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Pseudomonas Otitidis and *Bacillus Subtilis* from Saline Soil of Pakistan Exhibiting a Potential for Calcium Precipitation

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Abstract: Biomineralization is an important phenomenon reported in microbes of different environments. Present study deals with the analysis of mineralization potential of two salt tolerant species named IQ (*Pseudomonas otitidis*) and NAY (*Bacillus subtilis*) isolated from rhizosphere soil. These strains have the ability to show precipitation in acetate supplemented culture medium incubated for 21 days and showed positive results for urease tests. Increase in microbial precipitation was recorded in both strains. However, strain NAY (*Bacillus subtilis*) showed more precipitation as compared to other IQ (*Pseudomonas otitidis*). It was also observed that this precipitation was associated with increase in pH of the medium from 7.0 to 8.0 that started to increase on 4th day of culture incubation and increased slightly till 15 days. The FTIR analysis of the calcite crystals showed visible peaks at 711-1400 cm^{-1} wavelength. The visible bubbling appeared in acid test assay further confirmed the calcite precipitation. The mineral precipitation in both species under salt stress is significant for soil consolidation as well as adjusting soil pH under acidic conditions

Keywords: *Bacillus subtilis*, calcite precipitation, FTIR, *Pseudomonas otitidis*, urease tests.

Introduction

Heavy metal contamination is a major environmental hazard encountered by plants, animals, and humans, and demands serious focus (Mulligan et al. 2001). Ultimate effect of this process is environmental cleaning by the sequestration of CO_2, soil strengthening and reduction in soil porosity and heavy metals (Gower 2008; Meldrum and Cölfen, 2008). Many microbial species are involved in calcium carbonate precipitation e.g., through hydrolysis of urea, pH of media and the composition of the media (Wei et al., 2015). Soil acidification is one of the major land degradation phenomena resulting in low agricultural yield and nitrate leaching resulting in the addition of nitrogen fertilizers or organic matter within the soil (Bolan and Hedley, 2003). Ultimately, it results in the low nutrient availability to plant nutrients and reduced crop yield. Soil acidity can be corrected by the addition of liming (Goulding, 2016). Changes in pH influence the calcite precipitation in soil.

Carbonate precipitation by bacteria plays a significant role in the soil cementing and hence this process can be exploited for many biotechnological applications like remediation of metals and oil recovery (Zhu and Dittrich, 2016). The mineral precipitation has been reported to be significant for many environmental problems and in future this promising biotechnological approach should be focused to study the interaction of carbonate precipitating bacteria with its other native communities (Kim et al., 2016). On the basis of carbonate precipitation and its role in CO_2 sequestration, present study aims to analyze the precipitation response of two strains isolated from saline environment.

Materials and Methods

Bacterial Isolation

Both bacterial isolates used in the present study were obtained from rhizosphere of *Solanum surattense* from salt mine region. Soil sample was diluted (10^{-5}) and finally spreaded on LB- agar (Gerhardt et al., 1994) plates supplemented with 1 M or without sodium chloride. Plates were incubated at 37ºC for 24 hours. Seven to ten bacterial colonies were purified by repeated streaking based on their variable morphology.

Characterization and Analysis of Calcium Carbonates Crystals

Qualitative Assay for Precipitation

Urease Activity

Purified colonies obtained from both (salt supplemented and without salt) were used for initial screening of bacteria based on Urease activity by inoculating Christensen's Urea Agar (Merck) and incubating at 30ºC for 48 hours. The development of deep pink color was recorded as positive result (Atlas, 2010).

Mineral Precipitation Assay, Shift in Media pH

For analysis of mineral precipitation in media, acetate supplemented broth media B (Boquet et al., 1973) and media A (Sánchez-Román et al., 2007) were used. Sterilized (100 ml) media were inoculated (0.5 OD 600 nm) using fresh cultures (24 hours-37 ˚ C) of both isolates. Cultures were incubated at 30˚ C for 21 days in shaking incubator at 160 rpm at 28˚C temperature.

The change in pH was recorded after every 5th day of total incubation time of experiment (21 days). Control of the experiment was media without inoculation. The crystals obtained after 21 days of incubation were used for microscopy and FTIR analysis.

Microscopy of Crystals

For qualitative analysis of calcite crystals same method was followed as reported by Al Disi et al., 2017. Briefly, the crystals obtained from precipitation medium were observed under stereomicroscope microscope at 100x magnification. Crystals were confirmed using the Acid test method as described by King (2016). For this test crystals were placed on clean glass slide and 10 µL (optimized value) of cold HCL was dropped on crystals. A visible bubbling due to appearance of carbon dioxide gas was recorded. This bubbling of gas due to reaction of carbonate crystals and acid was finished after the carbonate crystals were completely dissolved. Same experiment was performed with isolates unable to precipitate minerals as a negative control experiment.

Fourier Transformed Infrared Spectroscopic (FTIR) Analysis for Precipitates

Calcium carbonate precipitates obtained from both isolates were washed repeatedly with sterile distilled water and oven dried. The dried precipitates were sent to LUMS for FTIR spectroscopy. The spectra were obtained in the range of wave number 500–3500 cm^{-1}

Bacterial Identification on the Basis of Cultural, Morphological and Molecular Characteristics

Bacterial strains were characterized on the basis of culture characteristics, staining behavior and biochemical tests following Bergey's Manual of Determinative Bacteriology (Holt *et al*. 1994). Isolates were finally identified on the basis of 16S rRNA gene sequence using commercial services of First BASE Laboratories Sdn. Bhd. (Shah Alam, Selangor, Malaysia). The nucleotide sequences thus obtained were compared for homology using Basic Local Alignment Search Tool (BLAST).

Identification

The 16S rRNA gene consensus sequences of strains were finally submitted to NCBI GenBank to get accession numbers. Phylogenetic association by neighbor joining method (Saitou and Nei., 1987) was constructed using MEGA 6.0 software (Tamura *et al*., 2013) with a bootstrap value of 1000 (Fig.1).

Statistical analysis

In the experiment, statistical data analysis was carried out from mean values of replicates and measuring standard errors of the means. The error bars are shown in each figure (Steel and Torrie, 1981).

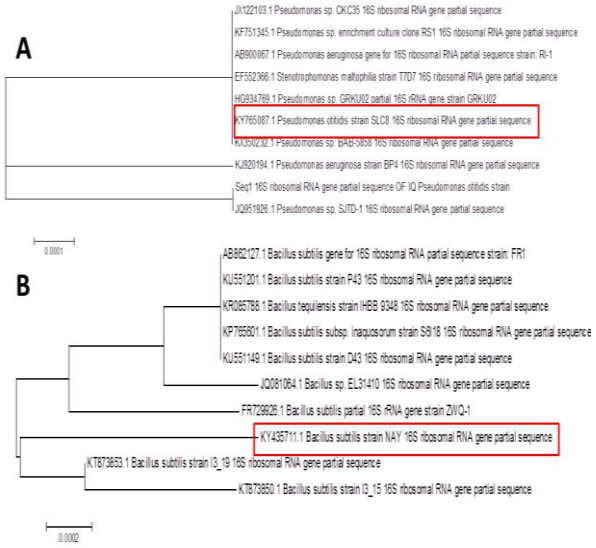

Fig. 1 Phylogenetic association using the Neighbor-Joining in MEGA6 software: (A) strain IQ (*Pseudomonas otitidis*) (B) NAY (*Bacillus subtilis*).

Results and Discussion

Bacterial Isolation, Screening and Mineral Precipitation

Two bacterial isolates were isolated from rhizosphere of Salt range soil of Pakistan and named as IQ and NAY. These strains were able to grow at 1 M NaCl supplemented Luria-Bertani Agar plates. Diversity of organisms like bacteria, fungi, algae and metazoan species have been reported in the precipitation of insoluble carbonate at earth (Gadd, 2010; Zhu and Dittrich, 2016) and influenced by different environmental conditions. Salinity, medium viscosity and ionic compositions are important environmental factors that upset the microbial mineral precipitation (Buczynski and Chafetz, 1991). This process is helpful for the environment in terms of metal precipitation, soil cementing and CO_2 sequestration (Achal et al., 2012; Zhu and Dittrich, 2016). On the basis of qualitative analysis of mineral precipitation, both strains were screened by results of Urease test, showing deep pink color as positive result. Many urea degrader soil bacteria have been reported to show carbonate precipitation e.g., *Pseudomonas calcis*, *Bacillus* sp., and *Pseudomonas denitrificans* (Boquet et al., 1973; Meyer et al., 2011; Chu et al., 2012). Another soil bacteria having urease enzyme results in reaction that show precipitation of calcite (Fujita et al., 2000; Hammes et al., 2003). *S. pasteurii* is one of the widely studied bacteria species due to its highly active urease enzyme, which catalyzes the reaction network toward the precipitation of calcite (Fujita *et al*., 2000; Hammes et al., 2003).

Quantification of mineral precipitation on the basis of weight of both strains IQ and NAY in acetate supplemented media showed that strain NAY produced maximum calcite (0.4 grams) crystals in acetate

supplemented media B as compared to strain IQ (Fig.2) in all tested media after 21 days of culture incubation. However, when media A was used, the maximum calcite crystals were recorded in strain IQ as compared to the other strain. Both strains showed visible crystals deposition at the bottom of the flasks after 4 days of culture incubation. Calcite crystals were obtained from cultured flasks at the bottom and after repeated washing white powdery mass of crystals were obtained (Fig. 2). Appearance of calcite minerals due to microbial precipitation can be the result of variation of exopolysaccharides (EPS) production, pH of the medium and presence of the variable functional groups (Braissant *et al*., 2003).

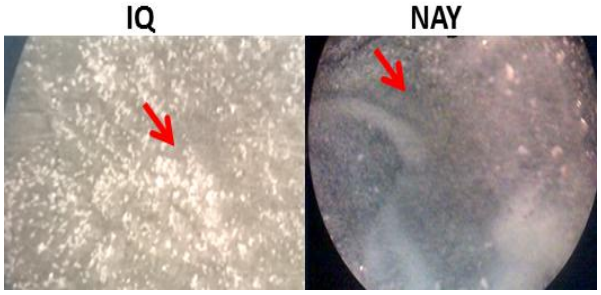

Fig. 2 Light microscopy of crystals obtained from strain IQ (*Pseudomonas otitidis*) and NAY (*Bacillus subtilis*).

There was a gradual increase in pH of the medium from pH 7.0 to pH 8.0 with the passage of time (Fig. 4). The increase in pH started at 4th day of culture incubation and increased slightly till 15 days and became consistent from then onwards. Visible bubbling on the addition of concentrated acid (HCL) was recorded from calcite crystals. Many researchers in previous and recent studies give evidence that precipitated minerals were of spherical shape and reported as microbial induced carbonates (Mckenzie and Vasconcelos, 2009; Bahniuk et al., 2015; Mettraux et al., 2015).

Microscopy of Crystals

The crystals obtained from precipitation medium were a white powdery mass as observed under stereomicroscope. These crystals showed visible bubbling due to carbon dioxide gas production on the addition of acid till all the carbonate crystals were completely dissolved. Our results of mineral precipitation quantification assay that indicated the precipitated minerals by strain IQ in acetate supplemented media A were higher as compared to media B after 21 days of culture incubation. However, media B was more favourable for increasing the calcite production for strain NAY (Fig. 3). Change in environmental conditions can trigger the increment in calcite formation. B4 medium has more buffering capacity for calcite precipitation compared to other media. A fluctuated balance in H+/OH– concentrations have been reported to influence bacterial growth and induce precipitation. These results were in line with the findings of the previous study showing Bacillus subtilus 168 and its association with calcite precipitaion (Marvasi et al., 2010).

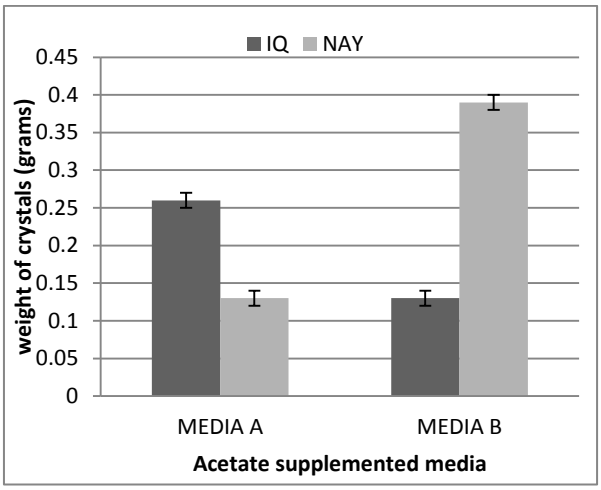

Fig. 3 Calcite precipitation in acetate supplemented media (A) and media (B) in strain IQ (*Pseudomonas otitidis*) and NAY (*Bacillus subtilis*).

Fourier Transformed Infrared Spectroscopic (FTIR) Analysis for Precipitates

IR spectrum of precipitated crystals produced by *Pseudomonas otitidis* (IQ) and *Bacillus subtilis* (NAY) showed the spectra ranging from wave number 500–3500 cm^{-1}. The three peaks located at range of 711-1400 cm^{-1} can be attributed to the C-O bonding of $CaCO_3$ (Chen and Xiang, 2009). The FT-IR spectra of $CaCO_3$ samples (Fig. 6) showed the absorption peak at wavenumber of 711 cm^{-1}, which was due to the presence of in-plane bending of carbonate ion while the absorption peak observed at 870cm^{-1} were attributed due to the out-of plane bending of carbonate ion. Furthermore, the peaks due to asymmetric extending of carbonate ions were recorded at the range of 1394 cm^{-1}. Slight peak region recorded at a range of 1399-1402.01 cm^{-1} corresponds to the OCH, COH and CCH groups (Fig. 5). The wave number recorded at 570 cm^{-1} is due to the presence of the C-O stretch of carbohydrates as previously reported (Chen et al., 2011; Zhao et al., 2012). Similar results with peak at wave number 711cm^{-1} have been reported as the carbonate group of calcite crystals (Addadi et al., 2003; Polowczyk et al., 2013). Similar results in *Pseudomonas* have been reported to be due to the presence of carbonate group. These peaks obtained in the region of 400-1400 cm^{-1} were common in both strains and also characteristic of carbonate groups as reported by others (Shafiu Kamba et al., 2013; Bharatham et al., 2014).

Bacterial Characterization

On the basis of cultural characteristics, strain NAY showed yellow colonies with irregular margins and umbonate elevation (with knobb like proturbance) (Table. 1). Cellular behavior after cell staining showed that cells of strain NAY were Gram positive capsule

and spore forming rods. While strain IQ showed brown colonies with regular margins, flat elevation and cells of IQ were gram negative rods with ability to form capsule but not the spore formation. Both strains were mucoidy in texture (Table. 1). The biochemical analysis of both isolates showed that the strain NAY showed positive results for Catalase, Oxidase, Starch Hydrolysis, Litmus milk test while negative for Methyl red test and Voges Proskauer test. Strain IQ showed positive results for Catalase and Litmus milk test while negative results for Oxidase, Starch Hydrolysis, Methyl red test and Voges Prokauer test. The 16S rRNA nucleotide sequences (fig. 1A) of both isolates revealed NAY as Bacillus subtilis under the accession number KY435711.1 and strain IQ as Pseudomonas otitidis with accession number MF136805.

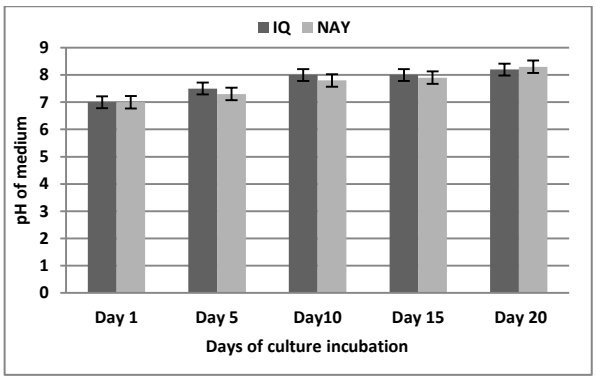

Fig. 4 Change in pH recorded in acetate supplemented media (A) and media (B) inoculated with strain IQ (*Pseudomonas otitidis*) and NAY (*Bacillus subtilis*).

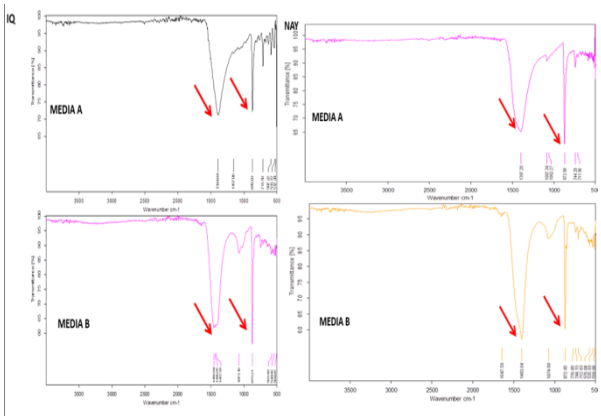

Fig. 5 FTIR analysis of precipitates of $CaCO_3$ produced from from two isolates in media A and media B.

This study presents the role of Pseudomonas otitidis in calcite precipitation. Pseudomonas otitidis has been reported to be associated which was firstly proposed and isolated by Clark et al. (2006) from clinical specimens of infected human ears and showed close resemblance to the Pseudomonas aeruginosa strain. Microbial diversity based on their different physiological parameters play a significant role in carbonate precipitation. Attributed to their diversities in physiology and metabolism, microbes from different taxa i.e., bacteria (Srivastava et al., 2015), cyanobacteria (Uma et al., 2014), fungi (Wei et al., 2013), and algae (Saghaï et al., 2015) were widely considered to play an important role in the formation of carbonate biominerals (Xiao et al., 2015).

Table 1 Cultural and morphological characterization of bacterial strains.

A Morphological characteristics

Isolates	Colour	Margins	Elevation	Texture	Simple staining	Gram staining	Capsule staining	Spore staining
IQ (*Pseudomonas otitidis*)	Brown	Regular	Flat	Mucoidy	Rods	Negative	Positive	Negative
NAY (*Bacillus subtilis*)	yellow	Irregular	Umbonate	Mucoidy	Rods	Positive	Positive	Positive

B Biochemical characteristics

Isolates	Catalase	Oxidase	Starch hydrolysis	Litmus milk test	Methyl red test	Voges prokauer test	Urease
IQ (*Pseudomonas otitidis*)	Positive	Negative	Negative	Positive	Negative	Negative	Positive
NAY (*Bacillus subtilis*)	Positive	Positive	Positive	Positive	Negative	Negative	Positive

Based on results of present study, microbes with bio mineralization abilities have a potential for carbonate precipitation in local saline microenvironments that influence plant growth. Further molecular studies were underway to answer the molecular mechanisms related to this phenomenon.

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