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ISOLATION OF PHOSPHATE SOLUBILIZING FUNGI IN SOIL FROM KANCHANABURI, THAILAND

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ABSTRACT

Phosphorus replenishment, particularly in smallholder agriculture, remains a challenge as it is mainly fertilizer dependent. While the user of soluble mineral phosphate fertilizers is the obvious best means to combat phosphate deficiency in soil, they were limited by high cost of fertilizers and availability at farmer's level. This research is to isolate and select phosphate solubilizing soil fungi from Kanchanaburi area. One hundred and forty five fungi isolates were incubated on Pikovskaya agar supplemented with 0.003% w/v rose bengal, isolates which gave the high ratio of clear zone were selected. Four fungal strains out of 30 strains, i.e., SA07P3332, SA22P3406, SA14P2418 and SA19P2120 solubilized tricalcium phosphate and showed the highest available phosphate in liquid medium. The available phosphate were 3.010, 2.993, 2.749 and 2.632 mg P_2O_5 ml^{-1}, respectively. All of the fungal strains are fast growing black colonies on potato dextrose agar with distinctive conidial heads characterized as *Aspergillus* sp. Their phosphatase enzyme activities were 0.029-0.102 unit ml^{-1} min^{-1} for acid phosphatase (pH 6.5) and 0.022-0.102 unit ml^{-1} min^{-1} for alkaline phosphatase (pH 11).

KEYWORDS: phosphate solubilizing fungi, phosphatase and available phosphate

1. INTRODUCTION

Phosphorus is second only to nitrogen in mineral nutrients most commonly limiting the growth of crop. Phosphorus is an essential element for plant development and growth making up about 0.2% of plant dry weight. Plants acquire phosphorus from soil solution as phosphate anions. However, phosphate anions are extremely reactive and may be immobilized through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+} and Al^{3+}, depending on the particular properties of soil. In these forms, phosphate is highly insoluble and unavailable to plants. As the results, the amount available to plant is usually in a small proportion [1].

The principle mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatases play major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganism. Production of organic acids results in acidification of the microbial cell and its surroundings [1].

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Some fungal strains are able to solubilize rock phosphate, aluminium phosphate and tricalcium phosphate [2]. Five isolates of *Aspergillus* were selected from rhizospheric soils of the Eucalyptus plantations which showed high solubilization of tricalcium phosphate. Isolates of *A. tubingensis* also showed more solubilization when grown in the presence of 2% rock phosphates than *A. niger* [3].

We studied the community of microorganism in the soils of Thongphaphum district, Kanchanaburi province, Thailand, where any plant can flourish. This study aimed to isolate the phosphate solubilizing fungi with the highest available phosphate.

2. MATERIALS AND METHODS

2.1 Fungal isolation and growth conditions

Fungal strains were isolated from 19 soil samples of Thongphaphum district, Kanchanaburi province, Thailand. Soil samples (2kg) were collected from the areas where any plant can flourish by digging the ground at 0-15 cm depth [4] and stored at 4 °C in refrigerator [5]. Each sample was added to 9 ml of 0.85% w/v saline with 10-fold dilution series. 0.1 ml dilution was plated on Pikovskaya agar (0.5 g $(NH_4)_2SO_4$, 0.5 g $MgSO_4.7H_2O$, 0.3 g NaCl, 0.3 g KCl, 0.03 g $FeSO_4.7H_2O$, 0.02 g $MnSO_4.H_2O$, 10.0 g $Ca_3(PO_4)_2$, 10.0 g Glucose and 15.0 g Agar) and plate count agar (5.0 g Tryptone, 2.5 g Yeast extract, 1.0 g Glucose and 15.0 g agar) by spread plate technique and incubated at 37 °C for 3-5 days. Various colonies on the 10^{-4}, 10^{-5} and 10^{-6} dilution plates which showed clear zone indicating the ability to solubilize tricalcium phosphate were selected and grown on Pikovskaya agar.The selected fungal strains were then re-streaked on the same medium [4].

2.2 Selection of fungi

One hundred and forty five fungi that solubilized tricalcium phosphate on Pikovskaya agar were inoculated on Pikovskaya agar supplemented with 0.003% w/v rose bengal. The fungal cultures were then incubated at 37 °C for 7 days [6].

2.3 Phosphate solubilization

Selection of fungal colony according to the ratios of clear zone were tested with z estimation on normal distribution at 95% confidence. Thirty strains of fungi were suspended with 0.85% w/v saline to obtain 10^6 spore ml^{-1}. One ml of spore dilution was added to 25 ml Pikrovskaya medium in Erlenmeyer flask and incubated in triplicate at 37 °C with 200 rpm for 3, 5 and 7 days. Autoclaved uninoculated medium was served as controls.

The culltures were harvested by filtration with Whatman paper filter no. 42. The supernatants were analyzed for available phosphate (P_2O_5) with the molybdo-vanado method [7], phosphatase enzyme activities [5] and pH estimation. Moreover, biomass and total ash were measured by drying mycelial mats at 80 °C for 24 hrs and at 550 °C for 2 hrs, respectively.

Potassium dihydrogen phosphate (KH_2PO_4) was standardized and spiked in the supernatants at about 0.400 mg ml^{-1}. The supernatants were divided into 5 lots such as lot 1 (SA11P3236, SA17P2217, SA19P2119 and SA192120) lot 2 (SA07P2118, SA11P2226, SA14P2211, SA14P2212, SA14P2213, S14P2315, SA14P2316 and SA14P2418) lot 3 (SA02P1204, SA16P2319, SA17P1205, SA17P3241 and SA22P3406) lot 4 (SA05P1201, SA05P2551, SA05P2553 and SA05P3255) and lot 5 (SA02P3412, SA05P3257, SA05P3259, SA05P3360, SA05P3363, SA07P3332, SA11P2225, SA11P3237 and SA14P3226). Percentage recovery was calculated by measuring available phosphate and relative percentage difference (% RPD) was also calculated.

2.4 Microscopic examination

Cell morphologies of the isolates were observed using a compound microscope after staining with lactophenol cotton blue.

2.5 Statistical analysis

Data (three replications) were subjected to analysis of variance (ANOVA) with complete randomize design using SPSS for windows version 11.5 software. DUNCAN was calculated at the 0.05 level of probability to determine difference among the means.

3. RESULTS AND DISCUSSION

One hundred and forty five phosphate solubilizing fungal isolates were obtained from the soil of Thongphaphum district areas (Table 1) and thirty of them appeared to have much higher ratio of clear zone (Table 2) when estimating with z test in normal distribution (Figure 1). Thirty strains were cultivated in liquid medium supplemented with tricalcium phosphate. Ten of them gave higher available phosphate in the following orders: SA07P3332, SA22P3406, SA14P2418, SA19P2120, SA14P2212, SA11P3236, SA16P2319, SA14P2315, SA02P1204 and SA02P3412, respectively (Table 3). Four strains (SA07P3332, SA22P3406, SA14P2418 and SA19P2120) showed the highest available phosphate in liquid medium without significant different at 3.010, 2.993, 2.749 and 2.632 mg P_2O_5 ml^{-1}, respectively (Table 3). KH_2PO_4 was standardized and spiked in the supernatants (about 0.400 mg ml^{-1}) showed percentage recovery of 93.06-105.08% and % RPD of 0.19-10.10% (Table 3; Figures 2 and 3). Moreover phosphatase enzyme activities were 0.029-0.102, 0.030-0.077, 0.032-0.082 and 0.040-0.041 unit ml^{-1} min^{-1} for acid phosphatase (pH 6.5), respectively and 0.029-0.102, 0.036-0.091, 0.022-0.038 and 0.033-0.042 unit ml^{-1} min^{-1} for alkaline phosphatase (pH 11), respectively (Table 4). Furthermore, the pH of supernatants at 25 °C reduced from pH ~ 6.297-6.674 (control) to pH 3.774-4.127, 3.267-4.056, 3.345-3.655 and 3.912-4.067 for SA07P3332, SA22P3406, SA14P2418 and SA19P2120, respectively (Table 5).

Fungal strains (SA07P3332, SA22P3406, SA14P2418 and SA19P2120) are fast growing and have black colonies on potato dextrose agar. Hyaline hyphomycete with distinctive conidial heads (flask-shaped) was observed (Figures 4, 5, 6 and 7) and characterized as *Aspergillus* sp.

The fungal strain SA07P3332 showed highest available phosphate when tricalcium phosphate was used. This fungus also showed maximum acid phosphatase and alkaline phosphatase. This result is in accordance with Achal *et al*. [8].

A. niger solubilized insoluble phosphate well in a liquid medium supplemented with tricalcium phosphate [9] and caused a remarkable drop in pH of culture media and solubilized considerable amounts of phosphate [10]. *Aspergillus* sp. solubilized 480 µg ml^{-1} of phosphorus showed diverse levels of phosphate solubilization activity in liquid broth culture in presence of various carbon and nitrogen sources. This indicates that absence of soluble phosphate in media induces the acid production [11].

Three isolates of *A. tubingensis* and two isolates of *A. niger* isolated from rhizospheric soils were tested on solubilization of different rock phosphates. All isolates of *Aspergillus* were capable of solubilizing all natural rock phosphates. *A. tubingensis* AT1 showed maximum percent solubilization in all rock phosphates tested when compared to other isolates. This isolate also showed highest phosphate solubilization when grown in the presence of 2% of rock phosphate [3].

Table 1 The numbers of fungi obtained from soil, Thongphaphum district, Kanchanaburi, Thailand which solubilized tricalcium phosphate on Pikovskaya agar

No.	Code of soil samples	The numbers of fungi
1	SA02	2
2	SA03	-[a]
3	SA05	26
4	SA07	6
5	SA08	1
6	SA09	-[a]
7	SA11	18
8	SA12	17
9	SA13	7
10	SA14	20
11	SA16	11
12	SA17	8
13	SA18	4
14	SA19	11
15	SA20	9
16	SA21	-[a]
17	SA22	1
18	SA23	3
19	SA24	1
Total		145

Note: [a] soil without fungi

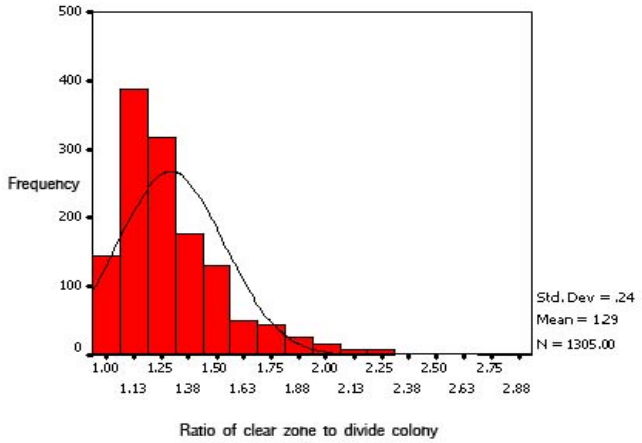

Figure 1 The ratios of clear zone of fungal colony estimated with z test in normal distribution

Table 2 The ratios of clear zone of fungal colony incubated on Pikrovskaya agar supplemented with 0.003% w/v rose bengal at 37 °C for 3, 5 and 7 days

No.	Fungal code	The ratios of clear zone		
		3 days	5 days	7 days
1	SA02P1204	1.6567	1.4633	1.3633
2	SA02P3412	1.5967	1.4233	1.3467
3	SA05P1201	1.7367	1.4667	1.3467
4	SA05P2551	1.5200	1.4433	1.5700
5	SA05P2553	1.5000	1.3500	1.4233
6	SA05P3255	1.8267	1.7433	1.5267
7	SA05P3257	1.5067	1.5700	1.5100
8	SA05P3259	1.4767	1.6333	1.5633
9	SA05P3360	1.6300	1.6567	1.5000
10	SA05P3363	1.4267	1.3500	1.4133
11	SA07P2118	1.5767	1.3267	1.3500
12	SA07P3332	1.6733	1.3867	1.3800
13	SA11P2225	1.3100	1.4133	1.3200
14	SA11P2226	1.9633	1.8100	1.4800
15	SA11P3236	2.0000	1.7700	1.6067
16	SA11P3237	2.1967	1.7100	1.5067
17	SA14P2211	1.8867	1.8600	1.8833
18	SA14P2212	2.0000	1.8100	1.7433
19	SA14P2213	2.2000	1.9767	1.8367
20	SA14P2315	1.9133	1.4467	2.8067
21	SA14P2316	1.9733	1.6300	1.5433
22	SA14P2418	1.7533	1.6267	1.4200
23	SA14P3226	1.5867	1.3800	1.3200
24	SA16P2319	1.7367	1.6067	1.4133
25	SA17P1205	1.7667	1.5767	1.3900
26	SA17P2217	1.5900	1.8633	1.8300
27	SA17P3241	1.7900	1.6767	1.6367
28	SA19P2119	1.5967	1.5033	1.4333
29	SA19P2120	1.7867	1.6100	1.5000
30	SA22P3406	1.5900	1.6900	1.4267

Note: Data are the means of triplicate experiments.

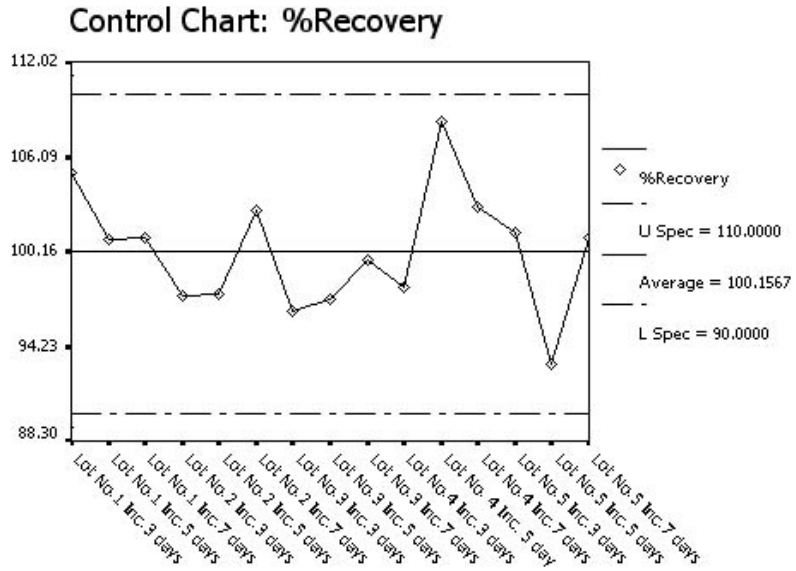

Figure 2 The control chart determined available phosphate for percentage recovery

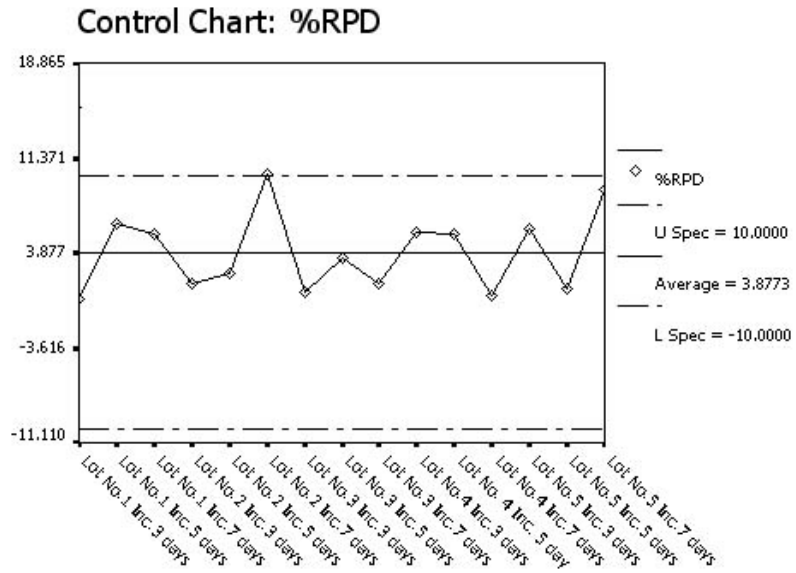

Figure 3 The control chart determined available phosphate for percentage RPD

Table 3 Fungal strains with high available phosphate on Pikrovskaya medium supplemented with tricalcium phosphate at 37 °C for 3, 5 and 7 days.

No.	Fungal code	3 days			5 days			7 days		
		Ava. P (mg P_2O_5 ml^{-1})	% Rec	% RPD	Ava. P (mg P_2O_5 ml^{-1})	% Rec	% RPD	Ava. P (mg P_2O_5 ml^{-1})	% Rec	% RPD
1	SA02P1204	1.652^{p}	96.39	0.75	2.219^{defgh}	97.14	3.49	2.104^{fghij}	99.64	1.47
2	SA02P3412	1.671^{p}	101.30	5.73	1.825^{p}	93.06	1.09	2.126^{fghij}	101.05	8.94
3	SA07P3332	1.260^{p}	101.30	5.73	2.182^{efghi}	93.06	1.09	3.010^{a}	101.05	8.94
4	SA11P3236	1.460^{p}	105.08	0.19	2.238^{defgh}	100.92	6.12	2.294^{defg}	101.00	5.42
5	SA14P2212	0.839^{p}	97.42	1.45	2.528^{cdef}	97.50	2.19	1.667^{p}	102.67	10.10
6	SA14P2315	0.735^{p}	97.42	1.45	1.734^{p}	97.50	2.19	2.233^{defgh}	102.67	10.10
7	SA14P2418	1.714^{p}	97.42	1.45	2.749^{abc}	97.50	2.19	2.571^{bcde}	102.67	10.10
8	SA16P2319	1.417^{p}	96.39	0.75	2.285^{defg}	97.14	3.49	2.141^{fghij}	99.64	1.47
9	SA19P2120	1.349^{p}	105.08	0.19	1.897^{p}	100.92	6.12	2.632^{abcd}	101.00	5.42
10	SA22P3406	1.781^{p}	96.39	0.75	2.932^{ab}	97.14	3.49	2.993^{b}	99.64	1.47

Note: Data are the means of triplicate experiments. Superscript letters a-j and p were calculated by DUNCAN for $P<0.05$. Superscript letter p referred to available phosphate less than 2.104 mg P_2O_5 ml^{-1}. Ava. P = Available phosphate; % Rec = % Recovery and % RPD = % Relation percentage difference.

Table 4 Phosphatase enzyme activities of ten isolates showing higher available phosphate on Pikrovskaya medium supplemented with tricalcium phosphate at 37 °C for 3, 5 and 7 days.

No.	Fungal code	Acid phosphatase pH 6.5 (unit ml^{-1} min^{-1})			Alkaline phosphatase pH 11 (unit ml^{-1} min^{-1})		
		3 days	5 days	7 days	3 days	5 days	7 days
1	SA02P1204	0.029^{p}	0.034^{p}	0.082^{c}	0.031^{q}	0.034^{q}	0.095^{b}
2	SA02P3412	0.030^{p}	0.056^{p}	0.092^{b}	0.029^{q}	0.061^{d}	0.092^{bc}
3	SA07P3332	0.029^{p}	0.058^{p}	0.102^{a}	0.029^{q}	0.062^{d}	0.102^{a}
4	SA11P3236	0.004^{p}	0.045^{p}	0.039^{p}	0.003^{q}	0.034^{q}	0.033^{q}
5	SA14P2212	0.036^{p}	0.050^{p}	0.075^{e}	0.027^{q}	0.029^{q}	0.039^{efg}
6	SA14P2315	0.037^{p}	0.044^{p}	0.080^{cd}	0.030^{q}	0.030^{q}	0.040^{ef}
7	SA14P2418	0.032^{p}	0.049^{p}	0.082^{c}	0.022^{q}	0.031^{q}	0.038^{efgh}
8	SA16P2319	0.028^{p}	0.038^{p}	0.073^{e}	0.034^{q}	0.035^{q}	0.088^{c}
9	SA19P2120	0.041^{p}	0.041^{p}	0.040^{p}	0.042^{e}	0.033^{q}	0.035^{q}
10	SA22P3406	0.030^{p}	0.034^{p}	0.077^{de}	0.036^{q}	0.033^{q}	0.091^{bc}

Note: Data are the means of triplicate experiments. Superscript letters a-h, p and q were calculated by DUNCAN for $P<0.05$. Superscript letter p referred to acid phosphatase less than 0.073 unit ml^{-1} min^{-1}. Superscript letter q referred to alkaline phosphatase less than 0.038 unit ml^{-1} min^{-1}.

Table 5 The pH and biomass of ten isolates showing higher available phosphate on Pikrovskaya medium supplemented with tricalcium phosphate at 37 °C for 3, 5 and 7 days.

No.	Fungal code	pH			Biomass (mg ml^{-1})		
		3 days	5 days	7 days	3 days	5 days	7 days
1	SA02P1204	4.145	3.964	4.694	5.1438^{a}	3.8778^{p}	3.6601^{p}
2	SA02P3412	4.142	4.066	4.043	2.8608^{p}	$4.2144^{abcdefg}$	$4.2438^{abcdefg}$
3	SA07P3332	4.127	3.877	3.774	1.7575^{p}	3.1915^{p}	4.4719^{abcdef}
4	SA11P3236	3.980	4.011	3.974	2.5555^{p}	3.8046^{p}	4.4150^{abcdef}
5	SA14P2212	4.311	3.999	4.233	2.4837^{p}	4.8196^{abc}	3.3216^{p}
6	SA14P2315	4.213	3.332	3.290	1.9484^{p}	3.6137^{p}	4.1785^{bcdefg}
7	SA14P2418	3.655	3.345	3.490	4.7569^{abcd}	5.0052^{ab}	4.6294^{abcde}
8	SA16P2319	4.057	3.998	4.893	4.6438^{abcde}	4.5006^{abcdef}	4.1568^{bcdefg}
9	SA19P2120	3.988	4.067	3.912	2.7176^{p}	3.0699^{p}	3.7294^{p}
10	SA22P3406	4.056	3.267	3.509	$4.0464^{bcdefgh}$	$4.1856^{abcdefg}$	4.5948^{abcdef}

Note: Data are the means of triplicate experiments. Superscript letters a-h and p were calculated by DUNCAN for $P<0.05$. Superscript letter p referred to biomass less than 4.0464 mg ml^{-1}.

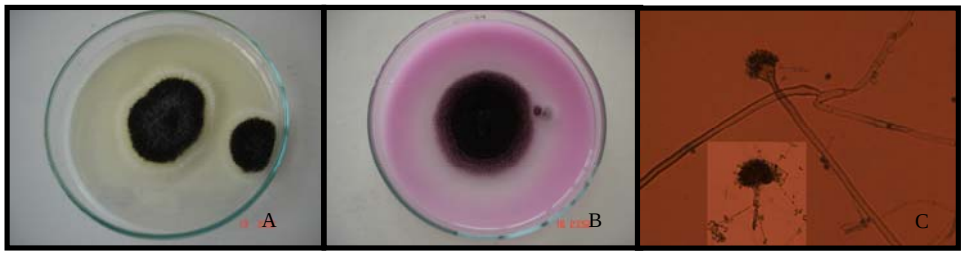

Figure 4 The fungal strain SA07P3332 incubated at 37 °C.
A: on potato dextrose agar
B: on Pikrovskaya agar supplemented with 0.003% w/v rose bengal
C: Morphological observation under compound microscope 400X.

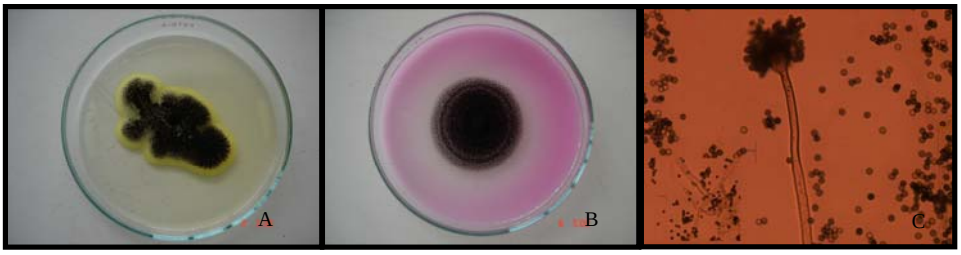

Figure 5 The fungsl strain SA14P2418 incubated at 37 °C.
A: on potato dextrose agar
B: on Pikrovskaya agar supplemented with 0.003% w/v rose bengal
C: Morphological observation under compound microscope 400X.

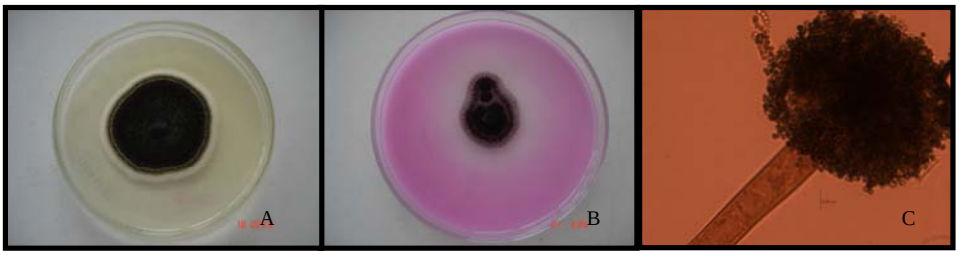

Figure 6 The fungal strain SA19P2120 incubated at 37 °C.
A: on potato dextrose agar
B: on Pikrovskaya agar supplemented with 0.003% w/v rose bengal
C: Morphological observation under compound microscope 400X.

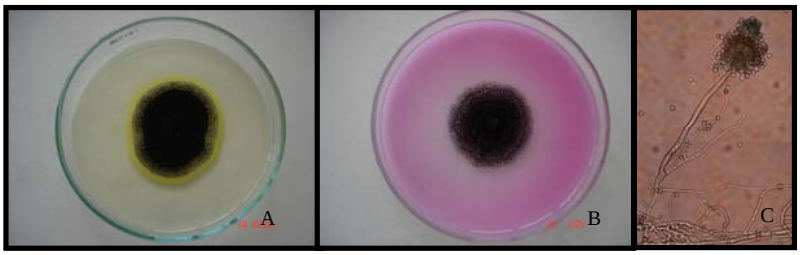

Figure 7 The fungal strain SA22P3406 incubated at 37 °C.
A: on potato dextrose agar
B: on Pikrovskaya agar supplemented with 0.003% w/v rose bengal
C: Morphological observation under compound microscope 400X.

4. CONCLUSIONS

Four fungal strains out of 30 strains, i.e. SA07P3332, SA22P3406, SA14P2418 and SA19P2120 could solubilize tricalcium phosphate and showed the highest available phosphate in liquid medium. All of fungal strains have black colonies with fast growing on potato dextrose agar and characterized as *Aspergillus* sp.

5. ACKNOWLEDGEMENTS

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