

Original Research Article

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Usage of Banana Pseudostem Waste for the Production of Potassic Biofertilizer using Cellulolytic Bacteria

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ABSTRACT

The present study aims to use banana pseudostem waste for the production of potassic biofertilizer using cellulolytic bacteria. Ten cellulolytic bacteria were isolated from soil samples collected from the College campus. After preliminary tests and biochemical tests, the cellulolytic bacteria were identified. The bacterial isolate which showed maximum cellulolytic activity (D8) was found to be *Bacillus aerius* by 16s rRNA sequencing. Its 16s rRNA sequence was submitted to Genbank and the accession number is KX530930. Appreciable amount of cellulolytic activity were shown by five different bacterial genera which were identified as *Bacillus circulans* (C3), *Pseudomonas stutzeri* (C4), *Serratia rubidaea* (C6), *Bacillus subtilis* (D1) and *Bacillus pumilus* (D2). These isolates were used alone and in consortium to degrade the banana pseudostem waste by solid state fermentation to obtain the fermented liquid. Analysis of the potassium content of the liquid by ICP-MS indicated that the potassium content is directly proportional to the cellulolytic activity of the bacteria. Tests done to check the effect of fermented liquid on plant growth revealed good increase in root length, shoot length, fresh weight, dry weight and chlorophyll content of fenugreek plants.

Keywords

Cellulolytic bacteria, Banana, Pseudostem, Solid state fermentation, Potassium.

Article Info

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Introduction

India is an agriculture based country. Agriculture along with fisheries and forestry is one of the largest contributors of Gross Domestic Product (GDP). Proper management and disposal of agro waste is necessary to prevent various environmental issues.

Agro waste is defined as the waste which is produced from various agricultural activities. Accumulation of agro wastes may cause health, safety, environmental and

aesthetic concern. (Lim and Matu, 2015) Agro wastes are rich in nutrients and can be used to improve soil fertility.

Cellulose is the major component of agro waste. It is an abundant natural polymer on earth. It is a linear polysaccharide of β -D-glucose residues with β -1, 4-glycosidic linkages. Cellulolytic bacteria are those bacteria that convert cellulose into soluble sugar. The complete degradation of cellulose is made by a cellulolytic enzyme system. It involves three enzyme systems. They are

endoglucanases, exoglucanases and β -D-glucosidase. Endoglucanases act internally on the chain of cellulose cleaving β -linked bond liberating non reducing ends. Exoglucanases removes cellobiose from this non reducing end of cellulose chain. β -D-Glucosidase splits cellobiose into glucose molecules.

Banana (*Musa sp*) is the second most important fruit crop in India. India leads the world in banana production with an annual output of about 14.2 million tonnes. The part of the plant which looks like a trunk is actually a false stem (pseudostem). It contains 50-60% of cellulose. According to the literature survey done, much attention was not paid on banana pseudostem as a source of potassium, although it contains 5.6-6.2% of potassium (Saibaba *et al.*, 2013).

The principle behind the present study is that cellulose degrading bacteria can be isolated from soil and can be allowed to degrade the cellulose in banana pseudostem waste by solid state fermentation. In this process, the potassium which is bound to cellulose fibres gets released and gets dissolved in the fermented liquid. The fermented pseudostem waste liquid contains high amount of potassium. This solution also contains cellulolytic bacteria. It can be used as liquid potassic biofertilizer. The potassium present in the liquid promotes plant growth and the cellulolytic bacteria will degrade other organic matter in soil and release various macronutrients and thereby promotes plant growth.

Using this simple and efficient method Banana pseudostem which is considered to be an agricultural waste can be routed back to enhance the agricultural productivity. There are many constraints in using potassium solubilizing bacteria (KSB) as potassic biofertilizers. Thus, Potassic

biofertilizer produced using cellulolytic bacteria with banana pseudostem waste as substrate can be used as an alternative.

Materials and Methods

Soil sample collection

Soil samples were collected from two different places in the college campus and they were labelled as soil sample 1 and soil sample 2 respectively. The soil samples were collected in a sterile petri plate and were aseptically brought to the lab.

Isolation of cellulolytic bacteria

Serial dilution and spread plating

1g of soil sample was taken and it was serially diluted using decimal dilution technique. 0.1 ml from 10^{-7} , 10^{-8} and 10^{-9} dilutions were inoculated onto sterile nutrient agar plates by spread plating. The plates were incubated at 37°C for 24 hours. The colonies with varied morphology were selected and were streaked on to separate nutrient agar plates. All the plates were incubated at 37°C for 24 hours.

Congo Red Assay

Congo red assay was carried out according to the method described by Kaur and Arora, (2012). This method serves as primary screening to isolate cellulolytic bacteria. The isolates that show positive results for primary screening were selected and were taken to secondary screening. Negative isolates were discarded.

Secondary Screening

CMCase assay serves as secondary screening for the determination of cellulolytic activity. It was done according to the method described by Khan *et al.*, 2011.

One unit of CMC_{ase} is expressed as micromoles of glucose liberated per ml enzyme used per minute.

Identification of cellulolytic bacteria

Identification of isolates

The isolates were subjected to preliminary tests such as gram staining, motility test, catalase test, oxidase test and endospore staining. Biochemical tests such as IMViC TEST, TSI test Urease test, Nitrate test, Carbohydrate fermentation test, PDA test, Arabinose test and Starch hydrolysis test were done. Growth of the isolates in 6.5% NaCl, basal media, differential media and selective media were checked. Based on the results of these tests the organisms were identified using Bergey's Manual of Determinative Bacteriology and Bergey's Manual of Systematic Bacteriology.

Identification of the best cellulolytic bacteria by 16 s rRNA sequencing

The identification of the isolate with maximum cellulolytic activity was confirmed by 16s rRNA sequencing. The protocol provided is as follows:

DNA Extraction

Bacterial Genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit – As per the kit instruction below procedure followed. An isolated bacterial colony was picked and suspended in 1ml of sterile water in a microfuge tube. It was centrifuged for 1 minute at 10,000–12,000 rpm to remove the supernatant. 200 µl of Insta Gene matrix was added to the pellet and was incubated at 56 °C for 15 minutes. It was vortexed at high speed for 10 seconds and the tube was placed in a 100 °C in heat block or boiling water bath for 8

minutes. Finally, the contents were vortexed at high speed for 10 seconds and were spinned at 10,000–12,000 rpm for 2minutes. 20µl of the supernatant was used per 50 µl PCR reaction.

PCR Protocol

Using 16s rRNA Universal primers, gene fragment was amplified using MJ Research Peltier Thermal Cycler. Universal primer details were shown in table 1.1µL of template DNA was added in 20 µL of PCR reaction solution. 27F/1492R primers were used for bacteria and then PCR reaction was performed with below conditions:

Initial Denaturation 94°C for 2 min and then 35 amplification cycles at 94°C for 45 sec, 55°C for 60sec, and 72°C for 60 sec. Final Extension at 72°C for 10 min. DNA fragments are amplified about 1,400bp in the case of bacteria. A positive control (*E.coli* genomic DNA) and a negative control were included in the PCR.

Purification of PCR products

Unincorporated PCR primers and dNTPs were removed from PCR products by using Montage PCR Clean upkit (Millipore).The PCR product was sequenced using the 518F/800R primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol

Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The fluorescent-labelled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were

resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems). Sequencing primer details were shown in table 2.

Bioinformatics protocol

The 16s r RNA sequence was blast using NCBI blast similarity search tool. The phylogeny analysis of our sequence with the closely related sequence of blast results was performed followed by multiple sequence alignment.

The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data, while being one order of magnitude faster. The program Tree Dyn 198.3 was used for tree rendering (Dereeper *et al.*, 2008)

Usage of Cellulolytic Bacteria to Degrade Banana Pseudostem Waste (Dabhi *et al.*, 2014)

Preparation of Banana Pseudostem Waste (Dabhi *et al.*, 2014)

Banana pseudostem waste was dried in sunlight. It was cut into 2 cm pieces.

Inoculum Selection and Preparation (Dabhi *et al.*, 2014)

Best 5 isolates of cellulolytic bacteria (C3, C4, C6, D2 and D8) were selected among

the 10 isolated cellulolytic bacteria on the basis of primary screening and secondary screening. A loop full of broth culture of the 5 cellulolytic bacterial isolates were separately inoculated into CMC broth tubes (5ml).

A consortium (a loop full of the best 5 cellulolytic bacteria) was also inoculated separately into CMC broth tubes (5 ml). All the tubes were incubated at 37°C for 48 hours. After 48 hours, the culture in these tubes serves as inoculum.

Solid State Fermentation

(Dabhi *et al.*, 2014), (Lim and Matu, 2015) 6.25 g of banana pseudostem waste and 18.75 ml of distilled water was added to 250 ml conical flask. It was cotton plugged and was sterilized by autoclaving. After cooling, 5ml of inoculum was added to it and was incubated at 37°C for 40 days.

Recovery of Fermented Banana Pseudostem Waste (Lim and Matu, 2015)

The fermented banana pseudostem waste was recovered by filtering the fermented waste using fabricated filters.

Biofertilizer Analysis (Lim and Matu, 2015)

pH Analysis

The purpose of the pH analysis is to find out whether the pH of the biofertilizer can support plant growth. pH analysis was done using pH meter.

Potassium Content Analysis

The Potassium content of Fermented Banana Pseudostem Waste Liquid (FBPWL) samples were analysed using Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS).

Efficiency of FBPWL on Fenugreek Plants

The efficiency of FBPWL as liquid potassic biofertilizer was checked by pot trials using fenugreek plants.

Seed Treatment (Lim and Matu, 2015)

Fenugreek seeds were soaked in FBPWL for 24 hours and they were sown in pots. Seeds soaked in FBPWL-Consortium were sown in pots labelled CONSORTIUM. Similarly seeds soaked in FBPWL-D8, FBPWL-D2, FBPWL-C4, FBPWL-C6 and FBPWL-C3 were sown in pots labelled D8, D2, C4, C6 and C3 respectively. Duplicates were made. Untreated seeds were sown in control pot. All the pots were kept in open space under sunlight. The plants were watered daily. Their growth was monitored for 3 weeks.

Plant Growth Analysis (Kapoor and Pande, 2015)

The root length, shoot length, fresh weight and dry weight of the plants were measured. The chlorophyll content of the leaves were measured.

Results and Discussion

Isolation of cellulolytic bacteria

Spread plating

From soil sample 1, thirteen colonies with varied morphology were isolated. They were named as C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11, C12 and C13 respectively. From soil sample 2, eight colonies with varied morphology were isolated. They were named as D1, D2, D3, D4, D5, D6, D7 and D8.

Congo Red Assay

In Congo red assay with CMC agar, isolates C3, C4, C6, C9, C10, C11, C12, D1, D2 and

D8 formed the zone of hydrolysis. Thus by Congo red assay with CMC agar, isolates C3, C4, C6, C9, C10, C11, C12, D1, D2 and D8 were identified as cellulolytic bacteria. The isolates that did not form the zone of hydrolysis were discarded. (i.e. Isolates C1, C2, C7, C8, C11, C13, D3, D4, D5, D6 and D7 were discarded). Its results were shown in table 3 and chart 1. The plate showing the zone of hydrolysis was shown in figure 2.

CMCase Assay

Among the isolates tested, D8 showed the highest enzyme concentration. The isolates in the descending order of CMCase production is as follows: D8, D2, C4, C6, C3, D1, C10, C9, C11 and C12. Its results were shown in table 3 and Chart 2.

Identification

On the basis of the results of preliminary tests and biochemical tests, the identification of isolates was made with reference to the Bergey's Manual of Determinative Bacteriology and Bergey's Manual of Systematic Bacteriology. C3 was identified as *Bacillus circulans*, C4 as *Pseudomonas stutzeri*, C6 as *Serratia rubidaea*, C9 as *Bacillus simplex*, C10 as *Bacillus coagulans*, C11 as *Pseudomonas fragi*, C12 as *Bacillus megaterium*, D1 as *Bacillus subtilis*, D2 as *Bacillus pumilus* and D8 as *Bacillus aerius*. The colony morphology of the isolates were shown in figure 1.

Confirmation of the Identification of the Best Cellulolytic Bacteria By 16s Rrna Sequencing

Among the 10 isolates, D8 was found to have the highest cellulolytic activity. By 16s rRNA sequencing D8 was identified as *Bacillus aerius*. Its phylogenetic tree was shown in figure 4. The 16s rRNA sequence of *Bacillus aerius* D8 was submitted to

Genbank and the accession number is KX530930.

Solid State Fermentation of Banana Pseudostem Waste with Cellulolytic Bacteria

Softening and water release was seen in the inoculated flasks. Water release occurred due to hydrolysis of cellulose in the banana pseudostem waste. Softening and water release was not seen in the control flask (control). After 40 days, 30-35ml of fermented liquid was recovered from each inoculated flask. The control yielded 7ml of liquid.

Bio Fertilizer Analysis

The results of pH analysis and potassium content analysis of FBPWL by Inductively coupled Plasma Mass Spectrophotometer were shown in table 4 and chart 3.

Pot Analysis

When compared to the FBPWL treated plants the germination and growth of the untreated plants were delayed. The results of plant growth analysis were shown in table 5 and chart 4. The root length and shoot length of the plants were shown in figure 3.

Selection of agrowaste

To produce a biofertilizer from agrowaste using microbial action, three factors have to be considered. They are as follows: 1. Type of bio fertilizer to be produced, 2. Type of agrowaste and 3. Type of culture. On the basis of the type of biofertilizer need to be produced, the agrowaste has to be selected and on the basis of the chemical and physical properties of the agrowaste the culture has to be selected. The objective of the present study is to produce potassic bio

fertilizer from agro waste. Banana pseudostem waste is the most abundant agro waste in India. It is rich in potassium (Li *et al.*, 2010; Saibaba *et al.*, 2013 and Kinekar, 2010). So it has been chosen for the study. Cellulose is the major component of banana pseudostem waste so cellulolytic bacteria is used to degrade the cellulose in banana pseudostem and thereby to release the potassium bound to it. Moreover cellulolytic bacteria are one of the common soil bacteria.

Isolation of Cellulolytic Bacteria from Soil

The present study, proves for the first instance, that *Bacillus circulans*, *Serratia rubidaea*, *Bacillus simplex*, *Pseudomonas fragi* and *Bacillus aerius* also possess cellulolytic activity. The isolated cellulolytic bacteria were the most common cellulolytic bacteria present in the soil. *Bacillus aerius* is an organism that has been discovered in 2006 by Shivaji *et al.*, 2006. It has been isolated from cryogenic tubes used for collecting air samples from high altitudes.

Till date it has not been isolated from any other source. In this study, it has been isolated from soil sample and was found to have the highest cellulolytic activity among the isolates.

Congo Red Assay and CMC Case Assay

The results of Congo red assay matches with the results of CMC Case assay. Isolate D8 (*Bacillus aerius*) was found to have the maximum cellulolytic activity in Congo red assay. Its zone diameter was 3.2 cm. In CMC Case assay, isolate D8 (*Bacillus aerius*) showed maximum cellulase production of 0.43 U/ml/min.

Solid State Fermentation of Banana Pseudostem Waste with Cellulolytic Bacteria

Softening and water release was seen in the inoculated flasks. Water release occurred due to hydrolysis of cellulose in the banana pseudostem waste.

Potassium Content of FBPWL

On analysing the results of potassium content of Fermented Banana Pseudostem Waste Liquid (FBPWL), it was found that as the cellulolytic activity increases, the potassium content in the fermented liquid also increases. Cellulolytic activity is proportional to the potassium content in the fermented liquid.

Isolate D8 has the highest cellulolytic activity among the isolates and its respective FBPWL has the highest potassium content of 10470 ppm. The potassium content of FBPWL of consortium (11400 ppm) is greater than that of FBPWL of D8 (10470 ppm).

This indicates that the organisms D8 (*Bacillus aerius*), D2 (*Bacillus pumilus*), C4 (*Pseudomonas stutzeri*), C6 (*Serratia rubidaea*) and C3 (*Bacillus circulans*) can coexist. As combined effect of the cellulolytic activity of these organisms is more, the potassium content in FBPWL-Consortium is more. When the potassium content of the liquid obtained from control was analysed, it was found that it contains 1300 ppm of potassium. The potassium

release in the control would have occurred due to diffusion. As the potassium in banana pseudostem waste is bound to the cellulosic fibres (Li *et al.*, 2010), it is difficult to remove the potassium from it without loosening the cellulosic fibres. Thus cellulolytic activity has loosened the cellulose fibres in banana pseudostem waste and have resulted in release of high amount of potassium in fermented liquid.

FBPWL As biofertilizer

The fermented banana pseudostem waste contains potassium and cellulolytic bacteria. Potassium will improve plant growth and cellulolytic bacteria being a normal flora of the soil will help in degrading the cellulosic wastes in the soil and release the bound nutrients and there by increases the growth of plants. The isolated cellulolytic bacteria were the most common cellulolytic bacteria present in the soil.

On analysing the results of plant growth studies with Fermented Banana Pseudostem Waste Liquid, it was found that as the potassium content in FBPWL increases, the shoot length, root length, fresh weight, dry sweight and chlorophyll content also increases. Plants grown with FBPWL-Consortium showed the highest shoot length, root length, fresh weight, dry weight and chlorophyll content among the plants under study.

Table.1 Universal Primer Details

Primer Name	Sequence Details	Number of Base
27F	AGAGTTTGATCMTGGCTCAG	20
1492R	TACGGYTACCTTGTTACGACTT	22

Table.2 Sequencing Primer Details:

Primer Name	Sequence Details	Number of Base
785F	GGATTAGATACCCTGGTA	18
907R	CCGTCAATTCMTTTRAGTTT	20

Table.3 Results of Congo Red Assay and CMCase assay

Isolate	C3	C4	C6	C9	C10	C11	C12	D1	D2	D8
Zone Diameter (Cm)	2.5	2.8	2.7	0.9	1	0.9	0.7	2	3	3.2
Enzyme Concentration (U/ml/min)	0.17	0.23	0.17	0.10	0.14	0.04	0.03	0.16	0.31	0.43

Table.4 Results of Biofertilizer analysis

Sample	pH	Potassium Content (ppm)
FBPWL-CONSORTIUM	7.02	11400
FBPWL-D8	7.18	10470
FBPWL-D2	7.23	10025
FBPWL-C4	7.30	7350
FBPWL-C6	7.31	6500
FBPWL-C3	7.45	6300
FBPWL-CONTROL	8.45	1300

Table.5 Results of Plant growth analysis

PLANT	SHOOT LENGTH (cm)	ROOT LENGTH (cm)	FRESH WEIGHT (g)	DRY WEIGHT (g)	CHLOROPHYLL CONTENT (mg/g)
PLANT-FBPWL-CONSORTIUM	16.9	8.3	15.2	1.24	3.669
PLANT-FBPWL-D8	16.0	8.0	13.9	1.02	3.438
PLANT-FBPWL-D2	15.5	7.2	12.7	0.98	2.995
PLANT-FBPWL-C4	14	6.9	10.9	0.94	2.862
PLANT-FBPWL-C6	13.7	6.7	10.6	0.96	2.862
PLANT-FBPWL-C3	11.2	5.0	9.7	0.90	2.036
PLANT-FBPWL-CONTROL	9.0	4.2	7.8	0.62	1.697

Chart.1 Results of Congo Red Assay

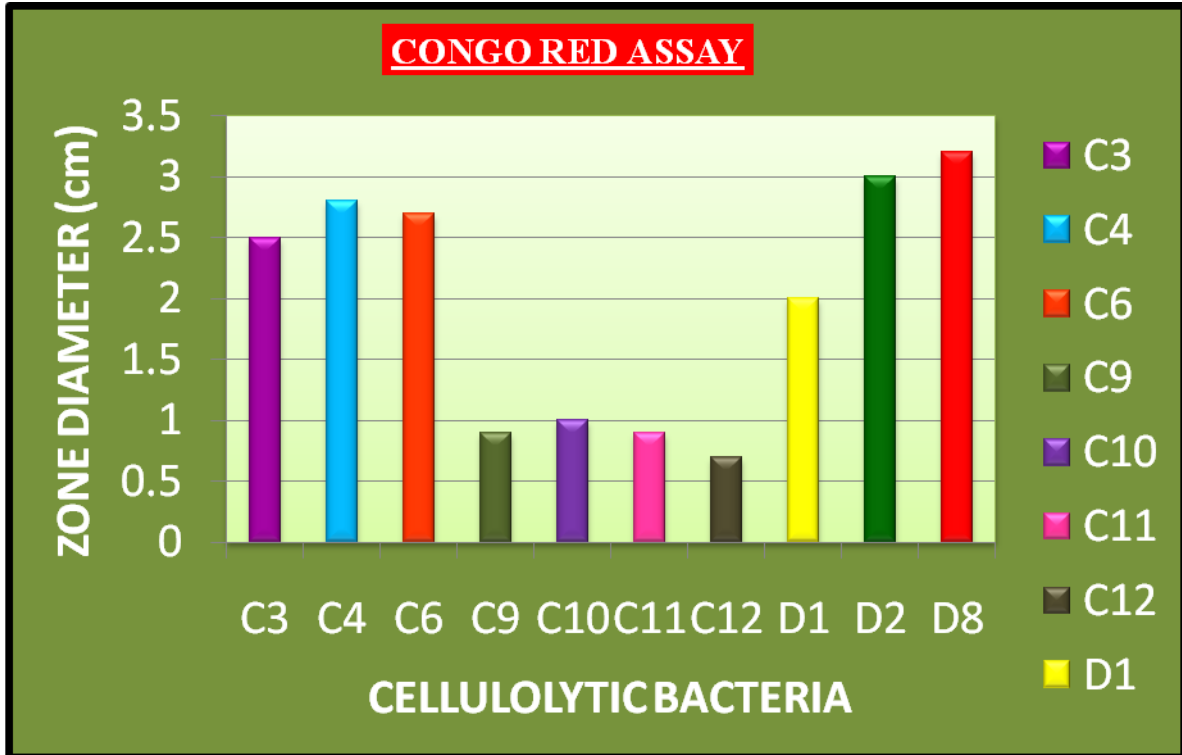


Chart.2 Results of CMCase Assay

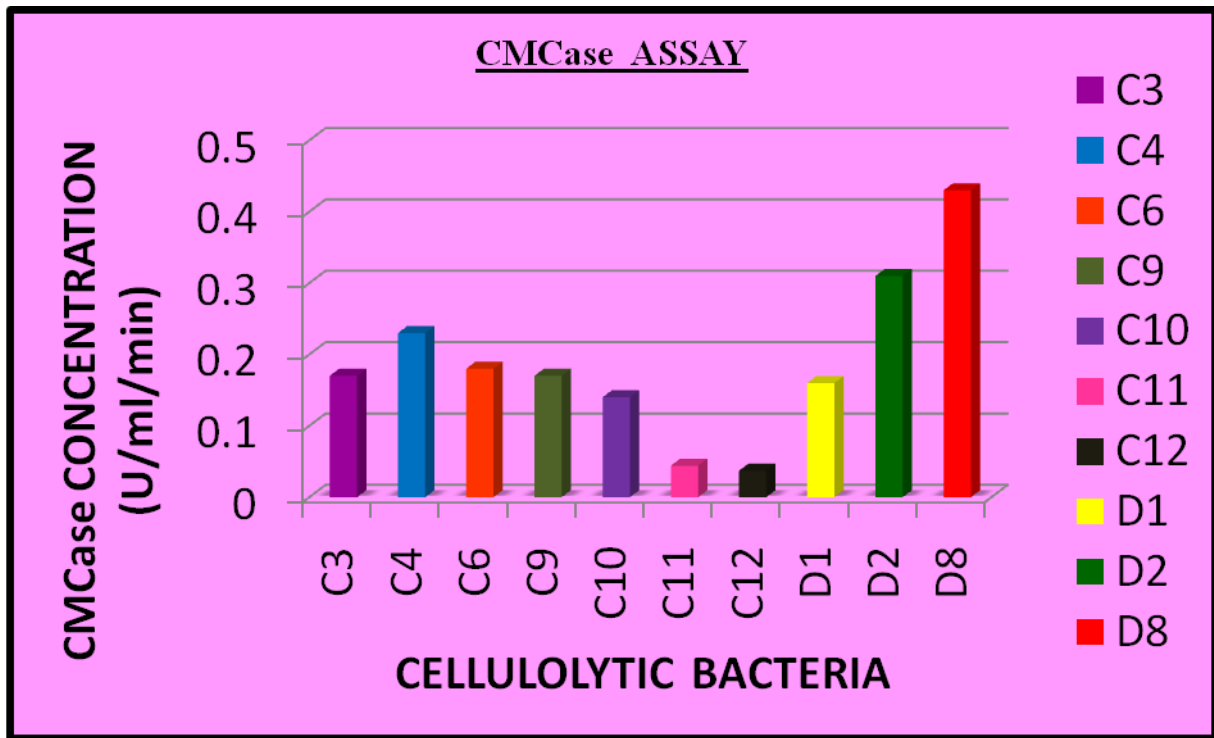


Chart.3 Results of Inductively coupled Plasma Mass spectroscopy Analysis

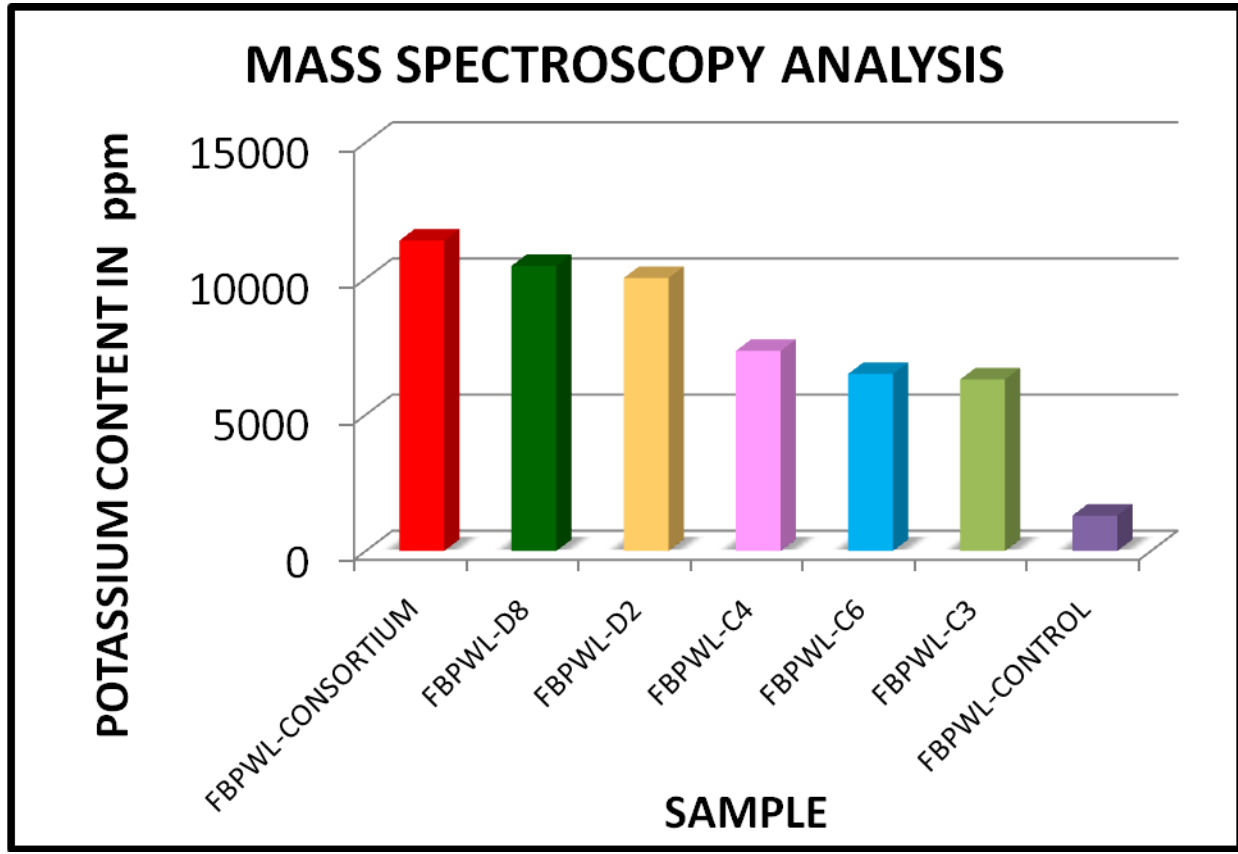


Chart.4 Results of plant growth analysis

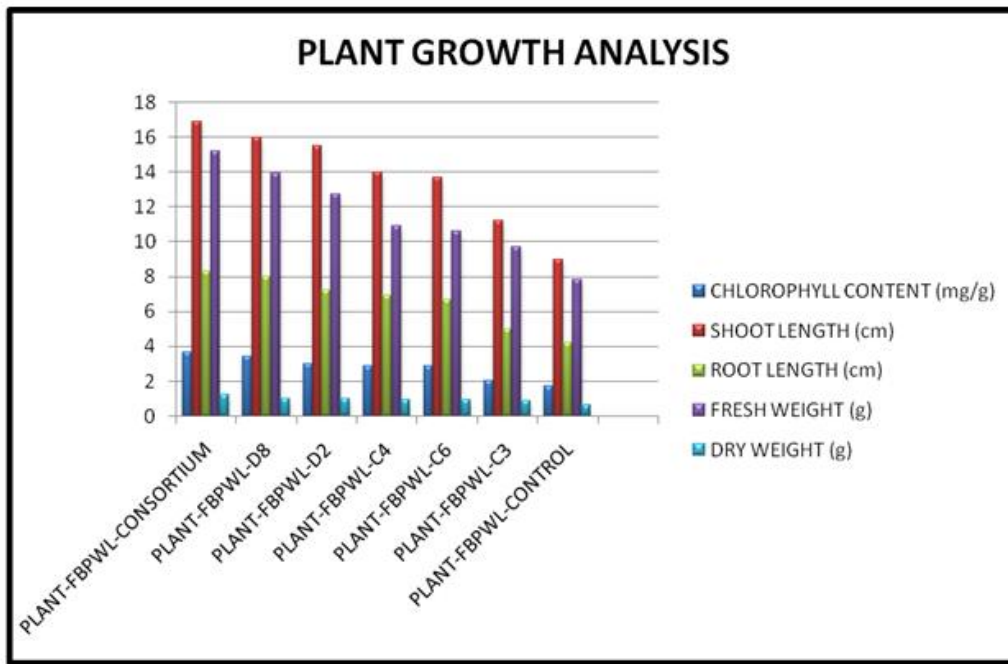


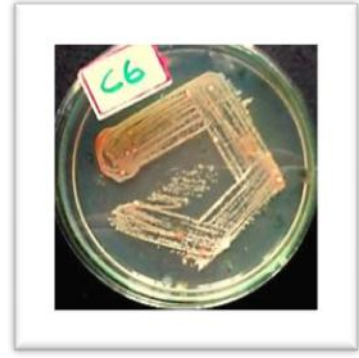
Figure.1 Colony Morphology of Isolates



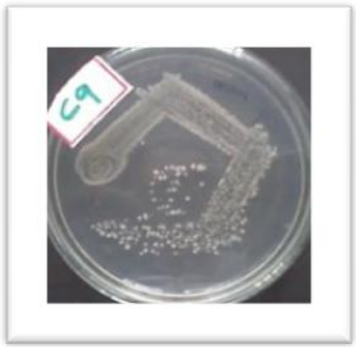
Bacillus circulans



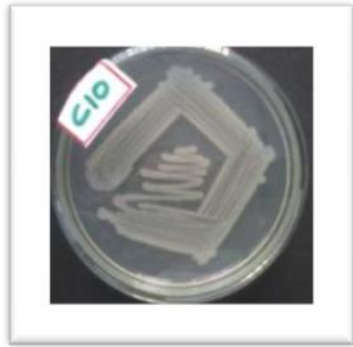
Pseudomonas stutzeri



Serratia rubidaea



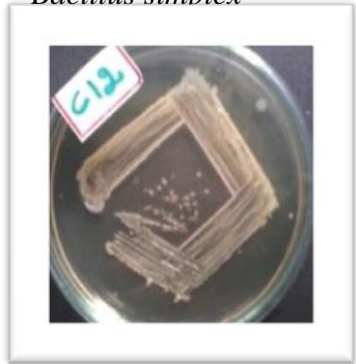
Bacillus simplex



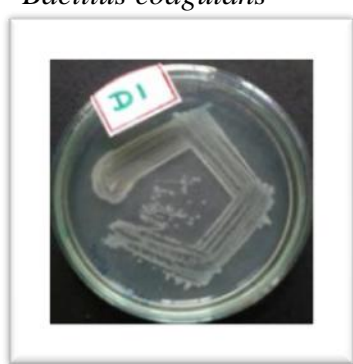
Bacillus coagulans



Pseudomonas fragi



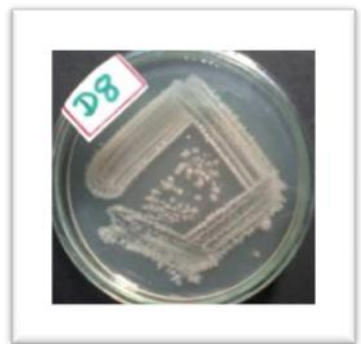
Bacillus megaterium



Bacillus subtilis



Bacillus pumilus



Bacillus aerius

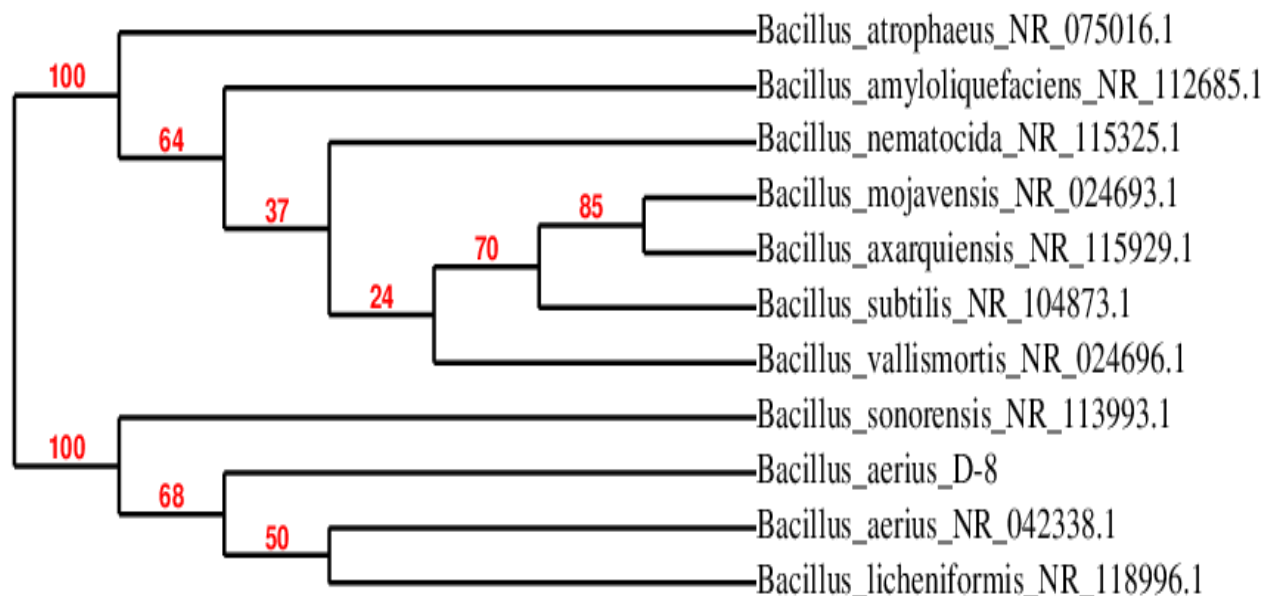
Fig.2 Zones formed by D8, D2 and C4 on Congo red assay



Fig.3 Root length and Shoot length of plants



Fig.4 Phylogenetic tree of *Bacillus aerius* D8



The present study shows that potassic biofertilizer can be produced from banana pseudostem waste and it was found to be effective in enhancing plant growth.

In conclusion, attempts to produce indigenous potassium fertilizer in India are being intensively made and research has been carried out at national and state level. Different approaches were followed to produce potassic fertilizers. But the use of an abundant potassium rich banana pseudostem waste for the production of potassic biofertilizer is an unexplored area and hence formed the basis of the study.

The present study proves that banana pseudostem waste can be used to produce potassic biofertilizer using cellulolytic bacteria as it degrades the cellulose present in banana pseudostem waste and releases the bound potassium. The methodology adopted is simple and cost effective. It also helps in turning a waste to wealth. Banana pseudostem waste which is abandoned in the field can be routed back to support

agriculture. Thus, the present study revealed the possibility of utilising banana pseudostem waste as a substrate for the production of potassic biofertilizer.

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