Screening and Characterization of *Achromobacter xylosoxidans* isolated from rhizosphere of *Jatropha curcas* L. (Energy Crop) for plant-growth-promoting traits

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Abstract

Plant growth promoting rhizobacteria (PGPR) colonizes almost all the ecological niches in and around the plant roots and enhances plant growth and show profound impact upon plants productivity. In the present study we have isolated large number of bacterial isolates from the rhizosphere of non-edible oil seed plant *Jatropha curcas* (Common name: Physic nut; Family: Euphorbiaceae). Out of large number of isolates we have selected only four bacterial isolates (AKDJ1, AKDJ2, AKDJ3, and AKDJ4) on the basis of their multifarious PGP traits (biofilm production, ammonia production, indole acetic acid (IAA), phosphate solubilization, catalase enzyme and cellulase enzyme production). Out of four, the isolate AKDJ2 was characterized by various biochemical utilization tests (Citrate, lysine, ornithine, urease, phenylalanine, H₂S production, nitrate reduction, glucose, lactose, adonitol, sorbitol, arabinose, and 35 different carbohydrate sources) and identified as *Achromobacter xylosoxidans* (Gene bank Accession no KX698100) which showed 99% similarity with *Achromobacter xylosoxidans* strain NBRC15126 by using 16S rDNA sequencing. We conclude that, bacterial isolates screened from the rhizosphere of plant could serve as a source of potential biofertilizer for improving the production of same and other crops under variety of stress conditions.

Keywords: Plant growth promoting rhizobacteria; Biofertilizer; 16S rDNA; Energy crop

Introduction

Jatropha curcas L. belongs to family Euphorbiaceae is a perennial, drought resistant, and multipurpose oil seed plant. It is often recognized as an potential source for future biodiesel production [1–4]. *Jatropha curcas* is a tropical plant and grow in wastelands, areas with low precipitation. *Jatropha* can be grown in boundaries to protect agriculture fields from grazing and soil erosion or they can be planted in the farms as a commercial crop. To improve its growth and production for biofuel generation, a number of agricultural management practice have been used by several researchers in the past. Kumar et al.[2,5–9] used different bioinoculants to improve its growth and yield under saline and alkaline soil conditions. The bioinoculants used in previous

studies are commonly screened and isolated from rhizosphere and commonly known as plant growth promoting rhizobacteria (PGPR). These PGPR promote plant growth and suppresses disease incidence which is solely resulted due to the synergistic effect of nutrients and phytohormones produced by these bacteria [10–12]. Bioinoculation of selected PGPR with seeds of *Jatropha* can improve growth of plant by providing resistance to plants towards different abiotic and biotic stress conditions. PGPR are often used for improving fertility and facilitates establishment of plant [13–15]. Great efforts have been made to investigate the beneficial role of PGPR on crop production under variety of stress conditions [16–20]. PGPR can stimulate plant growth by using direct and indirect mechanism of action. Direct mechanism of PGPR action includes fixation of atmospheric nitrogen, phosphate solubilization, Siderophore production, and production of plant hormones (like Auxins and Cytokines). Indirect mechanism of plant growth stimulation includes synthesis of some plant growth substances or facilitating the uptake of certain nutrients [21].

Soil is most dynamic and complex system that supports overall growth of the plant. The abiotic and biotic stresses are the major constrain for sustainable agricultural production. Most of these microbes are dependent upon different root exudates secreted by plants for their survival [21]. Evidences supports the fact that plants utilizes greater amount of nutrients that are present in the soil in modern intensive cultivation and often needs replenishment of the nutrients. Under these conditions microbes offers a good alternative strategy to replenish various nutrients. Kumar et al. (2015b) in his study isolated one hundred and six PGPR bacteria from the rhizosphere and endosphere of *Hippophae rhamnoides* L. (Sea-buckthorn). Theses bacterial isolates were then screened for different PGP traits. Results of their study showed 76.41 % of bacterial isolates, depict IAA or auxin production activity, 43.39 % of bacterial isolates depicts siderophore activity and 19.4 % of bacterial isolates shows HCN production activity.

The objectives of the present study was to isolate bacterial strains from the rhizosphere of the *Jatropha curcas*, and characterized them for morphological and physiological

attributes as well as identify them by using 16S rDNA sequencing. Graphical representation of work done is presented in Figure 1.

Figure 1: Flow chart of experimental work

Materials and methods

Collection of samples

The rhizosphere soil sample was collected from the Botanical garden located at Dr. Harisingh Gour University Sagar (M.P), India.

The location of the site is at 23°49'34 N latitude and 78°46'35 E longitude as shown in Figure 2. The rhizosphere soil was often collected after digging in depth up to 15 cm. These samples were placed in sterile polythene bags and brought to the lab. And stored at 4°C in refrigerator until use

Figure 2: Sample collection site (Google earth image)

Isolation of Rhizobacteria from the rhizosphere

For isolation of bacteria, 1g of rhizospheric soil was used and was plated on nutrient agar media (Peptone 10g/L, NaCl 5.0g/L, Yeast extract 10g/L, 1 M NaOH, 10ml/l, Agar 15g/l at pH 7). The plates were then incubated for about 24 hrs at 28 °C for further experiment. These bacterial isolates were further maintained at liquid nutrient broth and preserved in glycerol at -20°C

Morphological and biochemical characterization

Morphological characterization of each bacterial isolates was examined on nutrient agar plates. Three days old culture of bacterial isolates were used for determining the size, color, shape, surface, elevation, and margin of colonies. The Grams staining of the isolated strains was also carried out to find out the gram positive and gram negative strain as described by Vincent and Humphrey [23]. Light microscopy was used to observe the size and motility of the bacterial cell. Biochemical and carbohydrate test was conducted by using kits (KB002 HiAssorted™ Biochemical Test Kit and HiCarbohydrate™ Test kit KB009), respectively.

Molecular identification of potential bacteria and bioinformatic analysis

Total genomic DNA was successfully extracted from bacteria isolates by using Insta Gene™ Matrix Genomic DNA isolation kit. Final concentration of DNA was determined by using nano drop and visualized by running DNA gel electrophoresis. Isolated DNA was PCR amplified by using universal 27F forward primers with sequence (AGAGTTTGATCMTGGCTCAG) and 1492R reverse primer with sequence (TACGGTACCTTGTACGACTT). The PCR reaction was performed by using the method presented in Vyas et al. (2018). PCR products were then purified and sequenced using an ABI Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Grand Island, NY, USA). Obtained sequences were then compared with other sequences through NCBI BLAST at <http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>. Sequences were submitted to NCBI GenBank data base and obtained accession numbers (Accession number: KX698100). The phylogenetic analysis of sequences with the closely related sequence of NCBI blast results was performed by following multiple sequence alignment. A phylogenetic tree for these bacterial sequences were constructed by using iTOL (Interactive tree of life) after establishing relationship among the similar sequences analysis generated from Mega 5.05 software [27, 28].

Biochemical Assays

Solubilization of Insoluble Phosphate

200 µL of bacterial suspension was spot inoculated at the centre phosphate solubilizing agar plates or Pikovaskay's plate [24]. The plates were then incubated at 28°C for about 5 days and halos' zones produced were measured indicating varying levels of phosphate solubilisation.

Ammonia production

This test is based on the production of urease which break urea into ammonia and which in turn increase the pH of the medium. Freshly grown cultures of bacterial isolates were inoculated into urea broth containing peptone and incubated at 37°C for 24 hrs. Bacterial culture was then centrifuged. 1 ml of Nessler's reagent was added to the supernatant and change in color yellow to brown was a positive test for ammonia production.

Production of Indole-3-Acetic Acid

Bacterial cultures were grown on Dev tryptophan broth [25] on rotatory shaker at 37°C for 5 days. Bacterial suspension was then centrifuged at 1000 rpm for 20 min. 1ml of Salkowsky's reagent was added to 1 ml of supernatant and incubated in dark incubator for 1h. Then, development of pink color considered positive for IAA production and further measured at 536 nm by using microplate reader.

Biofilm formation

In this, ability of PGPR to form biofilm on root surface will be assayed using CV (crystal violet) by following standard protocol. For this assay bacterial isolates will be grown in nutrient broth and incubated at 37°C for 3 days. After incubation, samples were further stained by using 1% crystal violet solution and extracted with ethanol. The crystal violet stain will be then spectrophotometrically quantified by measuring the absorbance at 690 nm.

Catalase activity

1ml of bacterial culture taken into tubes and add few drop of H₂O₂ separately. The evolution of oxygen in the form of bubble indicates positive reaction for catalase production.

Cellulase activity

Cellulase production was determined by using the standard protocols. Agar medium (NaNO₃, K₂HPO₄, MgSO₄, KCl, Sodium CMC, peptone and Agar) with yeast extract plates were inoculated with individual bacterial isolates and incubated for 3-5 days at 28°C. Bacterial growth surrounded by clear halos was considered as positive indication of cellulose production. The incubated CMC agar plates were then flooded with grams iodine solution allowed to stand for 1 min at room temperature. 1M NaCl was thoroughly used for counter staining the plates. Clear halos zones were observed around growing bacterial colonies indicating hydrolysis of cellulose [26].

Results and Discussion

In this present study total of 4 bacterial isolates were successfully isolated from the rhizosphere of non-edible oil seed plant *Jatropha curcas*. These four bacterial isolates (AKDJ1, AKDJ2, AKDJ3, and AKDJ4) were identified morphologically on the basis of size, color, shape, surface, elevation, and margin, of colonies. The Grams staining was performed most of the bacterial species were gram negative. These bacterial isolates

were further screened for different plant growth promoting traits (biofilm production, ammonia production, indole acetic acid (IAA), phosphate solubilization, and catalase and cellulase production). Out of the four isolates (AKDJ1, AKDJ2, AKDJ3, AKDJ4), two isolates formed biofilm (AKDJ2, AKDJ3). Biofilm formation by rhizobacteria is an important trait, with respect to their beneficial activity. Two bacterial isolates (AKDJ3, AKDJ4)

showed positive result for ammonia production as shown in Figure 3. Two bacterial isolates (AKDJ2, AKDJ4) shows formation of clear halos zone when inoculated at PSB agar medium as shown in Figure 4. Two isolates showed positive result for IAA activity (AKDJ3, AKDJ4). All the four bacterial isolates (AKDJ1, AKDJ2, AKDJ3, AKDJ4), showed catalase activity as shown in Figure 5. Three bacterial isolates (AKDJ1, AKDJ2, and AKDJ3) showed cellulase producing activity as shown in Figure 6.

Figure 3: Bacterial isolates showing ammonia production activity

Figure 4: Bacterial isolates showing phosphate solubilizing activity

Figure 5: Bacterial isolates showing catalase activity

Figure 6: Bacterial isolates showing cellulase producing activity

Biochemical characterization test were performed for isolates AKDJ2. This isolate show positive result for ornithine, urease, nitrate reductase, Adonitol, lactose and sorbitol utilization and show negative result for citrate utilization, lysine utilization, phenylalanine deamination, H_2S , glucose and arabinose

utilization. This bacterial isolates (AKDJ2) characterized biochemically for 35 carbohydrates sources and showed positive test for 24 carbohydrate sources and negative for 11 carbon sources and utilized 7 biochemicals out of 12, as shown in Table 1 and Table 2.

Table 1: Utilization of carbohydrates by AKDJ2

S No.	Carbohydrate	AKDJ2
1.	Lactose	Negative
2.	Xylose	Positive
3.	Maltose	Positive
4.	Fructose	Positive
5.	Dextrose	Positive
6.	Galactose	Positive
7.	Raffinose	Positive
8.	Trehalose	Positive
9.	Melibiose	Positive
10.	Sucrose	Positive
11.	L-Arabinose	Positive
12.	Mannose	Negative
13.	Inulin	Negative
14.	Sodium gluconate	Positive
15.	Glycerol	Positive
16.	Salicin	Positive
17.	Dulcitol	Positive
18.	Inositol	Positive
19.	Sorbitol	Positive
20.	Mannitol	Negative
21.	Adinitol	Negative
22.	Arabitol	Negative

23.	Erythritol	Negative
24.	Methyl-D-glucoside	Positive
25.	Rhamnose	Positive
26.	Cellobiose	Negative
27.	Melezitose	Negative
28.	Methyl-D-mannoside	Positive
29.	Xylitol	Negative
30.	ONPG	Positive
31.	Esculin hydrolysis	Negative
32.	D-Arabinose	Positive
33.	Citrate Utilization	Positive
34.	Malonate utilization	Positive
35.	Sorbose	Positive
36.	Control	Negative

Table 2: Biochemical Utilization by AKDJ2

S No.	Test	AKDJ2
1	Citrate Utilization	Negative
2	Lysine Utilization	Negative
3	Ornithine Utilization	Positive
4	Urease	Positive
5	Phenylalanine Deamination	Negative
6	Nitrate reduction	Positive
7	H ₂ S Production	Negative
8	Glucose	Positive
9	Adonitol	Positive
10	Lactose	Positive
11	Arabinose	Negative
12	Sorbitol	Positive

AKDJ2 was identified as *Achromobacter xylosoxidans* (Gene bank Accession no KX698100) which showed 99% similarity with *Achromobacter xylosoxidans* strain NBRC15126 (Accession

number: KX698100) by using 16S rDNA sequencing. Phylogenetic tree was prepared by using iTOL tool for establishing relationship of this isolate with other closely related genera (Figure 7).

Figure 7: Phylogenetic tree created by iTOL software to present relationship between closely related species of *Achromobacter xylosoxidans* strain AKDJ2

Conclusions

PGPR are a group of bacteria that play an important role in plant growth promotion. Screening PGPR from rhizosphere of plants may be viable option to enhance the biomass production on limited soil conditions/marginal land. Additionally the potential isolates may be further utilized as tailor made biofertilizer for promoting growth of the other plants. The application of PGPR instead of chemical fertilizers offers a sustainable, safe, and eco-friendly approach to increase crop production and soil health.

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