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Application of *Acetobacter diazotrophicus* as a Biofertilizer to Improve the Qualitative and Quantitative Characteristics of Sugarcane Crop

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Abstract **– Present study was carried out to study the effectivity of *Acetobacter diazotrophicus* as a bio fertilizer to increase sugarcane yield. *A. diazotrophicus* are endophytic bacteria capable of synthesizing IAA growth hormone and nitrogen fixation. Endophytes are plant-associated prokaryotes that form association with their host plants by colonizing the internal tissues, which has made them valuable tool in agriculture for improving crop performance. Isolation was carried out from sugarcane stem by using LGI medium. Morphological and biochemical characterization of the isolates was performed. Inoculum build up and fermentation of A. diazotrophicus was performed for mass production. Two sugarcane varieties were used for this study viz. CO-86032, CO-671. Both varieties were cultivated by giving set treatment of A. diazotrophicus against a control field. A. diazotrophicus treated varieties were found to have greater internode length, more sugar content and greater yield as compare to the control field. The data obtained in this study could be utilized for further studies for development of A. diazotrophicus as an effective bio fertilizer for sugarcane crop.**

Keywords **– *A. diazotrophicus*, Endophytes, Bio Fertilizer, IAA, LGI Medium, Internode Length, Sugar Content.**

I. INTRODUCTION

In the recent years various chemical fertilizers are being extensively used in sugarcane cultivation. This is ultimately leading to increase in cost of production and soil pollution. The nutrient demand of Sugarcane crop is very high. It requires 450 kg/ha N, 170 kg/ha P, and 170 kg/ha K during its life cycle. Farmers apply heavy dose of chemical fertilizers in their field in sugarcane cultivation. Overuse of these fertilizers is leading to disturbed soil structure, reduced soil fertility, poor water holding capacity, reduction in soil microbial population, decrease in yield (Ref. No. 1). The organic philosophy, standard and practices restrict the use of chemicals and external inputs. As part nitrogen fixing bacteria could be used as bio fertilizers. Although several nitrogen fixing bacteria have been isolated from sugarcane, it still remains unknown which of these bacteria are the most important in the plant associated biological nitrogen fixation. Several field experiments have shown the efficiency of *A. diazotrophicus* as nitrogen fixer (Ref. No. 2). They could show well growth on different pH ranging from 3 to 6 and they are able to fix nitrogen below the pH 3. These bacteria could tolerate higher nitrate concentrations. They could utilize higher sugar concentration. All these factors make *A. diazotrophicus* most suited to sugar rich environment of cane crop (Ref. No. 3). They could transfer forty percent of fixed nitrogen immediately to the surrounding plant tissue (Ref. No. 4). In addition they could synthesize phytohormone such as IAA. This study is carried out to check the efficiency of *A. diazotrophicus* to fix the nitrogen and their ability of IAA synthesis and their effect on yield of sugarcane crop.

II. MATERIALS AND METHODS

Isolation was carried out from sugarcane stem. Samples were collected in sterile bags from sugarcane field of Latur district of Marathwada region Maharashtra. Collected samples were cut into small pieces and surface

sterilized initially with 1% bavistin solution and then with 0.1% HgCl2. 2 to 3 wash of sterile distilled water was given to remove any traces of disinfectant. All this procedure was carried out aseptically in laminar air flow cabinet.

2.1. *Media and Cultural Conditions: LGI Medium was used for the Isolation of A. diazotrophicus.*

Composition: Sucrose (6.0gm), K2HPO4 (0.2gm), KH2PO4 (0.6gm), $MgSO4.7H_2O$ (0.2gm), $CaCl_2.2H_2O$ (0.02gm), $Na2MoO4.2H_2O$ (0.02gm), Bromothymol blue (0.5%) 2ml, $FeCl_2$ (0.010gm), Agar (18gm), Vitamin Solution (1.5ml). pH was adjusted to 6. After surface sterilization small pieces of sugarcanes were crushed into sterilized mortar and pestle. The supernatant was inoculated on to solid LGI media with the help of spreader. The inoculated LGI plates were incubated at 30^0C for 8 to 10 days.

2.1.1. *Isolation:*

After eight days large, pale yellow colored colonies were observed (Ref. No. 9). These colonies were spot inoculated on fresh LGI medium.

2.2. *Morphological Test:*

Gram staining was performed to confirm the morphological characteristics of isolates that has been studied previously (Ref. No. 10). Suspension was prepared by suspending a colony in saline. A loopful of suspension was smeared on a clean slide. It is then air dried, heat fixed and stained by Gram's staining method. The Gram character and morphology were observed under light microscope using oil immersion lens.

2.3.1. *Antibiotic Sensitivity:*

Susceptibility of *Acetobacter* isolates to antibiotics was tested on LGI medium containing antibiotics (Penicillin-G 10 IU, Streptomycin 30 µg/ml and Rifampicin 40 µg/ml).

2.3.2. *Nitrate Reduction:*

Nitrate reducing ability of *Acetobacter* isolates was determined in nutrient broth supplemented with KNO3 (0.1% W/V). Saline suspension of the isolate was incubated into the medium and incubated for 3 days at 30'C. The test was detected by addition of 0.4 ml of the reagent freshly prepared. Equal volumes of each, Griess-Illosvay reagent I and II were mixed to prepare the test reagent. The tubes were observed for formation of the red color.

2.3.3. *Utilization of Various Alcohols and Organic Acids the Sole Source of Carbon:*

The ability of isolates to utilize different carbon sources was tested by incorporating these separately into carbon utilization agar. Isolates were inoculated on the medium and incubated at 30^oC. Results were recorded after 5 days by comparing growth with that on unsupplemented basal media and on positive control media. Carbon compound included in the medium under study were alcohols viz. ethanol and methanol and salts of organic acids viz. sodium acetate and sodium citrate compound concentration taken was 1% W/V.

2.3.4. *Sugar Fermentation:*

The ability of *Acetobacter* isolates to ferment various sugars into acids was tested by incorporating these sugars into LGI agar devoid of cane sugar. Isolates were spot inoculated on the medium and incubated at 30^0C

for *Acetobacter*. Bromothymol blue was the pH indicator present in the medium. Acid production was recorded after 5 days by comparing change in the color of the medium with that on supplemented basal media and on positive control containing sucrose. Sucrose tested for *Acetobacter* isolates were D-glucose, sucrose, mannitol, lactose, maltose. Concentration taken was 1% W/V.

2.4. *Mass Production:*

Fermentation was carried out for the mass production of *A. diazotrophicus*. A loop full culture was transferred into the freshly prepared LGI broth and kept for incubation at 30^0C for five days in shaking incubator. After five days turbidity was observed in the broth. Freshly prepared LGI medium was used for the mass production of *Acetobacter* Biofertilizer. Fermentation was carried out at 30^0C and pH of the medium was adjusted to 6.0. Inoculum was transferred aseptically into the fermenter vessel. After four days sterilized glycerin was added as cell protectant. Product was harvested after five days of inoculation and packed aseptically in sterile bottle.

2.4.1. *Set Treatment:*

Healthy, disease free sugarcane sets of CO-86032, CO-671, varieties were selected for plantation. Sugarcane sets were treated before planting by dipping in previously prepared *Acetobacter* biofertilizer. Internode length was recorded first after two months from planting of both the varieties CO-671 and CO-86032 and that of control. And then subsequently every after every month the internode length was recorded. And compared it with control (Ref. No. 6). At the time of maturity sugar content of treated and control varieties was calculated with the help of Brix saccharometer.

III. RESULTS AND DISCUSSION

Isolation of *A. diazotrophicus* was carried out from sugarcane stem. After 8 days sticky, pale colored colonies were observed on the LGI plates. Morphological characterization was done to confirm the genus of isolates. Gram staining was performed to study the gram character and morphology of isolates. Under light microscope using oil immersion lens isolates were found short, slightly curved and pink colored which confirm gram negative character. To study biochemical characteristics different tests were performed. Antibiotic sensitivity test was carried out by supplementing media with penicillin-G, streptomycin and rifampicin. Results were recorded after 4 days. (Table No. 1) it was observed that isolates were showing resistance to Penicillin-G and Streptomycin (slight growth observed) while sensitivity was observed for rifampcin where growth was not observed. Nitrate reducing ability of *Acetobacter* was studied by supplementing nutrient broth with KNO_3 (0.1% w/v). (Fig. No. 4) the tubes were showing formation of red color after the addition of Griess-Illosvay reagent I and II against the control where color formation was not observed. The ability of isolates to utilize various alcohols, sugars and organic acid as the sole source of carbon was studied (Fig. No. 3). Carbon compound supplemented in the medium for this study were alcohol, organic acids, sugars (Table No. 2). Plates containing ethanol were showing positive growth while methanol containing plate did not showed the growth of *Acetobacter*. In organic acid plate containing sodium acetate showed the positive growth and sodium citrate showed negative growth. LGI medium was supplemented with various sugars viz. D-glucose, sucrose, maltose, D-mannitol, lactose and results were recorded after 5 days. Isolates had showed positive growth on medium supplemented with D-glucose, D-fructose, Mannitol, Sucrose and Meso-Inositol. Set treatment was given using two sugarcane varieties. As shown in fig. No. 1 the treated varieties showed the increased internode length as

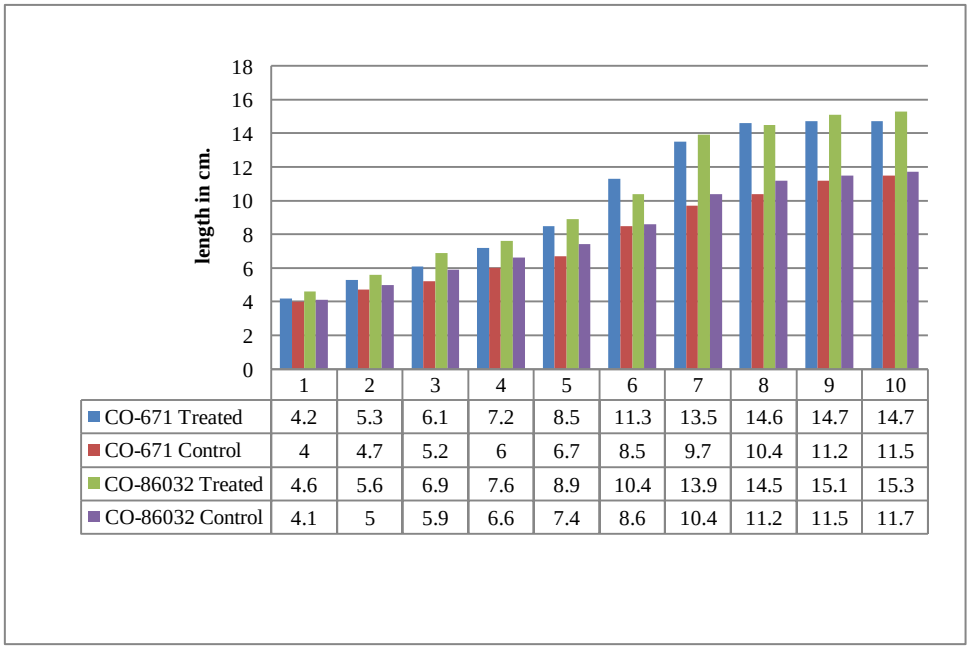
compare to control. We found tremendous increase in internode length of treated varieties after six months and onward as compared to control. The normal range of sugar content at the time of maturity in sugarcane should be 21-24^0 brix. Whereas the sugar content in sugarcane crop in Marathwada region was observed in the range of 17-19^0 brix. The *Acetobacter* treated sugarcane varieties CO-671 and CO-86032 selected for this study showed increased amount of sugar content 21.3^0 brix and 22.2^0 brix respectively. Sugar content in control CO-671 was found to be 17.60 brix and control CO-86032 was 180 brix. (Fig. No.2).

Table 1. Antibiotic Sensitivity Test.

Sole Carbon Source	Result
D-Glucose	Positive (+)
D-Fructose	Positive (+)
Sucrose	Positive (+)
Meso-Inositol	Positive (+)
Glycerol	Positive (+)
Ethanol	Positive (+)
Methanol	Negative (-)
Sodium Acetate	Positive (+)
Sodium Citrate	Negative (-)

Table 2. Sole Carbon Utilizing Test.

Antibiotic	Result
Penicillin-G	Negative
Streptomycin	Negative
Rifampicin.	Positive



	1	2	3	4	5	6	7	8	9	10
CO-671 Treated	4.2	5.3	6.1	7.2	8.5	11.3	13.5	14.6	14.7	14.7
CO-671 Control	4	4.7	5.2	6	6.7	8.5	9.7	10.4	11.2	11.5
CO-86032 Treated	4.6	5.6	6.9	7.6	8.9	10.4	13.9	14.5	15.1	15.3
CO-86032 Control	4.1	5	5.9	6.6	7.4	8.6	10.4	11.2	11.5	11.7

Fig. 1. Internode Length Comparison.

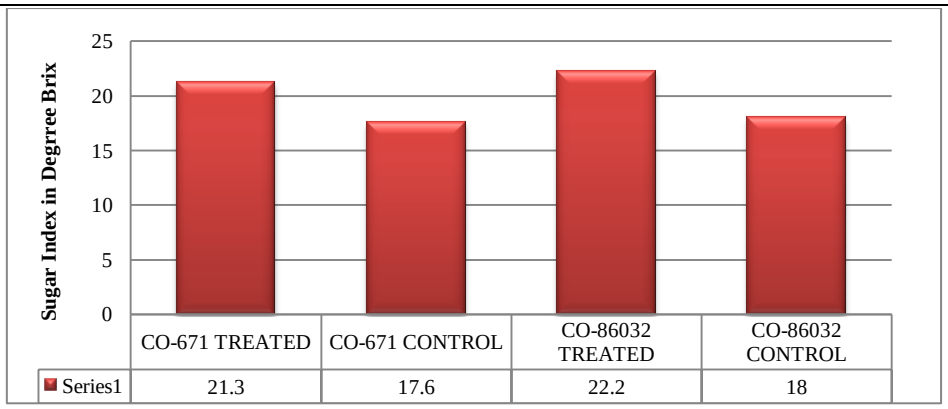


Fig. 2. Sugar Content Comparison.

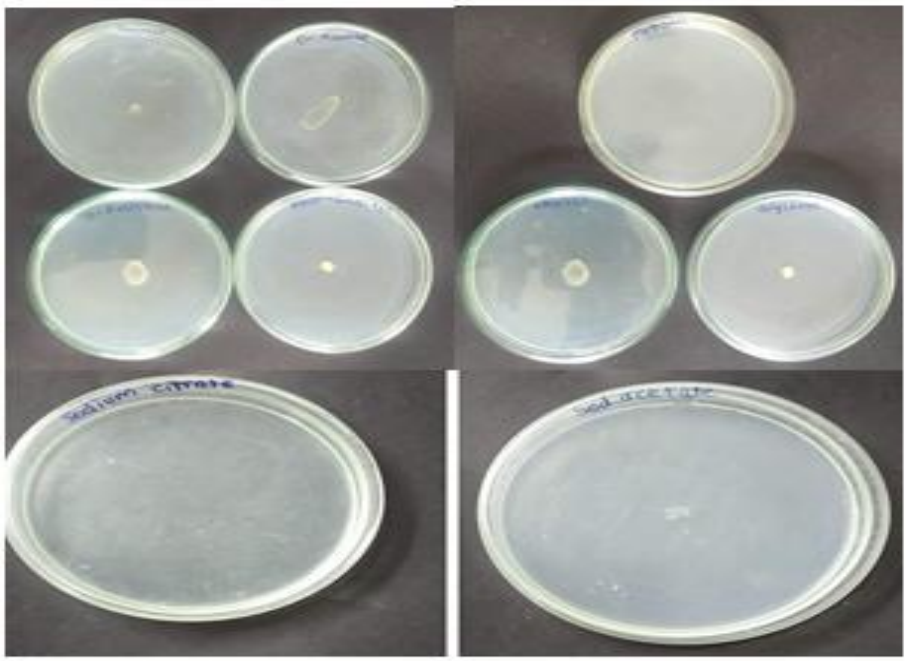


Fig. 3. Utilization of various Sugar, Alcohols, and organic acids.

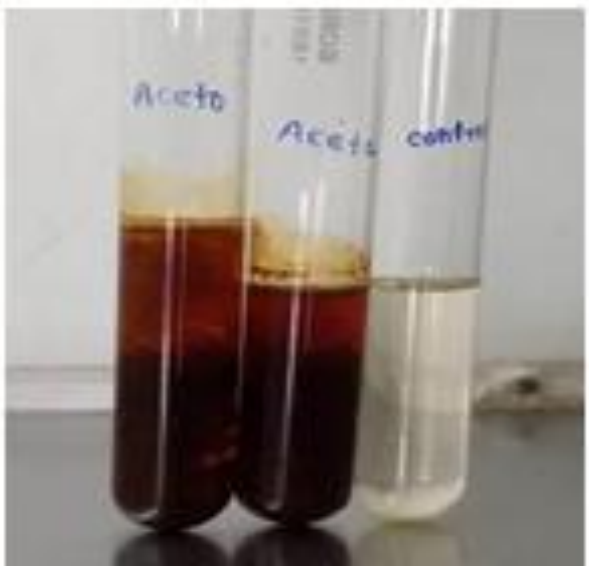


Fig. 4. Nitrate reduction test.

Fig. 4. Internode length.

IV. CONCLUSION

The endophytic bacteria isolated from sugarcane stem were *Acetobacter diazotrophicus.* LGI medium with given concentration was found to be effective as selective medium for the isolation of *Acetobacter diazotrophicus.* These species of endophytic bacteria were able to fix the atmospheric nitrogen to meet the nitrogen demand of sugarcane varieties used in this study. Application of *Acetobacter diazotrophicus* as biofertilizer has showed improved qualitative and quantitative characteristics in both CO-671 and CO-86032 sugarcane varieties evidenced by the field trial results.

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