



Research Article

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Study on effectiveness of selected bacterial isolates on groundnut cultivation

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Abstract

Four bacterial isolates were selected and screened for phosphate solubilizing and cellulolytic activities on Pikovaskaia and Berg's media respectively. S1 and P6 showed the 1.545 and 1.571 values of solubility index for tricalcium phosphate on NBRIP media. In quantitative determination, S3 was the best strain which showed phosphate solubilizing activity on tricalcium phosphate and rock phosphate at 136.01ppm and 29.35ppm respectively. The amounts of reducing sugar formation were determined by dinitrosalicylic colorimetric method (DNS). S1 can more solubilize cellulose 0.60 ppm, S2 on carboxymethylcellulose (CMC) at 0.55 ppm and S3 on rice straw and filter paper at 0.5 and 0.45 ppm on Berg's media respectively. According to the coexistence study, the four selected bacterial isolates were able to survival together and applied in combination for field trial. These isolates were studied the effectiveness on the groundnut cultivation. This experiment was carried out on the field trial of groundnut (Spanish 121) at Mandalay Technological University, Patheingyi Township. All treatments (S1, S2, S3, P6 and Com) were significantly better than Control (water only). According to soil analysis data, organic carbon, EC, humus, the total N, available P, K₂O, and K⁺ in all treatments were higher than control and those of soil parameters before cultivation. So, it can be assumed that the selected bacterial isolates had positive effect on soil physical and chemical properties.

Keywords: Phosphate solubilizing, Cellulolytic, Solubility index, Reducing sugar, Field trial.

INTRODUCTION

Quality of soil is determined by microbiological, biochemical, physical and chemical characteristics and it is very important for agricultural systems, due to it defining the use capacity of the soil, sustainable management and agricultural productivity^[1]. Microorganisms are major key players for sustaining the soil quality degraded by intensive use of synthetic chemicals for increasing crop production and therefore, use of them as inoculants or biofertilizer is an integral part of sustainable agriculture^[2].

Agricultural residues are a rich source of cellulose^[3]. Cellulases play an important role in carbon availability and so can be used to give a preliminary indication of some of the physical and chemical properties of soil, thus, easing agricultural soil management strategies^[4]. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system^[5]. Cellulase enzyme system comprises three classes of soluble extracellular enzymes: 1,4- β -endoglucanase, 1,4- β -exoglucanase, and β -glucosidase (β -D-glucosideglucohydrolase or cellobiase^[6]. Endoglucanase is responsible for random cleavage of β -1, 4-glycosidic bonds along a cellulose chain^[7]. Exoglucanase is necessary for cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose and β -1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose^[8]. Only the synergy of the above three enzymes gives the complete cellulose hydrolysis to glucose or a mineralization to H₂O and CO₂ possible^[9].

Due to its polymeric nature bioprocessing of cellulose is limited^[10]. Cellulolytic bacteria include aerobic species such as *Pseudomonas* and *Actinomyces*, facultative anaerobes such as *Bacillus* and *Cellulomonas* and strict anaerobes such as *Clostridium*. PSMs, found in the earthworm (*Eudriluseugenine*), casts proved cellulase activity and released phosphorus (P) using carboxymethyl-cellulose as carbon source in the medium^[11].

Increased mineralization of organic P occurs due to incorporation of crop residues, crop rotations and increases the rate of P cycling through the microbial biomass^[12]. Incorporation of organic residues

through legume rotation resulted in higher biological activity and increased microbial P uptake and release. However, the contribution of phosphorous released through these processes needs to be evaluated in relation to plant uptake ^[13]. Elucidation as to whether or not the availability of this P can be synchronized with plant requirements or be targeted to rhizospheres remains a significant challenge ^[14].

Phosphatases play a critical role in plant growth by enhancing the availability of phosphorus due to enhanced solubilization and remobilization of phosphate. Activities of these enzymes in agricultural soils are affected by several factors such as temperature, soil pH, water and oxygen content, chemical characteristics of organic matter and its location in the soil profile horizon ^[15]. Microorganisms are an integral component of the soil P cycle and are important for the transfer of P between different pools of soil P. Phosphate solubilizing Microorganisms (PSM) through various mechanisms of solubilization and mineralization are able to convert inorganic and organic soil P respectively ^[16] into the bioavailable form facilitating uptake by plant roots. It is important to determine the actual mechanism of P solubilization by PSM for optimal utilization of these microorganisms in varied field conditions. Hence it is necessary to better understand the plant-soil-microbial P cycle with the aim of reducing reliance on chemical P fertilizers. This has led to increased interest in the harnessing of microorganisms to support P cycling in agroecosystems ^[17]. Use of these microorganisms as environment friendly biofertilizer promotes to convert the much expensive phosphatic fertilizers ^[18]. Phosphorus biofertilizer could help increase availability of accumulated phosphate by solubilization efficiency of biological nitrogen fixation and increase availability of Fe, Zn etc., through production of plant growth promoting substances ^[19]. Phosphate solubilizing bacteria use as a biofertilizer for improving plant nutrient uptake, soil fertility and sustainable crop production in nutrient poor systems ^[20].

The major aim of this research was to study the effectiveness of selected bacterial isolates on the ground nut cultivation as a biofertilizer.

MATERIALS AND METHODS

Selection of bacterial isolates

Thirty nine isolated bacteria were collected from the Microbiology Laboratory, Biotechnology Research Center, Kyaukse and cultured on Pikovsakaia (PVK) and National Botanical Research Institute's Phosphate (NBRIP) media containing tricalcium phosphate and PVK media containing zinc phosphate for 3 days at 37°C. The bacterial isolates were selected according to their solubility index. For the selection of cellulolytic bacteria, thirty nine isolated bacteria were cultured on Berg's agar media containing 1% carboxymethyl cellulose (CMC) and 0.3% or 0.5% cellulose for seven days. At the end of the incubation, the agar medium was flooded with an aqueous solution of Congo red (0.1% w/v) for 30 minutes. The Congo red solution was then poured off, and the plates were further treated by flooding with 1M NaCl for 30 minutes. The formation of a clear zone of hydrolysis indicated cellulose degradation. The clear zone diameter of the bacteria was measured in millimeter. The bacterial isolates were selected according to the clear zone formation.

Quantitative determination of soluble phosphate by UV-VIS spectrophotometric method

The selected isolates were cultured on PVK media at 37°C and inoculated 50ml culture broth for quantitative measurement. 10ml of each bacterial culture broth was passed through the cation exchange resin. The tricalcium phosphate solution was diluted to 100ml solution

with deionized water. Then, 100ml of $(\text{PO}_4)^{3-}$ solution was mixed and shaken with 20ml of 0.1M $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ solution and 8 ml of hydrazine sulphate. After placing in boiling water bath for 10 minutes, the blue colour complex solution was measured the absorbance by UV-VIS spectrophotometer at 830nm wavelength ^[22].

Quantitative determination of cellulolytic activity by dinitrosalicylic colorimetric method

The cellulolytic activity was assayed by the determination of reducing sugar released from cellulosic substrates. The selected isolates were incubated in Berg's medium containing 0.5% cellulosic substrates (such as CMC, cellulose, filter paper and pretreated rice-straw) at 37°C for 7 days. During incubation period, cultures were harvested daily by centrifugation at 5000rpm for 20 minutes. Culture supernatants were used for assay of reducing sugar and determined by measuring absorbance against the reagent blank at 540 nm ^[23].

Characterization of bacterial isolates

To characterize the desired bacterial isolates, various biochemical tests were used and the results were compared with the results from Bergey's Manual ^[24]. The bacterial isolates were biochemically characterized by Gram reaction, motility, citrate utilization, gelatin liquefaction, methyl red, voges-proskauer, catalase, nitrate reduction and indole tests according to the instructions of Bergey's Manual of Determinative Bacteriology.

Coexistence study of selected bacterial isolates

Study on the coexistence of four selected isolates was carried out by using cross inoculation method. The selected isolates were inoculated on nutrient medium and incubated at 30°C for seven days. Then, the plates were checked whether the selected bacteria can coexistence or not daily.

Study on the effect of selected bacterial isolates on groundnut cultivation (*Arachis hypogaea*)

Characteristics of the experimental field were as follow. The groundnut field is located at 21° 58'N latitude and 86° 11' E longitude at Mandalay Technological University, Patheingyi Township, Mandalay Division during November to January of 2014-2015. The experiment was laid out in a Randomized Complete Block Design (RCBD) with four replications [21]. The number of plots was 24 and the each plot size was 144ft² (12' x 12'). The total plot size was 2304ft² (48' x 48'). There are six treatments in this study. They are Control (water only), S1, S2, S3, P6 and Com (combination of selected isolates S1, S2, S3 and P6).

Data were collected on the following aspects, 50% flowering days, number of pods per plant, number of grains per plant, 100 seeds weight and plant height. The data was subjected to analysis of variance (ANOVA) analysis and multiple comparisons of different treatments were carried out by the computerized SPSS program ^[25]. All differences in mean were done by Duncan's Multiple Range Test (DMRT) at 0.05% significant level.

RESULTS AND DISCUSSION

Selection of bacterial isolates

In this research, phosphate solubilizing activities of thirty nine bacterial isolates were detected by plate screening method using two different media; PVK and NBRIP media. All isolated bacteria were cultured on PVK medium with 0.5% insoluble tricalcium phosphate and incubated

at 30°C for 3 days. The data on the solubility index of the selected isolates on PVK and NBRIP media was shown in Table 1. According to plate screening method, the isolates S1, S2, S3 and P6 were phosphate

solubilizers in terms of the development of the clear zone around the colony.

Table 1: The solubility index of the P-solubilizing selected bacterial isolates

S No	Selected Isolates	Solubility Index		
		PVK Media		NBRIP media
		Tricalcium Phosphate	Zinc Phosphate	Tricalcium Phosphate
1	S1	0.25	0.35	1.545
2	S2	0.275	1.3	1.182
3	S3	0.2	0.475	1.167
4	P6	0.167	1.2	1.571

Similarly, cellulolytic activities of thirty nine bacterial isolates were determined by plate screening method using Berg's agar medium. The clear zone diameter of the bacterial isolates was shown in Table 2. The comparative study of the cellulase enzyme producing activity of S1, S2, S3 and P6 was carried out on Berg's media supplemented with 1%CMC and 0.3% and 0.5% cellulose. All of them grew and gave clear zone on

respective medium. Cellulolytic bacteria were able to utilize cellulosic materials as their sole carbon sources and decompose insoluble cellulosic substrates into renewable products.

The selected four bacterial isolates had the dual activity including phosphate solubilizing and cellulolytic activity.

Table 2: Screening the cellulolytic activity of four selected bacterial isolates

S No	Selected Isolates	Cellulolytic Activity		
		Cellulose		1%CMC
		0.3%	0.5%	
1	S1	+	-	+
2	S2	+	-	+
3	S3	+	-	+
4	P6	+	+	+

Quantitative determination of soluble phosphate by UV-VIS spectrophotometer

spectrophotometer at 830nm with a 24hr interval for 168hr. They were shown in Figure 1(a) and (b).

The phosphate solubilizing activities of the selected bacterial isolates S1, S2, S3 and P6 were quantitatively determined by UV-VIS

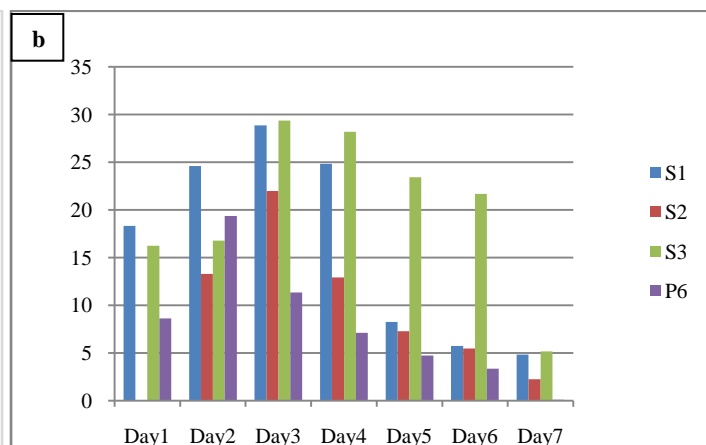
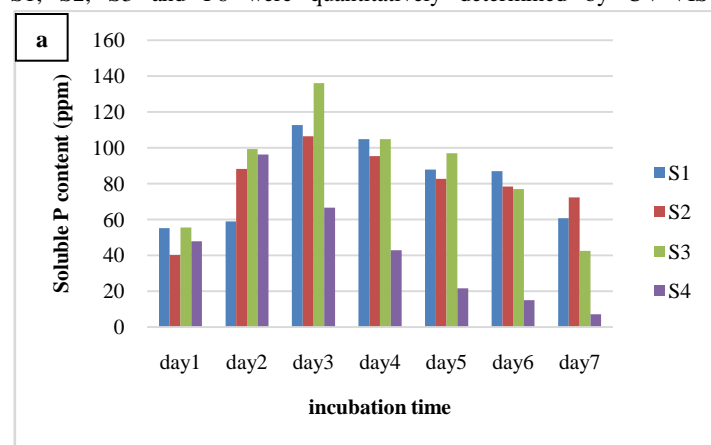


Figure 1: P-Solubilizing activities (ppm) of selected bacterial isolates on PVK medium supplemented with (a) 0.05% tricalcium phosphate and (b) 0.05% rock phosphate (initial pH= 7.8)

The quantitative measurements of solubilizing activities of S1, S2, S3 and P6 were carried out at 24hr interval for 168hr. In the presence of 0.05% tricalcium phosphate, the activities of S1, S2 and S3 were highest after 72hr incubation with an amount of 112.63, 106.43 and 136.01 ppm respectively.

The activity of P6 was highest after 48hr incubation with an amount of 96.20ppm. In the presence of 0.05% rock phosphate, the activities of S1, S2 and S3 were highest after 72hr incubation with an amount of 28.86, 22 and 29.35ppm respectively. The activity of bacterial isolates in P6 was highest after 48hr incubation with an amount of 19.35ppm.

Quantitative determination of cellulolytic activity by DNS method

The cellulolytic activities of the selected bacterial isolates S1, S2, S3 and P6 were quantitatively detected by dinitrosalicylic acid colorimetric method (DNS) using four cellulosic substrates with a 24hr interval for 168hrs. They were shown in Figure 2 (a), (b), (c) and (d). The cellulase enzyme producing activity was detected by determining the reducing activity using various cellulosic substrates such as 0.5% CMC, 0.5% cellulose, 0.5% filter paper and 0.5% rice-straw. By using CMC substrates, S2 showed the highest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.54 mg/ml at 3rd day. S3 showed the lowest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.38 mg/ml at 3rd day. By using cellulose substrates, S1 showed the

highest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.63 mg/ml at 3rd day. S2 showed the lowest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.41 mg/ml at 3rd day. By using filter paper substrates, S1 showed the highest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.51 mg/ml at 3rd day. S2 showed the lowest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.43 mg/ml at 3rd day. By using rice straw substrates, S3 showed the highest cellulase enzyme producing activity among the selected bacterial isolates with reducing sugar concentration of 0.49 mg/ml at 3rd day. S2 showed the lowest cellulase producing activity among the selected bacterial isolates with reducing sugar concentration of 0.34 mg/ml at 3rd day.

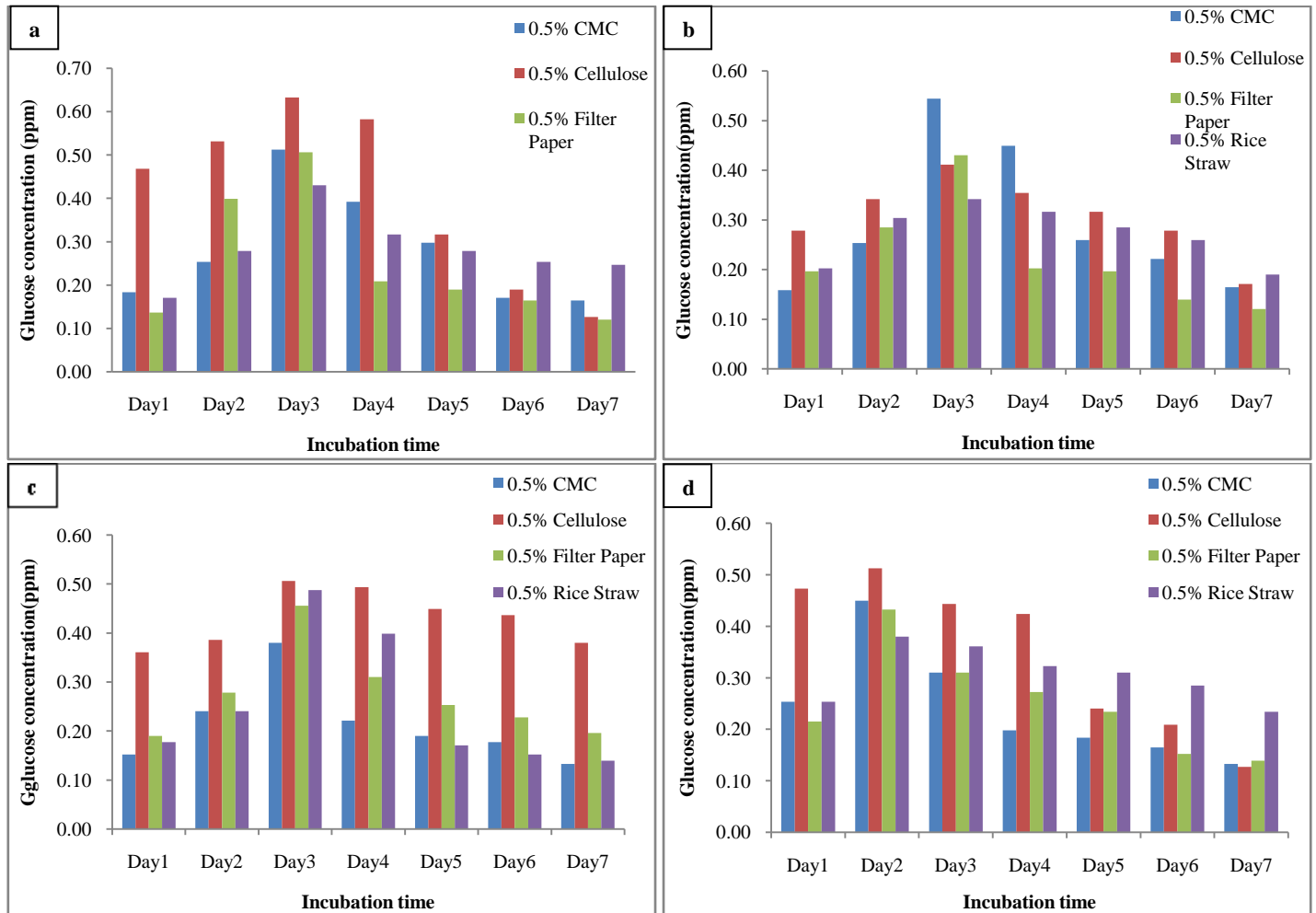


Figure 2: Comparison in cellulase enzyme activity of selected bacterial isolates (a) S1, (b) S2, (c) S3 and (d) P6 in Berg's culture media containing (0.5%) different substrates

Characterization of bacterial isolates

The morphological, cultural and biochemical characteristics of selected bacterial isolates were studied and the result are shown in Table 3. The colonies of S1, S2 and S3 were low convex, smooth, red colour and 1-2 µm in diameter on *Serratia* selective media but P6 was round, smooth, clear, slightly yellowish colour on King's B medium. All cells were small rod in shape. The results of the biochemical tests of the selected bacterial isolates were showed in table 3. Tests for catalase production, citrate utilization and gelatin liquefaction indicated that P6 differed from the other bacterial isolates. In methyl red test, all selected isolates showed negative reaction. In voges-proskauer and indole tests, S1, S2 and S3 showed the positive reaction but P6 showed negative reaction. So, S1, S2 and S3 were not similar to P6.

Coexistence study of selected bacterial isolates

This study was aimed to utilize the combination of the best isolates in bacterial inoculants for field trial. The selected bacterial isolates were studied for their coexistence in nutrient medium. According to the coexistence study, the four bacterial isolates were able to survive together and applied in the combination for field trial. Study on the coexistence of four selected bacterial isolates in nutrient medium was shown in Figure 3.

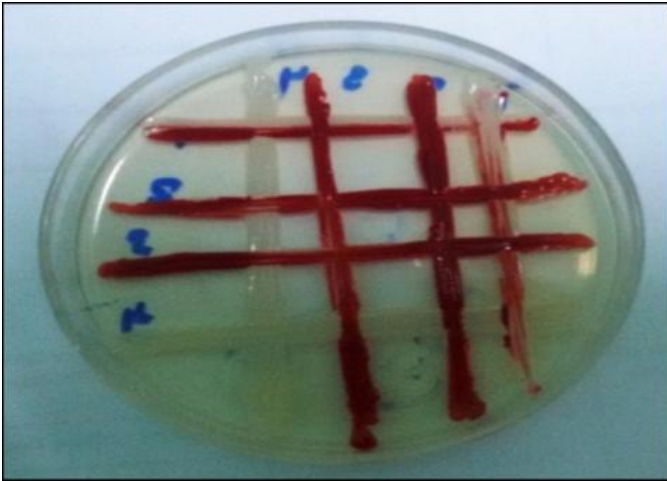


Figure 3: Coexistence of selected bacterial isolates on nutrient medium

Study on the effect of selected bacterial isolates on groundnut cultivation

Finally, in field trial experiment, all selected bacterial isolates were examined for their effects on the growth of groundnut plants. The growth parameters consist of two components. The effect of bacterial inoculants on groundnut plant growth and performance of the selected bacterial isolates were shown in Table 4 and 5.

Primary yield component parameters: In field trial, all treatments had no significant on the number of pods per plant and seeds per plant but there was significant difference in one hundred seeds weight. The maximum numbers of pods per plant was observed in Com treatment at 30.15 ± 10.16 pods and the number of seed per plant was also observed in Com treatment at 51.05 ± 19.23 . One hundred seed weight in S3 treatment gave the highest seed weight which was $62.38a \pm 2.25$ g.

Secondary yield component parameters: According to ANOVA analysis, the number of branch per plant, plant height and total length per plant were observed as no significant difference in all treatments but there was significant different in 50% flowering days which ranged from 24.25 ± 0.56 DAS to 26.5 ± 0.5 DAS. The maximum number of branch per plant was found in Com treatment at 8.98 ± 0.75 . The plant height ranged from 30.95 ± 3.45 cm to 35.13 ± 2.85 cm. The total length per plant ranged from 43.81 ± 5.85 cm to 51.01 ± 4.75 cm.

Soil analysis: Soil analysis was done on field trial before and after cultivation to detect the presence of OC, N, P and K and to determine the soil type. The analytical data of the groundnut cultivated soil was shown in Table 6. The field trial plots had silt clay loam soil that was moderately alkaline before cultivation. Soil analysis data showed that moisture (%), EC (m/s), organic carbon, humus (%), the total N(%), P(ppm), K_2O (mg/100gm), and K^+ (meq/100gm) were 3.27, 0.09, 2.1, 1.28, 0.13, 2.07, 45.54 and 0.38 respectively. After harvesting, the soil structure changes occur in moisture, EC, organic carbon, humus, total nitrogen, exchangeable cations (K^+), available P and K_2O . Moisture was decreased to lower than 3.27 because of the season. EC was increased to greater than 0.09 from the soil before experiment. Organic carbon was observed to increase to 3.17 for Com treatment. Available P content had increased to 3.67- 5.32 for S1, S2 S3, P6 and Com treatments higher than control. The percentage of total nitrogen in all treatments increased to greater than 0.13 from the soil before planting. The available nutrient K_2O (mg/100 g) increased to greater than 45.54 from the soil before planting. Exchangeable cations (K^+) in all treatments increased to greater than that of the soil before planting (meq/100g). These soil analysis data showed the soil quality was improved in groundnut cultivation treated with selected bacterial isolates. So, the selected bacterial isolates had good effect and improved soil fertility to the growth of groundnut plantation.

Table 3: Morphological, cultural and biochemical characteristics of selected bacterial isolates

Selected isolates	S1	S2	S3	P6
Colony Morphology	Low convex, smooth, red colony 1-2mm diameter	Low convex, smooth, red colony 1-2mm diameter	Low convex, smooth, red colony 1-2mm diameter	Round, smooth, clear, Slightly yellowish
Cell Morphology	Small rod	Small rod	Small rod	Small rod
Size	About 1.0-2.0 μ m in length and width	About 1.0-2.0 μ m in length and width	About 1.0-2.0 μ m in length and width	About 1.0-1.5 μ m in length and width
Gram reaction	-	-	-	-
Voges-proskauer test	+	+	+	-
Nitrate reduction	+	+	+	+
Indole test	+	+	+	-
Methyl red test	-	-	-	-
Motility test	+	+	+	+
Catalase production	+	+	+	+
Citrate test	+	+	+	+
Gelatin liquefaction	+	+	+	+
Nutrient Agar	Pink colour	Pink colour	Pink colour	Creamy colour

+ positive reaction; - negative reaction

Table 4: Primary yield components of groundnut plant after cultivation

Treatments	Pod /Plant	Seed/Plant	100 Grain Weight (g)
Control	21.48 ± 2.67	31.95 ± 5.34	45.35 ^c ± 5.26
S1	26 ± 11.17	43.56 ± 19.83	57.82 ^{ab} ± 4.48
S2	23.95 ± 5.39	36.9 ± 11.00	55.75 ^b ± 4.34
S3	27.13 ± 6.17	44.45 ± 12.16	62.38 ^a ± 2.25
P6	21.63 ± 5.20	33.78 ± 9.47	57.19 ^{ab} ± 1.48
Com	30.15 ± 10.16	51.05 ± 19.19	60.36 ^{ab} ± 3.41

Each value represents mean ± SD of four replicates per treatment.

abc Values with the same letter within row indicate no significant differences with $p \geq 0.0$

Table 5: Secondary yield components of groundnut plants after cultivation

Treatments	50% Flowering Time (DAS)	No of Branch per Plant	Plant Height(cm)	Total Length per Plant (cm)
Control	26.5 ^a ± 0.5	7.8 ± 0.67	30.95±1.36	43.81±2.30
S1	24.5 ^b ± 0.56	8.55 ± 1.13	31.96±1.02	46.88±1.84
S2	24.75 ^b ± 0.5	8.4 ± 0.56	31.75±1.32	48.46±1.89
S3	24.5 ^b ± 0.5	8.43 ± 0.33	35.13±1.12	51.01±1.87
P6	24.5 ^b ± 0.56	8.05 ± 0.72	31 ± 1.33	46.46±1.63
Com	24.25 ^b ± 0.56	8.98 ± 0.74	33.37±1.37	49.34 ± 1.8

Each value represents mean ± SD of four replicates per treatment.

ab Values with the same letter within row indicate no significant differences with $p \geq 0.05$.

Table 6: Soil structure of before plantation and after harvesting

Soil Samples	Moisture (%)	EC (ms/cm)	Organic Carbon	Humus(%)	Total N (%)	Exchangeable cations (meq/100mg)	Available nutrients	
						K ⁺	P (ppm)	K ₂ O (mg/100gm)
Before plantation	3.27	0.09	2.1	1.28	0.13	0.38	2.07	45.54
Control (water only)	1.86	0.12	2.56	3.12	0.24	0.5	3.67	45.54
S1	1.9	0.15	2.9	2.84	0.26	0.52	4.71	53.22
S2	1.85	0.13	2.84	2.74	0.25	0.50	5.32	59.46
S3	1.94	0.15	2.98	3.09	0.26	0.55	5.12	58.93
P6	2.01	0.10	3.04	3.28	0.25	0.49	4.22	61.34
Com	2.6	0.16	3.17	3.41	0.28	0.51	4.72	66.83

CONCLUSION

All thirty nine bacterial isolates were carried out to determine P-solubilizing and cellulase enzyme producing activities by plate screening method. From these bacterial isolates, four isolates were selected and they were used to determine enzyme activities quantitatively. All of isolates with reducing sugar concentration 0.63 mg/ ml at 3rd day. By using filter paper, four selected isolates could produce P-solubilizing and cellulase enzyme. After detection of the P solubilizing activities, S3 was found to have the P-solubilizing effect on two substrates such as tricalcium phosphate and rock phosphate at 136.01ppm and 29.35ppm. After detection of cellulolytic activities, S1, S2, S3 and P6 showed higher efficacy to degrade CMC in plates. By using CMC substrates, S2 showed the highest cellulase producing activity among the selected isolates with reducing sugar concentration 0.54 mg/ ml at 3rd day. By using cellulose substrates, S1 showed the

highest cellulase producing activity among the selected substrates, S1 showed the highest cellulase producing activity among selected isolates with reducing sugar concentration 0.51 mg/ ml at 3rd day. By using rice straw substrates, S3 showed the highest cellulase producing activity among the selected isolates with reducing sugar concentration 0.49 mg/ ml at 3rd day. So S1, S2 and S3 had the high activities to degrade the cellulosic substrates and biomass. In Studying on the field trial of groundnut plants for 3 months, treatments (S1, S2, S3, P6 and Com) were not found significantly difference in all agronomic character. But, there were significantly difference in one hundred seeds weight and 50% flowering days. In soil data, organic carbon in all treatments was higher than control and those of soil before treatment. The organic carbon served as a proxy for soil organic matter. So, it exerted positive effect on soil physical and chemical properties and the improvement of soil fertility compared with the control. Therefore, S1, S2, S3 and P6 had the effectiveness on the groundnut cultivation as a biofertilizer.

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