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Phosphate solubilization potential of indigenous rhizosphere fungi and their biofertilizer formulations

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Abstract. The harmful effects of chemical fertilizers on soil, plants, and eco-systems have stimulated the growth of the global biofertilizer market. However, biofertilizer use remains limited in developing countries due to inadequate research and poor technology. The use of readily available materials for biofertilizer production can be a good starting point. This study aimed to investigate phosphate-solubilizing potentials of soil fungi and the shelf-life of their biofertilizer formulations using sawdust and charcoal as carriers. Soil samples from the rhizosphere were cultured on Pikovskaya (PVK) agar, and the best phosphate solubilizers (*Aspergillus niger, Aspergillus fumigatus* and *A. flavus*) were screened for their phosphate-solubilization potentials on solid medium. Results obtained showed that *A. niger* had the highest solubilization index of 1.72, followed by *A. fumigatus,* and *A. flavus* with a solubilization index of 1.01 and 0.95, respectively. Optimization studies showed that after 5 days of incubation, *A. niger, A. flavus* and *A. fumigatus* solubilized 149, 112 and 126 mg L^{-1} of phosphate, respectively. These values increased to 549 mg L^{-1} on day 11 for *A. niger*, 379 mg L^{-1} on day 9 for *A. flavus* and 430 mg L^{-1} on day 9 for *A. fumigatus*. Furthermore, *A. fumigatus* and *A. flavus* proved to be better inoculants than *A. niger* as they maintained higher CFU g^{-1} counts throughout the experiment. Also, sawdust supported higher counts of the three inoculants than charcoal and was thus the best carrier. The findings demonstrated that these aspergilli can be harnessed for improving soil fertility and plant development.

Key words: *A. niger, A. flavus, A. fumigatus,* biofertilizer, optimization, phosphate solubilization, shelf-life.

INTRODUCTION

Phosphorus is the most vital element for the growth and development of plants after nitrogen. It carries out key roles in the growth of plants, and it is also the major plant growth limiting nutrient (Azubuike et al., 2016)). Plants naturally have abilities to obtain nutrients through the formation of beneficial relationships with microbes (Lema et al., 2012). However, they encounter different growth limiting factors throughout their lifecycle. They overcome these conditions through different natural processes such as development of ability to adapt to changes in their immediate environments, and by adopting alternative metabolic pathways. Plants face many obstacles during development and growth; one of such is their inefficient uptake of phosphate from soil due to the complex nature of phosphates present in the soil (Jones & Oburger, 2011). In addition to that, the low levels of phosphorus in the Earth crust further limit absorption. Hence, improving the absorption rate of phosphorus in the soil is a key issue in agriculture (Cordell et al., 2009).

Recently, there has been a significant rise in the use of fertilizers formulated using microorganisms (Nosheen et al., 2021). This has aided the race to minimize the deleterious effects of synthetic fertilizers on the environment and soil. Many studies revealed the potential role of microorganisms to solubilize phosphates. This includes mediating the bioavailability of soil phosphorus (Cong et al., 2020), aiding the mineralization of organic phosphates (Tate, 1984; Tamburini et al., 2012), solubilizing inorganic phosphate minerals into absorbable forms (Fixen & Johnston, 2012) and storing large amounts of phosphorous in biomass. Chen et al. (2006) reported that phosphorus-solubilizing microorganisms (PSMs) can secrete acids, which are of organic structure. These compounds then acidify the cellular components of microorganisms, leading to the solubilization of inorganically bound phosphates (PO_4^- or PO_4^{2-}) by dissociating them from any metallic element they are bound to. Many microorganisms with phosphate- solubilization potential have been isolated from soil. The most common groups of organisms are those of fungal (*Penicillium* and *Aspergillus*) and bacterial origin (*Pseudomonas* and *Bacillus*) (Wakelin et al., 2004). Inoculation of soil with microbes to improve soil fertility began in the late 19th century when microbes were sold for the purpose of producing fertilizers (Kilian et al., 2000).

Dittmar et al. (2009) described fertilizers as naturally occurring or synthetically formulated substances, which when incorporated into plants or soil, provide vital nutrients for plant growth and development. The two main forms of fertilizers are synthetically- (chemical fertilizers) and non-synthetically (biofertilizers) formulated fertilizers. The continuous use of inorganic synthetic fertilizers over the years has helped to improve soil fertility and crop yield. However, the adverse effects that these fertilizers have posed on the ecosystem, plants, and soil is getting alarming in these past few years (Islam et al., 2017). Due to the degrading effects of chemical fertilizers on the environment, scientists have sought for safe alternatives, which are not lethal, non-synthetic, and cheap. This search led to the discovery of biofertilizers. One of the merits of using chemical fertilizers, particularly those of nitrogen and phosphorus origin, is their direct and swift action when applied to the soil. Also, chemical fertilizers are quite cheap, hence affordable to farmers of all kinds. However, overdependence on these chemicals

has led to deterioration in soil fertility, destruction of useful organisms in the soil (e.g., microbes and earthworms) and eutrophication, which has exposed plants to different diseases (Majumdar, 2015). More so, increased acidity due to the prolonged use of chemical fertilizers can cause accumulation of heavy metals in the soil (Dai et al., 2021). This can consequently limit the replication of beneficial bacteria and fungi.

Microorganisms are very important components of biofertilizers as their use can improve soil fertility and proliferation of beneficial bacteria and fungi. They are used as N_2 fixers (*Rhizobium, Bradyrhizobium, Azospirillum* and *Azotobacter*), phosphate solubilizers (*Bacillus, Pseudomonas, Aspergillus*, *Penicillium*, *Fusarium, Trichoderma*, *Mucor*, *Ovularopsis*, *Tritirachium* and *Candida*), and phosphate mobilizers (*Mycorrhiza*) are some of the biofertilizer inoculants commonly used (Pal et al., 2015). Besides the microbial inoculants, the inert carriers are also an important component of biofertilizers. They serve as protectants for the microbes, especially when the immediate environment (soil) is hostile, which helps the living inoculants to remain viable for a longer period in the soil (Chaudhary et al., 2020). Materials that have been employed as bioinoculant carriers include peat, clay, wood ash, grain protective coverings (brans and husks), natural and synthetic polymers, compost and alginate (Mitter et al., 2021; Sakpirom et al., 2021). In recent times, the use of nanoparticles has also gained increased attention (Chuen et al., 2021). However, considering the abundance of sawdust and the practice of charcoal production in developing countries where farmers depend on less advanced technologies, the use of these materials as bioinoculant carriers is an attractive option.

This study analyzed the phosphate-solubilizing potentials of three different fungi (*Aspergillus niger, Aspergillus fumigatus* and *A. flavus*) were analyzed. Soil samples from the rhizosphere were isolated, characterized, and cultured on growth medium in order to quantify the amount of phosphorus that they are capable of dissolving in both solid and liquid medium. In addition, optimization of incubation conditions was carried out to investigate the best conditions for phosphate solubilization in fungi. Finally, the phosphate-solubilizing isolates were incorporated into biofertilizers as living inoculants, then packed and stored to ascertain their viability using sawdust and charcoal as carriers.

MATERIALS AND METHODS

Sampling site and soil collection

Soil samples were taken from the University of Ilorin, Ilorin, Kwara State, Nigeria (Lat. 8.492819, Long. 4.596161) and from the Nigerian Stored Product Research Institute, Ilorin, Nigeria (Lat. 8.454472, Long. 4555397). The samples were obtained from the rhizosphere of palm tree, banana tree, mango tree, *Moringa* tree. The sample collection was carried out based on the method described by Xiao et al. (2008). For this, 20 g of soil was collected at 0–5 cm around the root of the plants. The soil was then bagged into separate sterile Ziploc bags and kept in the refrigerator (4 °C) until use. Granulated rock phosphate (30 g) was obtained from the laboratory of the Department of Agronomy, University of Ilorin, Ilorin, Nigeria. One hundred grams of fine powder of calcium phosphate used throughout the course of this study was also obtained from the same laboratory.

Preparation of media

The two media used for this study were Pikovskaya (PVK) agar and Potato Dextrose (PD) Agar. PVK medium was formulated using the method described by Elias et al. (2016). For this, 0.5 g of $(NH_4)_2SO_4$, 0.5 g of $MgSO_4.7H_2O$, 0.3 g of NaCl, 0.3 g of KCl, 0.03 g $FeSO_4.7H_2O$, 0.02 g of $MnSO_4.H_2O$, 10.0 g of $Ca_3(PO_4)_2$, 10 g glucose and 15 g agar were used. The PD agar was prepared based on the description provided by the manufacturer. The medium was homogenized using a magnetic stirrer and further autoclaved at 121 °C for 15 minutes. Pikovskaya agar was prepared by weighing its constituents into a 1,000 mL conical flask, then adding 1,000 mL of distilled water into the mixture, this was then gently shaken and heated on a magnetic stirrer for 20 minutes until the mixture is well homogenized and all the calcium deposits are completely dissolved, the mixture was then autoclaved at 121 °C for 15 minutes. Pikovskaya broth was prepared using the same method above but without adding agar.

Isolation of rhizosphere fungi

This was done using the pour plate method as described by Nelofer et al. (2016). Separate serial dilutions of each soil sample were prepared. One gram of soil sample was introduced into 9 mL of sterile distilled water shaken vigorously and serial dilutions of the resulting suspension were prepared in separate tubes containing similar amount of sterile distilled water. Aliquots (1 mL) of appropriate dilutions were then dispensed into molten PVK agar (at 45 °C) and swirled for even homogenisation. The plates were incubated at 27 °C for 3–5 days.

All the distinct colonies that grew on PVK agar and that had zone of clearance (halo) around them were aseptically subcultured and incubated at 27 °C for 3–4 days. This method was repeated until a pure isolate of each isolate was obtained. The pure cultures were then transferred into a sterile solid agar slant (PDA) and kept at 4 °C until use.

Identification of isolates

Identification of the isolates was using a combination of colonial-microscopic characteristics (Fawole & Oso, 2004) and molecular identification. Molecular identification was done by sequencing of the ITS gene of the best three isolates from the screening process. Extraction of the genomic DNA of the isolates was done using fungal DNA isolation kit 26200 (Norgen Biotek Corp., Canada). Amplification of the ITS region of the genome was done using PCR with the primers ITS4: 5–TCCTCCGCTTATTGATATGC-3 and ITS5: 5-GGAAGTAAAAGTCGTAACAAGG-3. The PCR mix contained 10× PCR buffer (1.0 µL), 25 mM $MgCl_2$ (1.0 µL), 5 pmol of forward and reverse primers (0.5 µL each), DMSO (1.0 µL), 2.5 mM DNTPs (0.8µL), Taq 5U uL^{-1} (0.1 µL), 10 ng μL^{-1} DNA (2.0 µL), and H_2O (3.1 µL). The PCR reaction was run under the following conditions: initial denaturation was done for 5 min at 94 °C, followed by 30 sec denaturation at 94 °C; annealing was done at 54 °C for 35 sec, extension was at 72 °C for 45 sec (36 cycles), and final extension was at 72 °C for 7 min. Finally, the reaction was held under holding temperature of 10 C (∞). The PCR amplicon was then loaded on 1.5 % agarose gel using a 1 kbp plus ladder (Invitrogen). the estimated size of the amplicon was 850 bp. The product was purified and sequenced using the ABI genetic analyzer model 3500 automated sequencer (Applied Biosystems, USA). Obtained sequences were identified based on homology with the highest similarity using the BLASTN tool (www.ncbi.nlm.nih.gov:80/BLASTN/).

Screening for phosphate solubilization potentials on agar medium (PVK agar)

The isolates previously grown on PVK agar were screened to quantify the amount of phosphate solubilized in the solid medium. This was done by aseptically transferring fungal isolates from stock cultures into PVK agar. The samples were then incubated at 28 °C for 4 to 7 days. The diameter of halo formed was measured using a ruler and used to calculate the solubilization index (Equation 1), as described by Saxena et al. (2013):

$$\text{Solubilization Index (SI)} = \frac{\text{Colony diameter + Halo zone diameter}}{\text{Colony diameter}} \quad (1)$$

Preparation of fungal inocula

The isolates were cultivated on PD agar and incubated at a temperature of 27 °C for 7 days. An aliquot of 10 mL of sterile distilled water was then measured into each of the culture plates. The plates were swirled gently to dislodge conidia from the culture surface. The dislodged conidia suspensions were then collected in 250 mL conical flasks and filtered through a few layers of cheesecloth and then centrifuged. The resulting pellet was re-suspended in sterile distilled water and the concentration of conidia was adjusted to 1.0×10^8 CFU mL^{-1} using a Neuber haemocytometer (Niranjana et al., 2009; Mahadevamurthy et al., 2016).

Estimation and quantification of solubilized phosphate in liquid medium

Sterile PVK medium of 100 mL volume was prepared in 250 mL flask and was supplemented with either 0.5% calcium phosphate (CaP) or rock phosphate (RP). The pH of the medium was adjusted to 7.0 with 2 M NaOH or H_2SO_4. The sterilized PVK medium was inoculated with a 1.010^8 CFU mL^{-1} spore suspension of each isolate and incubated in a shaker at 150 rpm and 27 °C. The incubation was done in triplicate for each isolate and their results were recorded as average values (Elias et al., 2016). The amount of solubilized phosphorous and the decrease in pH were measured every 24 hours for 4 days. This was done by aseptically collecting 5 mL of culture broth and centrifuging it at 5,000 rpm for 10 minutes to separate suspended solids and mycelial fragments. The pH of the supernatant was then measured, and the amount of solubilized phosphate was quantified. Aliquots of the medium were taken every 24 hours and quantified for dissolved phosphate using the chlorostannous acid reduced molybdophosphoric blue colour method (Naik et al., 2013).

Determination of pH in the liquid medium

Changes in pH of the medium were measured with a pHep® Hanna pH meter using 5 mL samples collected every 24 hours.

Optimization of phosphate solubilization

The optimization of the growth conditions for phosphate solubilizing fungi was done by the method described by Yadav & Tarafdar (2003). A spore concentration of 1.0×10^8 cells per mL was inoculated into sterile PVK broth medium. The pH of the medium (before inoculation) was adjusted to 7 using 2 M NaOH or H_2SO_4. Incubation was carried out in a shaker at 150 rpm and 27 °C. Aliquots of the medium were taken every 24 hours and assayed for dissolved phosphate using the chlorostannous acid

reduced molybdophosphoric blue colour method. This was done continuously until a decline in phosphate levels was observed.

The phosphate solubilization capacity of the fungal isolates at varying concentrations of metallic phosphate was analyzed by incubating each isolate in Pikovskaya Broth medium which was supplemented with two phosphates sources *viz*. CaP or RP at the following concentrations: 1.5, 2.5, 5.0, 7.5, and 10 g L^{-1}. This was done by replacing the original CaP used in the formulation of PVK medium with varying quantities of CaP or RP.

Phosphate solubilization capacity at different initial medium pH was investigated by varying the pH of the Pikovskaya broth medium between 5 and 9 t. The flasks were incubated at 27 °C for 96 hours and changes in pH were recorded accordingly.

The effect of inoculum size on phosphate solubilization was studied by using varying concentrations of the spore suspension ranging from 0.5–2.0×10^6 spores mL^{-1} (Niranjana et al., 2009 and Mahadevamurthy et al., 2016).

Biofertilizer Formulations

The biofertilizer formulations were prepared based on the method described by Bhattacharjee & Dey (2014). Here, fungal isolates were grown on PDA and when sporulation was noticed (after 7 days) 9 mL of sterile distilled water was added to the agar plates and shaken until the spores became dislodged. The displaced spores were collected in a sterile conical flask, filtered through cheesecloth, and then centrifuged. The recovered spores were adjusted to 1.0×10^8 CFU mL^{-1} with the aid of a haemocytometer as described by Niranjana et al. (2009) and Mahadevamurthy et al., (2016). Fungal spores (9 mL) were then inoculated into 300 mL of sterile potato dextrose broth. A mass of 1 kg of sterilized carrier material (charcoal or sawdust) was then introduced into the mixture and blended. The mixture was allowed to dry at 27 °C. After drying, it was packed in Ziplock bags and saved at room temperature until use.

Statistical Analysis

Statistical significance was determined using one-way and two-way analysis of variance (ANOVA), while multiple comparisons between means were determined by Tukey's multiple comparisons test. All data were expressed as means of triplicates ± standard deviation, and values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Characterization of the phosphate-solubilizing fungi (PSF)

Three fungal isolates (*A. niger, A. flavus*, and *A. fumigatus)* were used in this study to investigate the potential of solubilizing phosphate in both solid and liquid medium. Fig. 1 shows the microscopic view of the three isolates utilized in this study. Hefnawy et al. (2009) reported that these fungal isolates are capable of solubilizing high quantities of phosphate even under low nutrient conditions.

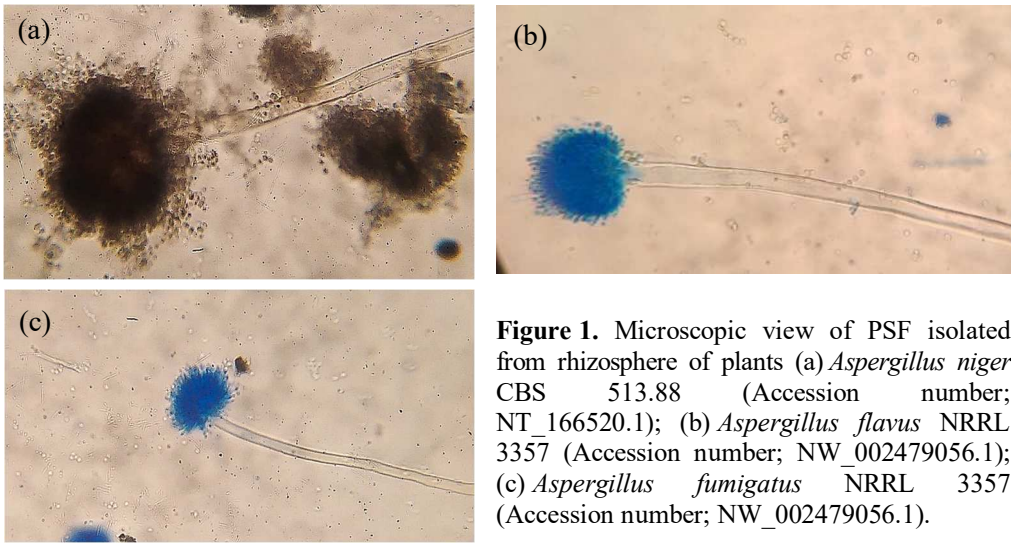

Figure 1. Microscopic view of PSF isolated from rhizosphere of plants (a) *Aspergillus niger* CBS 513.88 (Accession number; NT_166520.1); (b) *Aspergillus flavus* NRRL 3357 (Accession number; NW_002479056.1); (c) *Aspergillus fumigatus* NRRL 3357 (Accession number; NW_002479056.1).

Solubilization index of the phosphate solubilizing fungi

The amount of phosphate solubilized by *A. niger, A. flavus* and *A. fumigatus* on a solid medium (PVK agar) supplemented with calcium phosphate is reported in Fig. 2. As it can be seen from the figure, *A. niger* had the highest solubilization index (1.72 SI), followed by *A. fumigatus* (1.01 SI), and *A. flavus* (0.95 SI).

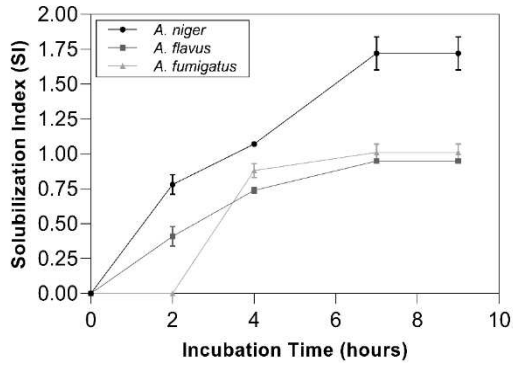

Figure 2. Solubilization Index of the three isolates on PVK agar after 9 days of incubation. Values are means of 3 replicates and error bars represent standard deviation.

The high performance of *A. niger* can be explained by the fact that this isolate produces organic acids that are highly ionizable and that can cause the solubilization of calcium phosphate compounds (Pradhan & Sukla, 2005). Additional studies performed by Yasser et al. (2014) and Seshadri et al. (2004) showed that *A. niger* solubilizes high quantities of phosphates from calcium phosphate supplements. These results can also be explained by the halo zone. A study performed by Hefnawy et al. (2009) has shown that *A. niger* formed a wider halo zone on Pikovskaya agar when supplemented with calcium phosphate than *A. flavus and A. fumigatus*. However, the same study reported that *A. fumigatus* had a higher phosphate solubilization index than *A. flavus*.

Effect of different pH levels on solubilized phosphate

The effect of different pH values (pH 5 to pH9) on the phosphate solubilization potential of the fungal isolates is shown in Fig. 3. The effects of different pH values (pH

to pH 9) on the solubilized phosphate 5quantities by the isolated fungi is shown in Fig. 3. From the results, overall, the pH of the liquid medium plays a vital role in the amount of phosphate solubilized, it was observed that pH 7 proved to be the best for phosphate solubilization, followed by acidic pH, while alkaline pH caused the least phosphate solubilization among all the 3 isolates. Ordinarily, an acidic pH should cause more phosphate solubilization because of the presence acids that could help ionize locked phosphates, however, fungi species of the *Aspergillus* genera grow best under neutral pH conditions than under low or high pH hence tends to solubilize more phosphate under neutral conditions.

Overall, the pH of the incubation process plays a vital role in the amount of solubilized phosphate generated. All the isolates had the maximum solubilized phosphate at pH 7, and the minimum at pH 9, except for *A. fumigatus*. These results can be explained by the fact that the *Aspergillus* genera can grow in a wide pH range, but the optimum growth is found at pH 7 (Wheeler et al., 1991). Other studies performed by Wu et al. (2012) and Anitha & Padma (2016) also reported pH 7 as the most favorable for solubilized phosphate production by fungal isolates.

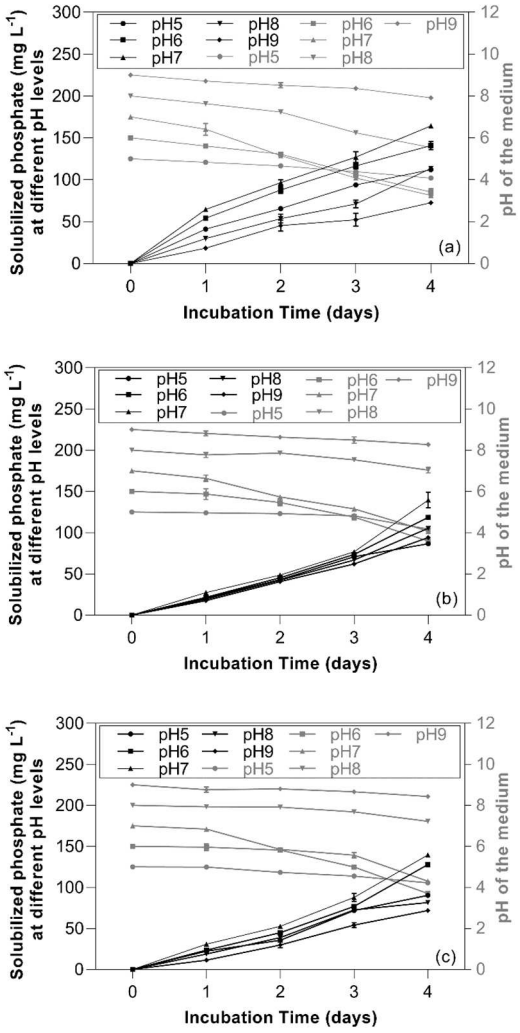

Figure 3. Effect of different pH levels on solubilized phosphate (a) *A. niger;* (b) *A. flavus*; (c) *A. fumigatus.* Values are means of 3 replicates and error bars represent standard deviation.

Effect of different phosphate sources on phosphate solubilization

The effect of different calcium phosphate concentrations on solubilized phosphate are reported in Fig. 4. For *A. niger*, the phosphate solubilization was higher for samples with a calcium phosphate concentration of 7.5 g L^{-1}, followed by samples with a concentration of 5.0 g L^{-1}, 2.5 g L^{-1}, 1.45 g L^{-1}, and 10 g L^{-1}. When it comes to *A. flavus* and *A. fumigatus,* both organisms had a similar performance. Samples with calcium phosphate concentration of 5 g L^{-1} had the highest amount of solubilized phosphate (between 148 and 152 mg L^{-1}), and samples with calcium phosphate concentration of 10 g L^{-1} had the lowest *amount* of solubilized phosphate (between 54 and 82 mg L^{-1}).

The effect of different rock phosphate concentration on the amount of solubilized phosphate is shown in Fig. 5. For *A. niger*, the highest (178 mg L^{-1}) and lowest (94 mg L^{-1}) amount of solubilized phosphate was achieved when the rock phosphate concentration was 5 g L^{-1}, and 10 g L^{-1}, respectively. For *A. flavus* and *A. fumigatus*, the maximum amount of solubilized phosphate (158–169 mg L^{-1}) was reported when the rock phosphate concentration was 7.5 g L^{-1}, and the minimum amount (34–60 mg L^{-1}) when the rock phosphate was 2.5 g L^{-1}.

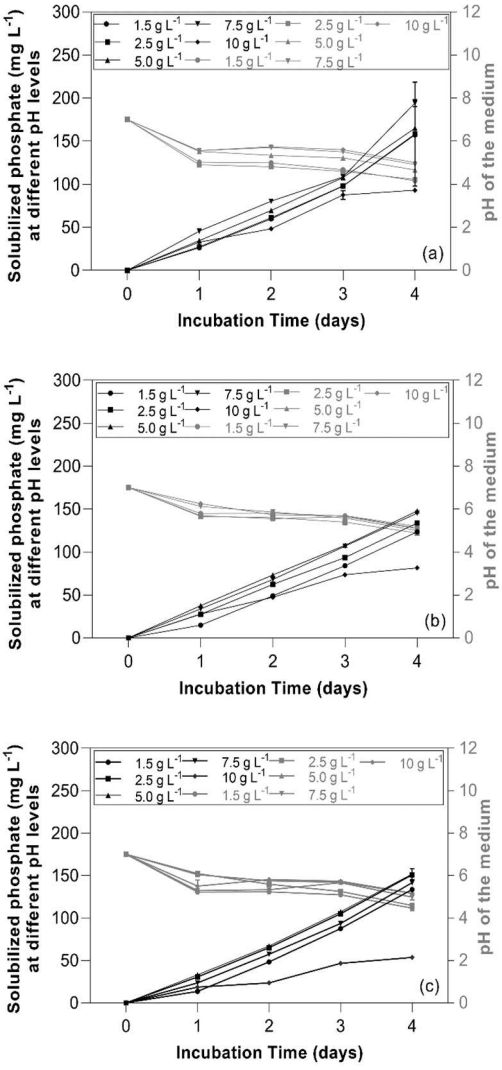

Figure 4. Effect of different concentrations of calcium phosphate on solubilized phosphate (a) *A. niger;* (b) *A. flavus;* (c) *A. fumigatus.* Error bars represent standard deviation of triplicate samples.

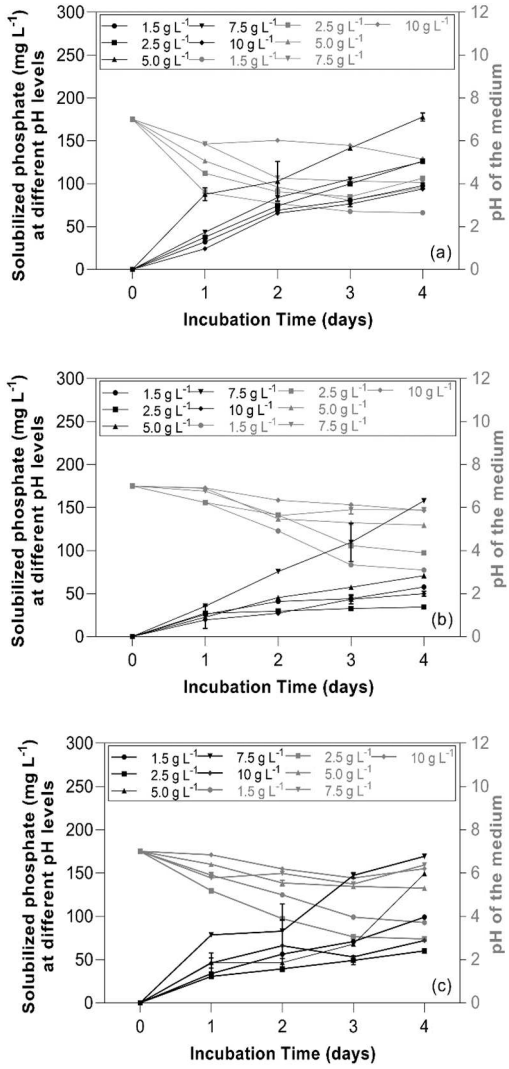

Figure 5. Effect of different concentrations of rock phosphate on solubilized phosphate (a) *A. niger;* (b) *A. flavus;* (c) *A. fumigatus.* Error bars represent standard deviation of triplicate samples.

In this section, two different sources of phosphate (calcium and rock phosphate) were used to investigate changes in the amounts of solubilized phosphate. Overall, the utilization of calcium phosphate has led to the highest solubilized phosphate yield, when compared to rock phosphate. This may be due to the complex nature of the atoms and compounds that comprise rock phosphate (Moawad et al. 1996 and Mahamuni et al., 2012). Pradhan & Sukla (2005) and Mahamuni et al. (2012) reported that one of the reasons why rock phosphate does not solubilize easily is because of the presence of strong apatite bonds in its molecules. These bonds make it hard for other compounds to bond to rock phosphate. Also, from our observations, *A. niger* solubilized phosphates more efficiently than *A. flavus* when calcium phosphate was used to supplement the growth medium. Similar findings were reported by Das et al., 2013.

Effect of inoculum size on phosphate solubilization

The effect of inoculum size on solubilized phosphate is presented in Fig. 6. For all the isolates (*A. niger, A. flavus,* and *A. fumigatus*) the highest amount of solubilized phosphate (188–234 mg L^{-1}) was obtained for samples with an inoculum size of 2.0×10^6 CFU mL^{-1}, while the lowest amount of solubilized phosphate (122–128 mg L^{-1}) was achieved for samples with an inoculum size of 0.5×10^6 CFU mL^{-1}.

These results show that the inoculum size of the isolates affects the amount of solubilized phosphate. Higher inoculum sizes shorten the time required for the phosphate solubilization, and also led to a high rate of phosphate solubilization. This is due to the higher proliferation of mycelia and increased activity of the associated enzymes at higher spore concentrations of the inoculum. The best inoculum size was at 2.0×10^6 CFU mL^{-1} for all the isolates and the trend for phosphate solubilization efficiency was *A. niger*, followed by *A. flavus*, and *A. fumigatus*.

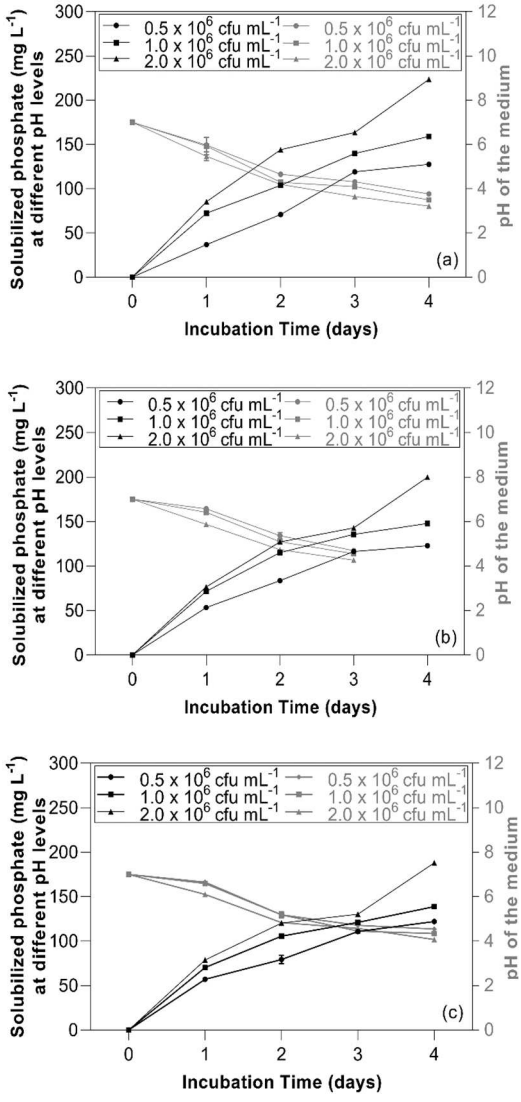

Figure 6. Effects of different inoculum size on the solubilized phosphate of (a) *A. niger;* (b) *A. flavus* (c) *A. fumigatus.* Error bars represent standard deviation of triplicate samples.

Effect of optimization on phosphate solubilization and fungal count

A pre-optimization study was carried out to quantify the phosphate solubilized by the three fungal isolates under normal conditions. The results obtained from the pre-optimization process were further compared with those obtained under optimized conditions. The results from the optimization process were then applied during the cultivation of the strain.

Fig. 7 (a) shows the effect of the optimization of incubation conditions on the (phosphate solubilization potential) PSP of PSF. Overall, unoptimized samples had the lowest amount of solubilized phosphate (78–100 mg L^{-1}), while optimized samples had the highest amounts (293–517 mg L^{-1}). For pre-optimized samples after 24 hours of incubation, *A. flavus* had solubilized 25 mg L^{-1}, *A. fumigatus* 32 mg L^{-1}, and *A. niger* 62 mg L^{-1}, while for optimized samples after 24 hours of incubation, *A. flavus* had solubilized 9 mg L^{-1}, *A. fumigatus* 84 mg L^{-1}, and *A. niger* 77 mg L^{-1}. For *A. niger*, the results obtained from the optimization studies showed that phosphate solubilization continued incrementally until the 11th day of incubation. When *A. flavus* and *A. fumigatus* were used, there was also an increase in the phosphate solubilization, but only until day 9. There was a wide gap in the amount of phosphate solubilized by the three isolates at pre-optimization and optimization stages, with significantly higher solubilization being recorded under optimized conditions. This underscores the importance of using optimal growth and culture conditions for phosphate solubilization. Previous studies similarly demonstrated that the use of optimal levels of pH, temperature, agitation and nutrients promoted maximum phosphate solubilization (Behera et al., 2017a; Behera et al., 2017b).

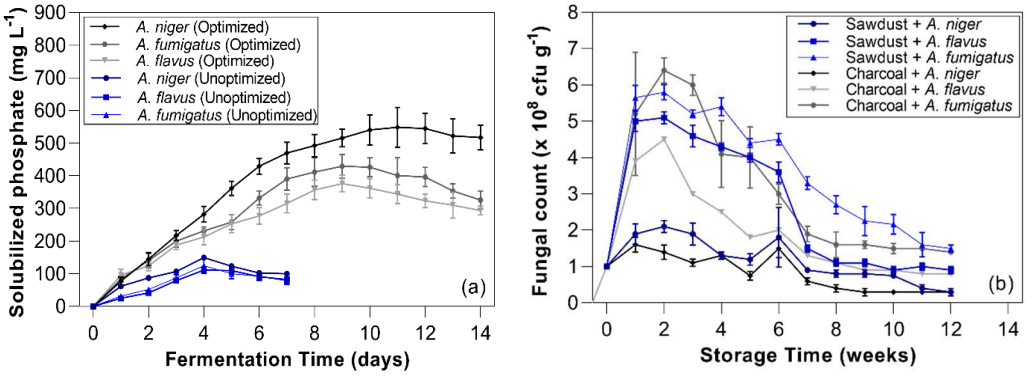

Figure 7. Effects of (a) optimization of incubation conditions on phosphate solubilization; (b) storage time on the viability of phosphate solubilizing fungi. Error bars represent standard deviation of triplicate samples.

The influence of storage time on the viability of fungal isolates of phosphate solubilizing potentials is reported in Fig. 7(b). As it can be seen from the figure, after 12 weeks of storage *A. fumigatus* had the highest fungal count with 1.0×10^8 CFU g^{-1} on sawdust and 1.4×10^8 CFU g^{-1} on charcoal. Next was *A. flavus* with 0.9×10^8 CFU g^{-1} on sawdust and 0.8×10^8 CFU g^{-1} on charcoal. The least count was recorded for *A. niger* with a count of 0.3×10^8 CFU g^{-1} on both charcoal and sawdust.

Similar results on the phosphate solubilization of *A. niger*, *A. fumigatus,* and *A. flavus* were reported by Kooliyottil et al. (2013). The authors concluded that *A. niger* solubilized higher amounts of phosphate when compared to *A. fumigatus* and *A. flavus*. In the same study, the authors showed that *A. fumigatus* has a higher solubilization potential when compared to *A. flavus*. However, this observation was not in line with the results obtained in our study since *A. flavus* solubilized more phosphate than *A. fumigatus*. Chang & Yang (2009) reported that *A. fumigatus* had a phosphate solubilizing potential of 113 mg L^{-1} after 7 days of incubation. In our study, the same isolate solubilized 125 mg L^{-1} of phosphate only after 4 days of incubation under unoptimized conditions, and 429 mg L^{-1} after 9 days of incubation under optimized conditions. A study by Das et al. (2013) found that *A. niger* and *A. flavus* solubilized 121 mg L^{-1} and 91 mg L^{-1} of phosphate, respectively after 14 days of incubation. However, in our study both isolates solubilized higher quantities in the same time interval. These differences can be explained by the natural tendency of strains of the same species originating from different sources to behave differently. Furthermore, the soils used in these studies were obtained from different climates, which may have different effects on the metabolism of these strains (Jain et al., 2014).

Effect of storage time and carrier material on the viability of biofertilizer inoculants

The influence of storage time on the viability of biofertilizer inoculants is reported in Table 1. After 8 weeks of storage, A. *fumigatus* stored with sawdust carrier had the highest fungal count (2.4×10^8 CFU g^{-1}) and *A. niger* stored with charcoal carrier had the least fungal count (0.4×10^8 CFU g^{-1}). Generally, it was observed that the best and most stable biofertilizer inoculant was *A. fumigatus* as it maintained relatively higher counts in both carrier materials throughout the study period compared to the other inoculants. On the other hand, *A. niger* appeared to be the least favourable as it had the least counts throughout. This may be due to the relatively higher growth rate of the other two strains.

Table 1. Total fungal counts ($\times10^8$ CFU g^{-1}) of biofertilizers formulated with different carriers during storage at 27 °C

Time	*A. niger*		*A. flavus*		*A. fumigatus*	
(weeks)	sawdust	charcoal	sawdust	charcoal	sawdust	charcoal
1	1.9 ± 0.2^a	1.6 ± 0.5^a	5.0 ± 0.4^a	3.7 ± 0.8^a	5.5 ± 0.4^a	5.0 ± 0.6^a
2	2.1 ± 0.4^a	1.4 ± 0.2^b	5.0 ± 0.1^a	4.3 ± 0.5^a	5.7 ± 0.8^a	6.2 ± 0.3^a
3	1.9 ± 0.2^a	1.1 ± 0.2^b	4.9 ± 0.3^a	3.3 ± 0.7^b	5.2 ± 0.5^a	5.9 ± 0.2^a
4	1.3 ± 0.2^a	1.3 ± 0.4^a	4.5 ± 0.4^a	2.3 ± 0.4^b	5.4 ± 0.6^a	4.4 ± 0.3^b
5	1.2 ± 0.2^a	0.8 ± 0.3^a	4.1 ± 0.2^a	1.8 ± 0.2^b	4.6 ± 0.4^a	4.2 ± 0.6^a
6	1.7 ± 0.4^a	1.3 ± 0.7^a	3.6 ± 0.6^a	1.7 ± 0.4^b	4.8 ± 0.2^a	3.3 ± 0.7^b
7	0.9 ± 0.3^a	0.6 ± 0.1^a	1.8 ± 0.2^a	1.3 ± 0.1^b	3.4 ± 0.6^a	1.9 ± 0.3^b
8	0.9 ± 0.2^a	0.4 ± 0.2^b	1.2 ± 0.2^a	1.1 ± 0.1^a	2.8 ± 0.2^a	1.4 ± 0.3^b

[a,b] Fungal counts of the same bioinoculant on the same row having the same subscripts are not significantly different (*t*-test, $p > 0.05$).

Also, it was observed that the sawdust carrier supported the fungal inoculants better than charcoal as the fungal counts of inoculants in the sawdust carrier were higher than those in charcoal carrier, especially in the final weeks of storage where the difference in counts between the two carriers was significant ($p < 0.05$) (Table 1). This can be

attributed to the fact that sawdust is a cellulolytic material, which serves as a nutrient source for the fungal inoculants. Furthermore, it can help improve porosity and help retain water (Lin et al., 2021). On the contrary, charcoal that contains low or no nutrient. Mahdi et al. (2010) reported that biofertilizer inoculants can only be viable for 6 months. However, the result from this showed that all the isolates started to lose viability after the eighth week of storage. This variation may be due to the different carriers, available nutrients, soil moisture levels and the microbial strains used in this study.

CONCLUSION

This study investigated the phosphate solubilizing potentials of three fungi from the genus *Aspergillus* (*A. niger, A. flavus,* and *A. fumigatus*). The results obtained show that *A. niger* had the highest solubilization index while *A. flavus* had the lowest. Optimization experiments showed that after 5 days of incubation *A. niger* was able to solubilize the highest amount of phosphate, while *A. flavus* solubilized the lowest. Overall, *A. niger, A. flavus,* and *A. fumigatus* have great potential for phosphate solubilization. Sawdust performed better as a carrier for all the inoculants and is thus recommended as a carrier of choice. These fungal isolates can be further converted into biofertilizers and used as a replacement for conventional chemical fertilizers. This will help to reduce environmental impacts and load caused by synthetic fertilizers in the soil, plants, and water bodies. Since phosphorus is an essential source of nutrients and is the least mobile nutrient available to plants, phosphate solubilizing fungi (*A. niger, A. fumigatus* and *A. flavus*) are recommended to be inoculated into the soil using sawdust as the carrier. This will help to dissolve phosphates that are locked up in the soil by converting them into soluble forms that can be utilized and absorbed by the plant roots.

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