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ISOLATION AND ENRICHMENT OF SUGAR PRESS MUD (SPM) ADAPTED MICROORGANISM FOR PRODUCTION OF BIOFERTILIZER BY USING SUGAR PRESS MUD.

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ABSTRACT

Sugar Press Mud (SPM) a by-product of the alcohol distillation originating from the fermentation of sugar cane molasses persists difficulties in handling due to its large volume productions and the huge amounts of organic matter that it contains which unable its unloading into the water sources. Thus there is a necessity of treating SPM to turn it in a valuable bio-fertilizer. The selected strains of plant growth promoting Rhizobacteria (PGPR) beneficial soil organism is adapted to the SPM in laboratory followed by enriching it with SPM which acts as a nutrient as well as Carrier material.

SPM a carrier of inoculant bacteria shows lower tolerance for physical stress during storage and particularly for temperature variations. Thus it can be used for soil application or seed treatment to make basic nutrients readily available and enhance soil fertility.

Key words: Sugar Press Mud (SPM), Organic matter, Soil Application, PGPR, Bio-fertilizer.

INTRODUCTION

India is highly dependent on the agricultural sector, which is the main income- and employment-generating sector of the economy. The most important crop from which sugar can be produced in commercial quantity are sugarcane. Sugarcane is grown for sugar production primarily. India is a largest sugar producing country. Today sugarcane is grown in over 110 countries. In 2008 an estimated 1,743 million metric tons were produced worldwide, with about 50 percent of production occurring in Brazil and India (Solomon, 2008). More than 45 million of sugar cane growers in the India and about 65% of the rural population depend on this agro-based industry. The sugar industry is the second largest agricultural industry followed after the textile industry. Maharashtra Sugar Industry is one of the most notable and large-scale sugar manufacturing sectors in the country. Due to energy crises, scientists and researchers have realized the value of sugarcane, it's by products and co-products. Sugarcane is processed to sugar and biomass. This biomass contains many components like lignin, fiber, pith and pentosans, which has plenty of applications in biochemical & microbial fields. (R.L.YADAV and S.SOLOMON 2006)

Molasses is one of the major components of growth media used in industrial process.

Due to its unique physical and chemical properties molasses has traditionally been used

as a major component in compound feeds, livestock feeds and silage additives and most widely used in various industrial processes. Molasses-based distilleries are one of the most polluting industries generating large volumes of high strength wastewater (Y. Satyawali and M. Balakrishnan, 2007).

Sugar Press Mud (SPM) is an industrial by-product of the process of ethylic alcohol distillation, produced by biological fermentation of the raw material (molasses), that presents a dark coloration and a great turbidity. The generated volume of this product is elevated, since are produced approximately 13lts of SPM for each one liter of alcohol obtained in the process. This by-product is highly corrosive and polluting of the water sources, presents in its chemical composition, high contents of organic material, potassium, calcium and moderate amounts of nitrogen and phosphorus, that give it an important commercial value with a great potential for diverse uses (Dimas Román et al., 2006).

It is characterized by a pH value between 4 and 5 and due to its high organic material content has a high biochemical demand of oxygen (BDO) that oscillates between 7.000 and 20.000mg/lt. This value become it in a pollution agent of the environmental, since it requires high oxygen concentrations for the oxidation of the organic material that it has; therefore when are discharged to the rivers, it exhaust the dissolved oxygen affecting the flora and fauna present in that ecosystem (Pande and Sinha, 1997).

This SPM is produced at a rate of 7-9% of total weight of sugar cane in Carbonation industries and 3-5 % in sulfitation industries. The composition of SPM used to produce biofertilizer is listed in Table No. 1

Sr. No.	Nutrients	%
1	Nitrogen	0.22
2	Phosphorus	0.2
3	Potassium	1.27
4	Moisture	50-65
5	Humic acids	19.75
6	Fiber	20-30
7	Crude wax	7-15
8	Sugar	5-12

Source: (Suneela Sardar et al., 2006).

Silicon, Iron, Manganese, Calcium, MgO & P2O5 is also detected in some appreciable amount in SPM. Compost fertilizer is always evaluated by the percentage of organic matters in the final product. Organic matter contains all types of fiber, wax, crude proteins sugar, and all other carbon containing components available in the final product (Suneela Sardar et al., 2006).

Sugar factory effluent produces obnoxious odour and unpleasant color when released into the Environment without proper treatment. Farmers have been using these effluents for irrigation, found that the growth, yield and soil health were reduced (Rahman *et al.*, 2002, Street *et al.*, 2007). With proper application of SPM to grounds of sugar cane production, improved the pH of them, diminishes the interchangeable aluminum and increase the potassium concentration. Also it's improved the cationic relationship, mainly of calcium and magnesium. These results allow concluding that SPM is excellent organic product. Nakajima-Kambe et al., (1999) studied that the various microorganisms were screened for their ability to decolorize molasses wastewater under thermophilic and anaerobic conditions. Isolation and identification of *Pseudomonas, Enterobacter, Stenotrophomonas, Aeromonas, Acinetobacter* and *Klebsiella* has more efficiency in reducing the chemical oxygen demand of spent wash (Ghosh et al., 2004).

The life in effluent is highly diverse and consists of interacting population of microorganisms and effluent fauna, and their activities affect physical, chemical and biological characteristics of effluent. Some fungal strains such as *Penicilliumchrysogenum, Alternaria gaisen, Aspergillus flavus, Aspergillus awamori, Fusarium monolifome, A.niger* were isolated from sugarcane industrial effluent (Pant and Adholeya 2007). In many areas SPM is directly transported to the fields from sugar mills as an organic enhancement to fields. Due to this practice, cane sugar yield has been increased causing decrease in demand of inorganic fertilizers (Sugar Tech, 2010). Organic wastes SPM are enriched with Nitrogen and

Phosphorous, which are the main part of nutrients of crop**.** To enhance the yield and growth of wheat, application of N fixing bacteria, fungi, K solubilizing bacteria and some microorganisms were reported by *Warana Biofertilizer*.

The selected strain of plant growth promoting Rhizobacteria (PGPR) beneficial soil organism adapted to the SPM in laboratory and enriched with SPM, SPM used as nutrient as well as Carrier material. They can be either for seed treatment or soil application. This bio fertilizer generate plant nutrients

The biofertilizer production technology is mainly based on inoculation technology. The success of inoculation technology depends on two factors such as the microbial strain and inoculants formulation. In practical terms, formulation determines potential success of inoculants(Fages,1992) The technical optimization of an inoculants formulation is independent of strains used, since most of the strains of same bacterial species share many physiological properties, it may be assumed that a technological progress developed for a particular strain is readily adaptable to another strain of same species with only minor modifications(Bashan,1998) In spite of a central role of formulation in successful commercialization of inoculants products, research in this area has been largely ignored. In addition to limited availability of published scientific information with regard to inoculant formulation, the information available is fragmented (Xavier *et al.,* 2004).

Formulation step is a crucial aspect for producing microbial inoculants and determines the success of a biological agent. Formulation typically consists of establishing viable bacteria in a suitable carrier together with additives that aid in stabilization and protection of microbial cell during storage, transport and at the target. The formulation should also be easy to handle and apply so that it is delivered to target in most appropriate manner and form, one that protects bacteria from harmful environmental factors and maintain or enhance the activity of the organisms in the field. Therefore, several critical factors including user preference have to be considered before delivery of a final product (Xavier *et al.,* 2004).

A suitable carrier as SPM plays a major role in formulating microbial inoculants. SPM is a delivery vehicle which is used to transfer live microorganism from an agar slant of laboratory to a rhizosphere. We use A good quality inoculant should be made of a superior carrier material. In SPM carrier based inoculants, bacteria have a lower tolerance for physical stress during storage, particularly for temperature variations. This also provides organic material for plant growth. They are often suppressing the unwanted contamination that can reduce the shelf life of the inoculant.

Now days research is going on mixed inoculants, microbial inoculants with multiple organisms (G.P. Brahmaprakash and Pramod Kumar., 2012) are not yet produced commercially. Until now, the research on mixed microbial inoculation was only confined to development and inoculation of each bacterium in separate formulation. In this direction concept of microbial consortium assumes greater importance for sustainable agriculture. Feasibility of production of microbial consortium using *Azotobacter* and *PSB* using lignite, liquid and alginate granules have been tested(Nethravathi *et al.,* 2005, Scaglia.,1991). The development of microbial consortium may minimize cost, labour and energy involved production of inoculants. But more and more single strains microbial inoculants must be registered, before inoculation industry can contemplate the development and commercialization of multi-bacterial inoculants (Polonenko., 1994).

MATERIALS AND METHODS

Chemicals

The chemicals used during this work were of analytical grade.

Microorganisms

Warana Biofertilizer, Warana sugar mill, Warananagar. Provided the microorganisms for the study. The microbes used in the study were

the fungus *Aspergillus niger, Aspergillus awamori, Penicillium crysogium, Tricoderma viridi* .All above strain maintained on potato dextrose agar(PDA). Bacterium *Azotobacter chroococcum* and *Acetobacter nitrogenifigens*, which was chosen because it is a nitrogen-fixing bacterium. It was thought that the presence of bioavailable nitrogen would aid in overall microbial biodegradation rates. *Azotobacter chroococcum* and *Acetobacter* were maintained on Azotobacter agr modified 1(pp166), Acetobacter medium (pp27, Handbook of Microbiological Media Fourth Edition, by Ronald M. Atlas). The Phosphate solubilizing bacterial (PSB) strains *Pseudomonas striata, Bacillus megaterium, Bacillus polymyxa* solubilize available in soil. This PSB were maintained on PSB agar. All strains were stored at 4°C. A spore suspension was made by adding 25 ml of sterile distilled water to a 5 days old slant and scraped aseptically with inoculating loop. A suspension, having spore concentration of about 1.3 x 107 cells ml-1, was used as inoculums for the subsequent inoculation.

Culture media

Isolation of sugar Press mud adapted cultures take place on the Petri Plate containing industrially used Media provided by the Warana Biofertilizer, in which we add 50% SPM. Isolated SPM adapted cultures further grown in the 250 ml Erlenmeyer conical flask containing broth with 100% SPM.

Isolation of sugar Press mud adapted culture

The lethal concentrations of SPM on bacterial strains were determined by growing them in medium having different concentrations of SPM (50–100) %. The microbial sample were prepared and spread plate technique was done on the individual selective media agar plates, in order to produce SPM resisting microorganisms on the media

Streak plate technique:

0.1ml of each organism sample was streaked on sterile selective media agar medium with the help of sterile wire loop in laminar airflow. Plates were incubated at 370 C for 24 to 72 hrs.

Transforming isolated SPM resisting organism colony:

A loop full suspension of log phase cultures prepared from isolated colonies of microorganism of lower dilutions from streak plates were transferred under aseptic conditions to the respective Enrichment medium and incubated at 28^0C. The plates were then incubated at room temperature for 72 hrs.

Formulation of the bacterial consortium

The experiment was conducted in the laboratory using five large plastic containers (58 × 43 × 18 cm). Each container was filled with 1 kg of SPM collected from Warana Sugar Mill, Warananagar. The treatments studied were microbial consortium sprayed over SPM. Bacterial and fungal counts in the soil were monitored periodically using pour plates of individual Enriched media. Also we measure Nitrogen, Potassium, Phosphorous; pH, Electric conductivity and total organic matter were analyzed.

Container No	**Bacterial mixture**
BAG 1	*Bacillus polymyxa, Pseudomonas striata, Bacillus megaterium*
BAG 2	*Azotobacter chroococcum, Acetobacter nitrogenifigens*
BAG 3	*Azotobacter chroococcum, Bacillus polymyxa, Pseudomonas striata, Bacillus megaterium*
BAG 4	*Acetobacter nitrogenifigens, Bacillus polymyxa, Pseudomonas striata, Bacillus megaterium*
BAG 5	*Azotobacter chroococcum, Acetobacter nitrogenifigens, Bacillus polymyxa, Pseudomonas striata, Bacillus megaterium*

Mass Production of Biofertilizer

Preparation of mother culture:

Inoculums were prepared by taking a loop full suspension in 50 ml medium. These medium were incubated in Rotary Shaker at 70-80 rpm, 28-30^0C for 48 hours. This culture refer as mother culture.

Preparation of Flask Culture:

The 5 % inoculums prepared was added to a flask containing 100 ml medium. These flasks

were incubated in Rotary Shaker at 90 rpm, 30-32^{0}C for 48 days.

Preparation of container Culture:

The production of mass culture was done by taking Flask Culture as Inoculums in the SPM in the given formulations. The container were incubated at 10^{0}C to 15^{0}C

Determination of moisture Content:

The moisture content was determined by using the "Gravimetric Method" which uses the difference in the mass before and after incubation. The desired range of moisture is 35-46 %. It helps in reducing the microbial contamination and keeps the cells active.

Total moisture content

(Z %) = (X - Y)*100 / X

Wt. of sample before incubation

(X) = B - A

Wt. of sample after incubation

(Y) = C - A

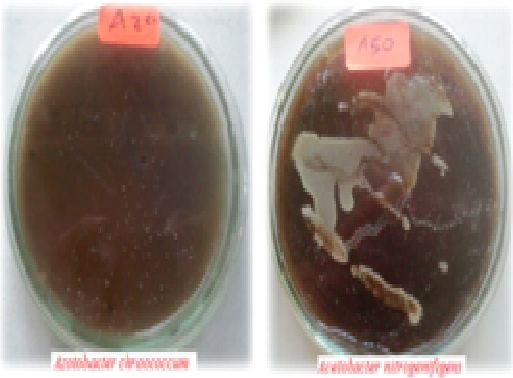
(Where A is Wt. of Empty Petri plate**,** B is Wt. of Sample + Petri plate before incubation and C is Wt. of Sample + Petri plate after incubation)

OBSERVATION

Isolation of SPM adapted cultures

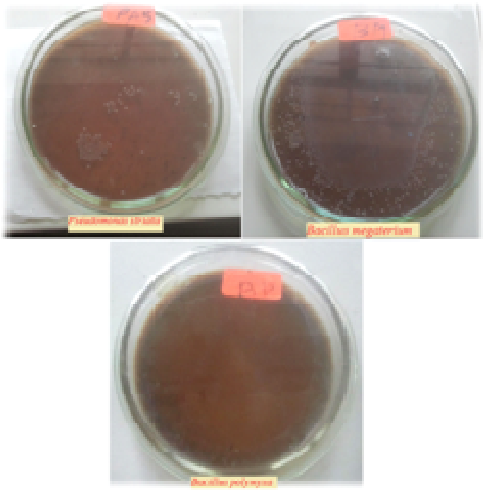
Azotobacter and Acetobacter-

Isolated SPM adapted *Azotobacter chroococcum, Acetobacter nitrogenifigens* bacteria on the respective industrial media containing media with 50% SPM



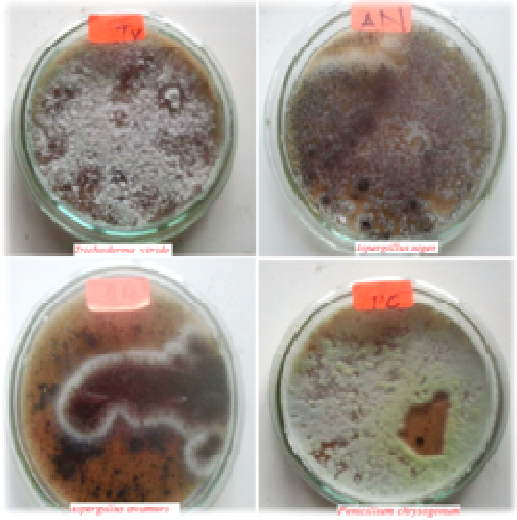
Phosphate Solubilizing Bacteria

Isolated SPM adapted Phosphate Solubilizing Bacteria on the PSB industrial media containing media with 50% SPM



Decomposing Fungi-

Isolated SPM adapted Decomposing fungi on the Decomposing industrial media containing media with 50% SPM



Viability of consortium Bag Culture using SPC(at Incubation)

	Dilution	**$10^{(-1)}$**	**$10^{(-2)}$**	**$10^{(-3)}$**	**$10^{(-4)}$**	**$10^{(-5)}$**	**$10^{(-6)}$**	**$10^{(-7)}$**
BAG 1	No. Of Colonies	TNTC	TNTC	88	51	29	16	5
BAG 2	No. Of Colonies	TNTC	TNTC	97	66	32	17	6
BAG 3	No. Of Colonies	TNTC	TNTC	93	59	28	11	3
BAG 4	No. Of Colonies	TNTC	TNTC	90	56	38	19	4
BAG 5	No. Of Colonies	Tmc	Tmc	89	57	34	16	5

Viable cell count of BAG Culture using Hemocytometer: (at Incubation)

	No. of Viable cells	No. of dead cells	Average No. of Viable cells	Average No. of Dead cells	Total cell count /ml (*10^4)
BAG 1	357	32	14.30	01.40	785
BAG 2	377	53	15.08	02.12	860
BAG 3	407	41	16.28	01.64	896
BAG 4	435	57	17.40	02.28	984
BAG 5	426	37	17.04	01.48	926

Note: No. of areas counted = 25

Calculations for Cell count:

A=10 µl (Sample collected)

B=05 (Dilution factor)

C= (Total no. of viable cells) / (No. of areas counted)

$C_1 = 357/25 = 14.30$

$C_2 = 377/25 = 15.08$

$C_3 = 407/25 = 16.28$

$C_4 = 435/25 = 17.40$

$C_5 = 426/25 = 17.04$

D= (Total no. of dead cells) / (No. of areas counted)

$D_1 = 32/25 = 01.40$

$D_2 = 53/25 = 02.12$

$D_3 = 41/25 = 01.64$

$D_4 = 57/25 = 02.28$

$D_5 = 37/25 = 01.48$

Total cell count per ml (T) = $A*B*(C+D)*10^4$

$T_1 = 10*5*(14.3+01.40)*10^4 = 785*10^4$

$T_2 = 10*5*(15.08+02.12)*10^4 = 860*10^4$

$T_3 = 10*5*(16.28+01.64)*10^4 = 876*10^4$

$T_4 = 10*5*(17.4+02.28)*10^4 = 984*10^4$

$T_5 = 10*5*(17.04+01.48)*10^4 = 926*10^4$

RESULT AND DISCUSSION

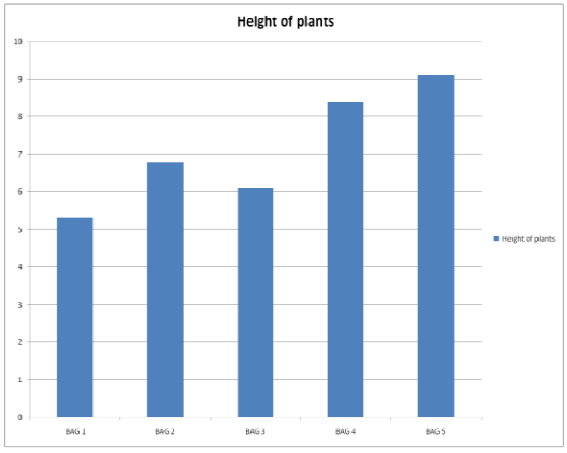
Large areas of cultivated land in Kolhapur District are deficient in phosphorus and potassium nutrient therefore to increase the available P and K in this soil we have preferred addition of phosphorus and potassium solubilizing bacteria. Mainly organism like atmospheric nitrogen fixing bacteria is used as biofertiliser. In addition we use some decomposting fungi like *Aspergillus niger, Aspergillus awamori, Penicilium crysogenum* and *Trichoderma viridiae*. Biofertillizers biologically fix nitrogen in the form of Ammonia, in adequate amounts to make it available for plant use; some Phytohormones (like Gibberellins, Auxins, Cytokines, etc.) are available in the soil in simple form. Other nutrients like phosphorus, zinc, copper, potassium and microelements like calcium, sulphur, manganese, chloride, bromide, iron etc. to some extent. Under certain conditions decomposing fungi exhibit anti-fungal activity to other pathogenic fungi and thereby protect the plants from pathogenic fungi. Fungi also transform complex organic matter into simple humic acid. Biofertilizers can improve soil structure (porosity) water holding capacity, enhance seed germination, water uptake in plants and produce organic glues which bind soil particles into semis table into aggregates.

India is a largest sugar producing country and sugarcane is grown all over. During the processing of sugarcane cane juice contains a large number of impurities which are in the form of precipitates and these impurities are referred as SPM. It is an impurity that has multiple uses like as a fertilizer, animal feed. This SPM is produced at a rate of 7-9% of total weight of sugar cane in Carbonation industries and 3-5 % in sulfitation industries.

In many areas SPM is directly transported to the fields from sugar mills as an organic enhancement to fields but SPM contains some essential nutrients which cannot be directly absorbed by the plants, to make these nutrients available beneficial organism like *Azotobacter chroococcum, Acetobacter nitrogenifigens, Bacillus polymyxa, Pseudomonas striata, Bacillus megaterium* are used which transform the complex nutrients into available form. We have prepared SPM adapted culture by using various concentrations of SPM , firstly we isolated the SPM adapted microorganism by using the commercially used media and then we replaced 50% essential nutrient with the SPM

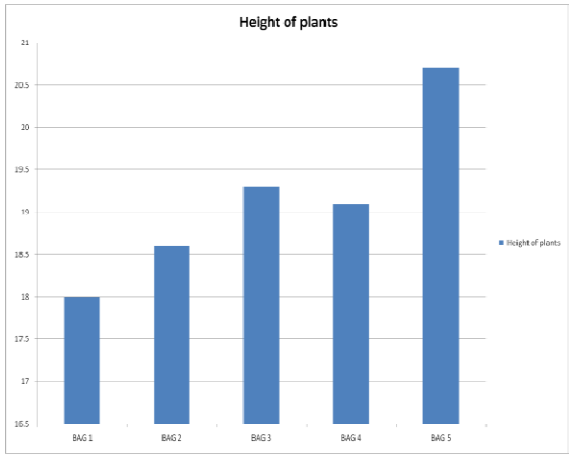
and finally isolated the SPM adapted miroorganism. Isolated organism was again recultured on the same media and then this culture was used for enrichment. Enrichment of microorganism take place by using 100% SPM . The success of inoculation depends on two factors, the microbial strain and inoculant formulations. The optimization of an inoculant formulation is independent on strains used since most of the strains of some bacterial species share many physiological properties and fungal species increase their own activity in the combination with each other. To optimize inoculation we prepare five consortium as shown in Table no.1. During the isolation of the efficient fungal–bacterial consortium in laboratory we check its efficiency on the various plants like **Chickpea** (*Cicer arietinum*) and **Wheat** *(Triticum aestivum)* for 21 days. The effectivity of each consortium of SPM adapted organism on **Chickpea** plants as listed in **Table no.2.**

Consortium No.	Height of plants
BAG 1	5.3
BAG 2	6.8
BAG 3	6.1
BAG 4	8.4
BAG 5	9.1



we find the consortium number of fifth shows the maximum growth with the root zone of plants are increased. The increase in size of plant rooting zone shows that the nutrient absorbing activity of plants is increased. The effectively of the each consortium is checked on **Wheat** and we find that, the same consortium i.e. the fifth shows highest effectively. **Table No.3** shows the effectively of each consortium of SPM adapted organism on plants.

Consortium No.	Height of plants
BAG 1	18.0
BAG 2	18.6
BAG 3	19.3
BAG 4	19.1
BAG 5	20.7



In the next stage we produce the isolated consortium on SPM. We check the nutrient level in the produced consortium at the Warana Biofertiliser. The following reports show the comparative increase in the nutrient level of consortium than SPM. Finally we confirm the effectivity of SPM Biofertiliser on two plants in the field as shown in following two fig.

Fig: comparison of Growth of Fenu greek on 15th day in fields with SPM Biofertilizer and with only SPM.

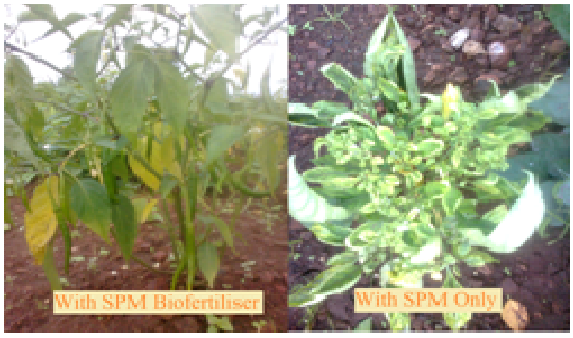


Fig: comparison of Growth of Red Chili pepper (*Capsicum baccatum)* on 30th day in fields with SPM Biofertilizer and with only SPM.

The fifth consortium was isolated and a comparative study with Fenugreek (Trigonella foaum-graecum) and Chilli pepper to obtain highest activity with SPM biofertlizer.

CONCLUSION

The SPM adapted cultures of microorganism were isolated, characterized and enriched in the respective broth. The microbial consortiums were produced using bacteria and fungi. The effectively of varied consortiums in form of SPM biofertilizer was checked on Wheat and Chickpea to obtain maximum growth by fifth consortium. The fifth consortium was isolated and a comparative study with Fenugreek and Chilli pepper to obtain highest activity with SPM Biofertilizer. This leads to a conclusion that the product is useful to increase the macro and micro nutrient of soil.

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