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Research Article

Isolation, Evaluation and Characterization of Free Living Nitrogen Fixing Bacteria from Agricultural Soils in Myanmar for Biofertilizer Formulation

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Abstract

Three *Azomonasagilis*, two *Azotobacter chroococcum* and one *Alcaligenes* sp. were obtained for the best nitrogen fixing activity. These all strains showed nitrogen fixing activity from screening using Viscolor Alpha Ammonium Detection Kit. Estimated ammonia concentration was obviously higher than other isolates. These strains were selected and evaluated for other beneficial activities. The ability of these strains was that they could grow at 45°C on nitrogen free glucose mineral media (NFGMM) but salt tolerance was low. Above 1% NaCl, they could not grow well on NFGMM. Besides nitrogen fixing activity, phosphate solubilizing activity was also detected. 11 ppm of soluble phosphate was given by *A.chroococcum* (Ey1) and it was the lowest concentration among all strains. *A.agilis* (M3) gave 20 ppm of soluble phosphate concentration, highest concentration. With the aim of biofertilizer formulation with effective nitrogen fixing bacteria for used in agricultural soils in Myanmar, these six nitrogen fixing bacteria were formulated as bioferilizer. After preparation of biofertilizer for one month, mineral contents of formulated biofertilizer were determined. Total nitrogen (1.49%), total phosphate (0.515) and total potassium (0.475) of formulated biofertilizer were detected. The mineral contents were higher than other fertilizers. Therefore, we postulate that biofertilizer formulated from the six nitrogen fixing bacteria may be effective for application in Myanmar's agricultural soils.

INTRODUCTION

Bio-fertilizers are products containing living cells of different types of microorganisms which promotes growth of plants and the structure of soils by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form when applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant [1]. In establishing of organic farming system, biofertilizers are important components to become successful organic farming and important in sustaining the crop productivity and improving the soil fertility [2]. Organisms that are commonly used as biofertilizers component are nitrogen fixers, potassium and phosphorus solubilizers, or with the combination of molds or fungi. Most of the bacteria used in biofertilizer have close relationship with plant roots. *Rhizobium* has symbiotic interaction with legume roots, and *Rhizobacteria* inhabit on root surface or in rhizosphere soil.

Biological nitrogen fixation (BNF) is one way of converting elemental nitrogen into plant usable form [3]. Nitrogen input through BNF can help maintain soil nitrogen reserves as well as substitute for nitrogen fertilizer to attain large crop yields [4].

Nitrogen-fixing bacteria (NFB) that function transform inert atmospheric N_2 to organic compounds [5]. Nitrogen fixer or N-fixers organism are used in biofertilizer as a living fertilizer composed of microbial inoculants or groups of microorganisms which are able to fix atmospheric nitrogen. They are grouped into free-living bacteria (*Azotobacter* and *Azospirillium*) and the blue green algae and symbionts such as *Rhizobium*, *Frankia* and *Azolla* [6]. Both groups play an important role to improve the structure of the soil [7-9].

While *Rhizobium*, Blue Green Algae (BGA) and *Azolla* are crop specific, bio-inoculants like *Azotobacter*, *Azospirillum*, Phosphorus Solubilizing Bacteria (PSB), Vesicular *Arbuscular Mycorrhiza* (VAM) could be regarded as broad spectrum biofertilizers [6].

The aim of this study was to develop effective biofertilizer for application in Myanmar's agricultural soils by isolation of effective nitrogen fixing bacteria having other beneficial activities. In biofertilizer preparation, use of many strains has difficulties due to different growth time and growth media. The use of same species of bacteria can overcome these problems because they can grow on the same media. Growth of one strain cannot be inhibited by other strains.

METHODS

Media used for isolation

Nitrogen Free Glucose Mineral Media (NFGMM) was used for isolation of free living nitrogen fixing bacteria. The NFGMM contained (g/L): KH_2PO_4 1.0, $CaCl_2$ 1.0, $MgSO_4.7H_2O$ 0.25, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, $Na_2MoO_4$0.01, Gluose 7.0, Agar 20.0.

Soil sampling

Soil samples were collected from soils of rice growing area, rhizospheric soils of rice during early growth and after mature. In addition, soil samples were collected from soils under decayed rice straw around Patheingyi Township, Mandalay Region, and Myanmar.

Isolation and growth conditions of nitrogen fixing bacteria

One gram of soil samples was added into250 ml conical flasks containing 100ml of NFGMM broth. The flasks were shaken once and incubated in water bath shaker at 150 rpm and 37ºC for one week. Here growth temperature was used at 37ºC because Myanmar' weather is rather hot. After one week incubation, 100µl of supernatant was spread on NFGMM and incubated at 37ºC. After two to three days, appearance of colonies on media were checked and purified for further study.

Screening on nitrogen fixing activity

Nitrogen fixing activities of isolated strains were screened by growing the strains on NFGMM for both liquid and solid media containing Bromothymol Blue (BTB) as an indicator. The strains that change the color of the BTB containing media to blue were assumed as nitrogen fixers, suggesting excretion of ammonia increased pH of the media and caused changing of the color of the media.

Excreted ammonium concentration released by isolated strains was estimated using the Viscolor Alpha Ammonium Detection Kit (Macherey-Nagel). Single colony of isolated strains was inoculated in NFGMM broth and incubated at 37ºC for one week. After one week incubation, culture broth was centrifuged at 10000 rpm for 5 mins and 1ml of supernatant was transferred into a test tube. Firstly, two drops of NH_4-1 were added into supernatant and mixed well, after which one-fifth of a spoon of NH_4-2 was added (spoon enclosed in the test kit). After mixing well, the mixture was left for 5 min at room temperature. Finally, one drop of NH_4-3 was added, mixed well and left for 5 min. The color development of supernatants was observed and the ammonium concentration was recorded by comparing with the color chart from the Viscolor Alpha Ammonium Detection Kit [10].

Identification of nitrogen fixing bacteria

Selected NFB were identified by 16s rDNA sequencing. Sequencing reactions were performed using the Big Dye Terminator Cycle Sequencing Kit (v.3.1) and the results were analysed in a GA 33130 sequencer. Nucleotide sequences were analysed using BLAST on the NCBI BLAST and Greengenes.IBI. gov.

Selected NFB were also characterized by their morphological and some biochemical characteristics according to Bergey' Mnaual of Systematic Bacteriology [11].

Determination of ammonia concentration

Accumulated ammonia concentration in NFGMM broth was determined by the indophenol method [12]. NFB were inoculated in NFGMM broth and incubated in water-bath shaker at 37ºC. Culture broth was withdrawn daily and centrifuged at 10000 rpm for 5 minutes. Ammonium presence in the supernatant was estimated by indophenol method.

Salt and temperature tolerance

Salt tolerance of selected NFB was detected using NFGMM media using 1%, 2%, 2.5% and 3% NaCl. Incubation was performed at 37ºC. For temperature tolerance was also studied at 40ºC, 45ºC and 50ºC. Six NFB were cultured on NFGMM media and incubated at above different temperature. Growth of these strains was observed after two to three days incubation.

Detection of phosphate solubilizing activity

Qualitative determination of phosphate solubilizing activity was assayed using National Botanical Research Institute Phosphate (NBRIP) media. Single colony of bacteria was spotted on NBRIP media containing BTB. Tricalcium phosphate was used as substrate. Halo zone diameter was recorded for phosphate solubilizing activity.

Selected strains were further evaluated for their phosphate solubilizing ability. Phosphate solubilization in Pikovaskaia's broth media was quantified in a flask (10 ml) and incubated in water batch shaker at 37°C for five days. Un-inoculated medium served as control. After incubation, the culture broth was passed through the cation exchange resin and $(PO_4)_3$- solution was reacted with color forming reagent (Sodium Molybdate and Hydrazium Sulphate). After blue color development, phosphate solubilizing activity was measured by UV-vis spectrophotometric method at 830 nm [13].

Mineral contents determination of biofertilizer

After studying of co-existence growth of six selected strains, all these strains were inoculated in NFGMM broth for three days. Carrier system was prepared using rice straw, cow dung and water hyacinth. After three days incubation, 500 ml of broth culture was mixed with1 kg of carrier system. Mixed carrier was packaged using black plastic bag and placed in a dry cool place. After one month, mineral contents of formulated biofertilizer were determined. Other fertilizers, biofertilizer based for flowering and fruits using *Bacillus megaterium*, other two liquid fertilizers were also determined for their mineral contents. Total nitrogen, phosphorus and potassium contents in different fertilizers were determined and compared.

RESULTS

Isolation of nitrogen fixing bacteria

Free living NFB were isolated from various soil sources of Myanmar's agricultural soils. NFB were found on NFGMM media from all soil sources.

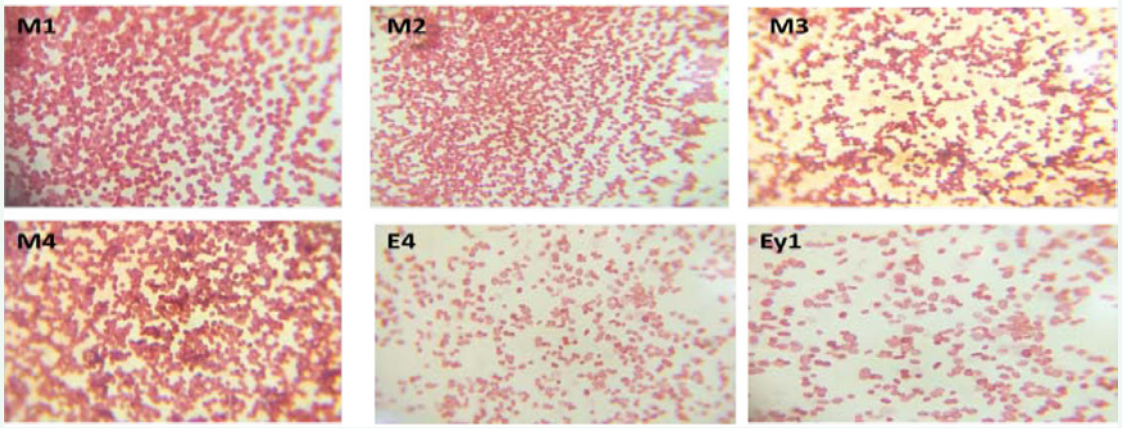

Figure 1 Microcopic morphology of six NFB.

Table 1: Identification of six NFB by 16s rDNA sequencing.

No.	Code	Sources	Method used for sequencing	Max identity	Identity
1.	M1	Soil under decayed rice straw	BigDye v.3.1	99.34%	*Azomonasagilis*
2.	M2	Soil under decayed rice straw	BigDye v.3.1	99.29%	*Alcaligenes* sp.
3.	M3	Soil under decayed rice straw	BigDye v.3.1	99%	*Azomonasagilis*
4.	M4	Soil under decayed rice straw	BigDye v.3.1	99%	*Azomonasagilis*
5.	E4	Soils before sowing of rice	BigDye v.3.1	99.87%	*Azotobacterchroococcum*
6.	Ey1	Rhizospheric soils of pepper	BigDye v.3.1	99.87%	*Azotobacterchroococcum*

Table 2: Some biochemical characteristics of six NFB.

No	Strains	Biochemical Tests									
		1	2	3	4	5	6	7	8	9	10
1	*A. agilis* (M1)	+	+	-	+	-	+	-	+	+	+
2	*Alcaligenes sp.* (M2)	+	+	-	+	-	+	-	+	+	+
3	*A. agilis* (M3)	+	+	-	+	-	+	-	+	+	+
4	*A. agilis* (M4)	+	+	-	+	-	+	-	+	+	+
5	*A. chroococcum* (E4)	+	+	-	+	-	+	-	+	+	+
6	*A. chroococcum* (Ey1)	+	+	-	+	-	+	-	+	+	+

Tests: 1-Triple Sugar Ion Agar; 2- Motility; 3- Nitrate reduction; 4- Citrate utilization; 5- Indole; 6- Methyl red; 7- Vogesproskauer; 8- Starch utilization; 9- Gelatin liquification; 10- Catalase

Table 3: Ammonia concentration accumulated in NFGMM broth by six NFB.

No.	Strains	Ammonia concentration by Indophenol method (ppm)	Ammonia concentration estimated by Kit (ppm)
1.	*A.agilis* (M1)	1.108	> 3
2.	*Alcaligenes* sp. (M2)	0.957	> 3
3.	*A. agilis* (M3)	1.005	> 3
4.	*A. agilis* (M4)	1.195	> 3
5.	*A. chroococcum* (E4)	1.071	> 3
6.	*A. chroococcum* (Ey1)	1.023	> 3

Screening on nitrogen fixing activity and strain selection

After screening of nitrogen fixing activity, color of media was changed for almost all isolated strains. But the best strains could not be determined by this screening.

When estimated by the Viscolor Alpha Ammonium Detection Kit, six NFB showed dark green color and it was estimated above 3 ppm according to standard color chart on Kit. So these six strains were selected for further study. All other isolates showed color development that was less than 3 ppm.

Identification by 16s rDNA sequencing

According to 16s rDNA sequencing, six NFB were identified. It was shown in Table 1. According to 16s rDNA sequencing, M1, M3, M4 isolates were in high similar with *A.agilis*. M2 isolate was

Table 4: Halozone diameter and soluble phosphate amount released by six strains.

No.	Strains	Halo zone diameter (mm)	Soluble phosphate concentration (ppm)
1.	*A. agilis* (M1)	20	11.7
2.	*Alcaligenes* sp. (M2)	31	16.1
3.	*A. agilis* (M3)	15	20
4.	*A. agilis* (M4)	14	16.2
5.	*A. chroococcum* (E4)	33	27
6.	*A. chroococcum* (Ey1)	10	11

Table 5: Mineral contents in biofertilizers.

No.	Biofertilizers	Total N (%)	Total P_2O_5 (%)	Total K_2O (%)	Total organic matter (%)
1.	T1	0.098	0.007	0.002	-
2.	T2	0.252	0.010	0.028	-
3.	T3	0.098	0.003	0.001	10.0.12
4.	T4	0.928	0.371	0.396	12.221
5.	T5	1.49	0.515	0.475	17.492

99.29% similarity with *Alcaligenes* sp. The colonial morphology of these four isolates was in similar with *Azotobacter* sp. E4 and Ey1 isolates were *A.chroococcum* with 99.87% similarity. Biochemical characteristics of these strains were not different among them.

Characterization of biochemical characteristics

Microscopic morphology (Figure 1) and some biochemical characteristics of six NFB were studied and their characteristics were characterized in Table 2.

Determination of ammonia concentration

From determination by Indophenol method, accumulated ammonia concentration was obvious after one week incubation in NFGMM. The ammonia concentration was lower than estimated ammonia concentration using Viscolor Alpha Ammonium Detection Kit. The accumulated ammonia concentrations of six strains are described in Table 3.

Salt and temperature tolerance

For the purpose of using as biofertilizer for Myanmar's agricultural soils, six NFB were detected for their ability to salt and temperature tolerance. All six strains grew well on NFGMM media containing 1% NaCl, but their growth rates were decreased at 2% NaCl. The growth of these six strains was not found at 3% NaCl.

These six NFB grew well at 40ºC and 45ºC but not at 50ºC.

Phosphate solubilizing activity

Besides the nitrogen fixing activity, the six strains possessed phosphate solubilizing activity. They solubilized tricalcium phosphate used as substrate. Halo zone diameter and soluble phosphate concentration released by the six strains was shown in Table 4.

Determination of mineral contents in formulated biofertilizer

After one month inoculation of the six NFB in carrier system, the mineral contents of formulated biofertilizer was determined. The results of mineral contents in different biofertilizers were characterized in Table 5.

DISCUSSION

Different soil samples were collected from agricultural soils in Myanmar. Free NFB were found in all soil types. In this study, the best NFB were obtained from soils under decayed rice straw that were remained for long time. This may be due to the fact that the soils were not applied with chemical fertilizers. Application of chemical fertilizers in agricultural soils can have adverse effect on growth and function of microbes in soils.

Broth and plate screening method was used using BTB as indicator. Before culturing of NFG on NFGMM, the color of media was green. But, after culturing of NFB on NFGMM for one week, the color of the media was turned from green to blue. Control media without the bacteria remained as green color. It was assumed that NFB accumulated excreted ammonia into media and pH of the media was increased. Consequently, this reaction caused the changing of the color of the media. The same method was used by some researchers [10,14,15]. The present results were in similar finding with their results.

When estimated by Viscolor Alpha Ammonium Detection Kit, the six NFB excreted above 3 ppm of ammonia concentration. This amount of ammonia concentration was obtained after one week incubation.

Like the results from screening, accumulated ammonia concentration of the six strains were given after one week incubation. During one week incubation, accumulated ammonia concentration was detected but it was too low.

Free-living diazotrophs fix dinitrogen sufficient for their own needs and do not generally excrete significant amounts of ammonium into their environment: fixed nitrogen is released after death and lysis of bacteria [16].

Phosphate solubilizing activity was evaluated for these selected NFB besides nitrogen fixing activity. Phosphate solubilizing activity was lower than other phosphate solubilizing bacteria in our Laboratory. Phosphate solubilizing activity of *Azotobacter* sp. was well documented but it is rarely found for

A.agilis. Nosratiet al. [17] studied phosphate solubilizing activities of *Azotobacter vinelandii* besides nitrogen fixing activity.

The aim of this research work was to formulate biofertilizer with effective NFB for use in Myanmar's agricultural soils. So, biofertilizer was formulated using these six NFB. After inoculation of NFB into carrier system, mineral contents of biofertilizer were determined. Total nitrogen, total phosphate and potassium were increased than other fertilizers. The increases of minerals in biofertilizer were due to function of inoculated fertilizing bacteria.

For the growth of plants, nitrogen is essential and other two elements such as phosphate and potassium also play important role. According to the results of mineral contents, formulated biofertilizer may be effective for agricultural soils in Myanamr.

CONCLUSION

Free living NFB were obtained from all collected soils samples in Myanmar's agricultural soil. But the best activity for nitrogen fixing was given from strains isolated from soils under decayed rice straw. The best six strains were selected and evaluated for other activities. After identification, three *A.agilis*, two *A.chroococcum* and one *Alcaligenes* sp. were obtained. These six strains gave higher ammonia concentration than other isolates. Salt and temperature tolerance was on benefits to be applied for agricultural soils in Myanmar. Besides nitrogen fixing activity, these six strains also possessed phosphate solubilizing activity. But it was also needed to test more experiments with and without nitrogen sources in media. After formulation of biofertilizer with selected NFB, mineral contents of formulated biofertilizer gave satisfactory results for application in agriculture.

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