




Isolation and characterization of phosphate solubilizing bacteria from agricultural soils for a potential use in cultivating *Capsicum frutescens*

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ABSTRACT

Phosphorus (P) is one of the essential macronutrients needed for the plant growth, other than nitrogen and potassium. Most phosphorus remains as insoluble form in soils and the plants only can uptake the phosphorus nutrient in soluble forms. Phosphate solubilizing bacteria (PSB) dissolves the phosphorus and make it available for plants. In this study, Soil samples were collected and screened for PSB on PK medium. PSB colonies with the highest phosphate solubilization ability were chosen and used for studying its rhizosphere effect on *Capsicum frutescens* by pot experiment.. It was evidenced that selected PSB strain could solubilize phosphate in PK medium and modified PK broth. Besides, it provided available phosphorus for plants and enhanced the plant growth in pot experiment.

Keywords: *Capsicum frutescens*; PSB; Phosphate solubilization; Plant growth; Eggshells and bones; PK medium.

1. INTRODUCTION

Plants need phosphorus (P) nutrients to optimize the growth and carry out various physiological activities, such as cell division, photosynthesis, develop a good root system and utilization of carbohydrates [1] and it is the major plant growth-limiting nutrients after nitrogen [2]. However, the issue of insufficient soluble phosphate in soil of Malaysia for crop production is often being raised [3]. This is due to the fact that about 95% of phosphorus is present in insoluble form in soils, where the plant only can absorb phosphate in soluble form [4]. The phosphorus also forms strong bonds with calcium and magnesium in alkaline soil pH; and with iron and aluminium in acidic soils [5]. Hence, it subsequently causes the mobility of phosphorus in soil to plants become very slow progress. Thus, the only way for plants to uptake phosphorus nutrient is by a slow and low solubility of phosphorus in natural cycle, otherwise by applying chemical fertilizers [6]. Therefore, the inoculation of phosphate solubilizing bacteria (PSB) to plant roots or soil to solubilize the insoluble phosphorus in soils are highly recommended and widely investigated by researchers. The use of PSB as biofertilizer can minimize the use of costly chemical fertilizer, increase crop yield and much more environmentally friendly if compared to chemical fertilizer.

The rhizosphere refers to the soil regions that surround the plant roots, where influenced by root secretion, mainly colonized by plant growth promoting rhizobacteria (PGPR) [7], and is the active activity areas of bacteria, earthworm and nematodes [8]. Hence, the rhizosphere soils are having different physical, chemical and biological properties than other region of soils, which are critically affecting the root's activities of plants. Among PGPR, phosphate solubilizing bacteria (PSB) is the main concern that can convert phosphate to soluble form. Gerretsen [9] showed that soil bacteria

could increase solubility of Ca-phosphates by decreasing the soil pH, subsequently increased the soluble P nutrient available for plants. The interaction between PSB (phosphate solubilizing bacteria), soil and plants are quite complicated; it may have detrimental, beneficial or neutral effects towards the plants. However, the focus is mainly on looking for beneficial effect of PSBs to plants, such as some PSBs can convert insoluble forms of nutrients into soluble forms, so the plants can take it up. Subsequently, it is enhancing the growth of plants, including faster ripening of fruits and flowering, also supports the immune system [5].

The phosphorus solubilization ability of bacteria is routinely screened by Pikovskaya (PK) medium that incorporated with tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) [10], which is a differential medium. However, the reliability of halo-based technique used in PK medium is questioned as many isolates that did not produce any visible halo zone on PK agar, thus a modified PK medium by using bromophenol blue to enhance the clarity and visibility of yellow colored halo zones technique was tested [11].

The aim of this study was to focus on isolation and characterization of phosphate solubilizing bacteria (PSB) from the rhizosphere soils of *Capsicum frutescens* plants (chili), and then the selected PSB was evaluated for the phosphate solubilizing ability. Different fertilization conditions, such as triple super phosphate (TSP), rock phosphate (RP) and eggshells and bones (EB) were designed and applied to the pot experiment to evaluate the rhizosphere effects of PSB on *C. frutescens*. The tested hypothesis is that the selected phosphate solubilizing bacteria (PSB) strain was capable of solubilizing phosphate sources and contribute to promoting the growth of *C. frutescens*.

2. MATERIALS AND METHODS

2.1. Collection of soil sample

Soil samples were collected in sealed sterile polythene bags from four different places around Malaysia (Labu, Kota Damansara, Sungai Buloh and Petaling Jaya); in order to investigate the rhizosphere effect of phosphate solubilizing bacteria (PSB) on chili plants (*C. frutescens*). The soil samples were collected along with the roots of chilies at a depth of about 10 cm and then kept in 4 °C for further analysis.

2.2. Soil analysis

The collected soil sample was sent to FRIM (Forest Research Institute of Malaysia) for soil analysis to identify the suitability of soil for cultivation and various physico-chemical parameters.

2.3. Seedlings germination for pot experiment

C. frutescens was being chosen and it was purchased from the commercial supplies. Total of 300 seedlings of *C. frutescens* were distributed randomly on pot at the beginning of research for germination phases and was grown for 25 days. Later, the better seedlings of *C. frutescens* were selected to do the PSB root inoculation and transplanted for a pot experiment.

2.4. Separation of rhizosphere and non-rhizosphere soil sample

The soil sample along with the root system of plant was taken out from the sealed polythene bag, and then gently shaken to remove the adhered soil. The loosely attached soil was known as non-rhizosphere soil, while the soil that still adhered to the root system after shaken was considered as rhizosphere soil. Then, the separated soils were used for further microbial analysis.

2.5. Isolation of PSB by PK medium

1g of rhizosphere and non-rhizosphere soil samples were weighed and separately mixed with 100ml of sterile saline water in conical flask and placed in orbital shaker for 30 min at room temperature. Then, serial dilution was performed to dilute the samples and 100µl of each sample was spread on Pikovskaya (PK) media [10] contained (g/l): glucose, 10; $\text{Ca}_3(\text{PO}_4)_2$, 5; $(\text{NH}_4)_2\text{SO}_4$, 0.5; NaCl, 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; KCl, 0.2; yeast extract, 0.5; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; agar-agar, 20, pH=7.2.

After that, the plates were incubated at 37 °C and observed for a week. The colonies formed with halo zones were considered as phosphate solubilizing bacteria due to the capacity of solubilizing tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) in PK agar. Hence, it was streaked on a sterile PK plate to obtain a pure culture of PSB. Other than that, it was also streaked on nutrient slant agar for further analysis.

2.6. Study of phosphate solubilization ability

Several PSB colonies with clear halo zones were noted. The diameter of the colonies and the halo zones were measured, in order to select a potential PSB strain with the highest phosphate solubilization ability to be used to study the effect on *C. frutescens* by using a pot experiment. The solubilizing efficiency of PSB was determined by the following formula [12].

$$\text{Solubilizing Efficiency} = \frac{\text{Diameter of halo zone/mm}}{\text{Diameter of colony/mm}} \times 100\%$$

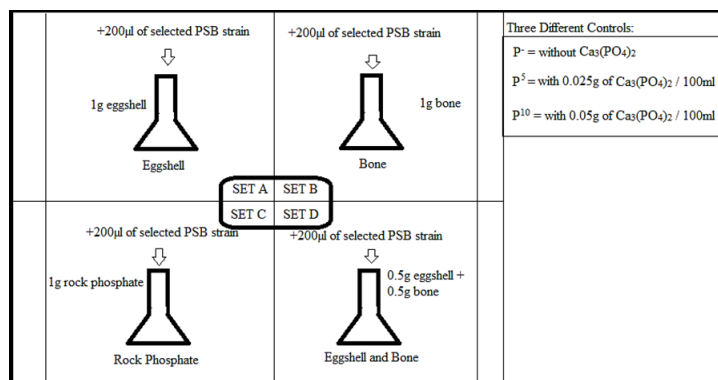


Figure 1. Different alternative phosphate sources of modified PK broth used in phosphate solubilization estimation (SET A – eggshell, SET B – bone, SET C – rock phosphate, SET D – eggshell and bone), and each set have 3 different $\text{Ca}_3(\text{PO}_4)_2$ concentrations of controls to conduct.

2.7. Morphological and biochemical characteristics of PSBs

The morphology of the selected PSB colony was observed on nutrient agar. Biochemical tests were done in order to identify the selected PSB strain.

2.8. A qualitative method for screening PSBs

A modified PK medium cooperated with bromophenol blue (BPB) was prepared and tested to enhance the clarity and visibility of halo zones formed; as some PSBs could not produce any clear halo zones on PK medium, or the capability of forming halo zones on PK medium declined with time [11,13].

The modified PK medium cooperated with bromophenol blue (BPB) contained (g/l): glucose, 10; $\text{Ca}_3(\text{PO}_4)_2$, 5; $(\text{NH}_4)_2\text{SO}_4$, 0.5; NaCl, 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; KCl, 0.2; yeast extract, 0.5; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; agar-agar, 20, pH=7.2; cooperated with 0.0025% of sterile purified bromophenol blue solution.

2.9. Phosphate solubilization estimation using modified PK broth

The modified PK broths were used with alternative phosphate sources (bone, eggshell or rock phosphate), instead of tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$) to test the capacity of selected PSB strain in solubilizing phosphate. There were 4 different sets of modified PK broth, each incorporated with different phosphate sources and labelled as SET A: eggshell; SET B: bone; SET C: rock phosphate and SET D: eggshell and bone as shown in the Fig. 1. Moreover, three different sets of control were prepared for SET A, B, C and D; in which each control have different concentration of tricalcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$), named P⁻ (without $\text{Ca}_3(\text{PO}_4)_2$), P⁵ (with 0.025g of $\text{Ca}_3(\text{PO}_4)_2$ / 100ml) and P¹⁰ (0.05g of $\text{Ca}_3(\text{PO}_4)_2$ / 100ml) as seen in Fig. 1.

Standard PK broth consisted of (g/l): glucose, 10; $(\text{NH}_4)_2\text{SO}_4$, 0.5; NaCl, 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1; KCl, 0.2; yeast extract, 0.5; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.002; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; agar-agar, 20, pH=7.2; while the concentration of $\text{Ca}_3(\text{PO}_4)_2$ was added according to different controls, and alternative phosphate sources (eggshell, bone or rock phosphate) was added according to different set of PK broth. In the end, the prepared PK broths were autoclaved. , 200 µl of selected PSB strain was inoculated into the sterile PK broths and incubated in shaking incubator (37 °C) over the period of 9 days.

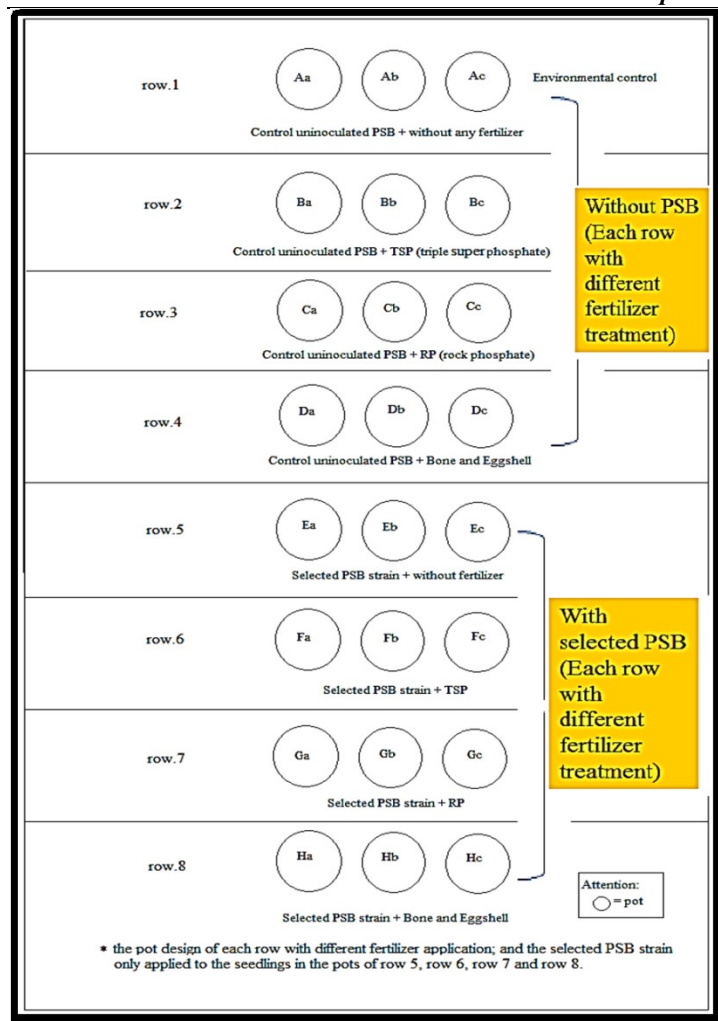


Figure 2. The illustration of pot experiment design conducted, each with different microorganisms and fertilizer conditions. (Row 1 – 4: soil without inoculation of selected PSB strain; Row 5 – 8: soil with inoculation of selected PSB strain), (Row 1 and 5: without fertilizer application; Row 2 and 6: with triple super phosphate; Row 3 and 7: with rock phosphate; Row 4 and 8: with bone and eggshell).

1 ml of the samples was taken out from each respective conical flask into Eppendorf tube for every 8 hrs until the total incubation time of 200 hrs. It was centrifuged for 5000rpm (10 min) and the supernatant was transferred into a new Eppendorf tube and stored at -20°C until analysis. The absorbance of soluble phosphate in the collected supernatant (by colorimetric method) was measured at 690nm by using the stock prepared (100 µl of sample + 4.9ml of double distilled water + 200µl ammonium molybdate + 30µl of stannous chloride). The final weight of bone and eggshell in modified PK broth (SET A, SET B and SET D) in dry forms was determined. The phosphate content was estimated by modified protocol of Trivedy and Goel [14].

2.10. Inoculum preparation for root inoculation

The selected PSB strain was cultured on nutrient broth for overnight. Then, 200 µl of it was pipetted out and added into 250 ml conical flask that contained 100 ml of sterile nutrient broth. Then, it was incubated (37°C) in shaker incubator for 24 hours. After that, the cultured PSB was centrifuged at 5000 rpm for 5 min; and the pellet was resuspended with 150 ml of tap water and mixed well. The root-dip method was used for the first time during transplantation of germinated *C. frutescens* seedlings to pot experiment for each pot on row 5, 6, 7 and 8 (refer to pot experiment design on Fig. 2). In addition, 10ml of selected PSB

strain was inoculated to treatment pots on row 5, 6, 7 and 8 every time one day before routinely 30 days of fertilizer application.

2.11. Pot experiment

Pot experiments of chilies were carried out at the MAHSA University Lab, located at Pusat Bandar Damansara, Kuala Lumpur (Malaysia). There were total 8 rows of plants, each row with different treatments and fertilization application, employing with block design of three replications and each pot with two plants on it. The pot experiment was performed using the *C. frutescens* seedlings and black polythene bags (18cm height, 15cm deep of soil) were used as the pots. The temperature of the environment ranged between 22°C-36°C which is suitable for plants to grow.

2.12. Experimental design

There were total 8 rows of pots with three replicated for each set of pot experiments, each row of pots was treated with different fertilization conditions as illustrated in Fig. 2.

2.13. Fertilizer application

For the first four rows (row 1-4) of pot design, it was without selected PSB; while the last four rows (row 5-8) of pot design, it was with selected PSB, each row of pot was with different fertilizer treatment.

For the pot experiment (there are 8 rows of pots with three replications), 80g of urea and 80g of potash were mixed together with 400ml of tap water to dissolve. It was added to every row of pots with 20ml/pot, except row 1 and row 5 (both are environmental control without fertilizer). For pots on row 2 and row 6, 20ml of triple superphosphate (TSP) was dissolved in 200ml of tap water and then added 20ml per pot. While for row 3 and row 7, 20ml of rock phosphate (RP) was mixed in 200ml tap water; and 20ml of it was added per pot. 200g of bone powder and 200g of eggshell powder were weighed and mixed; then 33g of it was applied to each pot of row 4 and row 8. The fertilizer application was carried out routinely for every 30 days to plants.

For the bone and eggshells preparations, it was collected from the nearby restaurant in a polythene bag, and it was washed and cleaned, meats and tendons were removed. After that, it was dispersed on a larger size of Petri dishes and dried in hot air oven at 60°C for few days.. Then, it was ground, crushed and blended into powder form and finally available to apply to plants.

2.14. Plant parameters

The plant parameters, such as number of flowers, number of fruits, number of leaves, weight of fruits, length of fruits and shoot length of plants, of each row of plants were recorded at regular interval. In addition, the root length of plants was also determined.

2.15. Chlorophyll content estimation

The whole chlorophyll content was estimated under dark and dim condition to avoid light sensitivity of chlorophyll. The sample's leaves were collected and weighed (500mg), and then transferred to a pestle-mortar. Then, several ml of 90% acetone and few acetone washed sands were added and ground until thoroughly macerated. After that, the ground sample was poured into a 15ml centrifuge tube. Mortar and pestle were rinsed with sufficient acetone and 0.2ml of MgCO₃ suspension was added to bring to a total volume of 10ml. Then, 15ml centrifuge tube was wrapped with aluminium foils in order to prevent the reaction between light and the content; then it was incubated at 4°C for 4-6 h for pigment

to elute. After that, the sample was centrifuged at 3000 rpm for 20 min at 4°C. 1ml of supernatant was used to read the absorbance at 630nm, 645nm and 663nm using UvLine 9400 spectrophotometer. Apart from that, the spectrum of sample (from 400nm-1100nm) was also measured [14].

2.16. Investigation of final rhizosphere effect

1 g of rhizosphere and non-rhizosphere soils were respectively collected from every different soil conditions of pot experiment (Fig. 2) in the final end of conducted research. The collected rhizosphere and non-rhizosphere soils were separately mixed with 100µl of sterile saline in conical flask by orbital

shaker for 30 min at room temperature. Next, the serial dilution was done to the samples and 100µl of it was spread on bromophenol blue (BPB)-PK medium. The cultured plates were incubated at 37 °C and observed for a week.

2.17. Statistical analysis

Analysis of Variance (ANOVA), T-test and standard deviation, was conducted using available statistical online software (GraphPad Prism 6). Compared among multiple treatment levels with the controls to test the significance of treatment means at $P \leq 0.05$.

3. RESULTS

3.1. Physico-Chemical Parameters of soil before cultivation

Before cultivation, the soil sample was sent to FRIM (Forest Research Institute of Malaysia) for soil analysis to determine the suitability of chemical constituents in soil for cultivation purposes (Table 1). The dry pH was 7.65, which was considered as slightly alkaline, may due to contained slightly higher saturation of base cations (Ca^{2+} , Mg^{2+} and K^{+}) or presence of limestone or carbonates.

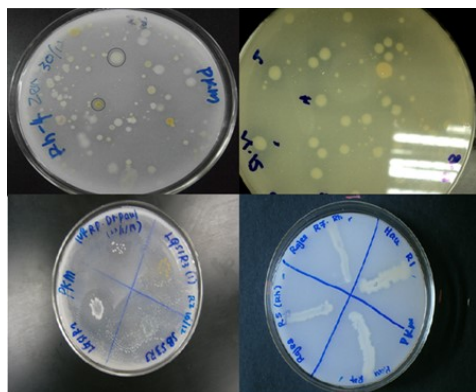


Figure 3. Different PSB colonies formed on PK agar with halo zones.

3.2. Distribution study of PSB in rhizosphere and non-rhizosphere soil.

The rhizosphere effects of PSB on *C. frutescens* between rhizosphere and non-rhizosphere soils from four different places around Malaysia (Labu, Kota Damansara, Sungai Buloh and Petaling Jaya) were determined as shown in Table 2. All different places of soils contained higher number of PSB count on rhizosphere soils when compared to non-rhizosphere, even though there was no significant difference ($P > 0.05$). In a research paper by Carolyn *et al.* [15], showed the same result with no significant difference between PSB counts in rhizosphere and non-rhizosphere soils.

3.3. Selection of potential PSB based on solubilization efficiency (%) on PK agar

The soil samples collected from different places were serially diluted and used for spread plate method to screen PSB on PK agar. The colonies formed with halo zones on PK agar after one week was considered as PSB. Fig. 3.

7 positive PSB colonies formed on PK agar with clear halo zones were noted and sub-cultured again on pure PK medium. The colony diameter, halo zone diameter and solubilization efficiency of noted 7 positive PSB isolates on PK medium after one week was tabulated in Table 3. PSB showed the highest solubilization

efficiency on PK media was 166.7% which was determined using the formula described by Edi *et al.* [12], with colony diameter of 3 mm and 5 mm of halo zones diameter. Then, that particular PSB strain (R2) was selected to study the effect on *C. frutescens* growth. The 7 isolates were able to show phosphate solubilizing zones ranging from 2.5-8mm diameter by utilizing the tricalcium phosphate in PK medium. Worapon *et al.* [16] also used the same concept to isolate potential PSB and the strain which displayed the highest solubilization efficiency was chosen to study the rhizosphere effects and further species identification.

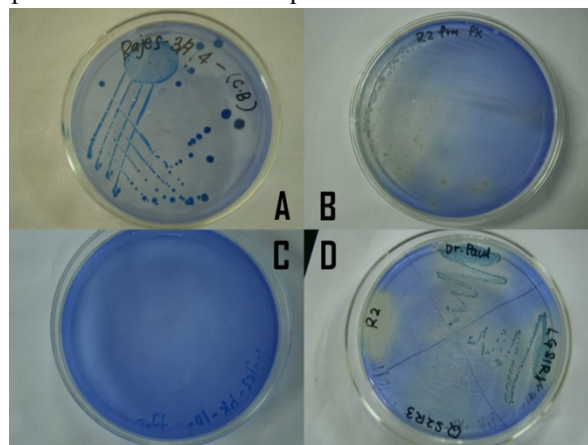


Figure 4. Different bromophenol blue (BPB)-PK medium patterns (A, B and D: random PSB cultured BPB-PK medium; D: original pure BPB-PK medium).

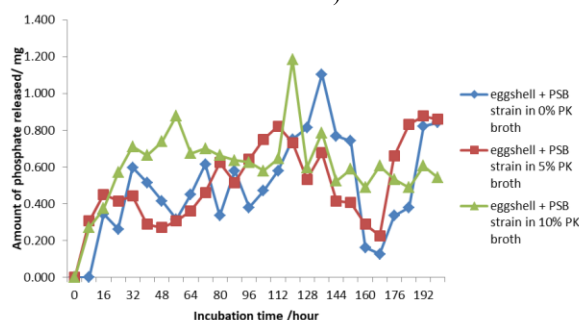


Figure 5. The amount of phosphate released in eggshell-modified PK broth along the time (hour).

3.4. Morphological and biochemical characterization of selected PSB strain

The selected PSB strain (R₂) was identified by a series of biochemical tests, and was identified as gram-positive, yellow-white color of small rod shape on PK agar, positive for oxidase, endospore, motility and starch hydrolysis, but negative for catalase test.

3.5. Qualitative screening of PSB by bromophenol blue (BPB)-PK medium

A modified PK medium containing bromophenol blue (BPB) had the capacity to enhance the clarity and visibility of halo zones formed by PSB isolates when compared with normal PK medium (Fig. 4). The changes in color of BPB-PK medium were quite obvious especially after 1week incubation of cultured medium.

3.6. Phosphate solubilization estimation by using modified PK broth

The amount of soluble P being released from modified PK broths was determined through the spectrophotometric method at 690 nm. Based on the result in Fig. 5 (eggshell-modified PK broth), from 0 – 104 h incubation period, the P released amount for three different controls showed a rise and fall to reach a peak at between range of 112 – 132 h incubation. After that, 3 different controls (Fig. 1) of eggshell-modified PK broths gradually declined and minimal rise was seen again before reaching a total incubation period of 200 h. .

The different controls of cultured bone-modified PK broth's results in Fig. 6 showed sharp and rapid increase in phosphate amount released and hit a peak with a range of 5.5 – 7.5 amount of phosphate released /mg at incubation time of 72 – 96 h. Then, the released phosphate (P) was found to drop and slightly rise again before complete 200 h.

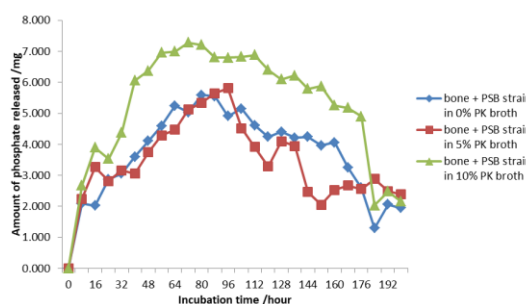


Figure 6. The amount of phosphate released in eggshell-modified PK broth along the time (hour).

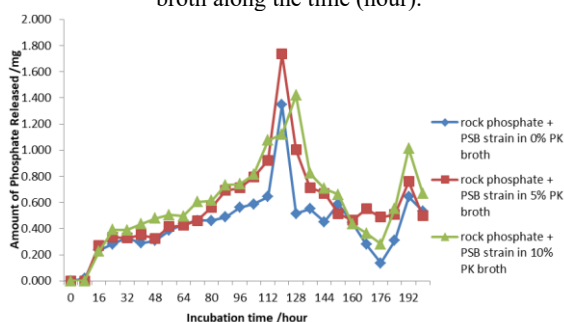


Figure 7. The amount of phosphate released in eggshell-modified PK broth along the time (hour).

While the phosphate amount released by cultured rock phosphate (RP)-PK broth (Fig. 7), three different controls of RP-PK broths showed a steady upward trend to reach a peak at incubation time of 120 – 128 h, then again a fall in phosphate released occurred and a minimal P was released before 200 h incubation.

The same happened in Fig. 8 (amount of P released in cultured bone + eggshell modified PK broths), a notably upward trend was seen and reached a peak at 72 – 88 h. Then, a reduction in P released occurred in 3 different controls of cultured bone +

eggshell modified PK broths and a minimal growth trend in line before 200 total incubation hours. Overall, all different alternative phosphate sources of modified PK broths (referred to Fig. 1) showed a considerable increase in P released before reaching a peak, after that it started to fall in release of P and a slight raise in P release was noticed before 200 h. Overall, phosphate (P) released detected by cultured eggshell-modified PK broth over time was the least amount when compared with other types of modified PK broth. While the selected PSB strain cultured in bone-modified PK broth acted as the most efficient one to release P from the content, then followed by rock phosphate (RP)- and bone + eggshell (BE)-modified PK broth.

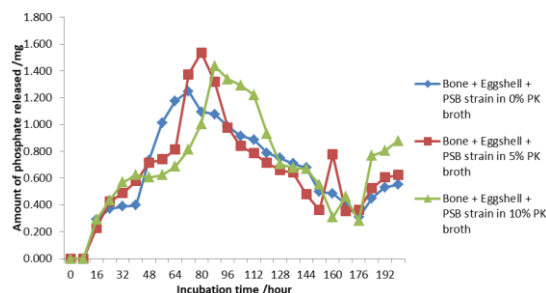


Figure 8. The amount of phosphate released in eggshell-modified PK broth along the time (hour).

Besides, 10% PK broth did have a higher amount of tricalcium phosphate than 0% and 5% PK broth for inoculated PSB to solubilize it. Hence, it supposed to have higher P content released than 0% and 5% PK broth, and it was shown in the case of bone + PSB strain in 10% PK broth (Fig. 6). However, for other types of modified PK broths, such as eggshell-, RP- and BE-modified PK broth, the content of tricalcium phosphate (0%, 5% or 10%) in modified PK broth was not a crucial factor in affecting P released amount; as the P released with different controls were inconsistent and less notable (Fig. 5, Fig. 7 and Fig. 8). However, statistical analysis showed a difference in control (amount of tricalcium phosphate presence in PK broth) thus significantly affecting the P released in eggshell-, bone- and RP-modified PK broth.

In short, selected PSB did significant result in solubilizing the tricalcium phosphate in modified PK broths and induced detectable amount of soluble P release. For instances, selected PSB strain caused significant ($P < 0.05$) P released in eggshell-modified PK broth; extremely significant ($P < 0.0001$) P release in RP- and bone-modified PK broth, but not significant in case of eggshell + bone modified PK broth ($P > 0.05$).

On top of that, the dry weight of eggshells and bones collected from eggshells-, bones- and BE-modified PK broth was determined after the experiment and the results in all three cases showed reduced final dry weight (Table 4), which then indicated some of the phosphate composition in either eggshell or bone was solubilized by inoculated PSB and then released into PK broth, which resulted in detectable level of soluble phosphate.

Interestingly, the percentage of P released from eggshell was very low when compared with chicken bones. The bone + PSB strain in 10% PK broth showed the highest percentage of P released (61%) ($P < 0.001$), followed by 5% bone-PK broth (55%) ($P < 0.01$), then cultured in 0% bone-PK broth (40%) ($P < 0.01$), while P release

from 0% eggshell-PK broth was found to be the least with 3 % ($P>0.05$).

The cultured eggshell + bone-PK broths with different controls have a range of 18 – 32 % of P released, and the 5% PK broth one was considered as significant ($P<0.05$) (Table 4). In spite of that, all different control of eggshell-modified PK broths were not significant ($P>0.05$) in certifying the P solubilizing capability of selected PSB strains.

3.7. Evaluation on effect of selected PSB strain on chilies (*C. frutescens*) plant parameters

The plant parameters (such as number of flowers, number of fruits, number of leaves, shoot length, weight of fruits and length of fruits) were recorded on Day 35 and was repeated until day 85 with 10 days interval. Besides, the root length of plants was also recorded on Day 85 and tabulated in Table 11.

From Table 5 – 10, the number of flowers, number of fruits, number of leaves, shoot length, weight of fruits and length of fruits were found to be higher in *C. frutescens* plants inoculated with selected PSB strains compared with uninoculated plants. On top of that, the growth of plants was found to be greater in soils inoculated with additional fertilizers, especially the most growth was noticed in the soil with eggshells and bones (EB) as fertilizer, followed by the plants in soil with rock phosphate (RP) and the least in triple super phosphate (TSP).

As a proof of that facts, treatment pot with PSB and eggshells and bones on Day 65 and Day 85 (Table 8 & 10) showed the highest number of flowers among all, with a significant difference value ($P<0.05$). Besides, treatment pot with PSB and eggshells and bone on Day 85 (Table 10) showed the highest number of leaves (574 units), which was significantly ($P<0.05$) higher than any others. Treatment pot with PSB and eggshells and bones always displayed the significant ($P<0.05$) highest shoot length at every 10 days interval of plant parameter measurements in this research. On Day 35 as illustrated in Table 5, Treatment pot with PSB and eggshells and bones displayed a significant highest value ($P<0.05$) of weight in fruits (2.231g) compared with other treatment pots. Interestingly, treatment pot with PSB and eggshells and bones again showed a significant highest value (7cm) ($P<0.05$) in length of fruits (Table 9) (Day 75 - after conducted pot experiment).

Table 1. Result of physico-chemical parameters of soil.

Chemical Components	Units
Dry pH	7.65
Organic Carbon	3.26%
Organic Nitrogen	0.10%
Available P	2115.00 ppm
Exchangeable Ca	4.66 cmol/kg
Exchangeable. Mg	2.72 cmol/kg
Exchangeable. K	2.62 cmol/kg

Regardless of the treatment pot with or without selected PSB, the treatment pot with eggshell and bones (EB) frequently showed significant values of plant parameters ($P<0.05$) than other treatment pots, (Table 10) (Day 85) every plant parameters of treatment pot with EB were significant, except shoot length,

number of flowers and fruits on treatment soil without PSB, but with EB.

In contrast, treatment soil without PSB and without fertilizer showed the lowest values in all plant parameters.. For examples, it was shown to have only one fruit harvested in the beginning of Day 35 conducted pot experiment (Table 5), then no more harvest infruits was seen till Day 85. Next, it only produced a few flowers (no more than three units) starting from Day 65 to Day 85. Moreover, it displayed a retarded plant growth, like the least total amount of leaves (range of 16 – 70 units) and least shoot length (20.8 – 21.7 cm) every time intervals.

Other than that, the treatment pots with triple superphosphate (TSP) could not achieve the same mean in plant parameters as pots with RP or EB. For instances, the number of flowers produced at every time interval (Day 35 – 85) was not significantly different than the control. The number of fruits produced was lesser than half when compared with treatment soil with RP or EB as can be seen in Table 8.

On comparing the plant parameters displayed on different day after conducted pot experiment (Day 35 – 85), it showed significant difference in plant parameters displayed on Day 65, 75 and 85 than on Day 35. On Day 35 (Table 5), only treatment pot with soil + PSB + eggshells + bones showed significant difference in plant parameters, that is shoot length (31.9cm) and mean weight of fruits (2.231g). Apart from that, the shoot length plant parameters generally displayed the most significant differences to determine the effect of selected PSB on growth of *C. frutescens* in this research, followed by the number of leaves, and length of fruits. There were the least significant figures displayed by comparing the number of flowers produced at different treatment soils.

All in all, the treatment soil with selected PSB strain overall showed higher values of plant parameters than uninoculated treatment soil, which was an advantageous fact to ensure the P solubilizing capability of selected PSB on promoting plant growth. Nevertheless, the uninoculated treatment pots of RP generally demonstrated higher plant growth than inoculated RP treatment pots as apparent facts particularly in Table 6 and 7, which was not the expected outcomes.

Data in Table 11 demonstrated that generally tested soil with selected PSB (except inoculated soil without fertilizer) indicated an increment in root length as compared with uninoculated soil, while inoculated soil with EB reported with highest root length (19.33cm) in this research, followed by inoculated soil with RP (17.68cm). Different uninoculated treatment of soil expressed a range of 11.58 – 16.57 cm of root length, yet soil with selected PSB revealed a range of 11.52 – 19.33 cm of root length. Generally, the differences showed in between the presence and absence of selected PSB strain in soil was not so significant ($P>0.05$), but different fertilization condition did significantly ($P<0.05$) affect the root length of plants.

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Table 2. Comparison of PSB rhizosphere effect on rhizosphere and non-rhizosphere soils in CFU/g.

Location	Sample No.	PSB (CFU/g)	
		Rhizosphere	Non-rhizosphere
Labu	1	1.31×10^6	1.97×10^5
	2	2.36×10^4	2.06×10^4
	3	3.95×10^4	1.94×10^3
Kota Damansara	1	3.20×10^5	6.78×10^4
	2	7.89×10^6	8.50×10^5
	3	6.55×10^8	3.42×10^7
Sungai Buloh	1	3.67×10^4	4.22×10^3
	2	4.04×10^6	3.41×10^6
	3	4.43×10^5	5.78×10^4
	4	2.79×10^4	2.14×10^4
	5	2.99×10^6	1.85×10^5
Petaling Jaya	1	1.86×10^5	2.39×10^4
	2	3.79×10^6	1.88×10^6
	3	2.33×10^6	3.44×10^5
	4	4.23×10^5	6.43×10^4

Table 3. Comparison of colony diameter, halo zone diameter and solubilization efficiency between several PSB isolates.

Strains no.	Colony diameter (mm)	Halo zone diameter (mm)	Solubilization Efficiency (%)
R ₁	5.0	7.0	140.0
R ₂ **	3.0	5.0	166.7
R ₃	3.0	4.0	133.3
R ₄	6.0	8.0	133.3
R ₅	3.0	4.0	133.3
Rh ₁	4.0	5.0	125.0
NR ₄	2.0	2.5	125.0

Strains number with labelled ** is the selected PSB strain to conduct in this research.

Table 4. The percentage (%) of P released at different type of modified PK broth.

Types of Modified PK Broth	Weight of Eggshells/Bones/Eggshell + Bone in dry forms (mg)			Percentage (%) of P released
	Initial Weight	Final Weight	Difference in Weight	
Eggshell + PSB strain in 0% PK broth	1	0.97	0.03	3
Eggshell + PSB strain in 5% PK broth	1	0.96	0.04	4
Eggshell + PSB strain in 10% PK broth	1	0.96	0.04	4
Bone + PSB strain in 0% PK broth	1	0.6	0.4	40**
Bone + PSB strain in 5% PK broth	1	0.45	0.55	55**
Bone + PSB strain in 10% PK broth	1	0.39	0.61	61***
Eggshell and Bone + PSB strain in 0% PK broth	1	0.82	0.18	18
Eggshell and Bone + PSB strain in 5% PK broth	1	0.68	0.32	32*
Eggshell and Bone + PSB strain in 10% PK broth	1	0.78	0.22	22

Values with * symbol considered as significant ($P < 0.05$); values with ** are very significant ($P < 0.01$) and values with *** are extremely significant ($P < 0.001$).

Table 5. Effect of seed treatment with selected PSB on growth of *C. frutescens* in pot experiment (Day 35 after conducted pot experiment).

Treatment	Number of flowers (units) ^{##}	Number of fruits (units) ^{##}	Number of leaves (units) [*]	Shoot length (cm) [*]	Weight of fruits (g) [*]
Soil without PSB and without fertilizer	0 ^a	1 ^b	16 ^c	20.8 ^d	0.127 ^e
Soil without PSB, with TSP	0 ^a	1 ^b	39 ^c	21.3 ^d	0.81 ^e
Soil without PSB, with RP	0 ^a	2 ^b	85 ^c	29.3 ^{dc}	0.897 ^e
Soil without PSB, with eggshells + bones	5 ^a	3 ^b	148 ^c	24.2 ^d	0.372 ^e
Soil + PSB	0 ^a	2 ^b	46 ^c	23.3 ^d	0.888 ^e
Soil + PSB + TSP	0 ^a	1 ^b	59 ^c	23.6 ^d	0.339 ^e
Soil + PSB + RP	9 ^a	6 ^b	168 ^c	24.8 ^d	0.371 ^e
Soil + PSB + eggshells + bones	0 ^a	9 ^b	178 ^c	31.9 ^{dh}	2.231 ^{eh}

Values under factors with symbol * are given as means for triplicate samples, while the values under factors with symbol ^{##} are given as total numbers for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at $P \leq 0.05$.

Table 6. Effect of seed treatment with selected PSB on growth of *C. frutescens* in pot experiment (Day 45 after conducted pot experiment).

Treatment	Number of flowers (units) ^{##}	Number of fruits (units) ^{##}	Number of leaves (units) [*]	Shoot length (cm) [*]	Length of fruits (cm) [*]
Soil without PSB and without fertilizer	0 ^a	0 ^b	33 ^c	21.2 ^d	-
Soil without PSB, with TSP	0 ^a	0 ^b	166 ^c	21.7 ^d	-
Soil without PSB, with RP	32 ^a	5 ^b	460 ^{cc}	30.3 ^{dc}	3.5 ^{lc}
Soil without PSB, with eggshells + bones	2 ^a	5 ^b	384 ^{cd}	25.8 ^d	2.8 ^f
Soil + PSB	0 ^a	0 ^b	47 ^c	22.1 ^d	-
Soil + PSB + TSP	0 ^a	0 ^b	161 ^c	24.3 ^d	-
Soil + PSB + RP	15 ^a	9 ^b	305 ^{cc}	27.2 ^{dc}	1.7 ^f
Soil + PSB + eggshells + bones	16 ^a	2 ^b	369 ^{cd}	33.1 ^{dh}	2.6 ^f

Values under factors with symbol * are given as means for triplicate samples, while the values under factors with symbol ^{##} are given as total numbers for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at $P \leq 0.05$.

Table 7. Effect of seed treatment with selected PSB on growth of *C. frutescens* in pot experiment (Day 55 after conducted pot experiment).

Treatment	Number of flowers (units) ^{##}	Number of fruits (units) ^{##}	Number of leaves (units) [*]	Shoot length (cm) [*]	Weight of fruits (g) [*]	Length of fruits (cm) [*]
Soil without PSB and without fertilizer	0 ^a	0 ^b	35 ^c	20.7 ^d	-	-
Soil without PSB, with TSP	10 ^a	0 ^b	198 ^c	21.5 ^d	-	-
Soil without PSB, with RP	41 ^{ac}	5 ^b	364 ^{cc}	31.2 ^{dc}	0.565 ^e	3.6 ^{lc}
Soil without PSB, with eggshells + bones	26 ^a	5 ^b	328 ^{cd}	25.1 ^d	0.59 ^e	2.6 ^f
Soil + PSB	1 ^a	0 ^b	56 ^c	21.6 ^d	-	-
Soil + PSB + TSP	1 ^a	1 ^b	189 ^c	26.1 ^{df}	0.535 ^e	2.1 ^f
Soil + PSB + RP	18 ^a	10 ^b	322 ^{cc}	26.8 ^{dc}	0.532 ^e	2.1 ^f
Soil + PSB + eggshells + bones	19 ^a	4 ^b	336 ^{cd}	34.7 ^{dh}	0.652 ^e	2.7 ^f

Values under factors with symbol * are given as means for triplicate samples, while the values under factors with symbol ^{##} are given as total numbers for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at $P \leq 0.05$.

Table 8. Effect of seed treatment with selected PSB on growth of *C. frutescens* in pot experiment (Day 65 after conducted pot experiment).

Treatment	Number of flowers (units) ^{##}	Number of fruits (units) ^{##}	Number of leaves (units) [*]	Shoot length (cm) [*]	Length of fruits (cm) [*]
Soil without PSB and without fertilizer	1 ^a	0 ^b	40 ^c	20.9 ^d	-
Soil without PSB, with TSP	11 ^a	1 ^b	209 ^{cb}	21.9 ^d	1.6 ^f
Soil without PSB, with RP	44 ^{ac}	8 ^b	497 ^{cc}	32.1 ^{dc}	3.4 ^{lc}
Soil without PSB, with eggshells + bones	34 ^a	9 ^b	334 ^{cd}	25.5 ^d	3 ^{ld}
Soil + PSB	1 ^a	1 ^b	65 ^c	22.1 ^d	1.4 ^f
Soil + PSB + TSP	9 ^a	1 ^b	259 ^{cb}	26.9 ^{df}	1.7 ^f
Soil + PSB + RP	37 ^a	13 ^b	394 ^{cc}	27.2 ^{dg}	2.9 ^{lc}
Soil + PSB + eggshells + bones	49 ^{ah}	13 ^b	407 ^{cd}	34.9 ^{dh}	3.5 ^f

Values under factors with symbol * are given as means for triplicate samples, while the values under factors with symbol ^{##} are given as total numbers for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at $P \leq 0.05$.

3.8. Role of PSB on chlorophyll content of plants.

As depicted in Table 12, the chlorophyll-a and chlorophyll-b content were measured by colorimetric method [14] and total chlorophyll content referred to sum of chlorophyll-a and chlorophyll-b. The treatment plant with selected PSB + TSP showed the highest total chlorophyll content (6.036 mg/g of leaves), followed by plant in RP treatment soil (5.926 mg/g) and then treatment plant with selected PSB + bone + eggshell (5.722mg/g); three of the highest values mentioned above also displayed significant differences ($P < 0.05$). While the uninoculated treatment plant (no selected PSB and no fertilizer/ environmental control) showed the lowest total chlorophyll content (2.949mg/g), which was similar to the results of Suharja and Sutarno [17].

Overall, the treatment pots with PSB showed higher total chlorophyll content when compared with the treatment pots without selected PSB, which Mehrvarz *et al.* [18], also showed the same outcomes by using Mycorrhiza along with *Pseudomonas putida*. Most of the treatment pots' plants with PSB (except plants on pot with soil + PSB, no fertilizer) showed significant differences ($P < 0.05$), especially soil with TSP and soil with EB was significantly different from both controls than without any

fertilizers: (a) soil without PSB and (b) soil with PSB. Besides, different fertilizer treatment did also influence the rhizosphere effect of selected PSB on the amount of total chlorophyll content obtained ($P < 0.05$).

3.9. The final rhizosphere effect study of selected PSB.

As depicted in Table 13, the total PSB count on inoculated soil was generally higher than the uninoculated soil, except the treatment soil with RP. Selected PSB inoculated soil with eggshells and bones as fertilizers showed the highest total PSB count in all the PSB inoculated groups and it was less in environmental control soil. However, the PSB count on rhizosphere and non-rhizosphere soil for different treatment soils was random and not significant.

3.10. Discussions.

PSB is capable of solubilizing insoluble phosphorus sources in soil, as a result it can promote the growth of plant and yield [9]. In fact, most of the phosphorus in soil as insoluble forms, in which the plants cannot directly utilize it [4]. Thus, the plants only can uptake phosphorus by a slow and low P solubilization in natural cycle or by applying chemical soluble P fertilizer. However, due to heavy rain in Malaysia, the soluble P in soil was easily washed

away and then lead to environmental impacts, especially river pollution [19]. As the results, most of the home gardening and small farms are using insoluble forms of rock phosphate as P fertilizer sources, but it is unavailable for plants to directly uptake it. Thus, the use of PSB as inoculants to plant's root/soil may be a promising agent to solubilize insoluble phosphate sources, subsequently can indirectly enhance plant growth and productivity. Therefore, PSB can be potential and practical use in agricultural fields.

In this work, the role in rhizosphere of selected PSB strain was assessed. Moreover, the distribution study of PSB in rhizosphere and non-rhizosphere soil also has been investigated. The selected PSB strain was partially characterized by some biochemical tests, along with the phosphate solubilization ability on different phosphate sources. Besides that, the relationship of selected PSB strain on the growth of *C. frutescens* was studied using pot experiment. In addition, the interactions of selected PSB with different fertilization conditions on *C. frutescens* growth were investigated.

The suitability of chemical constituents in soil was tested because the soil quality can reflect how well a soil can perform the functions of maintaining productivity, partitioning water and solute flow, filtering and buffering, nutrient cycling and provides support for plant. The pH of soil will affect most of the activity of plants this is because as concerned, the acid pH soils usually limited the nutrient contents in soils, such as calcium, magnesium and phosphorus [20]. Moreover, it sometime also can cause toxicity effects to plants [21], like aluminum toxicity. As described by Abedi *et al.* [21], aluminum toxicity will limit the plant root growth and seedlings germination. Apart from that, if the plant is not cultivated in ideal soil properties in the beginning, then it will induce plant stress, which subsequently misleading to inaccurate plant parameters.

In fact, the composition of rhizosphere and non-rhizosphere soils were different. As described by Yang *et al.* [22], it was showing that there was a difference in P content between both different soils. Besides, Xiong *et al.* [23] also stated that the concentration of arsenic, sulfur, phosphorus and iron in rhizosphere are all higher than non-rhizosphere soil samples. As a result, it may induce the difference in distribution of PSB in the soil. However, from this research results, the differences of PSB distribution in rhizosphere and non-rhizosphere soils were not so significant ($P > 0.05$) as Carolyn *et al.* [15], even though the readings were showing a higher number of PSB count on rhizosphere than non-rhizosphere soil. Moreover, the number of PSB found in each areas in this research was different due to the composition of microbial community is vary among temperature, nutrients, pollutants and other environmental controls as stated by Worapon *et al.* [16].

PSB strain R₂ was selected in this research to investigate the rhizosphere effects on the growth of *C. frutescens* based on the highest solubilization efficiency on PK media (166.7%) as compared with others isolates in this research. In addition, the general characteristics of R₂ isolates in this study which is corresponded with the *Bacillus* sp. [24]: gram positive, rod-shaped, positive for positive for oxidase, endospore, motility and starch hydrolysis, but negative for catalase test.

The solubilization of phosphate into soluble form was carried out with the presence of enzyme phosphatase (organic acid) that commonly secreted by various soil microbes, which described by Tabatabai and Bremner [25]. Then, the phosphorus solubilization process usually start with the chelation of cation (Al, Fe, Ca) that bound to phosphate by the metabolites released by microbes, then latter convert it to soluble forms. As mentioned by Mehta & Shekhar [13], 'they said most of the quantitative tests to assay relative efficiency of PSB are based on the lowering of pH, owing to production of organic acids into surrounding medium', and it was proved by Yu *et al.* [26] and Charana & Yoon [27] which reported significant ($P < 0.05$) decreased in soil pH and increased in available P content in soil after PSB strains (*P. agglomerans* or *B. anthina* or co-inoculation) inoculation. If the bacteria colony formed on PK agar that capable of solubilizing phosphate, then it will solubilize the phosphate around the colonies and a halo zones will be formed around the colonies [28].

The tricalcium phosphate was always the first choice as insoluble phosphates incorporated with PK medium, while BPB has been used earlier by Gupta *et al.* [11], in screening phosphate solubilization by bacteria and then later frequently tested by many researchers [13, 28]. While in this research, BPB-PK medium did efficiently show a clear halo zone of PSB isolates on it as shown in Fig. 4. In fact, bromophenol blue (BPB) is also known as pH indicator, with pK 4.10, approximate 3.0 – 4.6 pH range of color change (yellow in acid form and purple/blue in base form) (General Chemistry Online, 2012). Thus, the changes in color from blue to yellow or clear zones around PSB colonies hint some chemical reactions occurred in between PSB colonies and P sources in BPB-PK broth and affect pH.

As mentioned in results, all different alternative phosphate sources of modified PK broths showed a notably upward trend in P released before reached a peak, which may a sign of selected PSB started to solubilize the insoluble P content in modified PK broth to soluble form and became detectable by instruments, such as spectrophotometer. The peak in amount of phosphate released by modified PK broth may suggest the maximum available insoluble phosphate for selected PSB to solubilize it, eventually, there is a declined phase of P amount released right after that. Though there are some other factor result in declined phase of P released, likes the bacteria may reaches the decline phase, otherwise due to the active bacteria started to utilize the available phosphate. While a slightly rose in P amount released that always happened in four different modified PK broths before 200 h incubation time may indicate the bacteria used up some the available soluble phosphate sources as energy, and then started to solubilize insoluble phosphate again.

The reduction in dry weight of eggshell or bones in the end of estimating phosphate solubilization experiment is argued the fact that some of the weight or content of eggshell or bones was being solubilized by selected PSB into soluble form in modified PK broth, eventually showed a detectable level in P released amount.

In addition, the amount of P released in eggshell-modified PK broth was the lowest among different modified PK broths. In fact, eggshells consist of high level of calcium and strontium, while low in level of P [29], which could explain the reason that eggshells-modified PK broths were lesser in phosphate released. While the bone consists of high level in tricalcium phosphate as

described by Benjamin & Shear [30], and the effect of selected PSB strain did significantly ($P < 0.05$) in solubilizing the insoluble sources in three different controls of bone-modified PK broths, while the differences in P released by all eggshell-modified PK broths tested were not so obvious.

Generally, selected PSB strain inoculated plants were shown a higher plant growth than uninoculated plant and the effect was further enhanced when with additional fertilizers. This is because PSB can facilitate the plants growth by making soluble phosphorus, fixing atmospheric nitrogen [31], producing plant hormones such as auxins [32], cytokinins and gibberellins. As with research conducted by Sharma *et al.* [33], they also achieved the same conclusion with PSB promoted the growth of their selected plant (*Cicer arietinum*). Extra fertilizers were given to the soils, which means there were more available additional nutrients in soils and the plants could utilize it and showed more growth rate than without fertilizers one. While for the soil with selected PSB and additional fertilizers, the PSB could solubilize fertilizers and the plant growth rate could be more enhanced.

Moreover, the treatment pot with selected PSB and EB was quite significantly ($P < 0.05$) promoting the plant growth. It could be due to PSB solubilized the phosphate sources in bones and eggshell efficiently. Another reason why PSB inoculated EB soil caused more growth in plant is due to the bones consisted of high level tricalcium phosphate [30]. Hence with the assist of PSB, there could be even more soluble P available in soil for plants to uptake and promote plant growth.

While treatment soil (no PSB and no fertilizer) was always showing the lowest plant parameters' results, which may due to P nutrient deficiency in soil [34] and least PSB available in soil. The treatment pots with triple superphosphate (TSP) could not achieve the same mean length as pots with RP and bone + eggshell; might be due to TSP consists of soluble phosphate sources, that which easily being drained out by heavy rainfalls. As a result, the number of flowers produced was not significant and amount of fruits produced was lesser than half when compared to plants with fertilizers of RP or bone + eggshell.

On top of that, there were more significant differences shown in plant parameters at the end of pot experiment (like on Day 65, 75 and 85) than the beginning of conducted pot experiment (Day 35), which was due to the fact that PSB took time to solubilize the insoluble P in soils in the beginning, and the outcomes of soluble P in soils that available for plant uptake were expressed as significant plant growth rate later. Apart from that, a possible reason that uninoculated treatment pots of RP were showing the higher plant growth than inoculated RP treatment pots, was due to the soil always with many different PSB in it, hence maybe one of the PSB strain on treatment pots of RP that without PSB was capable to efficiently solubilize RP than the selected PSB.

In general, the selected PSB strain did influence the plant growth of *C. frutescens*, even though not all of the plant parameters taken from this research were shown significant differences, like number of flowers. However, shoot length, number of leaves and length of fruits did significantly show the rhizosphere effect of selected PSB strain on plants. In facts, PSB not only provide soluble phosphorus to plants, but also produce or

induce plant to synthesize plant hormones like auxins, cytokinins and gibberellins [27].

Auxins are not only in charge of cell enlargement, bud formation and root initiation; but also control the growth of stems, roots, fruits and flowers [35]. While cytokinins manipulate the cell division, shoot formation and leaf growth [36], and gibberellins induce elongation of stems, transport nutrients, new cell growths; some more promote the flowering and seed growth after germination as stated by Tsai *et al.* [37]. Thus, if an effective PSB was applied to soil with plants, it will cause a complicated pathway (included P solubilization and synthesis of plant hormones); eventually, lead to a change in plant parameters or plants growth.

Some other environment stresses, such as pest invasion and heavy rain, are also can severely affect the plant growth and lead to inaccuracy of the outcomes of plant parameters, which is unavoidable and encountered in this research. Hence, ladybugs (*Coccinellids*) were introduced to plants as a biological control for the pests (ScienceDaily, 2010) and it did effectively play its roles and resolved one of the plant stress sources. Moreover, it was much more environmentally friendly to environment and plants, as compared to chemical insecticides. Some more, it was also faithful to the ideas of organic farming recommended worldwide.

There are many articles were described the beneficial effect of PSB on promoting root length of plants [16,38,39]. As stated by Charana & Yoon [27], they described that increased root length associated with cell elongation and multiplication related to greater available of nutrients, especially phosphorus. Even more, their results were showing PSB to induce the increment in root length of tested tomato plant significantly. Though the plant root length differences between inoculated and uninoculated soil in this pot experiment were not so significant, but different fertilization treatment did significant influence root length of plants ($P < 0.05$). Interestingly, the root also synthesizes cytokinins in response to concentration of nutrients available in soil; eventually can control the growth rate of plant shoot length [40].

The chlorophyll content was the lowest for treatment plant on soil (no PSB and no fertilizer) due to there was no added nutrients applied to it, so the available nutrients in soil could have been absorbed by plant in the early growing stage. Eventually, when the time passed, the availability of nutrient in environmental control pots were limited, subsequently limited the absorption of nutrient thus, the formation of chlorophyll was disturbed.

The chlorophylla is the primary photosynthetic pigment of plants which gives the plant green color appearance; while the chlorophyll-b is the accessory pigment that collects energy and passes to chlorophyll-an as described by Suharja & Sutarno [17]. Moreover, the chlorophyll content of plant also can give the information about the plant stress level, which helps in determining whether the plants growth condition is ideal or not [34]. In which, the plant stress measurement is referred to quantification of environmental effects (eg: climate change, drought, floods, nutrient deficiency, air pollution, pH, chemical pesticides, disease and light) which can influence photosynthesis and plant health. As described by Carter & Knapp [41], it demonstrated that different level of total chlorophyll contents can be obtained from different level of plant stress.

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Even though the final rhizosphere results in this research were not significant as expected, but overall the total PSB count on inoculated soil was generally higher than the uninoculated soil. When the overall results obtained in this research analyzed together, it could tell that the selected PSB did effectively in solubilizing P in soil; eventually promoting the plant growth of *C. frutescens* tested in this research.

Further study can be done to improve the performance of PSB as bacterial inoculants to crops. Accordingly, much more investigation on the effects of the application of combinations of PSB with other PGPR should be carried out [42]. On the other hand, genetic manipulation of PSB to improve phosphate-

solubilizing abilities and the introduction of a trait in strains with other plant growth-promoting effects also offers a feasible approach [43]. Moreover, genetic manipulation by recombinant DNA technology (subcloning phosphatase encoding genes in appropriate vectors and transfer to host strains) to obtain an improved strain seem to be practically feasible in the future as stated by Rodrigues & Fraga [43]. Besides that, the use of genetic reporter systems, like bioluminescence genes [44] or green fluorescent protein genes [45] to study the stability and performance of PSB that have been inoculated to soil also should be done to know the survival and establishment of introduced strain [46].

Table 9. Effect of seed treatment with selected PSB on growth of *C. frutescens* in pot experiment (Day 75 after conducted pot experiment).

Treatment	Number of flowers (units) ^{##}	Number of fruits (units) ^{##}	Number of leaves (units) [*]	Shoot length (cm) [*]	Weight of fruits (g) [*]	Length of fruits (cm) [*]
Soil without PSB and without fertilizer	3 ^a	0 ^b	48 ^c	21.1 ^d	-	-
Soil without PSB, with TSP	19 ^{ab}	1 ^b	205 ^c	22.1 ^d	1.19 ^e	2.7 ^{fb}
Soil without PSB, with RP	6 ^a	23 ^{bc}	452 ^{cc}	33.1 ^{dc}	1.09 ^e	4 ^{fc}
Soil without PSB, with eggshells + bones	14 ^a	37 ^{bd}	324 ^{cd}	25.6 ^d	0.749 ^e	3.5 ^{fd}
Soil + PSB	2 ^a	0 ^b	57 ^c	22.4 ^d	-	-
Soil + PSB + TSP	6 ^a	3 ^{bf}	262 ^{cf}	26.9 ^{df}	-	4.6 ^{ff}
Soil + PSB + RP	22 ^a	23 ^{bc}	391 ^{cc}	27.4 ^{dg}	0.528 ^e	2.9 ^{fc}
Soil + PSB + eggshells + bones	34 ^a	21 ^{bd}	470 ^{cd}	36.3 ^{dh}	0.701 ^e	7 th

Values under factors with symbol * are given as means for triplicate samples, while the values under factors with symbol ^{##} are given as total numbers for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at $P \leq 0.05$.

Table 10. Effect of seed treatment with selected PSB on growth of *C. frutescens* in pot experiment (Day 85 after conducted pot experiment).

Treatment	Number of flowers (units) ^{##}	Number of fruits (units) ^{##}	Number of leaves (units) [*]	Shoot length (cm) [*]	Length of fruits (cm) [*]
Soil without PSB and without fertilizer	2 ^a	0 ^b	70 ^c	21.7 ^d	-
Soil without PSB, with TSP	3 ^a	0 ^b	216 ^c	22.9 ^d	-
Soil without PSB, with RP	18 ^a	1 ^b	544 ^{cc}	36.5 ^{dc}	1.4 ^f
Soil without PSB, with eggshells + bones	12 ^a	6 ^b	370 ^{cd}	25.8 ^d	3.5 ^{fd}
Soil + PSB	3 ^a	0 ^b	74 ^c	23.1 ^d	-
Soil + PSB + TSP	7 ^a	1 ^b	263 ^{cf}	31.1 ^{df}	0.8 ^f
Soil + PSB + RP	24 ^a	20 ^b	442 ^{cc}	28.2 ^{dg}	1.5 ^f
Soil + PSB + eggshells + bones	49 ^{ah}	23 ^{bh}	574 ^{cd}	39.6 ^{dh}	3.6 th

Values under factors with symbol * are given as means for triplicate samples, while the values under factors with symbol ^{##} are given as total numbers for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at $P \leq 0.05$.

Table 11. Effect of plant treatment with selected PSB strain on root length of *C. frutescens* at different fertilization conditions (Day 85 after conducted pot experiment).

Fertilization condition	Root length (cm)	
	Soil without selected PSB	Soil with selected PSB
Soil without any fertilizer	11.58	11.52
Soil with TSP	15.48	16.40
Soil with RP	17.48	17.68
Soil with EB (eggshells + bones)	16.57	19.33

Values are given as means for triplicate samples.

Table 12. The role of PSB on chlorophyll contents at different treatment conditions of plants.

Treatment pots	Total Chlorophyll Content mg/g of leaf		
	Chlorophyll a	Chlorophyll b	Total chlorophyll content
Soil without PSB and without fertilizer	2.187	0.762	2.949 ^f
Soil without PSB, with TSP	2.971	1.448	4.419 ^{rb}
Soil without PSB, with RP	3.441	1.993	5.434 ^{rc}
Soil without PSB, with eggshells + bones	3.343	1.415	4.757 th
Soil + PSB	2.852	1.044	3.896 ^f
Soil + PSB + TSP	3.516	2.52	6.036 ^{rt}
Soil + PSB + RP	3.511	2.415	5.926 ^{rc}
Soil + PSB + eggshells + bones	3.487	2.235	5.722 th

Values are given as means for triplicate samples. Within each column, means followed by same letter(s) are not significantly different at

$P \leq 0.05$.

Table 13. Effect of seed treatment with selected PSB on final rhizosphere effects (Day 85 after conducted pot experiment).

Treatment pots	PSB count (CFU/ml)	
	Rhizosphere soil	Non-rhizosphere soil
Soil without PSB and without fertilizer	1.40 x 10 ⁶	3.50 X 10 ⁵
Soil without PSB, with TSP	3.18 x 10 ⁶	4.80 x 10 ⁵
Soil without PSB, with RP	3.04 x 10 ⁶	2.38 x 10 ⁶
Soil without PSB, with eggshells + bones	2.96 x 10 ⁶	2.59 x 10 ⁶
Soil + PSB	9.20 x 10 ³	2.27 x 10 ⁶
Soil + PSB + TSP	2.26 x 10 ⁶	2.84 x 10 ⁶
Soil + PSB + RP	6.20 x 10 ⁵	2.26 x 10 ⁶
Soil + PSB + eggshells + bones	2.86 x 10 ⁶	1.99 x 10 ⁷

4. CONCLUSIONS

The results of this work showed that the selected PSB strains was capable of solubilizing phosphate sources into a soluble form, and significantly contributed in promoting the growth of *Capsicum frutescens*, such as enhanced the shoot length, increased the number of yields and leaves. Moreover, selected PSB did significantly solubilize the tricalcium phosphate

in modified PK broth to soluble form which was detected by their colorimetric absorbance at 690nm and significantly induced higher total chlorophyll content in leaves. Besides that, eggshells and bones powder as fertilizer along with selected PSB strain did significantly promote higher plant growth than TSP and rock phosphate as fertilizer.

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