

- 1 **Profiling Rhizosphere Microbes on the root of Maize (*Zea mays*) planted in an**
- 2 **Alfisol for Selection as Plant Growth Promoting Rhizosphere (PGPR)**

Abstract

Maize (*Zea mays* L. Merrill) root rhizosphere, being a metabolite-enriched niche was profiled with the objective of isolating Plant Growth Promoting Rhizobacteria (PGPR) for bio-fertilizer production. Isolation was carried out from the root surface and rhizosphere soil samples of the plant using standard procedures. Cultural, physiological and biochemical procedures were used to identify the isolates. Eighty (80) isolates obtained were screened for growth promoting attributes and sixteen (16) representative isolates selected, and further identified using molecular methods by sequencing of their 16S rDNA gene. Sixty three percent of the characterized organisms exhibited sequence homology level equal or greater than 90% with those of the gene bank. Microorganisms with pathogenic characteristics were eliminated. Thus, *Bacillus thuringiensis*, *Pseudomonas putida* and *Klebsiella varriicola* selected, were further screened *invitro* for Indole acetic acid (IAA), Gibberellic acid (GA) and Cytokinin production using standard assay methods. *Klebsiella varriicola* produced 19.697 mg/L of GA, 0.348 mg/L of IAA and 1.804 mg/L of Cytokinin. *Pseudomonas putida* had 2.693 mg/L of GA, 0.152 mg/L and of IAA and 5.066 mg/L of Cytokinin in solution while *Bacillus thuringiensis* excreted 15.091mg/L, 0.132 mg/L and 2.410 mg/L of GA, IAA and Cytokinin respectively. The 3 PGPR are considered suitable for biofertilization programme.

Keywords: Plant Growth Promoting Rhizobacteria (PGPR); Rhizosphere microbes; Maize; Alfisol

Introduction

Plant growth promoting rhizobacteria (PGPR) are a group of microorganisms that enhance growth parameters of host plants and can be used as bio-fertilizers. The rhizosphere is a rich niche of microbes and should be explored for obtaining potential plant growth promoting rhizobacteria (PGPR) [1]. These set of soil microbes play critical roles in Plant-Microbe relationships. The microbes enhance plant growth, development and nutrient uptake and yield [2]; [3]. The means by which PGPR enhance soil nutrient status were categorized into 5 [4]: i) biological nitrogen fixation, ii) increasing the availability of nutrients in the rhizosphere, iii) inducing increases in the root surface area, iv) enhancing other beneficial symbiosis of the host and v) a combination of the previously mentioned modes of action.

Common PGPR include the strains in the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Rhizobium* and *Serratia* [5]. Plant growth promoting rhizobacteria (PGPR), due to their potentials have shown importance and have gained acceptance for agricultural benefits worldwide. These microorganisms are the potential tools for sustainable agriculture and the trend for the future. The mode of action of many PGPR is by increasing the availability of nutrients for the plants in the rhizosphere [6] [7]. Several scientific researches which involve multidisciplinary approaches are being conducted to understand the application of PGPR to the rhizosphere, their mechanisms of root colonization, effects on plant physiology and growth, bio-fertilization, induced systemic resistance, bio-control of plant pathogens and production of determinants [8].

Phytohormone production by microbes can modulate the endogenous plant hormone levels and consequently have an enormous influence on plant growth and development [9] [10]. Non-conjugated indole-3-acetic acid (IAA) is the most abundant member of the auxin family. It has been estimated that up to 80% of the rhizosphere bacteria can synthesize IAA [11]; [12]. Bacteria which produce IAA can add to, or influence the levels of endogenous plant auxin [11]). It is assumed that plant growth promotion by exogenously added auxin acts by increasing root

growth, length and surface area, thereby allowing the plant to access more nutrients and water from the soil [4].

Plant growth regulators such as gibberellins and cytokinins are important biotechnological and economical products. Gibberellins (GAs) are a large group of important diterpenoid acids among commercial phytohormones [13]. They are endogenous hormones functioning as plant growth regulators and influencing a range of developmental processes in higher plants including stem elongation, germination, dormancy, sex expression and fruit senescence [14]. The objective of the study was to isolate and characterize some PGPR from the rhizoplane and rhizosphere soil of maize plant root and screen them for phytohormone production.

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130 **Materials and Methods**

131 **Collection of root and soil samples**

132 For the purpose of isolation, PGPR were isolated from the rhizoplane and rhizosphere soil of
133 maize (*Zea mays*) plant earlier screened by plant breeders to be tolerant to low nitrogen in soil.
134 Rhizosphere soil and root samples of the healthy maize (*Zea mays*) plants were aseptically
135 collected from established maize fields at the Institute of Agricultural Research and Training,
136 Moor plantation, Ibadan, Oyo State, South West, Nigeria (7° 23' N; 3° 51'E and 160 m above
137 mean sea level).

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139 **Isolation of rhizospheric bacteria**

140 Serial dilution agar plate method was used for further processing of the prepared samples'
141 suspension and suitable dilutions (10^{-3} , 10^{-5} and 10^{-8}) were plated on Tryptic Soy Agar (TSA:
142 Tryptic soy broth plus 5% agar), king's B medium (KBM: 20.0 g/L proteose peptone, 1.50 g/L
143 magnesium sulphate, 1.50 g/L dipotassium hydrogen phosphate, 15.0 g/L agar agar, 10.0 ml/L
144 glycerol, pH 7.2±0.2) and Jensen medium (JM: 20.0 g/L sucrose, 0.5 g/L Sodium chloride, 1.0
145 g/L Dipotassium hydrogen phosphate, 0.5 g/L magnesium sulphate, 0.1 g/L Ferrous Sulphate,
146 0.005 g/L sodium molybdate, 2.0 g/L calcium carbonate, 15.0 g/L Agar). Incubation of the
147 media plates was carried out at 35 °C ± 2°C for 24 hours after which growth was observed on the
148 respective media plates and individual colonies were picked and streaked on respective fresh
149 media plates for further purification. Pure colonies were observed and kept for identification and
150 further screening.

151 **Characterization of bacteria isolates**

152 Preliminary identification of the bacteria isolates was done using morphological, biochemical
153 and cultural characteristics [15]. Cultural characteristics such as elevation, colour and shape were
154 studied on agar plates. The cells were Gram stained, tested for motility, sugar fermentation and
155 other biochemical analysis including starch hydrolysis, indole, methy red, Voges- Proskauer,
156 citrate, catalase, oxidase, Spore staining , glucose hydrolysis, H₂S production and nitrogenase
157 tests. The isolates were identified based on the results of the tests using Bergey's manual of
158 Determinative Bacteriology.

Detection of Nitrogen Fixing Activity

The detection of Nitrogen fixing activity was observed using Glucose-Nitrogen free mineral medium (GNFMM: 20g/L Glucose, 1g/L K_2HPO_4 , 0.1 g/L $CaCl_2$, 0.5 g/L NaCl, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$, 0.01 g/L $Na_2MoO_4 \cdot 2H_2O$, 0.01g/L $MnSO_4 \cdot 5H_2O$, 20 g/L Agar) and BTB as color indicator. The bacterial isolates were inoculated in test tubes containing 10mls of the medium and these were incubated for a period of 7 days. After the incubation period, a colour change from green to blue indicated nitrogen fixing activity.

Molecular identification

Bacteria strains were identified via extraction of genomic DNA using (ZymoBacterial Genomic DNA extraction kit) from 24hrs bacteria culture incubated at 37°C in nutrient broth. The pure DNA was electrophoresed on 1% agarose gel containing 0.5µg/ml ethidium bromide. The DNA was visualized by UV trans-illuminator and photographed. 1500bp16S rDNA fragment was amplified as described [16]. Forward primer 16SF (5'-GTGCCAGCAGCCGCGCTAA-3') and Reverse primer 16SR (5'- AGACCCGGGAACGTATTCAC - 3') were used. Amplified fragment was extracted from 1.5% agarose gel and this was sequenced using Big Dye Terminator 3.1 sequenced kit. Sequences of partial 16S rDNA genes were determined in both the forward and reverse directions with the primers using the automatic sequencer ABI-PRISM 3100 Genetic Analyzer (Applied Biosystems). The reverse sequence obtained was converted to reverse complementary sequence using Bioedit software and aligned with sequence obtained with forward primers using Basic Local alignment search tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The aligned sequence were then submitted as a query to BLAST for comparison with the collection of 16S rDNA gene sequences present in the Gen Bank database.

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190 **Extraction and Purification of Indole Acetic Acid (IAA)**

191 The bacterial isolates were inoculated into 20 mL of nutrient broth supplemented with 0.2 %
 192 (v/v) of L-tryptophan and incubated at 30 °C. After incubation, the culture was centrifuged at
 193 3,000 rpm for 20 min and the supernatant was used for analyzing indole 3 acetic acid production.
 194 2ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 2ml of Salkowski
 195 reagent (50ml, 35% perchloric acid and 1ml of 0.5M FeCl₃).The development of the pink color
 196 was observed as the indication for positive result while un-inoculated growth medium was used
 197 as negative control [17].

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199 **Quantification of IAA by HPLC**

200 HPLC chromatograms were produced by injecting 10µl of the filter extracts onto a (C₁₈, 5µm 25
 201 x 0.46 cm) in a chromatograph equipped with a differential ultraviolet detector absorbing at
 202 280nm. Mobile phase was methanol and water (80:20 vol/vol) flow rate was 1.5ml / min.
 203 Retention time for peaks was compared to those of authentic standards added to the medium
 204 extracted by the same procedures used with bacterial cultures. Quantification was done by
 205 comparison of peak height.

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207 **Extraction and Purification of Cytokinin**

208 The bacteria isolates were inoculated in Nutrient Broth media and placed on a rotary shaker at
 209 150rpm for 72 h. The culture media was centrifuged at 16000rpm for 10 min at 4°C and the
 210 supernatant obtained was neutralized with 7N NaOH (pH 7.0) and extraction was done with
 211 equal volume of ethyl acetate [18]. Extraction was repeated three times, the filtrate was
 212 evaporated to dryness and reconstituted in HPLC grade methanol and subsequently fractionated
 213 by reverse phase high performance liquid using cytokinin as standards. Quantification was done
 214 by comparing peak area of the tested compound to the standard cytokinin and the wavelength
 215 used for detection of cytokinin was 280nm.

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217 **Extraction and Purification of Gibberellic acid**

218 Nutrient broth (50ml) was inoculated with 24hrs old bacterial culture and placed on a rotary
 219 shaker at 100rpm for 5 days after which the culture broth was centrifuged at 6000rpm for 15min,

the pellet was discarded and the pH of the supernatant was adjusted to 2.8 with 1 N HCl. Gibberellic acid (GA) was extracted using liquid-liquid (ethyl acetate/ NaHCO₃) extraction method [19]. GA was extracted two times from the supernatant with ethyl acetate (Volume 1:10) and the upper ethyl acetate fraction that contains the free GA was collected. To remove impurities, the ethyl acetate fraction was partitioned two times with 1 M NaHCO₃ (Volume 1:1) and the upper fractions were collected. The pH of the NaHCO₃ fraction was adjusted to 2.0 and the NaHCO₃ solution with the free GA was partitioned two times using ethyl acetate (Volume 1:10) and the upper fraction was collected. All the ethyl acetate fractions were composited together and evaporated to dryness at 35°C and the residue dissolved in 1500µl of pure methanol. The sample was analyzed on HPLC using UV detector and C₁₈ Column (39x300mm). For identification of hormones, a 100µl sample was filtered through 0.45 millipore filter and 20µl of the filtered extract injected into a 5µm reverse phase column. Pure Gibberellic acid dissolved in HPLC grade methanol was used as a standard for identification and quantification of bacterial hormone. Growth hormone was identified on the basis of retention time and peak area of the standard. The wavelength used for the detection of Gibberellic acid was at 280nm.

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Results and Discussion

Isolation and characterization of Plant Growth Promoting Bacteria

Plant rhizosphere is a rich environment that hosts a wide array of bacteria including PGPR [18], and in the present study, eighty (80) bacteria were isolated from the root rhizoplane and rhizosphere soil of maize plants tolerant to low nitrogen. Various studies have also reported the isolation of PGPR form the rhizosphere of different plants [20]; [21]; [22]; [23] including *Zea mays* [24]; [25]; [26] [27]. Probable identity of the bacteria was carried out by means of cultural techniques using morphological and biochemical characteristics. Sixteen representative bacterial isolates showing prolific growth, having different morphological appearance on agar medium as well as exhibiting different biochemical test results were selected. The isolates were stored and used for further studies on plant growth promoting abilities in vitro. The biochemical properties of the bacterial isolates based on the results are shown in Table 1. Sixty percent of the PGPR were isolated from the rhizoplane while 40% were obtained from the rhizosphere soils of maize (*Zea mays*). Dilution factors of 10^{-3} , 10^{-5} and 10^{-8} were used during the bacterial isolation process and out of the representative bacteria selected, 46.7%, 40.0% and 13.3 % were obtained from the 10^{-3} , 10^{-5} and 10^{-8} dilutions respectively indicating that the lowest dilution gave the highest diversity of bacterial isolates (Table 2). The sixteen isolated bacteria were positive for nitrogenase activity, this was indicated by a rapid and intense blue color change observed in the GNFM-medium from the second day of inoculation, suggesting the excretion of ammonia into the medium by the isolates.

Molecular identification of the PGPR

The amplification of the genomic DNA from the rhizobacterial isolates using the universal 16S primers and the yielded DNA fragments are shown in plate 1. Based on BLAST analysis of the 16S rDNA gene homology, the isolates were identified as presented in Table 2. Only 10 of the isolates (63%) exhibited sequence homology level equal or greater than 90% as shown in the table. The sequence analysis of 16S rDNA sequences of isolates RHZA , RHZa, RHZb, RHSc, RHZD, RHZE, RHZc, RHSe, RHSJ, RHSK, RHZO, RHZR, RHZV, RHZW, RHSX, RHSZ showed maximum identity of 98% to *Bacillus thuringiensis* (CP009651-1), 95% to *Chryseobacterium spp* (CP007626-1), 98% to *Klebsiella pneumonia* (AP014624-1), 98% to *Proteus vulgaris* (CPO1267J-1), 86% to *Bacillus cereus*(CPO09605-1), 85% to *Proteus vulgaris* (LN558631-1), 97% *Enterobacter spp* (CP005991.1), 96% to *Klebsiella pneumonia* (CPO14008-1), 90% to *Pseudomonas putida* (KRO58826-1), 92% to *Klebsiella varriicola* (CPO10523-1) , 87% to *Pseudomonas monteilli* (DQ071557-1), 93% to *Klebsiella pneumonia* (CPO10361-1), 84% to *Bacillus cereus* (LN559106-1), 98% to *Enterobacter asburiae* (CPO1262-1), 87% to *Myroides odoratimimus* (CPO13690-1) and 80% to *Bacillus cereus* (CPO09590-1) respectively. Therefore, the isolates can be considered as strains of the listed bacteria.

This result is comparable to numerous studies that have reported the isolation of *Bacillus* species (*B. thuringiensis* and *Bacillus cereus*), *Pseudomonas putida*, *Klebsiella* and *Enterobacter* [25]; [26]; [22] from the rhizosphere of *Zea mays* and other crops. The experimental work eliminated the use of *Chryseobacterium spp.*, *Klebsiella pneumonia*, *Proteus vulgaris*, *B. cereus*, *Pseudomonas monteilli*, *Enterobacter asburiae* and *Myroides odoratimimus* for further work due to their pathogenic characteristics. Various opportunistic bacteria pathogens including the listed ones have been identified as colonizers of plant rhizosphere and also described as opportunistic human pathogens [28];[29];[30]. Due to the potential of the isolated PGPR as bioferertilizers, three isolated PGPR namely; *Bacillus thuringiensis*, *Pseudomonas putida* and *Klebsiella varriicola* which are not likely to be opportunistic pathogens were selected for further work. Although all tested strains excreted phytohormones in the different complex growth medium, the levels of IAA, GA, and cytokinin production differed among them (Table 3).

All the selected strains of PGPR were able to produce IAA (indole-3-acetic acid) and the amount of IAA produced varied among the bacteria isolates, complying with earlier studies [31]; [32] and [33] which have shown that IAA production is very common among PGPR. Production of IAA by PGPR is an important mechanism of plant growth promotion because IAA has been implied in the promotion of root development and uptake of nutrients [34]. The production of IAA from tryptophan amended bacterial cultures was confirmed by HPLC bioassay and quantification was done by comparison of peak heights. The amount of IAA produced by *Klebsiella varriicola*, *Pseudomonas putida* and *Bacillus thuringiensis* was 0.348, 0.152 and 0.132 mg/L respectively indicating *Klebsiella varriicola* as the most effective IAA producer amongst the tested bacterial strains.

Production of gibberellic acid by the isolates were estimated and confirmed on high-performance liquid chromatography (HPLC). *Klebsiella varriicola* produced 19.697mg/L solution of Gibberelic Acid (GA), *Pseudomonas putida* excreted 2.693mg/L of GA while *Bacillus thuringiensis* produced 15.091mg/L of GA. As observed in the production of IAA, maximum concentration of GA was also recorded by *Klebsiella varriicola* while the lowest was recorded by *Pseudomonas putida*. Gibberellic acid improves plant growth through the promotion of primary root elongation and lateral root extension [35]; [36]), and the production of gibberellic acid by several PGPR has been documented [37]; [38]; [39].

The amount of cytokinin produced by the isolates also varied with a range of 1.804 to 5.066mg/L. Cytokinins are important plant growth hormones [40], exogenous cytokinin enhances cell division rate in plants. The amount of cytokinin produced by *Pseudomonas putida*, *Klebsiella varriicola* and *Bacillus thuringiensis* was 5.066mg/L, 1.804mg/L and 2.410mg/L respectively. Earlier reports have also documented the ability of isolated PGPR to produce cytokinin [41];[42];[43]. In the present study it was evident that *Pseudomonas putida* was the most efficient cytokinin secreting bacteria among the strains studied while *Klebsiella varriicola* produced the least amount of cytokinin.

It can be concluded that the screened PGPR isolated from the rhizoplane and rhizosphere soil of *Zea mays* were capable of producing some phytohormones. The screened PGPR showed multiple plant growth promoting traits namely; indole acetic acid, gibberellic acid and cytokinin. However, *in vivo* screening of the PGPR as plant growth promoters is required for further

confirmation. This is ongoing through greenhouse house and field trials to determine the potentialities of the PGPR as growth enhancers for development of biofertilizers. Further works will also confirm the suggestion that the PGPR might have been partly responsible for the ability of Low Nitrogen Tolerant Maize (LNTM) to grow optimally even when soil nitrogen is low. It is belived that development of PGPR into biofertilizer formulation will improve agricultural soil environment.

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523 **Table 1: Some Biochemical characteristics of representative bacteria isolates obtained from the rhizoplane and rhizosphere**
524 **soils of maize**

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543 MR; methyl red test, VP; Voges proskauer test, SH;Starch Hydrolysis, Lac; Lactose test, Glu ;Glucose test, Man; Mannose test, SS: Spore staining , CU ;Citrate
544 Utilization, GH; Glucose Hydrolysis, Mot ;Motility test,
545 H₂S; Hydrogen sulphide production, Nitro Act; Nitrogenase Activity
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Table 2: Molecular identification of 16S rDNA gene, accession numbers and the origin of representative plant growth promoting rhizobacterial strains

SN	Code	Identified Microorganisms	Location in root zone	Dilution	Identity (%)	Genbank Accession no
1	RHZA	<i>Bacillus thuringiensis</i>	<i>rhizoplane</i>	10^{-5}	98%	CP009651-1
2	RHZa	<i>Chryseobacterium spp</i>	<i>rhizoplane</i>	10^{-8}	95%	CP007626-1
3	RHZb	<i>Klebsiella pneumonia</i>	<i>rhizoplane</i>	10^{-3}	98%	AP014624-1
4	RHSc	<i>Proteus vulgaris</i>	<i>rhizosphere</i>	10^{-3}	98%	CPO1267J-1
5	RHZD	<i>Bacillus cereus</i>	<i>rhizoplane</i>	10^{-3}	86%	CPO09605-1
6	RHZE	<i>Proteus vulgaris</i>	<i>rhizoplane</i>	10^{-8}	85%	LN558631-1
7	RHZc	<i>Enterobacter sp.</i>	<i>rhizosphere</i>	10^{-5}	97%	CP005991.1
8	RHSe	<i>Klebsiella pneumonia</i>	<i>rhizosphere</i>	10^{-5}	96%	CPO14008-1
9	RHSJ	<i>Pseudomonas putida</i>	<i>rhizosphere</i>	10^{-5}	90%	KRO58826-1
10	RHSK	<i>Klebsiella varriicola</i>	<i>rhizosphere</i>	10^{-5}	92%	CPO10523-1
11	RHZO	<i>Pseudomonas monteilli</i>	<i>rhizoplane</i>	10^{-5}	87%	DQ071557-1
12	RHZR	<i>Klebsiella pneumonia</i>	<i>rhizoplane</i>	10^{-3}	93%	CPO10361-1
13	RHZV	<i>Bacillus cereus</i>	<i>rhizoplane</i>	10^{-3}	84%	LN559106-1
14	RHZW	<i>Enterobacter asburiae</i>	<i>rhizoplane</i>	10^{-3}	98%	CPO1262-1
15	RHSX	<i>Myroides odoratimimus</i>	<i>rhizosphere</i>	10^{-5}	87%	CPO13690-1
16	RHSZ	<i>Bacillus cereus</i>	<i>rhizosphere</i>	10^{-3}	80%	CPO09590-1

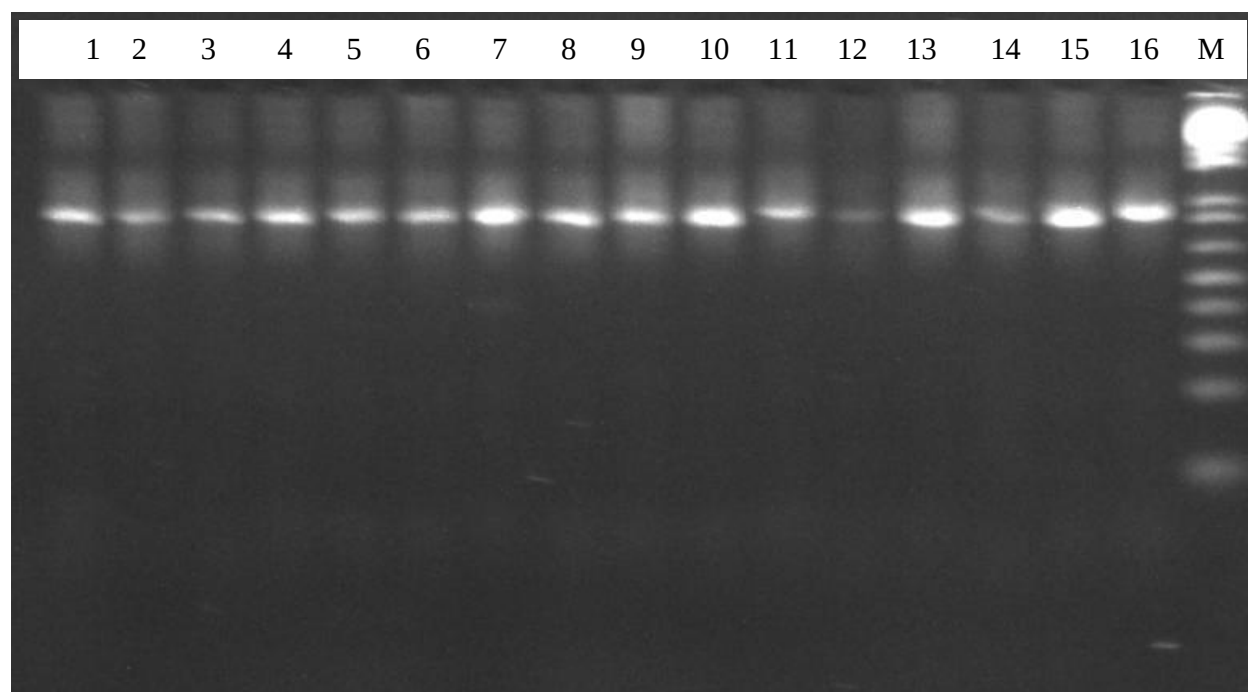


Plate 1: Agarose gel electrophoresis of DNA fragments specifically amplified from genomic DNA with 16S rDNA primers

Lane 1, *Bacillus thuringiensis* (CP009651-1); 2, *Chryseobacterium spp* (CP007626-1); 3, *Klebsiella pneumonia* (AP014624-1); 4, *Proteus vulgaris* (CPO1267J-1); 5, *Bacillus cereus* (CPO09605-1); 6, *Proteus vulgaris* (LN558631-1); 7, *Enterobacter spp* (CP005991.1), 8 *Klebsiella pneumonia* (CPO14008-1) 9, *Pseudomonas putida* (KRO58826-1); 10, *Klebsiella varricola* (CPO10523-1); 11, *Pseudomonas montei* (DQ071557-1); 12, *Klebsiella pneumonia* (CPO10361-1); 13, *Bacillus cereus* (LN559106-1); 14, *Enterobacter asburiae* (CPO1262-1); 15, *Myroides odoratimimus* (CPO13690-1) and 16, *Bacillus cereus* (CPO09590-1)

Table 3: Types and amount of Hormones produced by selected Plant growth promoting rhizobacteria

Microorganisms	Gibberelic Acid (mg/L)	Indole Acetic Acid (mg/L)	Cytokinin (mg/L)
<i>Klebsiella varriicola</i>	19.697	0.348	1.804
<i>Pseudomonas putida</i>	2.693	0.152	5.066
<i>Bacillus thuringiensis</i>	15.091	0.132	2.410