Arthrobacter Strains from Industrial Polluted Soil and its Oxidative Potential of Choline Oxidase Gene

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Abstract

Betaine is a trimethylglycine, serves as osmoregulator to prevent dehydration and plasmolysis under adverse hyperosmotic environments. Choline oxidase gene from Arthrobacter sp. catalyzes two step oxidation reaction of choline to betaine followed by betaine accumulation in cells which in turn help them to survive and thrive in harsh environmental condition. To identify potential choline oxidase gene source, a gram stain positive, rod shaped, catalase and oxidase positive, motile, aerobic bacterial strains designated as HYJE003 and HYJE005 was isolated based on the colony morphology, biochemical and molecular characterization from the industrially polluted soil samples of Hyderabad, India. Optimum growth of the isolated strains was observed at 32°C on nutrient agar media and was found that both the strains were capable of utilizing variety of sugars as carbon source. The 16S rRNA gene sequence analysis revealed that strain HYJE003 was closely related to Arthrobacter globiformis with pairwise sequence similarities of 99.85%, 99.63%, 98.76% and 98.12% respectively. The strain HYJE005 was closely related to Arthrobacter phenanthrenivorans with pairwise sequence similarities of 99.93%, 99.47%, 99.25% and 98.11% respectively. Choline oxidase gene potential of the isolates was studied by feeding the cultures with choline chloride and converted betaine was assessed by the formation of betaine reineckate. Findings revealed that the isolated strain Arthrobacter globiformis-HYJE003 has four times higher conversion rate of choline chloride to betaine than the strain Arthrobacter phenanthrenivorans HYJE005.

Keywords: Arthrobacter, BLAST, Pleomorphic, Reineckate, Betaine, Snapping division.
INTRODUCTION

Soil bacteria are considered as economical source of useful biocatalysts in numerous industrial processes. Arthrobacter are, a typical soil bacterium widely distributed in diverse environmental conditions. This community gained special interest when compared to other soil microorganisms due to their ability to perform key metabolic functions such as: recycling of elements, recycling of wastes, detoxification of hazardous chemicals, including heavy metal and organic pollutants. All Arthrobacter species are pleomorphic, since they are rod shaped during exponential growth phase and cocci during their stationary phase. Choline oxidase of Arthrobacter family is the only enzyme that catalyzes the two-step oxidation reaction of choline to betaine. Betaine acts as osmoprotectant and plays an instrumental role in the growth of Arthrobacter in very harsh environmental conditions. The enzyme choline oxidase has high importance because, betaine is one of the limited numbers of compatible solutes that can accumulate in cell cytoplasm at very high concentration in adverse hyperosmotic environments to prevent dehydration and plasmolysis.

Betaine is an organic base present in fruits, vegetables, grains and seafood, thus betaine rich diet potentially favors human health. Betaine is a product well known to improve meat characteristics, weight gain, growth, carcass characteristics and feed conversion ratio in domestic animals. Betaine allows higher protein synthesis and modulates the expression of methionine/cysteine from deamination and allows higher protein synthesis. It modulates the histone and DNA methylation for epigenetic gene expression. Various studies have demonstrated that betaine play critical role in embryonic and fetal development. Considering several prospective and retrospective studies on betaine, in 2011 European Food Safety Authority has authorized a health claim of betaine on homocysteine metabolism.

Natural betaine is currently extracted and purified from beetroot, sugar beet and its derivatives. Due to high demand, betaine is chemically produced in the form of betaine hydrochloride. Recent comparative studies between natural betaine and betaine hydrochloride clearly illustrates that the chemically synthesized betaine hydrochloride is not effective as the natural betaine. In view from the above findings, the current study is aimed at isolation, identification and characterization of Arthrobacter strains from bacterial consortium of industrially polluted soil to evaluate their choline oxidase gene potential of choline to betaine oxidation.

MATERIALS AND METHODS

Strain isolation and purifications

Five soil samples were collected from Jeedimetla industrial area, a well-known heavy metal polluted site in Hyderabad, India. Samples were aseptically sampled and grinded in lab by using a sterile mortar. From the homogenized soil samples, one gram each weighed and distributed in five respective sterile flasks with 99 ml of sterile saline solution (0.85% of NaCl). After incubation at 25°C for 15 minutes at 200rpm, each sample was diluted to tenfold and from fourth to fifth dilution series 0.1ml of inoculum was added to the surface of 1X, 0.5X, 0.25X and 0.1X strength SCDA (Soya bean Casein Digest Agar) culture medium and later incubated at 30°C for 2days. Based on the cultural condition, colony morphology and microscopic characteristics two Arthrobacter strains were isolated from the culture plate of 0.1X SCDA with 10^{-4} dilution plating. Further pure cultures were established with the isolates by two level streak plating on nutrient agar media.

Biochemical and molecular analysis

Isolated pure cultures were subjected to biochemical analysis such as catalase, oxidase, urease, nitrate reduction and indole test. To evaluate the sugar utilization profile of the isolated Arthrobacter strains, Hi-Carbohydrate kit from Hi-Media was used as per manufacture protocol. Later mobility test, gram staining and endospore staining were performed. The genomic DNA from the isolated strains was extracted from CATB DNA extraction method. Spectrophotometer and agarose gel electrophoresis was used to quantify the extracted DNA quality. By using 27F-AGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTAGCTCACCT primers nearly full length 16S rRNA gene was amplified.
from isolated strains. The PCR solution consists of 2X Go Taq Green mix 10µl, primers of 0.5µl each from 10µM stock, genomic DNA 1µl (10ng) and ddH2O was added to make up the final volume to 20µl. PCR condition of 94°C for 5 minutes and then 94°C for 20 seconds, 60°C for 30 second, 72°C for 1 minute for 35 cycles followed by final extension of 72°C for 5 minutes. The amplified PCR products were verified by agarose gel electrophoresis, followed by PCR clean up sent for sequencing. Sequencing was performed using Applied Biosystems DNA seqencer, the 16S rRNA sequences were BLAST analyzed (National Centre for Biotechnology Information) to determine the closest available database match20.

**Choline to betaine conversion of isolates**

Identified strains were further evaluated for their choline oxidase gene potential to convert betaine from choline chloride by feeding the cultures with 100 mM of choline chloride in the nutrient broth. Cells were harvested from the overnight grown culture, lysed by sonication and centrifuged at high speed of 18000 rpm to remove any cell debris. To the 10 ml of supernatant, 1 ml of 25% trisodium phosphate solution and 10 ml of ammonium reineckate (2%) reagent was added and incubated in ice bath for one hour. Centrifuged at 18000rpm, carefully supernatant was transferred into fresh 15ml centrifuge tube set aside in an ice bath for one hour after addition of 1ml hydrochloric acid (3N). Then centrifuged for twenty minutes at 18000rpm and the clear supernatant was discarded, betaine reineckate pellets were air dried and dissolved in 10ml of acetone water (75:25) mixture. Optical density of the solution was recorded at 525 nm in a spectrophotometer and the amount of the betaine conversion was calculated by a similarly treated betaine standard curve21 (6.25, 12.5, 25, 50, and 100 mg/L).

The PCR was performed to isolate and confirm the choline oxidase gene from the isolated *Arthrobacter globiformis HYJE003* strain. The choline oxidase gene was amplified by using oligonucleotide primers of COX-F_ AATTGAATTCGATGCACATCGACAACATCGAG and COX-R_ AGCTAAGCTTAGGCGAGGGCGCGCTCA. The PCR was carried with 5% DMSO, with the cycling condition of initial denaturation of one minute at 94°C, followed by 35 cycles of one of minute of cyclic denaturation at 94°C, 30 second at 50°C annealing, cyclic extension of 2 minute at 72°C and a final extension at 72°C for 10 minutes. 10 ng of template DNA, 2U of Pfu Taq DNA polymerase was used and the amplified product was agarose gel purified by QIAGEN kit following manufacture protocol. The amplified choline oxidase gene was then sequenced to confirm the gene sequence.

### Results and Discussion

Form the 0.1X strength SCDA media plating with 10^4 dilution (Fig. 1 and Fig. 2) two
strains (HYJE003 and HYJE005) were isolated based on colony morphology and snapping cell division (Fig. 3). *Arthrobacter* colonies on nutrient agar media have no distinctive pigmentation with undulate colony morphology appeared as smooth, circular with convex elevation, entire margin and nonsporulating (Tabel 1 and Fig. 2). In the early stage, cultures had rod shaped bacteria whereas in older culture the bacteria were coccoid. Microscopic observation reviled the size of the rod cells ranged between 0.5 to 0.8 by 1.5 to 2.0 µm and the late stage coccoid cells were 0.5 to 0.8 µm. The isolates showed growth in neutral to slightly alkaline pH and optimal growth was observed at 32°C temperature.

Both the isolated strains were recorded positive for grams staining, catalase test, indole test, urease test, nitrate reduction and gelatin hydrolysis assays (Table 2). On the other hand, both culture types produced negative result for crystal blue stain, motility test and bile esculin assays (Table 2). But strain HYJE003 produced...
positive result for oxidase test and on the other hand strain HYJE005 produced negative result and the nitrate reduction test was also scored positive in the current study. The sugar utilization result (Table 3, Fig. 4 and 5) reveals isolated strains were able to grow in a vast variety of sugar/carbohydrate source and this ability of *Arthrobacter* genus make them widely distributed and abundant in soils of harsh environmental conditions. Almost full length of 16S rRNA gene was amplified from the extracted DNA (Fig. 6 and 7) and read length of 1350 bp of high-quality sequences was obtained.

**Table 3.** Hi-Carbohydrate kit result for isolated strains

| Sugar Name   | HYJE003 | HYJE005 |
|--------------|---------|---------|
| Part-A       |         |         |
| Lactose      | Positive| Negative|
| Xylose       | Positive| Positive|
| Maltose      | Positive| Positive|
| Fructose     | Positive| Positive|
| Dextrose     | Positive| Positive|
| Galactose    | Negative| Positive|
| Raffinose    | Negative| Positive|
| Trehalose    | Positive| Negative|
| Sucrose      | Positive| Positive|
| Melibiose    | Positive| Positive|
| L-Arabinose  | Positive| Negative|
| Mannose      | Positive| Positive|
| Part-B       |         |         |
| Inulin       | Positive| Negative|
| Sodium gluconate | Positive| Positive|
| Glycerol     | Positive| Negative|
| Salicin      | Positive| Negative|
| Dulcitol     | Positive| Negative|
| Inositol     | Negative| Positive|
| Sorbitol     | Positive| Positive|
| Mannitol     | Positive| Positive|
| Adonitol     | Positive| Positive|
| Arabitol     | Positive| Positive|
| Erythritol   | Positive| Positive|
| Alpha-Methyl-D-glucoside | Negative| Negative|
| Part-C       |         |         |
| Rhamnose     | Negative| Negative|
| Cellobiose   | Negative| Negative|
| Melezitose   | Positive| Positive|
| Methyl-D-Mannoside | Positive| Positive|
| Xylitol      | Positive| Negative|
| ONPG         | Positive| Positive|
| Esculin      | Positive| Positive|
| D-Arabinose  | Positive| Negative|
| Citrate      | Positive| Positive|
| Malonate     | Positive| Positive|
| Sorbose      | Positive| Positive|

**Table 4.** Spectrophotometer reading for betaine estimation

| No. | Sample ID   | Mean O.D. Value | Standard Deviation | Standard Error |
|-----|-------------|-----------------|--------------------|----------------|
| 1   | STD-1(100mg/L) | 0.921           | 0.005508           | 0.00318        |
| 2   | STD-2(50mg/L)  | 0.458           | 0.012055           | 0.00696        |
| 3   | STD-3(25mg/L)  | 0.234           | 0.006083           | 0.003512       |
| 4   | STD-4(12.5mg/L) | 0.101          | 0.004933           | 0.002848       |
| 5   | STD-5(6.25mg/L) | 0.056         | 0.003215           | 0.001856       |
| 6   | HYJE003       | 0.085           | 0.005568           | 0.003215       |
| 7   | HYJE005       | 0.011           | 0.003055           | 0.001764       |
The obtained consensus sequences were analyzed and the 16S rRNA sequence were further used to investigate the identity of the isolates. Based on the concept of sequences similarity\(^\text{24}\) and similarity concept of 16S rRNA analysis\(^\text{25}\), the isolated strains had 16S rRNA sequence of 1350bp and HYJE003 shows the 99.85% similarity with the *Arthrobacter globiformis* and the second strain HYJE005 shows the 99.93% similarity with *Arthrobacter phenanthrenivorans*. The alignment results indicate a significant similarity of the putative strain HYJE003 DNA sequences.

![Fig. 6. Extracted DNA quality check on 1.0% agarose gel electrophoresis. M-1Kb DNA ladder, 1- λDNA 200ng, 2- HYJE003 and 3-HYJE005](#)

![Fig. 7. 16S rRNA PCR gel image of isolated strains showing amplicon size of around 1600 bp (M-1Kb DNA ladder, 1- HYJE003 and 2-HYJE005)](##)

![Fig. 8. Phylogenetic tree of *Arthrobacter* sp. HYJE003 and HYJE005 based on 16S rRNA sequences. Each branch represents bootstrap values (≥ 50%) for 1000 replications from pairwise neighbor joining](##)
The identified strain HYJE005 16S rRNA analysis indicate a significant similarity with Arthrobacter phenanthrenivorans strains IHB, TMV7-A3, SGR270 and S95\textsuperscript{20}. 16S rRNA sequence similarity and phylogenetic tree was constructed with MEGA X using Neighbor joining method, showed isolated strains was closely related to the genus Arthrobacter\textsuperscript{18} (Fig. 8). The 16S rRNA sequence information was deposited in NCBI GenBank with the consecutive accession numbers of MK968148 and MK968149 for the isolated strain HYJE003 and HYJE005 respectively.

Spectrophotometer observance of the betaine reineckate against standard curve relieved 9.8 mg/L and 1.8 mg/L of betaine in the cultures of HYJE003 and HYJE005 respectively\textsuperscript{4,21} (Table No-4 and Fig. 9). Choline oxidase gene from the HYJE003 strain was successfully amplified and sequenced. The sequenced choline oxidase gene from the strain HYJE003 was deposited in NCBI GenBank with the accession numbers of MK988621.

CONCLUSION
In conclusion, the data presented in this study extends our knowledge on the sequential isolation, biochemical and molecular characterization of Arthrobacter strains from industrially polluted soil samples and evaluation of their choline oxidase gene potential to convert betaine from choline chloride. Based on conversion result the strain HYJE003- Arthrobacter globiformis showed four-fold higher rate of choline to betaine conversion than the strain HYJE005- Arthrobacter phenanthrenivorans. Thus, the choline oxidase gene from isolated strain HYJE003 has shown higher rate of betaine conversion, further research needed for its purified enzyme activity and its commercial feasibility of betaine production.

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CONFLICT OF INTEREST
The authors declares that there is no conflict of interest.

AUTHORS’ CONTRIBUTION
All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY
All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT
This article does not contain any studies with human participants or animals performed by any of the authors.
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