Molecular Identification of *Azotobacter Chroococcum* Isolated From Different Wastes

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ABSTRACT

Poly-ß-hydroxy butyrate (PHB) is the best known polyhydroxyalkanoate. Considering the industrial and medical applications of PHB, this work had been undertaken for the screening of PHB producing bacteria from soil sources. In the present study, an attempt was made to isolate efficient PHB producing bacteria from diverse environmental samples. *Azotobacter chroococcum* is gram negative rod shaped bacteria which is economically useful and is capable to resist many heavy metals. *Azotobacter* has ability of nitrogen fixing and has many biotechnological applications. 25 soil samples were collected from rhizosphere soil of plants grown near many waste disposal areas such as effluents, tanneries, domestic wastes and 35 isolates were screened to confirm the *Azotobacter* spp and two isolates were further identified using 16s rDNA study and used for PHB production. Phylogenetic analysis was performed using neighbor–hood joining method which showed a common ancestor belonging to *Azotobacter* of Pseudomonadaceae family

Key Words: 16s rDNA, *Azotobacter*, screening, phylogenetic analysis and soil wastes.

INTRODUCTION

Aerobic bacteria belonging to the genus *Azotobacter* represent a diverse group of free-living diazotrophic microorganisms, which has the ability to use N₂ as the sole nitrogen source commonly occurring in soil. The genus *Azotobacter* includes 6 species, with *A. chroococcum* most commonly inhabiting various soils all over the world [1]. *Azotobacter* are gram negative bacterium strictly aerobic with oxygen as the terminal electron acceptor, live in soil and fresh water which exhibits pleomorphism, which vary in their morphology from rod shaped to cocci diameter ranging from 1.5 to 2.0 μm [2,3]. The young cells possess peritrichous flagella and are used as locomotive organs. Old populations of bacteria are encapsulated and have enhanced resistant to heat, desiccation and adverse conditions. *Azotobacter* is a useful organism in many ways as it can fix atmospheric nitrogen, producing plant growth hormones like gibberellins, auxins and cytokinins and can solubilize phosphate which reduces the use of fertilizers, [4, 5]. Beside nitrogen fixation and phosphate solubilization *Azotobacter* has ability to degrade various compounds. [6] reported that *Azotobacter chroococcum* has the ability to degrade 100% o-Xylene below 84 hours upto a concentration of 150 mg/l and 75% with 200 mg/l. *Azotobacter chroococcum* when used as a co-inoculant to degrade crude oil hydrocarbons, a maximum of 0.1472g/day of hydrocarbons were degraded [7]. They also produce extracellular biopolymers with
different nitrogen sources [8]. In conditions with nutrients and oxygen limitations Azotobacter accumulates large amount intracellular insoluble energy storage materials PHB [9, 10]. While considering the pollutions from plastics, the plastics are extensively used and the plastic waste increases every year and degradation is nearly impossible [11]. Thus the uses of biodegradable plastics which are produced naturally by microbes are preferred and are cost effective and pollution free. Poly-3-hydroxybutyrate (PHB) is intracellular polyester of the family of polyhydroxyalkanoates (PHAs), produced by numerous bacteria [12]. They are used in numerous biomedical applications such as medical implants for surgery, tissue engineering, novel materials for dentistry, etc [13]. The various bacteria producing PHBs are Ralstonia eutrophus, Methylobacterium rhodesianum, Bacillus megaterium, Azotobacter spp, etc. Many researchers isolated Azotobacter from different soil which was associated with plant growth and nitrogen fixation. [14] isolated Azotobacter chroococcum and Azospirillum brasilense from sugarcane roots collected from four different agricultural locations in Granada, Spain. Nitrogen fixing bacterium was isolated from soil samples beans farm land and maize farm lands in Enugu state, Nigeria and it was found that Azotobacter, Azospirillum and Clostridium species were present in these soils [15]. 43 isolates of Azotobacter were isolated which solubilize phosphate and fix nitrogen from rhizosphere soil samples of agricultural crops from Iran [4]. 31 soil samples from different regions of Poland were examined and 16 Azotobacter spp isolates were identified showing 51.6% presence of Azotobacter [1]. In this study Azotobacter chroococcum was isolated from waste soils and characterized by molecular techniques and its Phylogenetic study was performed.

MATERIALS AND METHODS

Soil
Various contaminated wastes (liquid) and contaminated soils were collected from different places of Tamil Nadu. The wastes are kitchen wastes (Tirupur), effluents contaminated soil from industries (Coimbatore), Textile industries (Tirupur), high fertilizer contaminated soil (Thanjavur) and Tanneries waste soil (Dindigul) were collected. The soil samples are collected from rhizosphere of plants growing in these contaminated places. 25 such soil samples were collected and these samples are taken to laboratory and stored in 4C until use.

Sample preparation
The sampled rhizosphere soil was mixed thoroughly to make a composite soil. Then 10 gm of sub-soil sample diluted to 100 ml that considered being 10⁻¹ dilution factor. Transferring of 1 ml of 10⁻¹ dilution to 9 ml sterilized water with the help of a sterilized pipettes yielded 10⁻² dilution. In this way, a series of up to 10⁻⁸ dilution was prepared under aseptic condition and plated.

Pure culture
Many slimy white colonies as well as pink colonies, dark brown colonies were selected and sub cultured in ashbys mannitol medium for the isolation of Azotobacter. 35 colonies from 25 samples were screened and 5 isolates which were suspected for Azotobacter were further processed with biochemical characterization.

Biochemical Characterization
Biochemical analyses of all isolates were carried out according to Bergey’s Manual of Determinative Bacteriology [16] and classified primarily through morphology, physiological and biochemical observation.

DNA isolation, PCR Amplification and sequencing of 16S rRNA encoding genes
The isolates were inoculated in Nutrient broth (Hi-media) and incubated overnight at 37 °C. The resulting bacterial suspension was pelleted at 10,000rpm for 5 min and the genomic DNA was extracted using PureLink genomic DNA mini kit (Invitrogen, USA). PCR amplification of 16s rDNA was performed with the isolated DNA. The primers used by [17], were used to amplify 464 bp partial 16s rDNA gene from the isolated bacterial genomic DNA. The 16S rRNA gene primers were [FP: 5'- CCTACGGGCGGCAGCAG- 3'and RP: 5'- GGATTAGATACCCTGGTAGTC- 3']. PCR was performed in Eppendorf Master Cycler (Eppendorf, Germany). PCR conditions were set as follows: initial denaturation at 95° C for 5 minutes, followed by 25 cycles of denaturation at 95° C for 40 seconds, annealing at 55° C for 2 minutes and primer extension at 72° C for 1 minute ending with final elongation step at 72° C for 7 minutes. PCR products were gel purified and sent for sequencing with 16S rRNA primer. The obtained sequences were trimmed to get a sequence which read 464 bp. Then the sequences were BLAST search analyzed on the National Center for Biotechnology Information (NCBI) to identify the isolate.
Phylogenetic analysis
The 16S rRNA gene sequences of the isolated strains of *Azotobacter chroococcum* were compared to sequences of other bacterial species of families *Pseudomonadaceae* and *Enterobacteriaceae*. Two sequences R3 and R4 were from this study and 24 reference sequences were retrieved from NCBI database. Phylogenetic and molecular evolutionary analyses using neighbor-joining method were conducted for the isolated strains of *Azotobacter chroococcum* using MEGA version 6 [18].

RESULTS

Biochemical test
Biochemical tests like Indole test, Catalase test, Citrate utilization test, Starch hydrolysis test, Fermentation of carbohydrate, Mannitol Motility test and Methyl red test are performed. According to Bergey’s manual and with the results obtained from biochemical test. The isolates R1, R3 and R4 were positive to Indole, Voges-prousker, Catalase and Oxidase test. The isolates R3 and R4 are motile and positive to urease and nitrate reduction test (Table 1).

Table 1: Biochemical Characterization of various isolates

<table>
<thead>
<tr>
<th>S. No</th>
<th>Biochemical tests</th>
<th>R1</th>
<th>R3</th>
<th>R4</th>
<th>R5</th>
<th>R7</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Gram’s Reaction</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Motility</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Indole test</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Methyl red test</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Voges-prousker test</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Citrate test</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Oxidase test</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Catalase test</td>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Urease test</td>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Nitrate reduction test</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PCR amplification and Sequencing
The PCR products was run on 1.5% Agarose gel and documented in gel doc system (BIO-RAD Gel doc XR, USA) (Fig.1). A bright band at 464 bp was observed. The amplified product was eluted and sequenced. Sequence result revealed that isolate R1 was identified as *Pseudomonas mendocina*, isolates R3 and R4 showed 98% sequence similarities to *Azotobacter* and 100% query cover. R5 isolate was identified as *Balneatrix* and R7 was identified as *Bacillus licheniformis*. The results showed that the isolates R3 and R4 were *Azotobacter* and the sequences were submitted to NCBI GenBank database (GenBank accession no. KU351180, KU595713).

Phylogenetic analysis
The sequences of 24 other bacterial species belonging to families *Pseudomonadaceae* and *Enterobacteriaceae* were retrieved from NCBI and neighbor-neighbor phylogenetic analysis was done. The neighbor-neighbor analysis of isolates R3 and R4 were related to *Azotobacter chroococcum* strain N21 and R3 showed a common ancestor with *Azotobacter chroococcum* strains of Mexico and Japan. A4 showed a common ancestor with *Azotobacter chroococcum* of

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Switzerland, India and Italy (Fig.2). The neighbor- 
hood analysis revealed that Azotobacter has the same lineage to the family Pseudomonadaceae.

DISCUSSION

The growth in Ashby’s medium reveals the presence of Azotobacter and then conformed by morphological and biochemical characters. Morphological studies of isolates R3 and R4 produced slimy, glistering, smooth, whitish convex colonies ranging from 2-8 mm in diameter. Gram staining revealed as G-ve rods and the isolates were motile. The colony morphology of isolated Azotobacter was large, shiny mucoid and watery appearance which correlates the study of [19, 20], where they isolated Azotobacter spp. from marine soils. The slimy white colonies, brown pigmentation and cyst formation was also identified by [21]. The 16s rDNA amplification and sequencing reveals that the species is Azotobacter chroococcum. In our study small fragment of 16s rDNA was amplified, but [22] amplified the 16s rDNA of 2 Azotobacter strains with size ranging from 1321 – 1410 bp. The phylogenetic analyses of these 2 strains were closely related to Azotobacter beijerinckii, whereas our strains R3 and R4 were related to Azotobacter chroococcum of Mexico and Switzerland respectively. The phylogenetic analysis shows the relationship of the isolated bacterium with the other species and strains of Azotobacter. [23] confirmed the Azotobacter isolates, isolated by him, using restriction enzymes RsaI and HhaI. In this study, since a small sequence of 16s rRNA amplification was used only BLAST similarity results were used for the identification of species.

CONCLUSION

On the basis of data obtained in the present work it can be concluded that two strains of Azotobacter chroococcum were isolated which was screened from 35 isolates from various wastes and was confirmed by biochemical and 16s rDNA
study. The sequences were submitted in NCBI (KU351180, KU595713) and phylogenetic analyses showed that *Azotobacter chroococcum* isolated had the common ancestor to *Azotobacter spp* of *Pseudomonadaceae* family. The *Azotobacter chroococcum* was further used in the production of PHB.

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**REFERENCES**