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BIOLOGICAL AUGMENTATION OF BIOGAS WASTE MICROBIOTA AS AN EFFECTUAL BIOFERTILIZER

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Abstract: Bio fertilizers significance and plant acceptance to environmental stress Abiotic and biotic stresses are the foremost limitations that are affecting the efficiency of the crops.

Our necessity on chemical fertilizers and pesticides has stimulated the flourishing of industries that are producing life-threatening chemicals and which are not only hazardous for human ingesting but can also disrupt the ecological stability. Biofertilizers can help solve the problem of nourishing an increasing global population at a time when agriculture is facing several environmental stresses. It is significant to appreciate the useful characteristics of biofertilizers and device its application to modern agricultural practices. The new technology developed using the powerful implement of molecular biotechnology can improve the biological pathways of production of phytohormones.

Keywords: Bacteria, microbioata, biofertilizer

INTRODUCTION

Biofertilizers are complex product of live microbial inoculants which are able to fix atmospheric nitrogen, solubilize soil phosphorus, decompose organic material or oxidize sulphur in the soil. Biofertilizers are artificially multiplied cultures of beneficial soil microorganisms that can improve soil fertility and crop productivity. They add nutrients through the natural processes of nitrogen fixation, solubilizing phosphorus, and stimulating plant growth through the synthesis of growth-promoting substances. They are made from biological wastes and do not contain any chemicals. The main sources of biofertilizers are bacteria, fungi and Cynobacteria (blue-green algae) (Hegde, 2008).

WHY BIOFERTILIZERS?

Biofertilizers offer a new eco-friendly technology which would overcome shortcomings of the conventional chemical based farming. Biofertilizers showed positive influence on both soil sustainability and plant growth. They gradually improve soil fertility by fixing atmospheric nitrogen. They increase the phosphorous content of the soil by solubilizing and releasing unavailable phosphorous. They help in restoring depleted nutrients of the soil. Growth promoting substances released by biofertilizers improve plant root proliferation. They also guard the plant against some soil-borne diseases. In addition to these advantages, biofertilizers are commercially promising too. They are comparatively cheaper than the chemical fertilizers. When used as a supplement to the chemical fertilizers, biofertilizers can decrease the dose of chemical fertilizers. It results in reduced cost of fertilization. They help in increasing the crop yield by 10-25%. Impact of Biofertilizers on Crops Biofertilizers can be put broadly into two groups viz., inoculants of specific organisms like *Rhizobia, Azotobacter*, Blue Green Algae, Phosphate Solubilizer, cellulolytic microorganisms, etc. and biomass producing organisms e.g. Azolla (Kannaiyan, 2002).

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Some of the popular biofertilizers and their impact on various crops

- *Rhizobium* inoculants are used for leguminous crops. These inoculants have ability to fix atmospheric nitrogen in symbiotic association with root-nodule forming plants.. Response to *Rhizobium* inoculation has been found beneficial for principal legumes such as Pigeon Pea (*Cajanuscajan*); Chickpea (*Cicerarietinum*), Green gram (*Vigna radiata*), Soyaben (*Glycine max)* and broad bean (*Vicia faba*).
- *Azotobacter* inoculants can be applied to many non-leguminous crops. *Azotobacter* can be used for crops like cereals, millets, vegetables, cotton (*Gossypium* spp.) and sugarcane (*Saccharum* spp.).
- *Azospirillum* is also a nitrogen-fixing micro organism used for non-leguminous plants. Besides ability to fix nitrogen, *Azospirillum* is known to secrete substances promoting plant growth. *Azospirillum* inoculations have been found beneficial mainly for millets, maize *(Zea mays),* sorghum, wheat (*Triticum spp.*) and sugarcane (*Saccharumspp*.).
- Phosphorus Solubilizing bacteria and fungi converts chemically fixed soil phosphorus into available form. PSBs are found useful for variety of crops such as wheat *(Triticum spp.*), sugarcane *(Saccharum spp.)* cotton *(Gossypium spp.)*, pulses, oilseed crops like caster, rice *(Oryza sativa)* and vegetables.
- Blue Green Algae (BGA) are photosynthetic nitrogen fixers and are free living. BGA fix atmospheric nitrogen and are used as inoculations for rice *(Oryza sativa)* crop.
- *Azolla* is an aquatic fern found in shallow water bodies and in rice fields. It has symbiotic relation with Blue Green Algae. The biomass is used as a green manure in rice *(Oryza sativa)* cultivation. So, *Azolla* can help rice or other such crops through green manuring (Vandergheynst, 2006).

Some of the microorganisms have the beneficial role of biological nitrogen fixation to supply nitrogen to crops, solubilizing insoluble phosphates to plant available (soluble) forms and synthesizing biomass for manuring crops like rice. A biofertilizer is not just any organic fertilizer or manure. It consists of a carrier medium rich in live microorganisms. When applied to seed, soil or living plants, it increases soil nutrients or makes them biologically available. Biofertilizers contain different types of fungi, root bacteria or other microorganisms. They form a mutually beneficial or symbiotic relationship with host plants as they grow in the soil. Biofertilizers have many advantages and a few disadvantages (Remesh, 2008).

Biofertilizers complement other fertilizers, but they cannot totally replace them. Biofertilizers lose their effectiveness if the soil is too hot or dry. Excessively acidic or alkaline soils also hamper successful growth of the beneficial microorganisms; moreover, they are less effective if the soil contains an excess of their natural microbiological enemies. Shortages of particular strains of microorganisms or of the best growing medium reduce the availability of some biofertilizers. To solve the above problem following objectives were designed-

1. Routine collection of biowaste from biogas plant
2. NPK, Ca and organic carbon analysis
3. Standardization of biofertilizer assessment parameters
4. Isolation of bacteria from all the samples
5. Assessment of bacterial strain for enhancement of biofertization parameter
6. Comparative analysis of freely suspended and consortia of isolated bacteria as biofertilizer

Sampling sites

In this study foursampling sites were selected which are located in Jabalpur, Madhya Pradesh, India. The geographical locations of Jabalpur is 23° 10′ 0″ N, 79° 56′ 0″ E. The details of sampling sites are listed below.

Site A: Gaurighat

Site B: KhandariNala

Site C: Tilheri area

Site D: poultry farm

Site E: poultry farm

Site F: Richai

Sample Collection

The Samples were collected in a 5L pre-sterilized plastic container bottles. During sampling, sample bottles were rinsed three times with sampled water before filling the bottles to the brim from depths of 1 m below the surface of each designated sampling sites. The temperatures and pH of the samples were measured at the timeof sampling site using portable thermo meter and pH meter, respectively. The entire sample were to avoid any alteration in physico-chemical properties, all the samples were stored and carry in ice coldcondition. For furtheranalysis to Microbiology Laboratory, Department Of Botany and Microbiology , St. Aloysius' college Jabalpur (M.P.).All analysis were performed inless than 72 h from sampling (Osuolale and Okoh, 2015; Igbinosa and Okoh, 2009).

Determination of nitrate by phenoldisulphonic method: The concentration of NO3- is determined using a colorimeter or spectrophotometer.

Phosphate (reactive, ortho) - Stannous Chloride Method: In an acidic solution, ortho-phosphate reacts with ammonium molybdate to form molybdophosphoric acid, which is then reduced by stannous chloride to the intensely colored molybdenum blue. The resulting blue color is directly proportional to the phosphate concentration. Results are expressed in ppm (mg/L) phosphate as PO4 or P. To convert results from ppm PO4 to ppm P, divide by 3.06.

Analysis of Potaassium Permanganate solution with Sodium Thiosulphate

Sodium thiosulphate solution is standardized against potassium dichromate in presence of hydrochloric acid and potassium iodide. Potassium dichromate oxidizes the iodide ion in acidic medium to equivalent amount of iodine. The iodine formed in the reaction oxidizes sodium thiosulphate giving sodium tetrathionate ion and the end point is detected by starch solution.

Biofertilizer sample dilution technique for microbial count

1) Diluent preparation: Put 90 ml of 0.85% NaCl solution in erlenmeyer or bottle and put 9 ml of 0.85% NaCl solution in test tube according to different required dilution ratio. Then sterilize by autoclave at 121 °C for 20 minutes.
2) Weigh 10 g of biofertilizer sample or pipette 10 ml of liquid biofertilizer and put in the 90 ml physiological solution, then shake the sample at 180-200 rpm for 30 minutes (10-1 dilution).
3) Using the sterile pipette, transfer 1 ml to the test tube with 9 ml diluent (10-2 dilution). Vortex it and transfer 1.0 ml to other test tube with 9 ml diluent (10-3 dilution). Repeat this procedure and make serial dilution until 10-5 dilution or higher depend on the population of microbes in the 4 biofertilizer. Use a new sterile pipette for every dilution prepared.
4) Count the microbe number contained in diluted biofertilizer by different methods.

Quantification of microbesin biofertilizer by theplate counting technique

Quantification of rhizobia by using the Most Probable Number (MPN) technique

Seed germination: Sterilize the seed of siratro or other legumes specified for rhizobia used for the biofertilizer production to disinfect their seed coat surfaces. Seed of legume with hard seed coats can be sterilized and softened by soaking in concentrated H2SO4 for 10 min, and then rinsed with sterilized distilled water 6 times. Soak the seed in sterilized distilled water and keep in the refrigerator at 4 °C for 3-4 hours. In case of seed of legumes with soft coat, such as soybean, rinse the seed in 95% ethanol for 10 second to remove waxy material. Seeds are sterilized by soaking in 5% H2O2 (hydrogen peroxide) solution or 2.5% NaClO (sodium hypochlorite) solution for 15 to 20 min, and rinsed with sterilized distilled water 6 times. Then soak the seed in sterilized distilled water and keep in the refrigerator at 4 °C for 3-4 hours. After that, place the seeds on sterilized cotton with moderate humidity in the petri dish with lid by evenly spreading the seeds. Then incubate the seeds in the plate at the temperature of approximately 28 °C. Leave the seeds until the root proliferation appears to be 0.5-1.0 cm long.

Planting of the seeds in the growth pouches: Place the sterilized growth pouches on the rack, and add 30 ml of sterilized nitrogen-free nutrients solution into each growth pouch. Make hole at the folded edge of the straw paper by using the sterilized forceps. Then use the sterilized forceps to grip the seed with root proliferation and insert the root into the hole of the growth pouch. Inoculate the biofertilizer solution with different degrees of dilution, starting from less to more, that means starting from 10-8 dilution to 10-1 dilution. To inoculate rhizobia contained in the biofertilizer onto the roots, the biofertilizer solution should be in contact with the root surface as much as possible by dropping 1 ml of diluted biofertilizer solution into each growth pouch of four replications in each degree of dilution. There must be one growth pouch to which rhizobia biofertilizer is not added. This growth pouch will be used as a control. After planting the seeds, place the growth pouches on the 9 light shelf that provides enough strength of light to the plant. The light must be at the distance of 15-17 cm from the top of the bags and provides the light for 12 hours a day at 25 °C. Add the nitrogen-free nutrients solution over time when needed for a period of 3 weeks.

Quantification of nitrogen -fixing bacteria in aerobic species

Colony morphology of Azotobacter shows a creamy mucilaginous appearance, while Beijerinckia produce raised mucilaginous viscous colonies.

Quantification of phosphate -solubilizing microbes: Pikovskaya medium for phosphate-solubilizing microbes (Pikovskaya, 1948)

Quantification of potassium -solubilizing microbes: Aleksandrov medium for potassium-solubilizing microbes (Hu et al., 2006)

Growth curves for an unknown bacterial species: Transfer 1ml of your culture to a cuvette. Using a spectrophotometer at 600' 700nm, zero (blank) the colorimetric spectrophotometer with the LB broth from step 1. Save the blank LB broth. You may need it in next step. Measure the Page 6 of 12 absorbance of the culture sample. This is your zero time absorbance of culture density.

Identification (Krieg and Holt., 1984; Bryant, 2003; Jenda and Abbott, 2006): The bacterial isolates exhibited having maximum waste water pollution reduction efficiency were identified on the basis of morphological, cultural and biochemical characteristics (Mac-Faddin, 1980) with the help of Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984) and Probabilistic Identification of Bacteria (PIB) computer kit (Bryant, 2003).

Analysis of NPK solubilization by bacteria: Bacterial culture in pure form was inoculated in nutrient broth and absorbance was noted. Minimal broth with NPK was inoculated with isolated bacteria. After 24 hour NPK solubilization was assessed. Bacteria with high solubilization ability was selected for further study of pot experiment.

Pot Experiment: Treatments and experimental design There were three treatments, with duplicates, for each of the three soil amendment systems.

RESULT

(1) Assessment of N, P, K value of various soil samples: Determining the nutrient concentration for nitrate, phosphate and potassium can revealed how a soil is functioning in regards to its intended use in crop production.

Analysis of soil sample revealed N, P, K values in different soil samples from biogas plant I Jabalpur. Sample collected from biofertilizer farm. Liquid sample both concentrated and diluted and solid samples processed (fine) and unprocessed was collected showing phosphate value from 0.1- 0.6 mg kg^{-1}, same samples showed nitrate value concentration from 0.2- 1.3 mg kg^{-1} and the same samples showed potassium value from 0.7-0.5 mg kg^{-1}.Samples collected from 18/2/18 to 3/5/18 showed different N, P, K value in table

Table 2: NPK analysis of liquid and soil samples collected from biogas plant

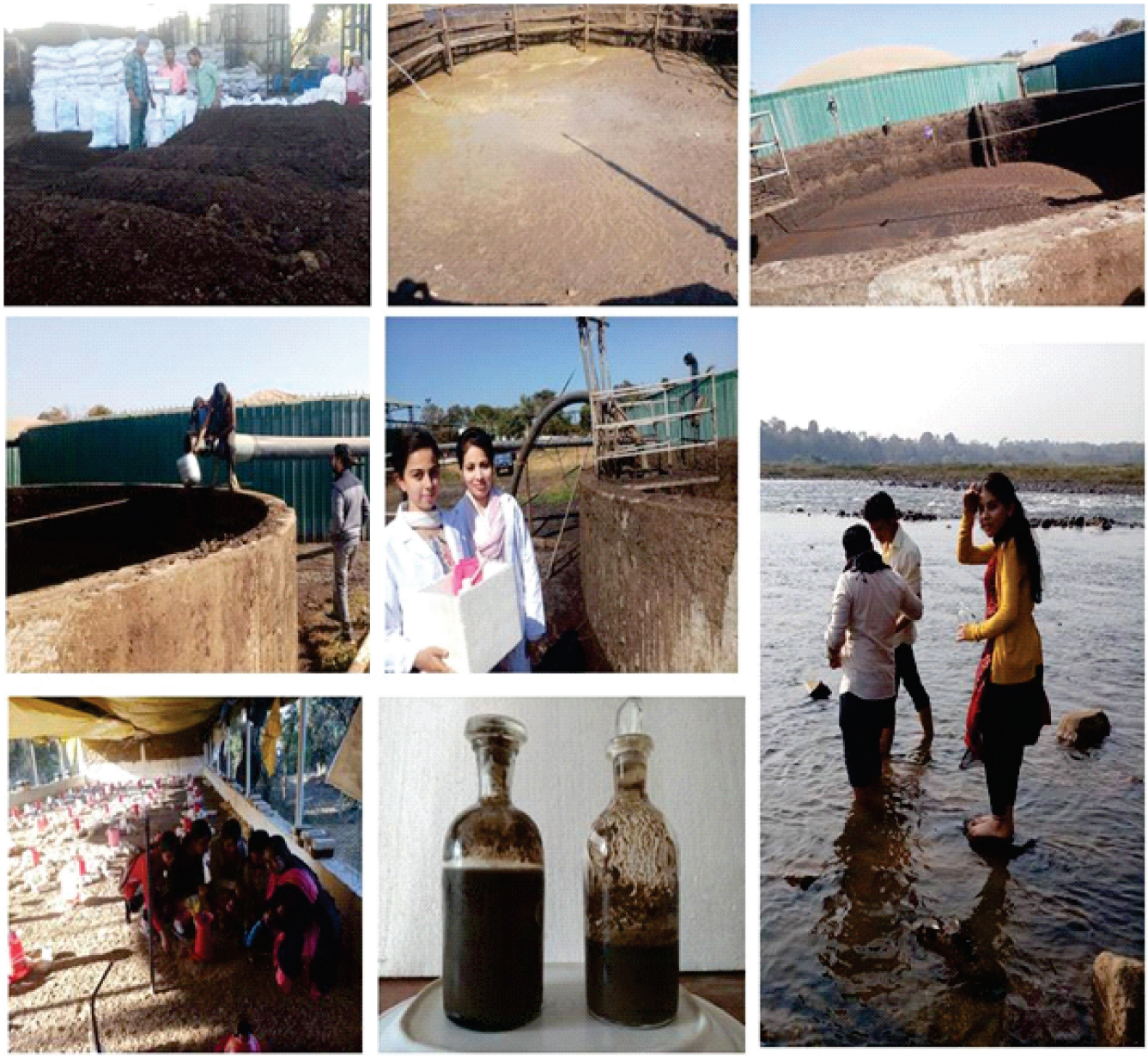
Date	*N mg kg^{-1}.*	*P mg kg^{-1}.*	*K mg kg^{-1}.*
7/2/18	0.46	0.344	O.46
7/2/18	0.535	0.341	0.87
15/2/18	0.615	0.310	0.56
12/3/18	0.310	0.047	0.87

Date	*N mg kg^{-1}.*	*P mg kg^{-1}.*	*K mg kg^{-1}.*
23/3/18	0.078	0.085	0.34
23/3/18	0.063	0.084	0.13
10/4/18	0.105	0.057	0.42
17/4/18	0.112	NA	0.43
3/5/18	NA	NA	0.34
3/5/18	NA	NA	0.23

Biofertilizer sample has C, N, P and protein content showed in table -3 showing highest solid content on third day similar results was shown by carbon and nitrogen percent with 36.2 and 0.89 respectively.

Table 3: Chemical analysis of processed and unprocessed soil samples

Date	*7/2/18*	*12/3/18*	*10/4/18*	*17/4/18*	*3/5/18*
Total solid	32.3	21.2	32.4	22.4	32.8
Carbon %	23.6	24.8	36.2	21.3	32.8
Nitrogen %	1.23	1.43	0.89	0.43	1.23
Phosph-orus%	0.74	0.63	0.43	0.54	0.83
Protein %	12	11	10	11	10




S4
S5 X

Identification of potent bacterial strain: On the basis of morphological characteristics, isolates were identified as *Pseudomonas, Bacillus, and Rhizobium* etc.

Different type of bacteria was identified under following steps-

Table 4: Biochemical analysis of the microbial strains isolated form plant soils

Test	*Strain 1*	*Strain 2*	*Strain 3*
Gram stain	-	-	-
Crystal blue stain	No spore	No spore	No spore
Anaerobic growth	Strict aerobe	Facultative aerobes	Strict aerobe
Motility	+	+	+
Glucose oxidation-fermentation	+	+	+
Ethanol oxidation	+	+	+
Gelatin hydrolysis	-	-	-
Catalase	+	+	+
Oxidase	+	+	+
Manitol fermentation	-	-	-
Salt tolerance	+	+	+
Growth at 4°C	Weak	-	Weak
Growth at 41° C	-	-	-
Pyocyanine production	-	-	-
Starch hydrolysis	-	-	-
Nitrate reduction	+	+	+
Methyl red test	-	-	-
Indole test	-	-	-
V-P test	-	-	-
Arginase	+	+	+
Ornithine decarboxylase	-	-	-
Phenylalanine deaminase	-	-	-
Hydrogen sulfide test	-	-	-
Lecithine hydrolysis	-	-	-
Denitrification	-	-	-
Citrate utilization	NT	+	NT
Malonate utilization	NT	-	NT
Urease test	NT	+	NT
Fermentation with fructose and glycerol	NT	+	NT
Fermentation with arabinose , D- mannose, dulcitose, ribose , D-xylose and galactose	NT	-	NT
Species identified	*Pseudomonas putida*	*Pseudomonas nitroreducens*	Ps. putida

Standard for N, P, K analysis: N, P, K standard was prepared through spectrophotometric analysis determining the concentration of substrate. They are obtained by relating a measured quantity to concentration of unknown substance having following values

1. Standard curve of nitrate- Intercept equation- y=0.0176x-0.0034 and R^2=0.9937
2. Standard curve of phosphate- Intercept equation- y=0.0049x+2.7786 and R^2=0.9587
3. Standard curve of potassium- Intercept equation-y=0.0183x+0.0996 and R^2=0.9625

Selection of bacterial strain with N, P, K solubilization potential: A3, A11 and A8 identified as *Rhizobium, Pseudomonas* and *Bacillus* species was selected for further study at pilot scale. They showed increase in NPK values when assessed spectrophotometrically for mg/Kg. among the three species Pseudomonas showed highest solubilization potential followed by Rhizobium and Bacillus respectively.

Field experiment to access ability of liquid biofertilizer produced: Soil sample collected from garden had N, P, and K value as 0.6, 0.5, 0.3 respectively and water sample had 0.1, 0.18, and 0.70 respectively. This sample (1gm) when added in nutrient broth having five different type of bacterial strain at log phase showed an increase in N, P, and K value.

Table 5: N, P, K solubilization potential enhancement by selected bacterial strains

Bacteria + soil sample	*N*		*P*		*K*	
	Before treatment	*After treatment*	*Before treatment*	*After treatment*	*Before treatment*	*After treatment*
Pseudomonas	0.650	0.820	0.180	0.462	0.227	0.348
Bacillus	0.126	0.306	0.423	0.540	0.198	0.216
Rhizobium	0.280	0.510	0.402	0.414	0.173	0.233

S.No	*Set up*
1.	Collection of garden soil.
2.	Selecting the best seeds of moong and wheat.
3.	Pots-
	Autoclave-1
	Biofertilizer – 1
	Control – 1
	Consortia – 1
	Culture-3*3
4.	Culture used- A3, A8 and A11

Garden soil when treated with samples A5, A11, A4, A3, A1 showed an increase in N, P, K value revealing N, P, K solubilization by these bacterial strains, potent among these was selected for field experiment.

First harvest study by growing moong bean (lantents) in garden soil was carried out with controlled , sample soil + biofertilizer, sample soil + bacterial culture and sample soil + consortia of above bacterial strain. Result showed 9.96cm shoot length, 5.63cm root length, 0.204gm weight for A3, 9.83cm shoot length, 4.66cm root length, 0.229gm weight for A8, 9.06cm shoot length, 3.76cm root length, 0.20gm weight for A11. Study was continued till 10 days showing 21.5cm shoot length, 5.75cm root length for A3. 21.25cm shoot length, 7.5 root length for A8. 16.25 shoot length, 3.75cm root length for A11.

Before second harvest N, P, K value was analyzed showing N= 0.705, P=0.66, K=0.4 for A3. For A8 N=0.374, P=0.414, K=0.173 and for A11 N=0.530, P=0.720, K=0.496.

Non leguminous plant (wheat) was grown to check the fertility of soil showing 2.5cm shoot length, 2.75cm root length, 0.061gm weight for A3. 2.1cm shoot length, 1.75cm root length, 0.094gm weight for A8. 1.8cm shoot length, 2.5cm root length, 0.126gm weight for A11.

Table 6

Moong bean (Vigna radiata) harvest 14/5/18					
Culture	*Shoot length*	*Root length*	*Leaves*	*Colour of Leaves*	*Weight*
A3	10.5cm	4.5cm	Two joint	Light green	0.192 gm
	10.2cm	4.5cm	Two joint	Light green	0.193 gm
	9.2cm	7.9cm	Two joint	Light green	0.228 gm
A8	9.5 cm	4.0 cm	Two joint	Light green	0.236 gm
	9.2 cm	4.5 cm	Two joint	Light green	0.220 gm
	10.8 cm	5.5 cm	Two joint	Light green	0.233 gm
A11	5 cm	2.5 cm	Two joint	Light green	0.10 gm
	10.2 cm	4.8 cm	Two joint	Light green	0.24 gm
	12 cm	4 cm	Two joint	Light green	0.26 gm
Control	2.2 cm	1.8 cm	Two joint	Light green	0.101 gm
Biofertilizer	3.5 cm	2.5 cm	Two joint	Light green	0.120 gm
Consortia	11.5 cm	2.5 cm	Two joint	Light green	0.176 gm

Table 9: Wheat (*Triticum aestivum*) 17/5/18

Culture	*Shoot length*	*Root length*	*Weight*
A3	3 cm	4cm	0.042 gm
	2cm	1.5cm	0.080 gm
A8	2.5 cm	1cm	0.080 gm
	1.7cm	2.5 cm	0.109 gm
A11	0.6 cm	1 cm	0.104 gm
	3 cm	4 cm	0.149 gm

Table 10: Wheat (*Triticum aestivum*) 18/5/18

Culture	*Shoot length*	*Root length*
A3	9.5 cm	4.5cm
	7cm	2.8cm
A8	8 cm	3.5cm
	11cm	6 cm
A11	7 cm	4.5 cm
	8 cm	3.5cm

Table 11: Wheat (*Triticum aestivum*) 21/5/18

Culture	*Shoot length*	*Root length*	*Weight*
A3	8.5 cm	6cm	0.205 gm
	6.5cm	6cm	0.185 gm
A8	9 cm	3cm	0.230 gm
	8cm	5.5 cm	0.215 gm
A11	7 cm	2.5 cm	0.195 gm
	7.2 cm	3.5cm	0.20 gm

Table 12: Second harvest16/5/18

Culture	*N*	*P*	*K*
A3	0.916	1.619	0.945
	1.190	1.716	0.260
A8	1.550	1.758	0.154
	1.158	1.513	0.208
A11	0.917	0.825	0.225
	0.730	0.875	0.729
Control	0.710	1.790	0.756
Biofertilizer	0.338	0.330	0.103
Consortia	1.419	2.110	0.685

DISCUSSION

To meet the steady demand of food supply, application of fertilizer is indispensable in modern agriculture. Role of fertilizers has already been proven by many countries with green revolution and by attaining food self-sufficiency within short period of time. Actually, application of synthetic/chemical fertilizers not only supplies essential nutrients to food crops but also provides them in an easily available manner. Therefore, these fertilizers can quickly enhance the growth and productivity of food crops and are quick to gain popularity. However, extensive use of such fertilizer leads to serious environmental concerns. Nitrate leaching and surface/ground water pollution due to increased use of fertilizer is directly related to human health problems. Similarly, freshwater contamination by chemical fertilizer/fertilizer residue is one of the major causes of eutrophication. Likewise, increased greenhouse gas emission as well as heavy metal uptake and accumulation by food crop could be considered as other environmental problems emerged due to synthetic fertilizers. Moreover, chemical fertilizer could eliminate the beneficial microbial as well as insect community of soil. Alternatively, many of these problems can be surmounted by utilization of biofertilizers. It may not be a realistic idea to completely replace the chemical fertilizers by biofertilizer; however, biofertilizers have the potential to supplement the synthetic fertilizers and to significantly reduce its use. In general, biofertilizers are living microorganisms, unlike chemical fertilizers; they themselves are not the source of nutrients but can help the plants in accessing the nutrient available in its surrounding environment.

Therefore, the development of a more efficient and sustainable agriculture, guaranteeing food supply for an expanding world population and minimizing damage to the environment, is one of the greatest challenges for humankind today. Promotion of the use of bacteria in biofertilizer is one possible way to achieve the goal. Most soils are well inoculated with the organisms involved in the general decomposition processes taking place there. Consumers demand more and more organic food, and most countries have developing policies to reduce the use of chemical fertilizers.

As a result, the commercialization and application of bacterial biofertilizers on agricultural fields or in arboriculture are increasing year by year. Nevertheless, their use is still far from that of chemical fertilizers. Demand from farmers is one of the most critical steps required for the promotion of biofertilizers. Farmers may be undecided as to whether they should adopt new technologies or trust biofertilizer efficiency. Therefore, governmental and international policies promoting this type of farming are needed urgently.

Also, coordinated work by bacteriologists, chemists, geneticists, agronomists and farmers could allow the adaptation of bacterium-based biofertilizers to the different agricultural systems by making them more efficient in the field. Consortia of various organisms with different benefits for crops can be integrated to combine different microbial capabilities into one product with several yield-promoting effects. Additionally, advances in new technologies leading to the enhancement of biofertilizer shelf-lives, facilitating their distribution and application, are essential for their use to be extended.

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