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Dhanni Devi
Department of Agricultural Microbiology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

SB Gupta
Department of Agricultural Microbiology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

BK Mishra
ICAR-National Research Centre on Seed Spices, Ajmer, Rajasthan, India

MK Sheshma
Department of Plant Pathology, SKRAU, Bikaner, Rajasthan, India

Uma
Department of Agricultural Microbiology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Corresponding Author
Dhanni Devi
Department of Agricultural Microbiology, Indira Gandhi Krishi Vishwavidyalaya, Raipur, Chhattisgarh, India

Isolation and identification of phosphate solubilizing filamentous fungi from semi-arid region of Rajasthan (India)

Dhanni Devi, SB Gupta, BK Mishra, MK Sheshma and Uma

Abstract
Phosphorus is an essential nutrient element required for plant growth and development. Low phosphorus availability in soil is one of the major constrains for crop production. Phosphate solubilizing fungi enhance available phosphorous released from soils and contribute to fulfill the plants phosphorous requirement. This investigation aimed to isolate and identify potential phosphate solubilizing fungi from arid and semi-arid soils for environment friendly biofertilizer development. Six fungal isolates were isolated and identified as Aspergillus spp. and Penicillium spp. from arid and semi-arid soil based on phosphate solubilization index, morphological studies. Subsequently, fungal isolates having excellent phosphate solubilization efficiency were selected by their potential in broth containing insoluble $Ca_3(PO_4)$. Interestingly, isolate DCU-201 (*Penicillium spp.*) have marked phosphate solubilization ability followed by DCU-203 (*Aspergillus spp.)*. The maximum, solubilization index (2.6) and solubilized P (383 µg mL^{-1}) were associated with DCU-201 (*Penicillium spp.*). In addition, there was inverse proportion between the pH and phosphate solubilizing capacities. These excellent properties of isolates suggested that they have a great potential for agricultural utilization as environmentally sound biofertilizer. In this study, phosphate solubilization by filamentous fungi is reported for the first-time in and semi-arid region of India.

Keywords: phosphorous, *Penicillium spp.*, biofertilizer, eco-friendly nutrient management, semi-arid soil

Introduction
Phosphorus (P) is one of the major nutrients for crop production (Reena *et al*., 2013) [26]. This nutrient play important physiological and bio chemical activities of plants, like, photosynthesis, energy and sugar production, nucleic acid synthesis, and promotes nitrogen fixation in legume plants (Saber et al., 2005) [28]. Phosphorus promotes the strength of grain crop straw, flower initiation and fruit settings, root development and seed formation (Sharma et al., 2013) [30]. fixation into an unavailable form (Zhou *et al*., 1992; Khan *et al*., 2010) [36, 16]. In order to provide this nutrient, farmers use chemical fertilizers. The most widely used fertilizers are obtained from the acidification of rock phosphates with strong acids which not only represent a major cost of agricultural production but also impose adverse environmental impacts on overall soil health, terrestrial, freshwater and marine resources (Sing *et al.*, 2011) [33]. Large amount of soluble P fertilizers is widely used in order to increase agricultural production world widely (Bo *et al*., 2011) [5]. Moreover, the efficiency of applied P fertilizers in chemical form rarely exceeds 30% due to its fixation, either in the form of iron/aluminium phosphate in acidic soils or in the form of calcium phosphate in neutral to alkaline soils (Lindsay *et al.*, 1989) [19]. According to the latest estimates, the global reserve of P could become depleted within 50-100 years (Heppel *et al.*, 2016) [16]. Besides the efficient use of P reserves, it is also important to reduce the current wastage of P fertilizers and to recover applied P. The realization of all these potential problems associated with chemical P fertilizers has led to the search for environmentally compatible and economically feasible alternative strategies for improving crop production in low or P-deficient soils (Zaidi *et al*., 2009) [34]. The microbial inoculants (biofertilisers) function as key player in sustainable agriculture by improving soil fertility and crop productivity (Deepak *et al*., 2014) [6]. Especially, fungi are able to penetrate in to deep underground and show good attachment to insolubilized P particles as results of its hyphal structure compared to bacteria and actinomycetes. Furthermore, fungi are good acid producer and consequently show greater phosphate solubilization activity than bacteria (Deepak *et al.*, 2014; Jose *et al*., 2010) [6, 15]. Among these, *Aspergillus spp.*, *Penicilium spp.*, *Talaromyces spp*. and *Eupenicilium spp.* are considered "key organisms" in the P cycle (Jose *et al*., 2010) [15].

However most of the fungal species solubilize inorganic calcium phosphate and have a limited capacity to solubilize aluminium or iron phosphate. There are few in vitro studies concerning the solubilization of other phosphates by fungal species. To address this limitation, the present study aims to isolate and identify new isolates of indigenous phosphate solubilizing filamentous fungi which could be potential to solubilize both tricalcium phosphate, aluminium phosphate and iron phosphate.

Materials and Methods

The experiment was done at Microbiology laboratory, Division of crop production, ICAR-National Research Centre on Seed Spices, Tabiji, Ajmer, Rajasthan during the year 2017-18 and 2018-19**.** The samples were collected in winter from cumin rhizosphere soil of Ajmer District, located at 26º 03' 29" N, 74 o 46' 10" E to 26º 21' 28" N, 74 o 37' 61" E. Its climate is semi-arid, In winters the minimum temperature (12^0) and maximum temperature (27^0), rainfall (529.83). Soil and plant samples were collected from ten different locations using sterile auger. One-hundred-gram soil was taken from each sampling point and it makes a total of 500 g composite sample (five points from each location make one composite sample). The samples were transferred to laboratory in sterile sealed polythene bag under aseptic condition and stored at room temperature. Then microbiological study was done as early as possible.

For isolating phosphate solubilizing fungi Pikovaskaya's (PKV) agar medium was used. Pikovaskaya's (PKV) agar medium consisted of 10.0 g glucose, 5.0 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4$.$2H_2O$, 0.2 g NaCl, 0.2 g KCl, 0.0001 g FeSO4, 0.0001 g $MnSO_4$, 0.5 g yeast extract, 15.0 g agar and 1000 mL distilled water (Synthetic pikovaskaya's media, Himedia). In this medium Ca_3 $(PO_4)_2$ was used as a source of insoluble phosphate. The medium was autoclaved at 121 °C for 15 min. About 20 ml of the sterilized medium poured into each petri dish and allowed to solidify before inoculation. Chloramphenicol was also used to avoid bacterial growth. Isolation of phosphate solubilizing fungi using serial dilution plate technique. One-gram soil sample was diluted in to 10 ml of sterile water. It was vigorously shaken until to get homogenous suspension and serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. From each dilution, 200 µL was plated on Pikovskaya's agar. The phosphate solubilizing fungi were identified by the presence of a clear halo around the colonies after 7 days incubation at 25 °C (Rao, 1982) [25]. The experiment was performed in triplicate. Phosphate solubilizing fungi of the soil samples were isolated and purified by transferring into new plates. The pure cultures were preserved on potato dextrose agar slants at 4 °C for further study. Phosphate solubilisation index was measured using the following formula (Birhanu *et al*., 2017) [4]:

SI = [Colony diameter + Halo zone diameter] / colony diameter

Quantitative estimation of phosphate solubilization

It was carried out using Erlenmeyer flask containing 40 ml Pikoveskaya's (PKV) broth medium supplemented with 0.5% tricalcium phosphate [Ca_3 $(PO4)_2$]. After sterilization, the medium of each flask was inoculated with the 5% (v/v) spore suspension of a particular fungal isolate containing 10^6 spores mL^{-1}. Sterile distilled water inoculated flaks was treated as control. Three replicates were maintained for each test isolate and mean value was recorded. Incubation was done at 25 °C in an incubator shaker at 120 rpm up to 15 days. The samples were autoclaved and centrifuged at 8000 rpm for 15 min at 4 ˚C to remove any suspended solids and mycelial parts. Then the cultures were filtered through 0.45 µm pore size syringe filter unit (Himedia, Mumbai). The filtrates were used for analysis of soluble phosphate and pH value. The pH value of the culture supernatants was determined by a pH meter equipped with a glass electrode. The amount of soluble phosphorus in culture supernatants was measured by molybdenum blue method and expressed as mg/L (Morphy and Riley, 1962) [23]. Samples cultured for 5, 10 and 15 days were compared. After calculation of mean phosphate degradation ability from 6 isolates of each day, we selected the adequate period for the comparison depending on the substrate. All experiments were conducted in triplicate and data were analyzed using Microsoft Excel program. The mean values were compared by significant differences were detected at $p<0.05$ level. Correlation between solubilized phosphate and pH of the medium was determined by using correlation studies.

Results

Screening and identification of phosphate solubilizing fungi

A total of 6 fungal isolates showed phosphate solubilizing activities. The isolates were 3 3 *Penicillium spp*. and *Aspergillus spp*., identified based on colony morphology and microscopic observation (Table 1). Apart from P solubilization, DCU-201 (*Penicillium spp.)* have also the ability to solubilize potassium from substance potassium alumino silicate and DCU-401 (*Aspergillus sp.*) can also solubilize both K and Zn from the substances of potassium alumino silicate and zinc oxide, respectively (Table 1).

Table 1: List of fungal strains isolated from semi-arid region of Rajasthan

S.N.	Isolates	Genus	P Solubilization	K Solubilization	Zn Solubilization
1.	DCU-201	*Penicillium sp.*	+	+	-
2.	DCU-202	*Aspergillus sp.*	+	-	-
3.	DCU-203	*Aspergillus sp.*	+	-	-
4.	DCU-204	*Penicillium sp.*	+	-	-
5.	DCU-205	*Penicillium sp.*	+	-	-
7.	DCU-401	*Aspergillus sp.*	+	+	+

Qualitative phosphate solubilization

Six fungal isolates showed significant phosphate solubilization in Pikovskaya agar medium using tricalcium phosphate as the substrate. The phosphate solubilization index (PSI) ranged from 1.3 to 2.6 (Table 2). Isolate DCU-201 (*Penicillium spp*.) produced highest PSI; 2.6 (Table 2), whereas; the smallest PSI of 1.3 was achieved from DCU-205 (*Penicillium spp*.). Both fungal isolates, DCU-201 and DCU-205 were found different in colony color. The maximum P solubilization zone recorded 27 mm (Figure 1) and the

minimum P solubilization zone (13mm) were recorded in DCU-201 (*Penicillium spp*.) and DCU-205 (*Penicillium spp*.), respectively. Phosphate Solubilization index (PSI) of the culture medium exhibited the positive changes. It increased with the increased of zone of clearance in the medium. Correlation studies showed a significant positive relationship between PSI and zone of clearance of the culture medium (Figure 2). The strongest positive correlation was (r= 0.93) observed.

Table 2: Qualitative assay for P Solubilization by rhizofungal isolates

S.N.	Isolates	Genus	Solubilization zone (mm)	Solubilization index (SI)
1.	DCU-201	*Penicillium sp.*	27*	2.6*
2.	DCU-202	*Aspergillus sp.*	18	1.9
3.	DCU-203	*Aspergillus sp.*	24	2.3
4.	DCU-204	*Penicillium sp.*	21	2.5
5.	DCU-205	*Penicillium sp.*	13	1.3
6.	DCU-401	*Aspergillus sp.*	15	1.5
	SEm		2.128	0.096
	CD (5%)		4.426	0.195
	CV		7.831	5.294
	Mean ± Sd		19.6 ± 5.3	2.01 ± 0.53

An asterisk (*) indicated outstanding values of solubilized phosphate. It was higher than sum of mean and standard deviation of P solubilized by 6 fungal isolates Values given are the mean ± standard deviation of P solubilized by 6 fungal isolate.

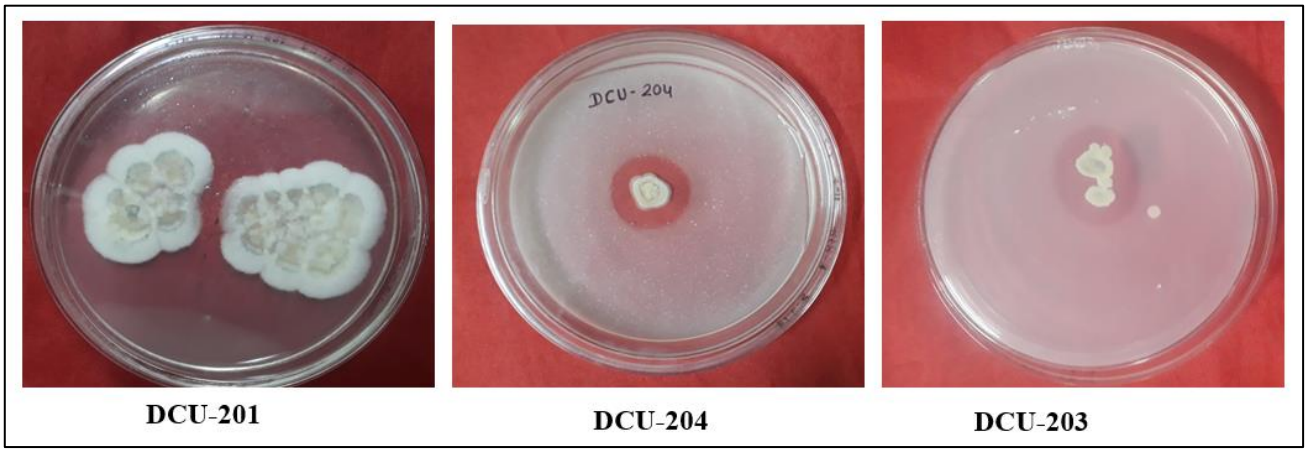

Fig 1: Clear halo formation by representative fungal isolates in Pikovskaya agar plates (A: *Penicillium spp*., B: *Penicillium spp*., and C: *Aspergillus spp*.)

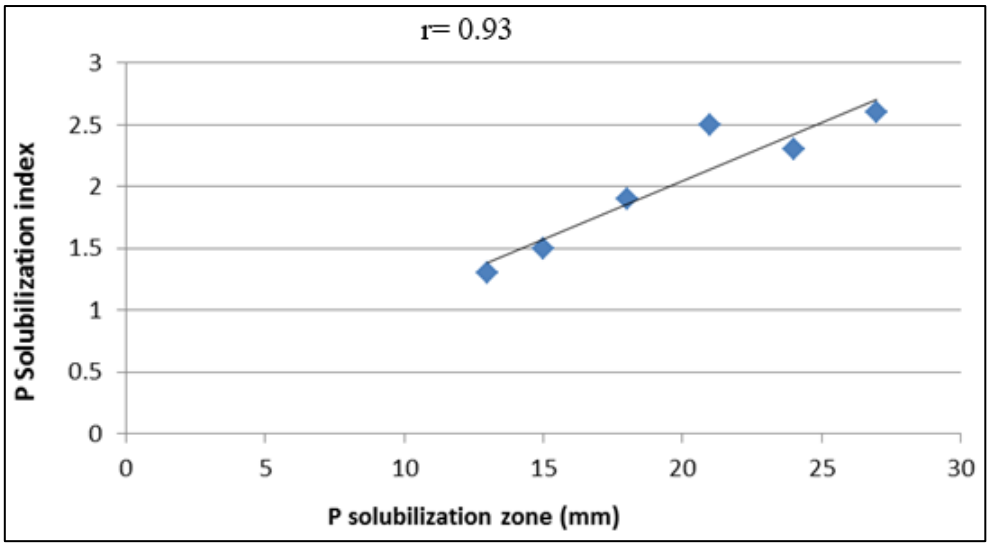

Fig 2: Correlation between solubilization zone and solubilization index of the culture medium containing tricalcium phosphate by P solubilizing fungal isolates

Quantitative phosphate solubilization

Phosphate solubilizations by the isolated fungi were analyzed in Pikovaskaya's broth medium using substrate of recalcitrant phosphate compounds: tricalcium phosphate [$Ca_3(PO_4)_2$]. The P-solubilizing ability of fungal isolates varied with incubation period and substrates. The best period of observation was

selected considering their mean P solubilization, which 15 days for $Ca_3(PO_4)_2$.

The strongest phosphate (P) solubilization effect was found in the medium containing $Ca_3(PO_4)_2$ at 15 days of inoculation (DI) followed by 10 and 5 DI (Table 3). The solubilized P ranged between 65-96 µg /mL, 135-187 µg /mL and 203-383 µg /mL at 5, 10 and 15 Days of inoculation respectively. Among the isolates the highest amount of P was solubilized by *Penicillium spp*. followed by *Aspergillus spp*. Finally, DCU-201 *Penicillium spp*. was considered as outstanding isolate because solubilized P was higher than sum of the mean and standard deviation of P solubilized by 6 isolates. The amount of solubilized P from at 5 days of inoculation (DI) was 98, 91 and 96 µg /mL; at 10 days of inoculation was 187, 185 and 180 µg /mL and at 15 days of inoculation was 383, 277 and 265 µg /mL respectively (Table 3). DCU-201 *Penicillium spp*. showed outstanding performance at both 10 and 15 days of solubilization but in case at 5 days, it was close to the outstanding.

Table 3: Comparison of phosphate solubilization at time interval by phosphate solubilizing fungal strains

S.N.	Isolates	Genus	5 Days		10 Days		15 Days	
			Soluble P (µg /ml)	pH	Soluble P (µg /ml)	pH	Soluble P (µg /ml)	pH
1.	DCU-201	*Penicillium sp.*	88	5.39	187	5.21	383*	4.16
2.	DCU-202	*Aspergillus sp.*	78	5.00	180	4.57	263	4.67
3.	DCU-203	*Aspergillus sp.*	91	5.12	185	4.37	265	4.23
4.	DCU-204	*Penicillium sp.*	81	5.27	172	5.11	214	4.86
5.	DCU-205	*Penicillium sp.*	65	5.38	135	5.13	203	4.63
6.	DCU-401	*Aspergillus sp.*	96*	5.33	168	5.24	277	4.19
	SEm		6.625		15.957		9.983	
	CD (5%)		13.416		32.492		20.731	
	CV		6.381		9.251		8.472	
	Mean ± Sd		83.16 ± 11.05		171.16 ± 19.17		267.5 ± 63.99	

An asterisk (*) indicated outstanding values of solubilized phosphate. It was higher than sum of mean and standard deviation of P solubilized by 6 fungal isolates

Values given are the mean ± standard deviation of P solubilized by 6 fungal isolate

pH value of the culture medium

pH of the culture medium exhibited the opposite changes. It decreased with the increased amount of soluble P in the medium. Correlation studies showed a significant inverse relationship between soluble P and pH of the culture medium (Figure 3). The strongest negative correlation was observed at all stages (5,10 and 15 days) of broth culture, and the strongest negative correlation (r= -0.72) was found at 15 days where the maximum solubilized P observed (Figure 3).

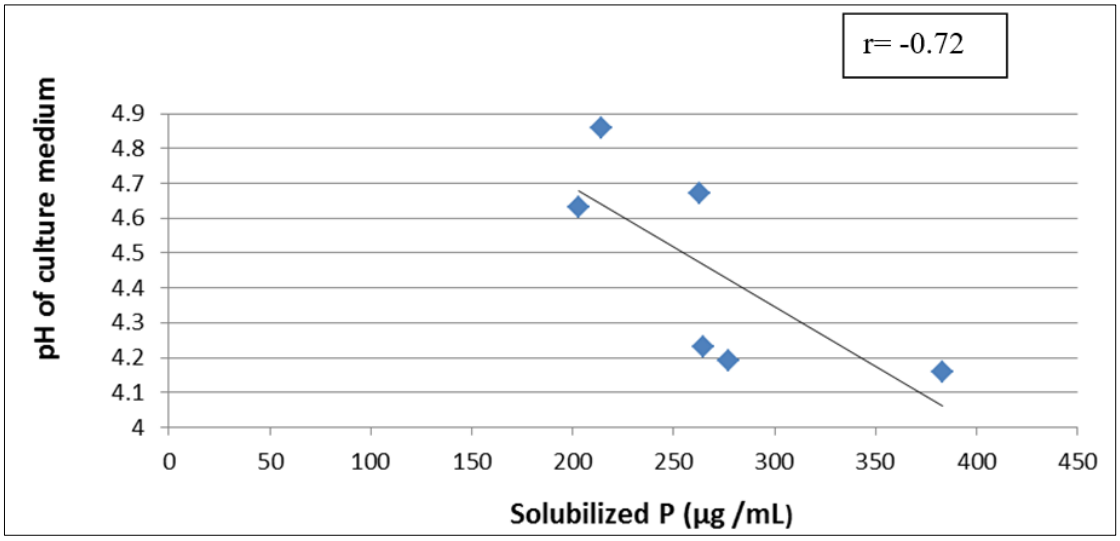

Fig 3: Correlation between soluble phosphate and pH of the culture medium supplemented with [Ca3(PO4)2]

Discussion

The six P solubilizing fungal strains were isolated from semi-arid region (Ajmer) of Rajasthan, India. The isolates were belonging to the genera of *Penicilium* and *Aspergillus*. According to Zang *et al*. (2018) [35], Mendes *et al*. (2014) [22] and Ruangsanka (2014) [27], there were diversities on the isolation rate of phosphate solubilizing fungi depending on area. They reported that the most dominant genera of P solubilizing filamentous fungi are Penicilium, Aspergillus and Talaromyces, however, there were large variations in phosphate solubilizing abilities among fungal species (Barrooso *et al*., 2006; Iman M. 2008; Mahamuni *et al*., 2012; Alam *et al*., 2002) [21, 2]. At the present studies, strains DCU-201 identified as *Penicilium*, showed excellent P solubilizing abilities regardless of the phosphate substrates. It suggested that phosphate solubilizing abilities in *Penicilium spp*. is an universal property.

Among the filamentous fungi *Penicillium spp*. is important in the natural environment as well as food and drug production. Some members of the genus produce penicillin, a molecule that is used as an antibiotic, which kills or stops the growth of certain bacteria spp. Other species are used in cheese

industries (https://en.wikipedia.org/wiki/Penicillium). *Penicillium oxalicum* produces secalonic acid D, chitinase and oxalic acid (https://en.wikipidia. org/wiki/ *Penicillium oxalicum*). Pandey *et al*. (2008) [24] reported fungal strains of *Penicillium oxalicum*, *Penicillium citrinum* and *Penicillium purpurogenum* to solubilized P in broth medium containing tri calcium phosphate as insoluble P compound. They also revealed that *Penicillium citrinum* and *Penicillium purpurogenum* were found more potential than *Penicillium oxalicum.*

Interestingly, beside *Penicillium spp*. (DCU-201 and DCU-204), *Aspergillus spp*. (DCU-203) also showed an excellent phosphate degradation ability on halo assay. According to Jain *et al*. (2014) [13], Alam *et al.* (2002) [2], Elias *et al*. (2016) [7] and Jain *et al*. (2017) [14] solid medium using agar plates performed better phosphate degradation ability than those in liquid media. Thus, it is impossible to ignore the *Aspergillus spp*. (DCU-203). *Aspergillus spp*. are widely used for the production of fermented foods, organic acids and enzymes (Wongwicharn *et al*., 1999) [33]. Especially, *A. niger* has a long history of industrial usage, which means many strains already have a GRAS ("generally regarded as safe") status (Wongwicharn *et al*., 1999) [33]. It has been used for commercial production of many enzymes, e.g. pectinase, glucose oxidase, glucoamylase, hemicellulase, glucanases, acid proteinase, catalase (Aguilar and Huitron, 1993; Liu *et al*., 1999; Garhartz, 1990) [20, 1, 9] and citric acid (Friedrich *et al.*, 1989; Gokhale *et al*., 1991; Lee *et al*., 1989) [8, 10, 17].

The mechanisms of phosphate solubilization by microorganisms are very complex and are not completely known yet (Bo *et al.*, 2011) [5]. The very common mechanisms are acidification, chelation and exchange reactions (Bo *et al*., 2011) [5]. Organic acids play an important role in phosphate solubilization processes, which can help the release of P by providing protons and complexing anions, or ligand exchange reactions or complexion of metal ions release to solution. Zang *et al*. (2018) [35] and Scervino *et al*. (2013) [29] reported that organic acids production depends on the interaction of P source and fungi.

In this study, DCU-201 (*Penicillium spp*.) showed the highest efficiency in P solubilization by decreasing pH of the culture medium, which indicated higher amount of organic acid production. Silva *et al*. (2014) [31], Li *et al*. (2016) [18] and Barroso *et al*. (2006) [3] reported that *A. niger* produce higher amount of organic acids and enhance phosphate solubilization. Zang *et al*. (2018) [35] reported that solubilization of the different P sources mostly depended on the amount of organic acids production by fungi. Tricarboxylic acids such as citric acid, oxalic acid and other lower molecular weight organic acids are considered to be the main contributors to phosphate solubilization and a decrease in pH of the medium (Bo *et al.*, 2011; Gong *et al*., 2014) [5, 11].

Conclusions

Isolates DCU-201 (*Penicillium spp*.) and DCU-401 (*Aspergillus spp*.) have unique capabilities to solubilized insoluble phosphate compounds (Tri calcium phosphate) and may become an important bio resource for soil fertility management as well as sustainable crop production and pollution free environment.

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