International Journal of Advanced Biotechnology and Research
ISSN 0976-2612, Online ISSN 2278–599X,
Vol 4, Issue 1, 2013, pp 85-95
http://www.bipublication.com

EFFECT OF DIFFERENT CARBON SOURCES ON PRODUCTION AND STABILITY OF BIOFERTILIZER

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[Received-15/11/2012, Accepted-21/01/2013]

ABSTRACT

Biofertilizers are ready to use live formulation of beneficial microorganism which on application to soil mobilize the availability of nutrients by their biological activity in particular and help build up the micro flora and in turn the soil health. Microbes convert the atmospheric N_2 into biologically usable Ammonia. The Phosphorous nutrition along with the Phytoharmones like Gibberellins & Cytokines is also provided.
The focus was on production of "*Azotobacter, Rhizobium, and Azospirillum.*" The desired microorganisms were isolated from soil & root nodules on specific medium. They were confirmed by studying their morphological, cultural & biochemical characteristics. Pure isolated samples were cultured on agar slants. The growth was studied on different substrates of Rice, *Echinochloa Colonum* (Bhagar, Moraiyo seeds or Samo seeds), *Solanum tuberosum* (Potato) & Jaggery and optimum concentration was determined.

Keywords: Biofertilizers, *Azotobacter, Rhizobium, Azospirillum*, Phytoharmones.

INTRODUCTION:

India is the most populous country in the world. Agriculture is one of the most prominent sectors of the economy and provides employment for near about 60% population in India. Considering India's growing population several steps were taken to increase the food production like use of hybrid seeds, and fertilizers. With the increasing population, the cultivable land resource is shrinking day to day. To meet the food, fiber, fuel, fodder and other needs of the growing population, the productivity of agricultural land and soil health needs to be improved. Green Revolution in the post independence era has shown path to developing countries for self-sufficiency in food but sustaining agricultural production against the finite natural resource base demands has shifted from the "resource degrading" chemical agriculture to a "resource protective" biological or organic agriculture. The chemical fertilizers were found harmful to environment. The results of using chemical fertilizers include plants putting their energy into growing bigger quicker. Chemically fertilized plants often perform poorly in terms of producing fruits. The succulent growth

provided from artificial fertilizers leaves the plants more vulnerable to diseases and pests. Using heavy amounts of chemical fertilizers to maximize crop yields leads to increased leaching of nitrates into groundwater. The nitrogen-rich substances found in fertilizer run-off are the main cause for the serious depletion of oxygen in many parts of the ocean. Chemical fertilizers can be harsh on plant physiology and devastatingly terminal on colonies of microorganisms. To overcome this problem Biofertilizers were preferred[1,3]. Biofertilizers are ready to use live formulation of such beneficial microorganism which on application to soil mobilize the availability of nutrients by their biological activity in particular and help build up the micro flora and in turn the soil health in general. Biofertilizers may be defined as the microbial inoculants or carrier based preparations, containing living or latent cells of efficient strains of nitrogen fixing, phosphate solubilizing and cellulose decomposing micro-organisms. Organic fertilizers (manure, compost, vermicompost) are also considered as Biofertilizers, which are rendered in available forms due to the interactions of micro-organisms or their association with plants[4,6]. In recent year's use of microbial inoculums as a source of Biofertilizers has become a hope of most of countries as far as economical and environmental issues are concerned.

Biofertilizers biologically fixes nitrogen to an adequate amount of nitrogen in the form of Ammonia to plants, some Phytohormones[7](like Gibberellins, Auxins, Cytokines, etc.) and other Nutrients (like Phosphorus, Zinc, copper, Potassium), Microelements (like Calcium, sulphur, Manganese, Chloride, Bromide, Iron, etc.) to some extent[8]. Under certain conditions Biofertilizers exhibit anti-fungal activities and thereby protect the plants from pathogenic fungi[9,10]. Biofertilizers can improve soil structure (porosity) and water holding capacity, enhance seed germination and water uptake in plants, and produce organic glues which bind soil particles into semistable into aggregates. The physicochemical properties of soil such as soil structure, texture, cation exchange capacity and pH are increased[11,12].Some biofertilizers produce metabolites which change ability of plant to induce roots from woody cuttings and increase root development during vegetative propagation. The problem of high cost of fertilizers can be solved which indirectly helps in saving economy of nation. It also consumes about 25 – 30 % less energy than normally done by chemical process[1,13] .

Rhizobium was isolated by Beijernick in 1888 from the root nodules of legumes and named it *Bacillus radicicola* now in genus *Rhizobium.* Bacteria belonging to genus live freely in soil and root region of both leguminous and non-leguminous plants. They infect and live in symbiotic association with only leguminous plants, forming nodules in them and fix atmospheric nitrogen. It is estimated that *Rhizobium* fixes 50-80 Kg N_2 / hectare[14,18].

Azotobacter is a free living, aerobic, Nitrogen fixing bacterium and is a non-symbiotic nitrogen fixer. The aerobic bacteria capable of fixing nitrogen belong to entire different families and have been isolated from different habitats viz. Soil, Fresh and Marine water, animals and others. *Azotobacter* is heterotrophic and depends on energy derived from degradation of plant residues. Beijernick was the first to isolate and describe *Azotobacter* (*A. chroococcum*, *A. agilis*) in 1901. *Azotobacter* species are characterized by the relatively large size of the individual cells as seen in the wet preparation under the phase contrast microscope, often motile with peritrichous or polar flagella and production of walled microcysts. *Azotobacter* fixes 20-40 Kg N_2 / hectare per annum [19,21].

Azospirillum was described a nitrogen fixing bacterium in 1952, found in root of digit grass in Brazil and was named *Spirillum lipoferum.* In 1978, Tarrand and co-workers renamed it as *Azospirillum* (i.e. Nitrogen fixing Spirillum). *Azospirillum* is Gram variable and form symbiotic

or non-symbiotic association with the host plants as it is present both inside as well as outside roots. *Azospirillum* fixes 30-50 Kg N_2 / hectare. It is found in xylem vessels of sugarcane and black-gram[22,27].

The carbon sources that provide energy are malate, succinate, lactate and private.hence it grows well in the media containing these carbon sources. The substrates which can provide these carbon sources directly or indirectly are potato, jiggery, samo/moraiyo seeds, rice, etc.

Potato: Potato is commonly called batata, alu in native places. Potato is a tuber grown underground on a specialized plant part (subterranean stem) known as stolon. Therefore, it is a modified stem in a strict botanical sense. A potato tuber is usually oval to round in shape. It consists of an inner flesh and an outer protective cover known as a skin. There is a great variation in flesh color and skin finish. The eye-shaped depressions on a potato tuber is known as its eyes, and actually these are the dormant buds, which give rise to new shoots under suitable conditions. These white to creamy white or pigmented new shoots are known as sprouts. And that is why the process is known as sprouting. This is a very important process in potato, because a sprouted potato is not acceptable for consumption. These sprouted potatoes can be used as a medium for microbial growth as it contains near about all nutrients in simpler form required for their growth.

Potato nutritive value per 100 gm -

Energy	**471 KJ**
Carbohydrates	66.90 gms
Fat	19.90 gm
Protein	7.1 gms
Fibre	5.90 gm
Minerals	2548.96 mg
Vitamins	79.62 mg

Source: (http://www.nutritionanalyser.com/food_composition/?fid=19422)

Jaggery: Jaggery or “Gur” or whole sugar is a pure, wholesome, traditional, unrefined, whole sugar. It contains the natural goodness of minerals and vitamins inherently present in sugarcane juice & this crowns it as one of the most wholesome and healthy sugars in the world. Magnesium strengthens the nervous system & potassium is vital to conserve the acid balance in the cells and combats acids and acetone. Jaggery is very rich in iron, which, a composite of hemoglobin prevents anemia. Jaggery is rich in carbohydrates and minerals but the quantity of proteins is negligible, so it can enhance the growth of Nitrogen fixers as they can consume the required nitrogen for growth from the atmosphere[28,30].

Jaggery nutritive value per 100 gm –

Energy	**383 kCal**
Moisture	04 gms
Protein	00
Fat	00
Minerals	01 gm
Fibers	-
Carbohydrates	95 gms
Calcium	80 mg
Phosphorous	40 mg
Iron	03 mg

Source: (http://www.nutritionanalyser.com/food_composition/?fid=19422)

Samo/Moraiyo seeds (*Echinochloa Colonum*):

The samo seeds or wild rice is known as Bhagar, Wari, etc. in local areas. Samo seeds (varicha bhat) are seeds of a grass *Echinochloa Colonum*. It was described in 1833, as a type of wild grass which originated from tropical Asia. It was formerly classified as a type of *panicum* grass. It grows amongst the rice paddy as it requires damp and moisture laden soil. With digestible fibers, high nutritional content and excellent nourishment, bhagar is immensely popular amongst the Indian majority. Jungle rice is found in the Central Valley, San Francisco Bay region, western South Coast ranges, southern Sierra Nevada foothills, southwestern region inhabiting the summer-irrigated crop fields and other disturbed, moist sites. It germinates throughout the summer. It contains good amount of carbohydrates, fats, fibres, lipids, proteins. These nutrients can support the growth of microbes when

properly boiled so that nutrients are in simpler form.

Samo/Moraiyo seeds nutritive value per 100gm:

Energy	**515.2 KJ**
Fat	2.32 gm
Protein	8.94 gm
Ions	15.18 mg
Carbohydrates	79.48 gm
Crude Fibre	0.23 gm
Lipids	1.8 gm
Salts	1.3 gm
Vitamins	3.98 mg
Moisture	6.11 %

Source: (http://www.nutritionanalyser.com/food_composition/?fid=19422)

Raw Rice (*Oryza sativa*):Rice is the seed of the monocot plants *Oryza sativa* .As a cereal grain, it is the most important staple food for a large part of the world's human population, especially in East and South Asia, the Middle East, Latin America, and the West Indies. It is the grain with the second-highest worldwide production, after maize (corn). Rice is normally grown as an annual plant, it is named as Bhat, chawal, saal, etc. in different areas.The traditional method for cultivating rice is flooding the fields while, or after, setting the young seedlings. Rice can be suitable for the growth of microbes as it can provide all the basic nutrients in simpler forms when boiled required for the growth [31,34].

Raw Rice nutritive value per 100 gm -

Energy	**344 KJ**
Carbohydrates	77.8 gm
Fat	0.5 gm
Protein	6.8 gm
Water	12.88 gm
Fibre	1.4 gm
Lipids	0.6 gm
Mineral	1.606 mg
Vitamins	2.273 mg

Source: (http://www.food-allergens.de/symposium-vol1%284%29/data/rice/rice-composition.htm)

[II] METHODOLOGY:

2.1 Collection of Samples:

Rhizobium:

1. The fields in local areas were surveyed for the availability of plants.
2. The plants (Groundnut, Soybean, and Fenugreek) were plugged out directly and with farming instruments (Khurpi).
3. The plants were kept in polythene bags and brought to laboratory [35,39].

- **Azotobacter:**

1. Potential fields rich in *Azotobacter* population were surveyed and selected.
2. Soil samples were collected from different regions in sugarcane fields.
3. The samples were immediately placed in a sterile bottle and packed properly.
4. These were brought to laboratory [40].

- **Azospirillum:**

1. The soil samples were collected from rice fields from different places.

2. These samples were immediately placed in the sterile bottle packed properly.

3. These were brought to the laboratory [41,42]

2.2 Isolation of the microorganisms:

- **Rhizobium:**

1. The root nodules were thoroughly washed under tap water to remove soil.
2. Healthy nodules (i.e. pink colored and unbroken nodules) were selected and thoroughly washed with 0.1% Hg_2Cl_2 for 5 min to surface sterilize these. Washing was repeated 3-4 times with 0.1% Hg_2Cl_2 and Autoclaved Distilled water alternatively.
3. Place the nodules in 70% Alcohol for 3 min to remove traces of 0.1% Hg_2Cl_2.
4. The nodules were crushed in 1 ml Autoclaved Distilled water with a sterilized glass rod.
5. The crushed samples were serially diluted upto 10^{-7} by dissolving 1 ml sample in 9 ml Autoclaved Saline Water in respective tubes.

6. 0.1ml of sample from the dilutions was spread plated on respective "Congo Red Yeast Extract Mannitol Agar" plates.
7. The plates were incubated at Room Temperature (25^0-28^0 C) for 7 days.
8. The isolated colonies were tested for morphological and biochemical tests.
9. The confirmed colonies were cultured on agar slants and stored in refrigerator[43,46]

• **Azotobacter and Azospirillum:**

1. The soil samples collected from different areas were mixed by taking 1 gm each in a sterile petri plate.
2. Serial dilutions were done upto 10^{-6} by dissolving 1 gm soil sample from petri plate in 10 ml Autoclaved Saline Water and then transferring 1 ml sample to 9 ml Autoclaved Saline water in other tubes.
3. 0.1 ml of the dilution was spread plated on the respective "Nitrogen Free Medium (Azotobacter)" and "Nitrogen Free Basal Medium (Azospirillum)" plates.
4. The plates were incubated at Room Temperature (25^0-30^0 C) for 3 days.
5. The colonies grown after incubation were selected and colony characters were studied.
6. The biochemical tests of *Azotobacter* and *Azospirillum* spp. were done.
7. The isolated confirmed colonies were cultured on the Nutrient Agar slants and incubated and then stored in the refrigerator[47,49]

2.3 Determination of effective (Optimum) Substrate Concentration:

1. The substrates (Potato and Jaggery, Rice medium, Samo/Moraiyo seeds) were measured in the 0.05 gm range upto 1 gm and then dissolved in respective 10 ml distilled water containing conical flask.
2. These flasks were autoclaved a 121.1^0C for 20 min.
3. The respective flasks were inoculated with the isolated organisms.
4. These flasks were incubated at 28^0C for 48 hr in shaking incubator.
5. The cell concentration was determined by measuring absorbance at 600 nm against nutrient broth as blank.[50]

2.4 Mass Production of Biofertilizer:

Stage 1: Preparation of Inoculum:

Inoculum was prepared by taking a loop full suspension in 5 ml medium (Yeast Extract Mannitol Broth for Rhizobium, Nitrogen Free Medium for Azotobacter, Nitrogen Free Basal Medium for Azospirillum). These medium were incubated in Rotary Shaker at 70-80 rpm, 28-30^0C for 48 hours [35]

Stage 2: Preparation of Flask Culture:

The 5 % inoculum prepared was added to a flask containing 100 ml medium (Nutrient agar for Azospirillum, Yeast Extract Mannitol Medium for Rhizobium, Ashby's medium for Azotobacter). These flasks were incubated in Rotary Shaker at 90 rpm, 30-32^0C for 48 days.

Stage 3: Preparation of Mass Culture:

The production of mass culture was done by taking Flask Culture as Inoculum in the respective medium (Potato and Rice, Rice medium, Samo seeds/Morayo Seeds medium). The flasks were incubated in Rotary Shaker for 100 rpm, 32^0C for 72-76 hrs.

Stage 4: Viable cell determination:

The viability of the cells is determined by using Standard Plate Count (SPC).

2.5 Field Application:

The biofertilizer were applied to fields of fenugreek and wheat. It was taken 100 gm and mixed with 2 liter water in bucket.

Three methods were adopted for application.

1. The seeds were mixed with the biofertilizer and then sowed in the soil.
2. The seeds were sowed and biofertilizer was applied externally.
3. The land was digged 3-4 inches, biofertilizer was added and then seeds were inserted.
4. The effect on wheat and fenugreek was determined by comparing them in field with and without biofertilizer

Wheat:

- Heights of the plant.
- Length of seed head i.e. Flares.
- Quality of seeds.
- Strength of stalk.

Fenugreek:

- Height of plant.
- Number of legumes.
- Strength of stem.[53,57]

[III] OBSERVATIONS

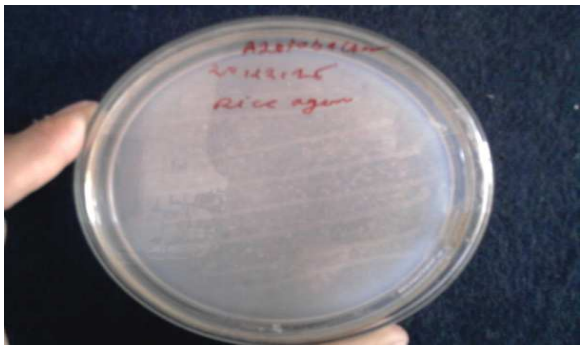


Fig. Azotobacter Growth on "Rice Agar"

Fig. Rhizobium Growth on "Rice Agar"

Fig. Azospirillum Growth on "Rice Agar"

Fig. Rhizobium Growth on "Samo/ Moraiyyo Seed Agar"

Fig. Day 21

(Fig: comparison of wheat growth after21th day on plants with and without Biofertilizer)

Fig. Azospirillum Growth on "Samo/ Moraiyyo Seed Agar"

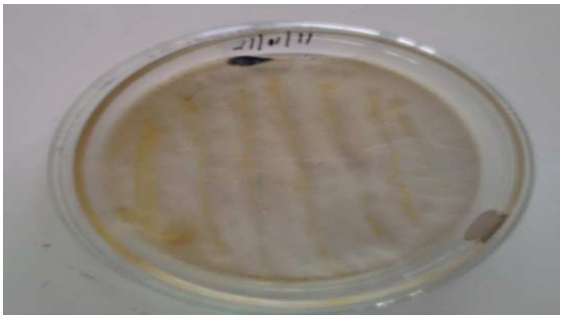


Fig. Azotobacter Growth on "Samo/ Moraiyyo Seed Agar"

Fig. Day 42
(Fig: comparison of wheat growth after 42th day on plants with and without Biofertilizer

[IV] RESULT AND DISCUSSION

Colony Characters (Selective Medium And Nutrient Agar):

Organism	Media	Size (mm)	Shape	Colour	Margin	Elevation	Opacity	Consistency
Azotobacter	N2 Free medium	4-5	Circular	White Mucoid	Undulated	Flat	Opaque	Moist
Azotobacter	Nutrient Agar	2-3	Circular	White	Regular	Convex	Opaque	Moist
Rhizobium	YEMA Medium	1-2	Circular	White	Regular	Raised	Opaque	Moist
Rhizobium	CRYEMA	1-2	Circular	White	Regular	Raised	Opaque	Moist
Rhizobium	Nutrient Agar	1-2	Circular	White	Regular	Convex	Opaque	Moist
Azospirillum	N2 Free medium	2-3	Circular	White	Undulated	Raised	Opaque	Moist
Azospirillum	Nutrient Agar	2-3	Circular	White Mucoid	Regular	Convex	Opaque	Moist

Effective substrate Concentration:

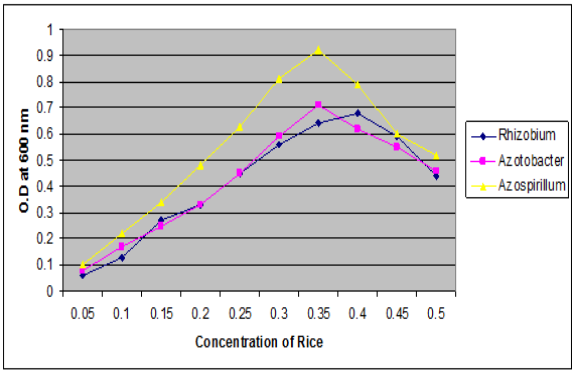


Fig. determination of optimum concentration Rice medium

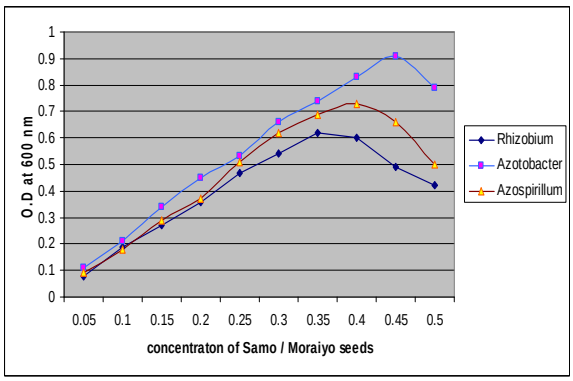


Fig. determination of optimum concentration of Samo/Moraiyoo seeds.

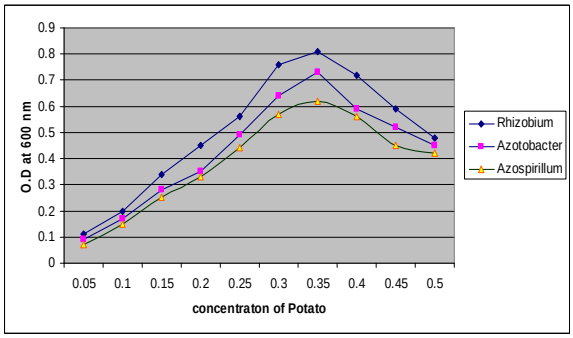
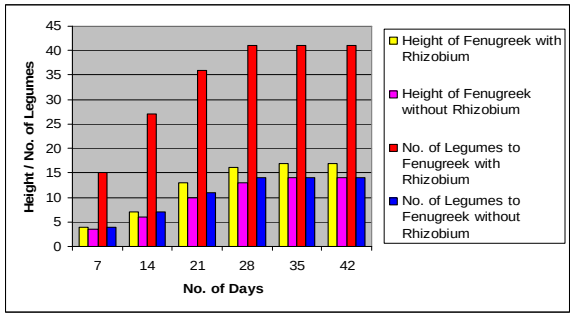


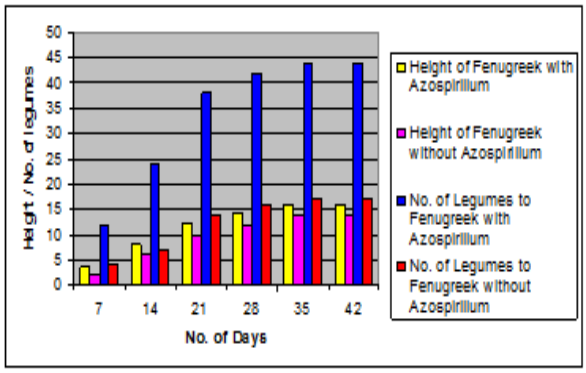
Fig. determination of optimum concentration of potato

Results of Field Trials:

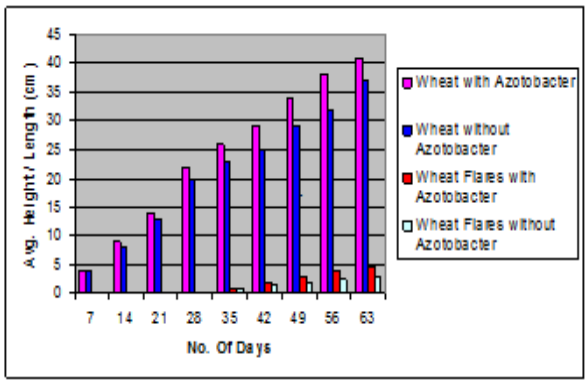


Rhizobium Effects on Fenugreek:

Viable cell count of Flask Culture using SPC:



Azospirillum Effects on Fenugreek:



Azotobacter Effects on Wheat:

Viable cell count of Flask Culture using SPC:

Rhizobium:

Dilution	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	$10^{(-5)}$	$10^{(-6)}$	$10^{(-7)}$
Nutrient Broth	Tmc	Tmc	92	57	30	13	3
Morayo seeds	Tmc	Tmc	86	62	27	11	5
Rice Agar	Tmc	Tmc	85	68	26	9	2
Jaggery & Potato	Tmc	Tmc	79	54	32	11	1

(**Note:** Tmc: Too many colonies)

Azotobacter(No. Of Colonies):

Dilution	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	$10^{(-5)}$	$10^{(-6)}$	$10^{(-7)}$
Nutrient Broth	Tmc	Tmc	88	51	29	16	5
Morayo seeds	Tmc	Tmc	97	66	32	17	4
Rice Agar	Tmc	Tmc	93	59	28	11	4
Jaggery & Potato	Tmc	Tmc	86	56	38	19	2

(**Note:** Tmc: Too many colonies)

Azospirillum:

Dilution	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	$10^{(-5)}$	$10^{(-6)}$	$10^{(-7)}$
Nutrient Broth	Tmc	Tmc	89	60	29	13	1
Morayo seeds	Tmc	Tmc	93	69	33	10	2
Rice Agar	Tmc	Tmc	95	71	28	11	1
Jaggery & Potato	Tmc	Tmc	90	61	30	13	3

(**Note:** Tmc: Too many colonies)

Viable cell count of Mass Culture using SPC:

***Azotobacter*:**

Dilution	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	$10^{(-5)}$	$10^{(-6)}$	$10^{(-7)}$
Nutrient Broth	Tmc	Tmc	91	58	37	18	5
Morayo seeds							
Rice Agar	Tmc	Tmc	91	54	30	16	4
Jaggery & Potato	Tmc	Tmc	89	59	41	20	7

(**Note:** Tmc: Too many colonies)

Rhizobium:

Dilution	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	$10^{(-5)}$	$10^{(-6)}$	$10^{(-7)}$
Nutrient Broth	Tmc	Tmc	97	54	26	12	4
Morayo seeds	Tmc	Tmc	89	61	30	8	5
Rice Agar	Tmc	Tmc	83	69	29	9	2
Jaggery & Potato	Tmc	Tmc	92	62	36	15	7

(**Note:** Tmc: Too many colonies)

Azospirillum:

Dilution	$10^{(-1)}$	$10^{(-2)}$	$10^{(-3)}$	$10^{(-4)}$	$10^{(-5)}$	$10^{(-6)}$	$10^{(-7)}$
Nutrient Broth	Tmc	Tmc	89	59	29	11	1
Morayo seeds	Tmc	Tmc	94	64	22	9	2
Rice Agar	Tmc	Tmc	95	68	35	13	5
Jaggery & Potato	Tmc	Tmc	90	63	19	7	2

(**Note:** Tmc: Too many colonies)

[VI] CONCLUSION

The pure culture of *Rhizobium*, *Azotobacter*, *Azospirillum* were isolated, characterized and stored.The Biofertilizers were produced using different substrates (Potato and Jaggery, Rice medium, Samo seeds/ morayo Seeds medium) and Carriers (Lignite, Baggasse, rice husk, Ground nut shell).Azotobacter show maximum growth on Morayo Seed (4.5%) medium whereas Rhizobium on Potato (3 %) and Jaggery (2.5%) Medium and Azospirillum on Rice (3.5%) medium.Wheat and Fenu greek showed productive growth after application with Biofertilizers.

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