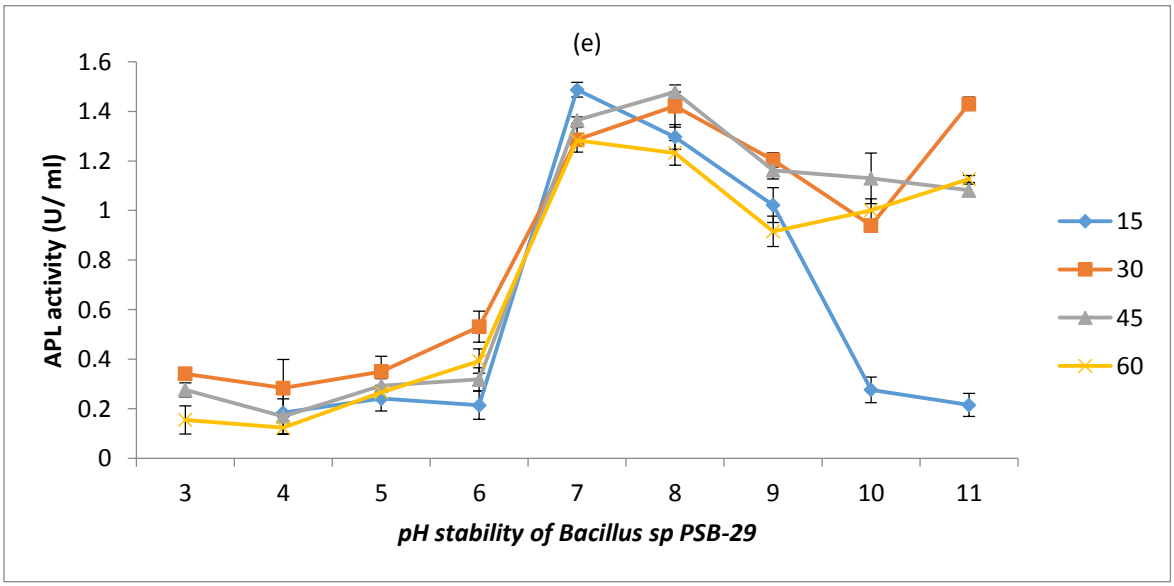
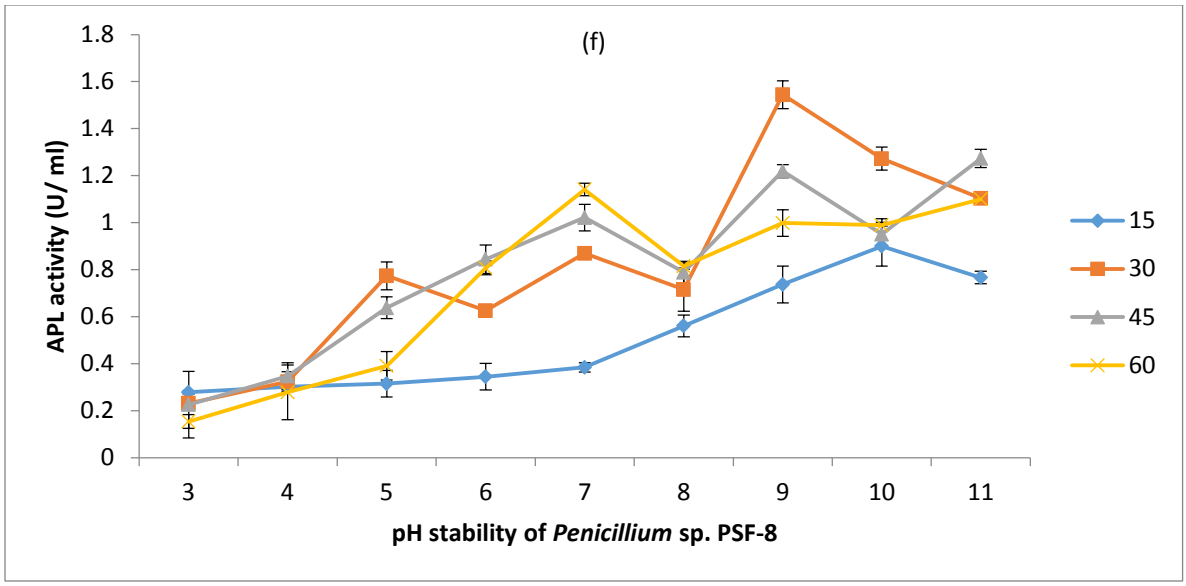


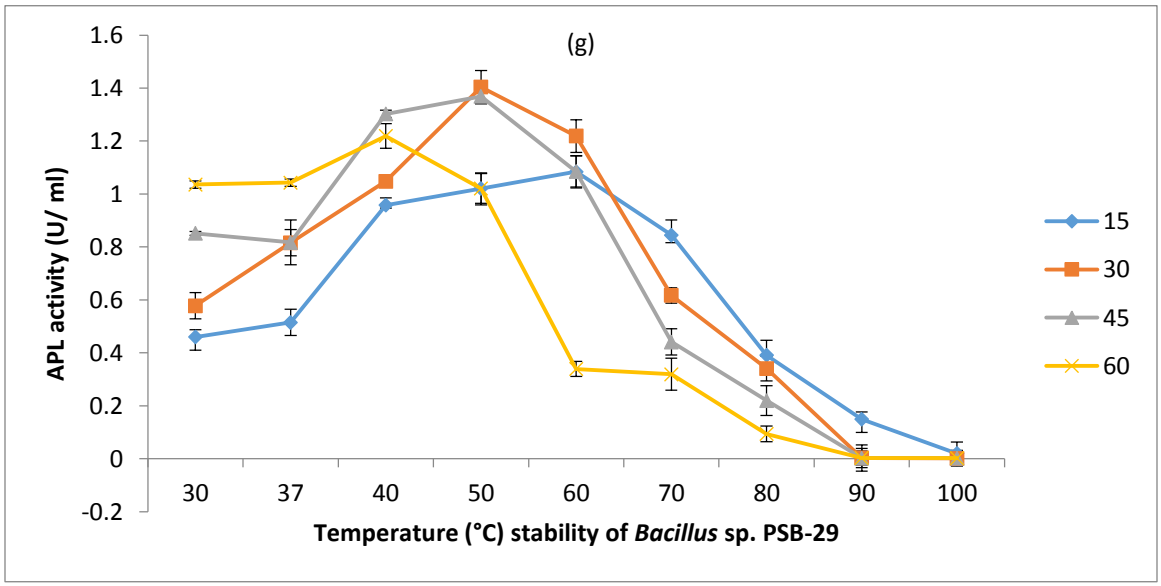
Fig. 5: Effect of pH on production of ALP by (a) *Bacillus* sp. (PSB-29) and (b) *Penicillium* sp. (PSF-8)

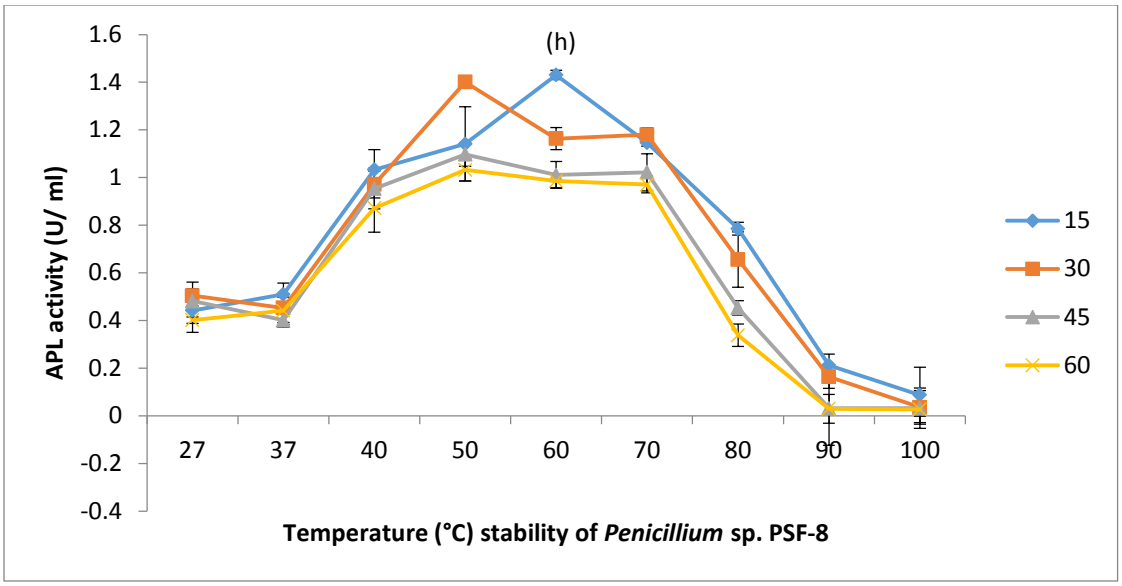



(b)
ALP activity (U/ ml)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
27
37
40
50
60
70
80
90
100
Temperature (°C)
Bacillus sp. PSB-29
Penicillium sp. PSF-8


(e)
APL activity (U/ ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3
4
5
6
7
8
9
10
11
pH stability of Bacillus sp PSB-29
15
30
45
60


(f)
APL activity (U/ ml)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3
4
5
6
7
8
9
10
11
pH stability of *Penicillium* sp. PSF-8
15
30
45
60


(g)
APL activity (U/ ml)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
30
37
40
50
60
70
80
90
100
Temperature (°C) stability of *Bacillus* sp. PSB-29
15
30
45
60



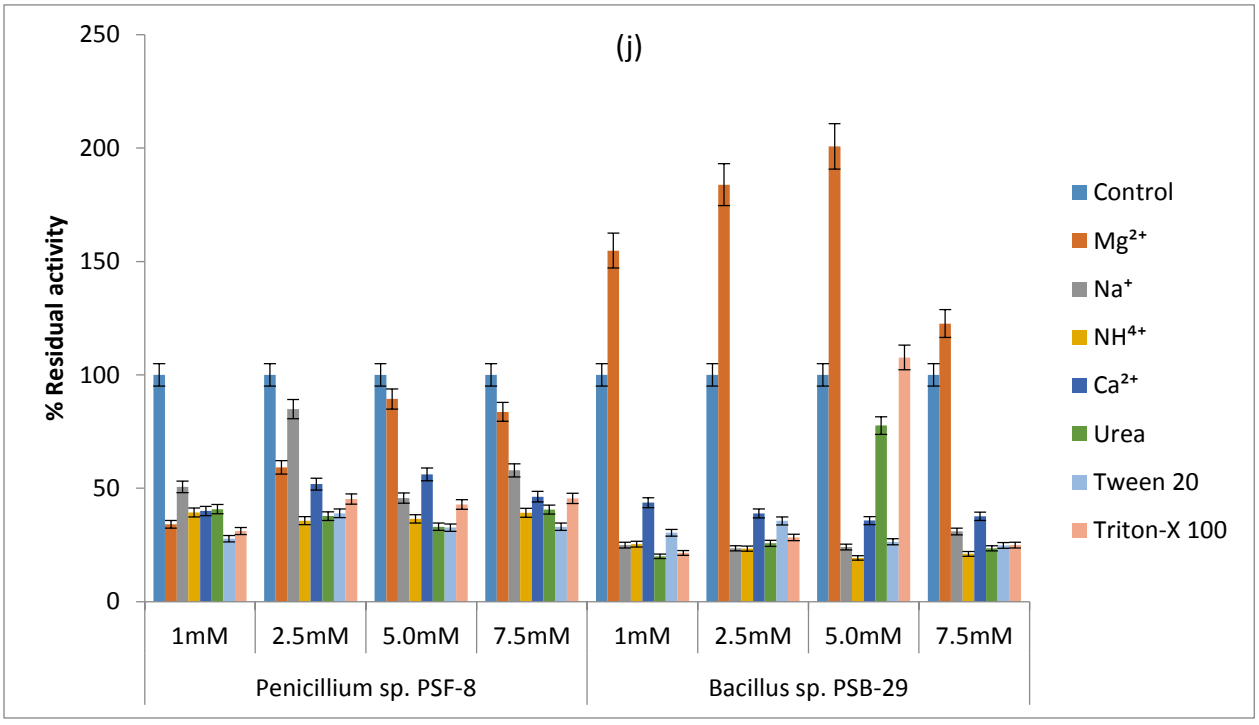


Fig. 6: Effect of (a) pH: (e, f) stability; (b) Temperature: (g, h) stability, and (j) Metal ions and inhibitors on ALP activity of *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29)

Table 3: Plant growth promoting effects of *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) on *A. hybridus* and *C. olitorius* plants

Treatment	Shoot height (cm)	Root length (cm)	SFW (g)	SDW (g)	RFW (g)	RDW (g)	No of leaves	No of capsules	P (µg/ mg)
DWTS	12.83±0.8	11.6±1.0	27.87±2.5	12.87±0.5	5.98±0.1	2.11±0.1	15.67±1.2	NA	143.43±2.0
DWTJ	34.33±2.5	12.1±0.4	33.25±0.7	16.34±0.6	10.22±0.5	4.65±0.3	14.33±0.6	2.5±0.5	103.42±1.4
BTS	14.00±1.0	13.5±1.2	35.76±1.1	16.54±0.6	6.43±0.4	3.31±0.3	21.67±2.1	NA	254.21±4.2
PTS	4.00±0.2	10.7±0.3	15.65±0.4	6.56±0.2	5.01	2.21±0.3	4.00±0.2	NA	26.75±0.3
BTJ	42.33±1.5	15.3±0.6	49.27±1.3	24.87±0.8	22.61±1.0	12.76±0.3	25.7±3.2	3.6±1.2	200.23±2.7
PTJ	22.67±0.1	12.1±0.6	31.74±0.3	15.51±0.3	13.05±0.5	7.21±0.1	15.5±1.5	2.5±0.5	67.98±0.1
PTBTS	-	-	-	-	-	-	-	NA	-
PTBTJ	17.00±0.9	11.4±0.2	34.6±1.0	18.87±1.8	11.47±0.3	6.98±0.6	14.0±0.6	2	24.66±0.4

Where; Dist. water treated spinach seeds (DWTS), Dist. water treated jute seeds (DWTJ). *Penicillium* sp. (PSF-8) treated spinach seeds (PTS), *Penicillium* sp. (PSF-8) treated jute seeds (PTJ), *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29) treated spinach seeds (PBTS), *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29) treated jute seed (PBTJ)), *Bacillus* sp. (PSB-29) treated spinach seeds (BTS), *Bacillus* sp. (PSB-29) treated jute seeds (BTJ), shoot fresh weight (SFW), shoot dry weight (SDW), root fresh weight (RFW), root dry weight (RDW), none applicable (NA), organic phosphorus (P), (±): Standard deviation

A better overall growth enhancement was observed on treatment with *Bacillus* sp. (PSB-29), compared to *Penicillium* sp. (PSF-8). Amongst the treatments, maximum enhancements of growth promotion were recorded in the *Bacillus* sp. (PSB-29) treated Jute seeds, recording 23.3 %, 26.4 %, 48.2 %, 52.2 %, 21.23 %, 174.4 %, 79.34 %, 44 % and 93.6 % improvement of the shoot height, root length, shoot fresh weight, shoot dry weight, root fresh weight, root dry weight, number of leaves, number of capsules and total organic P; respectively, compared to the untreated control. In the *Penicillium* sp. (PSF-8) treated *C. olitorius* plant; however, a 27.7 %, 55.1 % and 8.16 % increase in the root fresh weight, root dry weight and number of leaves was recorded compared to the control. On the other hand, co-inoculation of the seeds with the microbial isolates did not enhance the plant shoot and root length. Fungal treatments of the spinach and jute seeds resulted in 81.3 and 34.3 % reduction of the P content in the plants, compared to the control, whereas the bacterial treatment enhanced the P content in both *C. olitorius* and *A. hybridus* plants. The only growth promoting potential of *Penicillium* sp. (PSF-8) on the spinach plant was recorded on the dry root weight (a 4.74 % increase); however, the shoot length was reduced 3 times, compared to the control.

4. Discussion

In the current study, phosphate-solubilizing bacteria and fungi were isolated from the soil and water samples; evidenced by the formation of halo zones around the microbial colonies on the PVK and NBRIP-agar medium, due to the production of organic acids into the surrounding medium (Nautiyal, 1999, Mehta and Nautiyal, 2001; Kalayu,

2019). The microorganisms solubilized Tricalcium phosphate more efficiently on NBRIP medium than on PVK medium; as they produced clear and visible halos on NBRIP only, in accordance with results of the previous study conducted by Sanjotha and Manawadi, (2016). It was observed that the addition of a pH indicator dye (Bromocresol purple) that changed the medium color when the pH decreased, revealed a visual means for screening the microbial phosphate-solubilizing activity, and hence, aided in the selection of efficient isolates cultivated on the PVK and NBRIP media (Khan *et al*., 2014; Li *et al*., 2019).

The maximum bacterial and fungal phosphate solubilization index was exhibited by *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8), respectively. This high phosphate-solubilizing potential is possibly attributed to the microbial types, and to the diffusion of the organic acids secreted by the microorganisms (Yadav *et al*., 2011; Oteino *et al*., 2015; Li *et al*., 2019, Odeniyi and Itaba, 2020). Several authors had reported the production of organic acids as a solubilization mechanism of inorganic phosphorus by the microorganisms (Kishore *et al*., 2015; Elias *et al*., 2016; Kalayu, 2019). This is possibly due to the production of varied organic acids from glucose, which dissolve the phosphate compounds and/or chelate cations/ phosphorus ions (PO_4^{3-}), to release P into the solution (Yadav *et al*., 2011). The drop in pH of the cultures has been reported in several studies (Nautiyal, 1999, Malviya *et al*., 2011; Elias *et al*., 2016); in addition, the protons associated with microbially-secreted extracellular polysaccharides might also support the phosphate dissolution into the growth medium, as explained by Kishore *et al*., (2015); Li *et al*., (2019). The highest bacterial P concentration (848.88 µg/ ml) was released by *Bacillus* sp. (PSB-29), and similar phosphate-solubilization efficiency (876 µg/ ml) by *Pseudomonas* sp., was previously reported by Sharma *et al*., (2018).

The optimum temperature for maximum ALP production was at 42^{o}C after 48 h of incubation for *Bacillus* sp. (PSB-29) and after 72 h for *Penicillium* sp. (PSF-8). In accordance, another documented example of high ALP producing bacterium after 48 hours of incubation at 45^{o}C was *Serratia* sp., observed in the previous study conducted by Behera *et al*., (2017). Temperature is an important parameter to which the microbial responses may vary, since it affects the interior of the microbial cell, as well as the biological activity of soils (Behera *et al*., 2017). Microorganisms tend to grow and multiply rapidly at warmer temperatures (20-45^{o}C), as their molecules tend to move faster; the enzymes increase their rate of action and the cells rapidly increase in size (Khan *et al*., 2014).

The types of carbon and nitrogen sources utilized by the microorganisms may affect the amount of the enzyme produced. In this study, both *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) showed maximum ALP production in medium supplemented with glucose. *Bacillus* sp. (PSB-29) produced highest level of ALP on growing on ammonium sulfate as a nitrogen source, while *Penicillium* sp. (PSF-8 occurred) recorded higher levels in yeast extract medium. Previous studies conducted by Zhang *et al*., (2017); Li *et al*., (2019) reported that the phosphate solubilizing efficiency of the microorganisms is frequently related to their ability to secrete organic acids; the nature and quantity of these acids varies within the different sole carbon sources used and the microbes. In agreement with our study, Nautiyal, (1999) observed that when glucose was used as a carbon source, the microorganisms produced higher amounts of organic acids, which resulted in higher solubilization of insoluble phosphate. Similarly, Tahir *et al*., (2017) added that increasing the concentrations of the energy sources will enhance the phosphate-solubilizing potency of the microorganisms.

Alkaline phosphatase produced by *Bacillus* sp. (PSB-29) exhibited maximum activity at pH 8 on using *p*-NPP as substrate. In accordance, Dhaked *et*

al., (2005); Mahesh *et al*., (2010) also reported an optimum pH of 8 by *Bacillus sphaericus* P9 and a *Bacillus* sp., respectively. ALP of *Bacillus* sp. (PSB-29) had a relatively higher activity at pH 8 than pH 9 and 10, which may be attributed to the enzyme different sensitivity to Tris–HCl buffer (Chu *et al*., 2019). As the pH of the medium increased; the enzyme activity decreased, due to the effect of the charges on the amino acids present in the active site that inhibited the formation of an enzyme-substrate complex. Maximum activity of ALP of *Penicillium* sp. (PSF-8) was recorded at pH 9, which was the maximum defined ALP activity also of *Alcaligenes faecalis* (Behera *et al*., 2017), and with *Rhizopus microsporus* var. *rhizopodiformis* (Raheb *et al*., 2006).

The enzymes require cofactors such as metal ions for their structure, activation and assist in the enzyme-substrate binding (Chu *et al*., 2019). Maximum enzyme activity occurred in the presence of Mg^{2+} in *Bacillus* sp. (PSB-29), which agrees with the previous works of Behera *et al*., (2017); Sharma *et al*., (2018); Li *et al*., (2019); Tang *et al*., (2019), which reported the relevance of divalent metals in promoting the enzymatic activities. In accordance, ALP from several other microorganisms was enhanced by Zn^{2+}, Ca^{2+} and Mn^{2+} (Behera *et al*., 2017; Chu *et al*., 2019). All concentrations of Urea and Tween 20 inhibited the ALP activities of *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8). In the recent study of Li *et al*., (2019), other substances have been reported to inhibit ALP activity including EDTA and Flouro-oxalate.

The temperature influences the rate and activity of the enzymes through accelerating the velocity of the enzyme catalyzed reaction upon the increase in temperature (Tang *et al*., 2019), and the degree of this temperature varies from organism to organism. In this study, maximum ALP activity was observed at 40°C for *Bacillus* sp. (PSB-29) and at 50°C for *Penicillium* sp. (PSF-8). A previous work of Chu *et al*., (2019) reported 37°C as the optimum temperature for the ALP purified from *Lactobacillus casei*, whereas Zhang *et al*., (2018) revealed that the ALP purified from *P. aeruginosa* exhibited its optimum activity at 40°C. On the other hand, Dhaked *et al*., (2005) reported a much lower optimum temperature of 25°C for *B. sphaericus* P9, while Zhang *et al*., (2007); Mahesh *et al*., (2010) reported optimum temperatures of 62°C and 65°C for *Geobacillus thermodenitrificans* T2 and a *Bacillus* sp. respectively.

This study revealed that ALP of both *Penicillium* sp. (PSF-8) and *Bacillus* sp. (PSB-29) had higher substrate specificity to *p*-NPP, and the enzyme activity was amplified with the increase in the substrate concentration. In accordance, Chu *et al*., (2019) reported similar K_m and V_{max} with the ALP produced by *L. casei* 355 on using *p*-NPP as a substrate.

According to Seenivasagan and Babalola, (2021), the use of microbial inoculants as "live microbial biofertilizers" provides a promising alternative to the chemical fertilizers and synthetic pesticides. Solubilization of inorganic phosphate is one of the major mechanisms of plant growth promotion by the plant associated microorganisms, in addition to the production of plant growth hormones such as IAA. Oteino *et al*., (2015); Naziya *et al*., (2019) suggested that microbial release of organic acids into the soil solubilize the phosphate complexes, thus convert them into soluble phosphate that becomes available for the plant up-take and utilization. Currently, the detected amount of the produced IAA, which is an indicator of plant growth-promoting activities, was similar to that reported by Myo *et al*., (2019), but lower than that reported by Bunsangiam *et al*., (2021). These differences may be attributed to the difference in environmental sources and to variability among the microorganisms.

Bacillus sp. (PSB-29)-treated spinach (BTS) and jute (BTJ) seeds produced the highest biomass (i.e. root and shoot weights) and organic P for both plants, compared to the control. It was also observed

that *Penicillium* sp. (PSF-8) did not support the growth of *A. hybridus* as it supported the growth of *C. olitorius*. This suggests that *Bacillus* sp. (PSB-29) more efficiently solubilized the insoluble phosphate present in the soil; causing the release of soluble P, which was then easily absorbed by the plants resulting in enhanced plant growth.

It has been suggested that co-inoculation of the plant seeds with two or more microbial strains tends to significantly increase the plant growth, compared to single inoculations (Li *et al*., 2019). In this study, co-inoculation of *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8) did not enhance the growth of *A. hybridus* over the control plants; conversely, there was a decline in the growth of *A. hybridus*. However, for *C. olitorius*, co-inoculation of both microbial isolates resulted in higher fresh and dry root and shoot weights compared to the control, but was lesser than single inoculation with *Bacillus* sp. (PSB-29). Previous studies conducted by Yadav *et al*., (2011); Kalayu, (2019) revealed the synergistic effects of two or more plant growth promoting microorganisms. In the study of Kalayu, (2019), *P. striata* and *B. polymyxa* that were co-inoculated along with a strain of *Azospirillum brasilense*; significantly improved the grain and dry matter yields; in addition to an increase in the N and P contents, compared to single inoculation with each strain. In agreement with the current results, Yadav *et al*., (2011) carried out co-inoculation treatments with strains of *Trichoderma harzianum* and *Aspergillus niger* and reported significant increases in the shoot and root length and in the dry weights, compared to single inoculations and\or the control; along with an enhanced soil fertility. In accordance with the current results, Youssef *et al*., (2020) evaluated the growth and yield responses of Jute (*C. olitorius*) seeds on inoculation with a biofertilizer, which contained molasses as organic material in addition to mixed cultures of *Bacillus*, *Candida* and *Trichoderma* spp., and recorded supported and improved plant growth parameters over the control.

Conclusion

The current study showed that P; as an essential element in plant growth and nutrition, was successfully solubilized from insoluble phosphates by *Bacillus* sp. (PSB-29) and *Penicillium* sp. (PSF-8), with a high PSI. The alkaline pH and mesophilic temperature range enhanced both the ALP production and activity. Both microbial isolates produced the growth hormone IAA into the rhizosphere of the jute and spinach plants, in addition to causing the release of soluble phosphate that was subsequently assimilated by the plants, thereby improving the crop productivity. The use of efficient PSMs creates a better prospect for soil health, and therefore more studies on the PSMs should be undertaken to enhance the crop productivity.

Conflict of interest

The authors declare no conflict of interests.

Funding source

The current study did not receive any financial funds.

Ethical approval

None applicable.

5. References

Adnan, M.; Shah, F.; Zamin, M., Shah, S.; Mian, I.A.; Danish, S.; Zafar-ul-Hye, M.; Battaglia, M.L.; Naz, R.M.M.; Saeed, B.; Saud, S.; Ahmad, I.; Yue, Z.; Brtnicky, M.; Holatko, J. and Datta, R. (2020). Coupling phosphate-solubilising bacteria with phosphorus supplements improve maize phosphorus acquisition and growth under lime induced salinity stress. Plants. 9(900): 1-19. https://doi.org/10.3390/plants9070900

Alori, E.T.; Glick, B.R. and Babalola, O.O. (2017). Microbial phosphorus solubilisation and its potential for use in sustainable agriculture. Frontiers

in Microbiology. 8(971): 1-8. http://doi.org/10.3389/fmicb.2017.00971

Behera, B.C.; Yadav, H.; Singh, S.K.; Sethid, B.K.; Mishrad, R.R.; Kumarie, S. and Thatoi, H. (2017). Alkaline phosphatase activity of a phosphate solubilizing *Alcaligenes faecalis*, isolated from mangrove soil. Biotechnology Research and Innovation. 3: 1-11. http://doi.org/10.1016/j.biori.2017.01.003

Boubekri, K.; Soumare, A.; Mardad, I.; Lyamlouli, K.; Hafidi, M.; Ouhdouch, Y. and Kouisni, L. (2021). The screening of potassium- and phosphate-solubilizing actinobacteria and the assessment of their ability to promote wheat growth parameters. Microorganisms. 9: 470-485. https://doi.org/10.3390/microorganisms9030470

Bunsangiam, S.; Thongpae, N.; Limtong, S. and Srisuk, N. (2021). Large scale production of indole 3 acetic acid and evaluation of the inhibitory efect of indole 3 acetic acid on weed growth. Scientific Reports. 11: 13094-13107. https://doi.org/10.1038/s41598-021-92305-w

Cheesbrough, M. (2005). District laboratory practice in tropical countries Part 2, UK, Cambridge University Press, Cambridge, UK. 56-70.

Chen, J.; Zhao, G.; Wei, Y.; Dong. Y.; Hou, L. and Jiao R. (2021). Isolation and screening of multifunctional phosphate solubilizing bacteria and its growth-promoting effect on Chinese fir seedlings. Scientific Reports. 11: 9081-9093. https://doi.org/10.1038/s41598-021-88635-4

Chu, Y.H.; Yu, X.X.; Jin, X.; Wang, Y.T.; Zhao, D.J.; Zhang, P.; Sun, G-M. and Zhang, Y-H. (2019). Purification and characterization of alkaline phosphatase from lactic acid bacteria. Royal Society of Chemistry Advances. 9: 354-360. http://doi.org/10.1039/C8RA08921C

Dandessa, C. and Bacha, K. (2018). Review on role of phosphate-solubilising microorganisms in sustainable agriculture. International Journal of Current Research and Academic Review. 6(11): 48-55. http://dx.doi.org/10.20546/ijcrar.2018.611.006

Dhaked, R.K.; Alam, S.I.; Dixit, A. and Singh, L. (2005). Purification and characterization of thermo-labile alkaline phosphatase from an antarctic psychrotolerant *Bacillus* sp. P9. Enzyme and Microbial Technology. 36(7): 855-861. http://doi.org/10.1016/j.enzmictec.2004.11.017

Ebrahimian, M. and Motamedi, H. (2019). Phytase producing *Klebsiella oxytoca* fish 12 isolated from Carp farming sediment. South Western Journal of Horticulture, Biology and Environment. 10(1): 29-43.

Elias, F.; Woyessa, D. and Muleta, D. (2016). Phosphate solubilisation potential of rhizosphere fungi isolated from plants in Jimma Zone, Southwest Ethiopia. International Journal of Microbiology. 2016: 1-11. http://doi.org/10.1155/2016/5472601

EPA. (2021). Issue: Nutrient Pollution: US Environmental Protection Agency, Nutrient Pollution. http://www.epa.gov/nutrientpollution/issue

Holt, J.G.; Krieg, N.R.; Sneath, P.H.A.; Stanley, J.T. and Williams, S.T. (1994). Bergey's Manual of Determinative Bacteriology (9th Edition), Williams & Wilkins, Co., Baltimore. 559-565.

Ingle, K.P. and Padole, D.A. (2017). Phosphate solubilizing microbes: an overview. International Journal of Current Microbiology and Applied Sciences. 6(1): 844-852. http://doi.org/10.20546/ijcmas.2017.601.099

Jathoth, K.; Shantipriya, A.; Mangilal, T. and Junapudi, S. (2015). Optimization for the production of extracellular alkaline phosphatase

from *Bacillus subtilis*. International Journal of Current Microbiology and Applied Sciences. 4(10): 829-838.

Kalayu, G. (2019). Phosphate solubilizing microorganisms: promising approach as biofertilizers. International Journal of Agronomy. 2019: 1-7. http://doi.org/10.1155/2019/4917256

Khan, M.S.; Zaidi, A. and Musarrat, J. (2014). Phosphate Solubilizing Microorganisms: Principles and Application of Microphos Technology. Springer: Cham, Switzerland. pp. 1-307. http://dx.doi.org/10.1007/978-3-319-08216-5

Kishore, N.; Pindi, P.K. and Ram Reddy, S. (2015). Phosphate-Solubilizing Microorganisms: A Critical Review. In: Bahadur, B., Venkat Rajam, M., Sahijram, L., Krishnamurthy, K. (Editions) Plant Biology and Biotechnology. Springer, New Delhi. 307-333. http://doi.org/10.1007/978-81-322-2286-6_12

Li, Y.; Zhang, J.; Zhang, J.; Xu, W. and Mou, Z. (2019). Characteristics of inorganic phosphate-solubilizing bacteria from the sediments of a eutrophic lake. International Journal of Environmental Research and Public Health. 16: 1-15.http://doi.org/10.3390/ijerph16122141

Li, Y.; Liu, X.; Hao, T. and Chen, S. (2017). Colonization and maize growth promotion induced by phosphate solubilizing bacterial isolates. International Journal of Molecular Sciences. 18: 1-16. http://doi.org/10.3390/ijms18071253

Mahesh, M.; Neha, G.; Rajesh, T.S.; Somashekhar, R. and Puttaiah, E.T. (2010). Isolation and characterization of extracellular thermostable alkaline phosphatase enzyme from *Bacillus* spp. International Journal of Applied Biology and Pharmaceutical Technology. 1(1): 21-33.

Malviya, J.; Singh, K. and Joshi, V. (2011). Effect of phosphate solubilizing fungi on growth and nutrient uptake of ground nut (*Arachis hypogaea*) plants. Advances in Bioresearch. 2 (2): 110-113. http://doi.org/10.5539/jas.v8n10p204

Mehta, S. and Nautiyal, C.S. (2001). An efficient method for qualitative screening of phosphate-solubilizing bacteria. Current Microbiology. 43: 51-56. http://doi.org/10.1007/s002840010259

Mohamed, A.E.; Nessim, M.G.; Abou-el-seoud, I.I.; Darwish, K.M. and Shamseldin, A. (2019). Isolation and selection of highly effective phosphate solubilizing bacterial strains to promote wheat growth in Egyptian calcareous soils. Bulletin of the National Research Centre. 43(203): 1-13. https://doi.org/10.1186/s42269-019-0212-9

Murphy, J. and Riley, J.P. (1962). A modified single solution method for the determination of phosphate in natural waters. Analytica Chimica Acta. 27: 31-36. https://doi.org/10.1016/S0003-2670(00)88444-5

Myo, E.M.; Ge, B.; Ma, G.; Cui, H.; Liu, B.; Shi, L.; Jiang, M. and Zhang, J. (2019). Indole-3-acetic acid production by *Streptomyces fradiae* NKZ-259 and its formulation to enhance plant growth. BMC Microbiology. 19(155): 1-14. https://doi.org/10.1186/s12866-019-1528-1

28 Nautiyal, C.S. (1999). An efficient microbiological growth medium for screening phosphate-solubilizing microorganisms. FEMS Microbiology Letters. 170: 265-270. https://doi.org/10.1111/j.1574-6968.1999.tb13383.x

Naziya, B.; Murali, M. and Amruthesh, K.N. (2019). Plant growth-promoting fungi (PGPF) instigate plant growth and induce disease resistance in *Capsicum annuum* L. upon infection with *Colletotrichum capsici* (Syd.) Butler and Bisby.

Biomolecules. 10(1): 41. https://doi.org/10.3390/biom10010041

Niu, L.; Zhang, H.; Wu, Z.; Wang, Y.; Liu, H. and Wu, X. (2018). Modified TCA/acetone precipitation of plant proteins for proteomic analysis. PLoS ONE 13(12): 343-349. https://doi.org/10.1371/journal.pone.0211612

Obidi, O.F.; Awe, O.O.; Igwo-Ezikpe, M.N. and Okekunjo, F.O. (2018). Production of phosphatase by microorganisms isolated from discolored painted walls in a typical tropical environment: a non-parametric analysis. Arab Journal of Basic and Applied Sciences. 25(3): 111-121. http://doi.org/10.1080/25765299.2018.1527277

Odeniyi, O.A. and Itaba, L. (2020). Production and characterization of an *Aspergillus niger* GS1S6 phytase cultivated in phytic acid-rich agricultural substrates. Fungal Territory. 3(3): 9-15. https://doi.org/10.36547/ft.2020.3.3.9-15

Oteino, N.; Lally, R.D.; Kiwanuka, S.; Lloyd, A.; Ryan, D.; Germaine, K.J. and Dowling, D.N. (2015). Plant growth promotion induced by phosphate-solubilising endophytic *Pseudomonas* isolates. Frontiers in Microbiology. 6: 745-760. https://doi.org/10.3389/fmicb.2015.00745

Pikovskaya, R.I. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiology. 17: 362-370.

Pilar-Izquierdo, M.C.; Ortega, N.; Perez-Mateos, M. and Busto, M.D. (2012). Barley seed coating with free and immobilized alkaline phosphatase to improve P uptake and plant growth. The Journal of Agricultural Science. 150(06): 691-701. https://doi:10.1017/s0021859612000019

Raheb, J.; Naghdi, S. and Flint, K.P. (2006). Alkaline phosphatase activity of *Flexibacter chinensis* under starvation stress in water microcosms. Enzyme and Microbial Technology. 40(1): 13-16. http://doi.org/10.1016/j.enzmictec.2005.12.024

Rodriguez, H. and Fraga, R. (1999). Phosphate-solubilising bacteria and their role in plant growth promotion. Biotechnology Advances. 17: 319-339. https://doi.org/10.1016/S0734-9750(99)00014-2

Sanjotha, G. and Manawadi, S. (2016). Isolation, screening and characterization of phosphate-solubilizing bacteria from Karwar costal region. International Journal of Research Studies in Microbiology and Biotechnology. 2(2): 1-6. http://doi.org/10.20431/2454-9428.0202001

Seenivasagan, R. and Babalola, O.O. (2021). Utilization of microbial consortia as biofertilizers and biopesticides for the production of feasible agricultural product. Biology. 10(11): 1111-1133. https://doi.org/10.3390/biology10111111

Sharma, R.; Thakur, R.K. and Kaur, M. (2018). Purification and characterization of alkaline phosphatase from *Pseudomonas aeruginosa* isolated from apple rhizosphere. International Journal of Chemical Studies. 6(6): 1156-1159.

Tahir, H.A.; Gu, Q.; Wu, H.; Raza, W.; Hanif, A. and Wu, L. (2017). Plant growth promotion by volatile organic compounds produced by *Bacillus subtilis* SYST2. Frontiers in Microbiology. 8: 171-179. https://doi.org/10.3389/fmicb.2017.00171

Tang, Z.; Chen, H.; He, H. and Ma, C. (2019). Assays for alkaline phosphatase activity: progress and prospects. Trends in Analytical Chemistry. 113: 32-43. https://doi.org/10.1016/j.trac.2019.01.019

Yadav, J.; Verma, J.P. and Tiwari, K.N. (2011). Plant growth promoting activities of fungi and their effect on chickpea plant growth. Asian Journal of

Biological Sciences. 4(3): 291-299. http://doi.org/10.3923/ajbs.2011.291.299

Youssef, A.F.; Youssef, M.A.; Ali, M.M.; Ibrahim, M.M.; Xu, Y. and Mauro, R.P. (2020). Improved growth and yield response of Jew's mallow (*Corchorus olitorius* L.) plants through biofertilization under semi-arid climate conditions in Egypt. Agronomy. 10(1801): 1-14. https://doi.org/10.3390/agronomy10111801

Zhang, Y.; Chen, F.S.; Wu, X.Q.; Luan, F.G.; Zhang, L.P. and Fang, X.M. (2018). Isolation and characterization of two phosphate-solubilising fungi from rhizosphere soil of Moso Bamboo and their functional capacities when exposed to different phosphorus sources and pH environments. PLoS ONE. 13(7): 1-14. https://doi.org/10.1371/journal.pone.0199625

Zhang, J.; Zhang, Q.A.; Wang, P.; Fang, L.; Yan, C. and Chen, J. (2017). Isolation and characterization of phosphate-solubilising bacteria from mushroom residues and their effect on tomato plant growth promotion. Polish Journal of Microbiology. 66(1): 57-65. https://doi.org/10.5604/17331331.1234993

Zhang, H.; Kim M.S.; Krishnamachari, V.; Payton, P.; Sun, Y.; Grimson, M.; Farag, M.; Ryu, C.M.; Allen, R.; Melo, I. and Pare, P. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. Planta. 226: 839-851. https://doi.org/10.1007/s00425-007-0530-2