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Original Article

Segregation and Identification of Nitrogen Fixing, Phosphate and Potassium Solubilizers microbes from Sugarcane pressmud

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Abstract

Growth of plants is dependent upon optimum availability of various micronutrients and macronutrients. Inorganic nitrogen (N), phosphate (P) and potash (K) are the most influencing macro-nutrients for plant growth. Microbial supplementation of these minerals through N2-fixation, P- and K-solubilization is gaining importance. Pressmud, a sugarcane waste, acts as an important source of fertilizers. The biofertilizer production is mainly based on inoculation technology. The success of inoculation technology depends upon the isolation, identification and inoculants formulation of selected microbial strain. Isolation of indigenous micro flora capable of phosphorus solubilization and nitrogen fixation is an important procedure when studying their inherent capacity to benefit crops probably because of their superior adaptability to the environment than the introduced strains. Hence from pressmud, total three nitrogen fixers and phosphate solublizer were isolated by using selective media. Nitrogen fixers identified were viz. Bacillus weihenstephanesis (O), Candidatus Rhizobium massiliae (T) and Bacillus flexus (G). Phosphate solublizer isolated was Candida tropicalis (f). On analysis it was observed that, N2 fixing capacity of O, T and G isolates was 0.56%, 0.42% and 0.39% respectively. Also the phosphate solubilisation capacity of (f) was 6 µg/ml. Similarly indol acetic acid production of (f) was estimated as 600 µg/ml. Further, f was studied for solubilisation of potassium. Result shows that f has the capacity to solubilise Potassium. Details are discussed in the paper.

Keywords: *Sugarcane Pressmud, Nitrogen, Phosphate, Potash, Micronutrient and Microorganism.*

Introduction

Sugar is produced in large scale in country like India. Though other crops like sweet beet/sugar beet are also used worldwide for production of sugar, India obtains sugar and ethanol from sugarcane crop only. The sugar processing and alcohol manufacturing produce mainly four major types of waste products; residue of cane left in field after cane harvesting, bagasse, pressmud and spent wash (Partha and Sivasubramanian, 2006). Sugarcane is pressed to obtain juice which is later used for sugar production. The obtained juice is clarified. The waste remained after clarification of cane juice is termed as pressmud. As it is rich in various nutrients, it is considered as a good source of fertilizer. In India it is estimated that about twelve million tons of filter cake/ pressmud is produced from sugar industries which follows the process of double sulphitation. As the obtained pressmud is in large quantity it is generally it is disposed off in open fields. At some places farmers tend to buy it to apply as soil amendment for crops. Pressmud is also subjected for energy production due to availability of many combustibles as important constituents. Biogas is also generated from it as it contains about 5–15% of sugar. The composition and nutrient availability of pressmud determines its quality to be used as a fertilizer. As it is rich in various micronutrients, they are widely applied for many agricultural crops and also at horticulture garden (Partha and Sivasubramanian, 2006; Partha and Krishnan M.R.V, 2000; Saravane *et al*, 2005). Pressmud is the compressed sugar industry waste produced from the filtration of the cane juice (Bakulin, *et.al.*). As the pressmud contains high percentage of nutrients it can also be used as fertilizers. Pressmud is a good source of organic matter, NPK and important micronutrients and has proved its importance in improving fertility, productivity and other physical properties of agricultural soil (Jamil *et al.*, 2008). Rapid vegetative growth especially leaf and stems is usually promoted by availability of Nitrogen (N) content from the soil. Nitrogen also plays an imperative role in the formation and functioning of chlorophyll which is a key element to impart green colour to the plants and promotes photosynthesis process.

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Nitrogen al Amino acids are synthesized by nitrogen which later forms the protein, building block of living organisms. Uptake of other nutrients is mostly regulated by nitrogen element in the soil. Most important it is a basic ingredient of vital compounds - Nucleic acid and enzymes.

Phosphorus (P) is a second important micronutrient in the soil. The phosphorous content from soil stimulates early root formation and growth of plants. This helps the plants to develop a proper root filter system in the soil which efficiently absorbs the nutrients and water from the soil. Ultimately all this leading to the exact growth of the plant system. Phosphorus (P) is essential for plant strength and stamina. It plays a vital role in various physiological processes that contribute to a plant's overall health and ability to withstand stress. Specifically, phosphorus supports root development, stem strength, and disease resistance, all of which contribute to a plant's stamina. Phosphorus also plays a key role in converting starch into sugars which also accelerates the maturity of plant. Phosphorous is pivotal in stimulating blooming and developing seeds. It also significantly supports energy transformation and conversion processes where sugars are converted to hormones, protein and energy which leads to the formation of new leaves and fruit. It is also one of the forms of nucleic acids i.e. DNA and RNA. Phosphorus is imperative for photosynthesis process and cell division.

Potassium (K) is another macronutrient which has a significant role in growth of plants and soil enrichment. Potassium is important as it aids the development of stems and leaves of plant for healthy growth. The plants may sometimes undergo wilting if there is inadequate supply of potassium and lot more presence of moisture. Hence, at such situation Potassium impacts the water intake by cells of plants. Similarly it also increases iron intake capacity by acting as a catalyst. The wear and tear of plant system is also regulated by Potassium availability as it increases disease resistance and hardiness of vegetation. It also strengthens the cell walls, causing grass to stand up and reduces lodging. Potassium also improves the size and quality of fruit, grains and tubers as it leads to the formation and translocation of protein, starches, sugar and oil. Along with these important nutrients, other macronutrients such as calcium, magnesium, sulfur and micronutrients such as iron, zinc, manganese are crucial for proper growth of plants.

Materials and Methods:

1. **Isolation and screening of Nitrogen fixers**

The press mud was collected from KumbhiKasari S.S.K. Kuditre sugar factory which is situated about 12 km west of Kolhapur city having its crushing capacity of 3500 TCD and distillery of 45000 LPD. 1gm of pressmud was added into 10 ml sterile, fresh Ashby's broth containing mannitol (2gm), K_2HPO_4 (0.02gm), $MgSO_4.7H_2O$ (0.02gm), NaCl (0.02gm), $FeCl_3$(0.0003), $MnSO_4.5H_2O$ (0.0003) in 100ml distilled water. This was incubated at Room temperature, for 24 hrs. On next day 1ml of from enriched Ashby's broth was added into fresh sterile Ashby's broth. The remaining broth was used for serial dilution for obtaining pure culture. Then plating was done for SPC on the respective solid media. Same procedure was repeated for 10 times. Depending on difference in morphology/colony characteristics, the isolates were purified by single colony streaking on solid media. SPC for each batch was calculated.

2. **Isolation and screening of Phosphate solubilizers**

By using pikovasky's broth, of composition glucose (1gm), $CaCO_3$ (0.5gm), $(NH_4)2SO_4$ (0.05gm) $MgSO_47H_2O$ (0.01gm) KCl (0.02gm), traces of $MnSO_4$ and FeSO4 yeast extract (0.05gm) into 100 ml distilled water was used for isolation of phosphate solubaliser. The procedure same as above was repeated.

3. **Isolation and screening of Potassium solubilizers**

Samples were inoculated after serial dilution up to 10^{-6} on Aleksandrov agar medium constituted 1% glucose, 0.05% MgSo4.7H2O, 0.0005% FeCl3, 0.01% CaCO 3, 0.2% CaPO4 and 0.5% potassium aluminium silicate, agar 3 % pH=6.5 (Sugumaran and Janartham, 2007) and incubated at 370 C for 5 – 6 days. Colonies exhibiting clear zone of potassium solubilization were selected as potassium solubilizers from the 10^{-4}, 10^{-5} and 10^{-6} dilutions containing plates were identified.

4. **Determination of Nitrogen fixation capacity**

1ml enriched culture taken and 50 mlof 10 % NaCl solution added. By heating stirrer it for30 min. The solids allowed to filtered and extracted supernatant taken in 250 ml volumetric flask and the volume adjusted to 250 ml with 10 % Nacl. Transfer this content to kjeldahl flask. To it 10 ml of 1:1 HCl and 5 drops of phenolphthalein indicator was added. The kjeldahl distillation assembly set up and 50 % NaOH added till the solution neutralizes. Pink color distilled off ammonia from the nitrogen. 50 ml boric acid contains 5 drops of mixed indicator and during the distillation boric acid mixture turns blue due to dissolution of ammonia. 150 ml distilled solution collected and titrate it with 0.02N H_2SO_4 until blue color changes to pink.

5. **Determination of Phosphate solubilizing capacity**

Efficiency of phosphate solubliser was checked by Inorganic phosphate estimation phospho molybdenum method. Standard quantitative inocula of each isolate obtained from nutrient broth were added into separate sterile fresh pikovasky's broth. It was incubated at room temperature for 8 days. After incubation 1.5 ml broth was withdrawn in to eppendroff tube from each isolate and it was centrifuged at 10000 rpm for 10 min.1ml supernatant was used for estimation . The estimation was carried out as follows:

A reaction mixture containing1ml supernatant of each isolate 3 ml acetate buffer, 0.5 ml ammonium molybdate (5%) and 0.5ml mettol (2%) was added in 1 mL eppendroff and was incubated for for 10 min at room temperature and after that optical density was measured at 880 nm on spectrophotometer. Unknown concentration was calculated from Standard graph of by Inorganic phosphate estimation phospho molybdenum method.

6. **Production of Indole acetic acid**

Indole acetic acid is a very essential plant growth harmone which elongates shoot of plant. Production of indole acetic acid from phosphate solublisers was studied. Standard quantitative inocula of each isolate obtained from nutrient broth was added into separate sterile fresh Czpek-Doxbroth.Assay was carried out by Salkowisk reagent method.10000 rpm for 10 min and used supernatant was separated this supernatant was used for assay, into this supernatant 2 drops of orthophosphoric acid,4 ml salkowisk's reagent was added and then tube were incubated for 30 min in dark. The concentration of compound was determined by measuring optical density at 530 nm. Unknown concentration was calculated from Standard graph of Indole acetic acid.

7. **Evaluating the potential of Phosphate solubilizers and Nitrogen fixers as fertilizers**

Result and Discussion

Cell density by nephalometer:

Table no. 2 indicates that the no. of organism "c" is more than other organisms.

	Optical density	No. of Bacterial volume	Bacterial no./100 ml
a	0.96	2380	2380×10^8
c	1.03	2500	2500×10^8
d	0.28	700	700×10^8
e	0.80	2000	2000×10^8
f	0.54	1350	1350×10^8
G	0.79	1950	1950×10^8
O	0.67	1650	1650×10^8
T	0.67	1650	1650×10^8

Gram staining of Phosphate solubilizing bacteria:

organism	Gram nature
PSB a	Yeast
PSB c	Yeast
PSB d	Yeast
PSB e	Yeast
PSB f	Yeast

Standard Plate Count of Nitrogen fixer and Phsphate solubilizer:

Formula used : $\dfrac{\text{No. of colonies} \times \text{dilution factor}}{\text{No.of dilution}}$

Table no. 3 : SPC of sample after one week:

a	c	d	E	F	G	O	T
220×10^8 CFU/ml	213×10^8 CFU/ml	945×10^8 CFU/ml	1313×10^8 CFU/ml	1256×10^8 CFU/ml	142×10^8 CFU/ml	1581×10^8 CFU/ml	1296×10^8 CFU/ml

SPC of sample after two weeks:

A	c	d	e	f	G	O	T
1290×10^8 CFU/ml	56×10^7 CFU/ml	318×10^8 CFU/ml	770×10^8 CFU/ml	533×10^8 CFU/ml	708×10^8 CFU/ml	120×10^8 CFU/ml	1312×10^8 CFU/ml

SPC of sample after three weeks:

A	C	d	e	f	G	O	T
91.6×10^8 CFU/ml	85×10^8 CFU/ml	86.6×10^8 CFU/ml	105×10^8 CFU/ml	63.3×10^8 CFU/ml	30×10^8 CFU/ml	42×10^8 CFU/ml	68.3×10^8 CFU/ml

Above tables shows that the count of viable cells is more after one week than two and three weeks, i.e.it would be good to apply fertilizer after one week.

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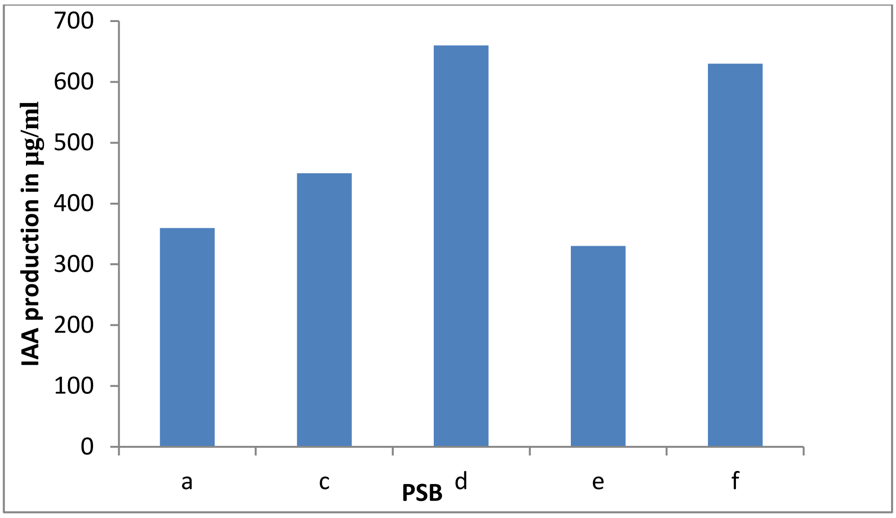
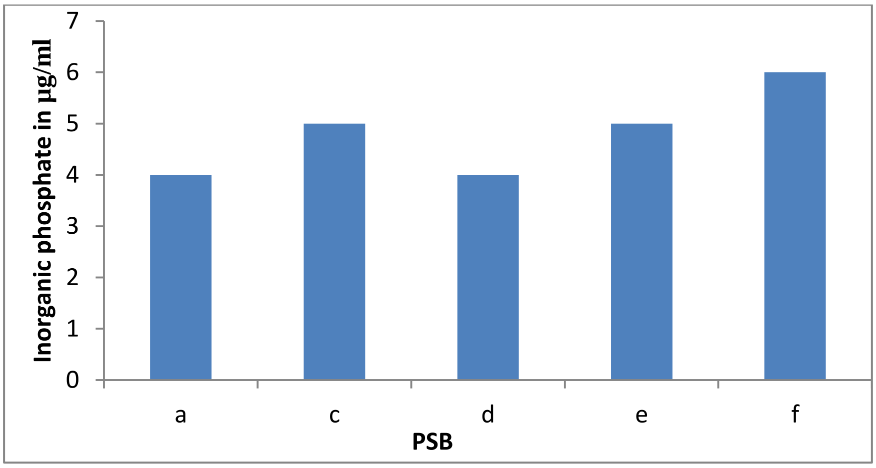
Evaluation of phosphate solubalizer for the production for Indol Acetic acid:



Indole acetic acid production property was checked for the phosphate solubalizing isolates. The efficiency of this phytoharmone production by this isolate was monitored by salkowisk's method for definite time of interval figure shows that the difference in product concentration. Whereas isolate'd' shows maximum yield of IAA. Hence this isolate was used for extraction of IAA.

Estimation of Inorganic phosphate



To check the potency of inorganic phosphate Solubilization by each phosphate solubalizer each isolate was monitored for its phosphate Solubilization efficiency as described in material method section it was observed that isolate *f* shows yield of 6 µg/ml of inorganic phosphate after incubation of 8 days.

Estimation of nitrogen fixation capacity by nitrogen fixing bacteria:

Formula used : $\frac{14 \times \text{Normality of acid} \times \text{titrate value}}{\text{wt of sample}}$

Isolates	Nitrogen fixation capacity in %
G	0.39
O	0.56
T	0.42

Table shows all nitrogen fixer fixes nitrogen in high amount (>0.2 %). Among them "O" fixes more nitrogen than other. (Bremner, 1965).

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Isolation of Potassium solubilising bacteria:

Isolation and screening Colonies exhibiting zone of clearance indicating Potassium solubilization were selected. The colonies were selected which were morphologically distinct. Total 05 bacterial isolates were isolated as potassium solubiliser.

Table-1: Potassium solubilization values of bacterial isolates by Khandeparkar's selection ratio

Isolates	Diameter of zone of clearance (D) mm	Diameter of growth (d) mm	D/d (ratio)
KSB 1	13	10	1.3
KSB 2	13	10	1.3
KSB 3	20	7	2.8
KSB 4	13	6	2.1
KSB 5	11	8	1.3

Conclusion:

The outcome of present study revealed that isolated microorganism from pressmud have ability to fix Nitrogen and also they are Phosphate Solubilizers .Some micro organisms have capacity of Potassium solubilisation and Indol Acetic acid secretion which helps to increase the fertility value of pressmud and growth of plant

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Conflicts of interest
The authors declare that there are no conflicts of interest regarding the publication of this paper.

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