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Biochemical and Molecular Characterization (16s rRNA) of Bacteria Isolated From Soil Sample of Govind Sagar Lake, Himachal Pradesh and Baga Beach, Goa, India.

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ABSTRACT

Soil is consortium of different micro-organisms which is diversified by altering geographic location. In this current investigation, collection of samples were done from Govind sagar lake, Bilaspur, Himachal Pradesh and Baga beach, Goa. After growth of bacteria on nutrient agar, pure cultures are prepared on Eosin Methyl Blue (EMB) agar and then isolated bacterial colonies were utilized for carrying out biochemical tests and molecular characterization. The biochemical tests include Gram staining, motility test, phenol red test, citrate and urease test, fermentation of carbohydrates, triple sugar iron test, antibiotic sensitivity test. The physiochemical characteristics in addition to the morphological characteristics were also studied by checking growth of bacteria in varying pH [4, 7, 8.5, 10], temperature [37^{0}C, 50^{0}C, 60^{0}C,70^{0}C, 80^{0}C] and salt concentration [2%, 4%, 6%, 8%]. Pure culture of bacterial isolates Govind Sagar (GS1, GS2) and Baga beach, Goa (G1, G2) were prepared and then molecular characterization was carried out by 16s rRNA sequencing. The results of biochemical and molecular characterization confirmed that in Govind sagar sample, *Inquilinus limosus* was isolated in both the samples while in Baga beach, Goa sample *Stenotrophomonas maltophilia* and *Pseudomonas alcaliphila* were present. Basic Local Alignment Search Tool (BLAST) and phylogenetic analysis and dendrogram development was carried out to know relation of the bacteria obtained from different environment.

Keywords: Biochemical, phylogenetic tree, 16srDNA, BLAST, microbial diversity.

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INTRODUCTION

Lithosphere has wide array of the microbes present in it and the isolation and identification of those microbe is essential in order to know the type of microbial biomass present in it. Soil is source of millions of diverse bacterial species and isolation of a novel bacterium and their characterization provide details about the composition of bacterial biomass of the soil. Soil of Govind sagar, artificial lake on Satluj River is selected because of its unique physiological conditions because the area remains submerged for half of the year and other half of year there is plant growth on this soil. Due to this diversity, there is possibility of occurrence of some unique bacteria. On contrary the second soil sample is taken from Goa beach side, adding to large geographic and physiological diversity in both of the soil samples. Both the samples were collected in second week of January 2014. Proper strile package is utilized for collection of the soil sample with proper details about the location on it [1-3]. Although top soil was not taken but the sample was collected after digging 30-40 cm below the top surface to avoid the external presence of microbial flora which may be carried out by other means. The sample is collected from two or three nearby regions so that the generalized bacterial population is included in the soil sample taken [4, 5]. The pH of the soil samples is also one of the significant factors for analysis of microbial diversity, in specific pH condition specific group of bacteria are present. Basic tests for presence of potassium and phosphorous is performed in order to access the nature of the soil. Doubling time of the bacteria is very small, so by initializing from very small amount of soil sample we were able to perform several biochemical tests. Bacteria present in the soil are grown on Nutrient agar, so that by utilizing the nutrients, microbes were able to sustain their continuous growth. The morphology of the colonies obtained was observed and Bergey's manual of systematic bacteriology was utilized to get the details about the isolated bacteria.

MATERIAL AND METHOD

Soil sample was collected from depth of 30cm to 40cm in sterile air tight bags by using a sterile spatula [1-3] from Govind sagar, Himachal and Baga beach, Goa. For isolation of bacteria nutrient agar medium was prepared by taking 28g of the powdered nutrient agar in 1000 ml of distilled water in asceptic conditions. Individual bacterial colonies that appear on the nutrient agar were identified by the morphological characterization and biochemical techniques by utilizing the illustrated taxonomy scheme of "Bergey's Manual of Determinative Bacteriology [6]", which is used to identify microbe as to their genus and species. Colony are then picked and its pure culture is prepared. The colonies are streaked on EMB and Asby mannitol agar. Four pure cultures are obtained and named GS1, GS2 for Govind sagar sample and G1, G2 for Goa sample.

Gram staining was performed to analyze the morphological appearance of bacteria under microscopic observation [7, 8]. Spore staining and motility test was performed for detection of bacterial endospores and mobilization potential in isolates by standard protocol.

The effect of environment provided to the bacteria is studied by checking the growth in varied pH, temperature, salt and heavy metal concentration. These are carried out to get knowledge about the optimum conditions in which the isolated bacteria are able to grow [9, 10].

To study effect of pH nutrient broth is adjusted using ph meter by utilizing hydrochloric acid and Sodium hydroxide. The pH was maintained at 4, 7, 8.5, 10 and then autoclaved and finally to all the tubes with varying pH, bacterial isolates were added. After 24 hour incubation the test tubes were checked for growth.

Further temperature test was performed to find the thermal death point of bacteria; temperature was maintained at refrigeration temperature, 37^0C, 50^0C, 60^0C. After 24-36 hours incubation, spectrophotometric analysis was performed.

Salt tolerance was also performed by adding sodium Chloride concentration of 0.5%, 1%, 2%, 4%, 8%, 16% to nutrient broth .Following inoculation of bacteria, growth characteristics of bacteria were observed after 24-36 hours of incubation through spectrophotometer.

Heavy metal resistance analysis were also conducted, concentration ranging from 10 to 30µg/ml for each isolates. We utilized mercuric chloride, copper sulphate and ferrous chloride followed by inoculation of bacteria and checked for any growth of bacteria [11].

Antibiotic sensitivity test was performed by taking 4 antibiotics tetracycline, gentamycin, chloramphenicol, penicillin on Muller hinton agar by disc diffusion method then zone of inhibition was observed as per standard protocol.

To perform indole test, organism was inoculated into tryptone broth. Tryptophanase is generally produced by indole positive bacteria, which shows degrading behaviour against tryptophan, resulting into production of indole and other products. When Kovac's reagent is added to a broth with indole in it, a dark pink color develops. Tryptone broth is taken and put into test tubes which are then autoclaved. Pure sample was inoculated into broth by using inoculation loop. Tubes were then incubated for 24-48 hours at 37^0C, followed by addition of 1ml Kovac's reagent in tubes and observed the colour. Methyl red test was performed for glucose, lactose and sucrose to know whether bacteria present in sample is able to ferment these carbohydrates. Methyl red was added with initial red colour as pH indicator. Catalase producing organisms were identified by catalase test; this enzyme targets hydrogen peroxide and resulting into water and oxygen gas. The presence of oxygen bubbles indicates a catalase positive result. For this putted a drop of hydrogen peroxide on the glass slide containing the bacterial isolate, after 10-15 seconds results were observed.

Urease producing bacteria were screened by using differential media, which can hydrolyze urea into ammonia and carbon dioxide. In this test phenol red was used as indicator, which turns into yellow in acidic condition and into fuchsia colour in alkaline environment. For performing this test urea broth was prepared and bacterial isolates were inoculated into it in different tubes. It was then kept for incubation for 24 hours at 37 ^{0}C. Urease is enzyme which acts on urea. It helps in distinguishing members of genus proteus from gram negative pathogens [12].

Oxidase test was performed to test whether the glucose utilization in bacteria takes place in presence or in absence of it. Although by utilization of glucose there is fall in pH.

When nitrate is provided in medium, it is reduced to ammonia or free nitrogen by certain bacteria. For this test, nitrate broth was prepared with potassium nitrate (0.1%), peptone and yeast extract in Durham tube. For carrying out molecular characterization by 16s rRNA sequencing for confirming the bacteria present in sample, standard protocol was followed (Courtesy: Yaazh xenomics Mumbai, India) [13-15].

RESULTS

Four samples were found to be Gram negative with rod shaped morphology. Also no endospores were found in any samples. The bacteria present in samples were motile proving the presence of flagella in them which was confirmed by motility test. No growth was observed on heavy metal plates of mercuric chloride, copper sulphate and ferrous chloride proving that the bacteria is sensitive to all of these heavy metals. Zone of inhibition was observed in plates of different antibiotics. The sensitivity and resistance of the antibiotic is then analysed using standard protocol. For Govind sagar samples the best growth was observed between 2% to 6% and 2% to 7% of salt concentration for Goa sample respectively. Best growth characteristics of bacteria in all the samples were observed at pH 7 and 8.5. Thermal death point for Govind sagar and Goa sample was 50^0C. Methyl red fermentation test was performed where in most of the samples Glucose and sucrose were not utilized but lactose was utilized by bacteria except G2 sample. The results for indole test was negative for all samples, catalase test was positive for all samples , oxidase test was positive for GS1 and GS2 while it was observed negative for G1 and G2, urease was negative for all samples and nitrate reduction test was observed positive against G1 and rest samples were negative. On performing the 16s rRNA sequencing the bacteria in Govind sagar was found to be *Inquilinus limosus* in both GS1 and GS2. In G1 the bacteria was *Stenotrophomonas maltophilia* while in G2 bacteria obtained was *Pseudomonas alcaliphila.* Biochemical tests also proved the properties of these bacteria which were analyzed by using Bergey's manual of classification. Phylogenetic analysis was also performed for all samples after 16s rRNA sequencing and BLAST analysis. Accession number for bacterial samples have been obtained from NCBI, they are KM248328 (for GS1 and GS2), KM248329 (for G1), and KM248330 (G2).

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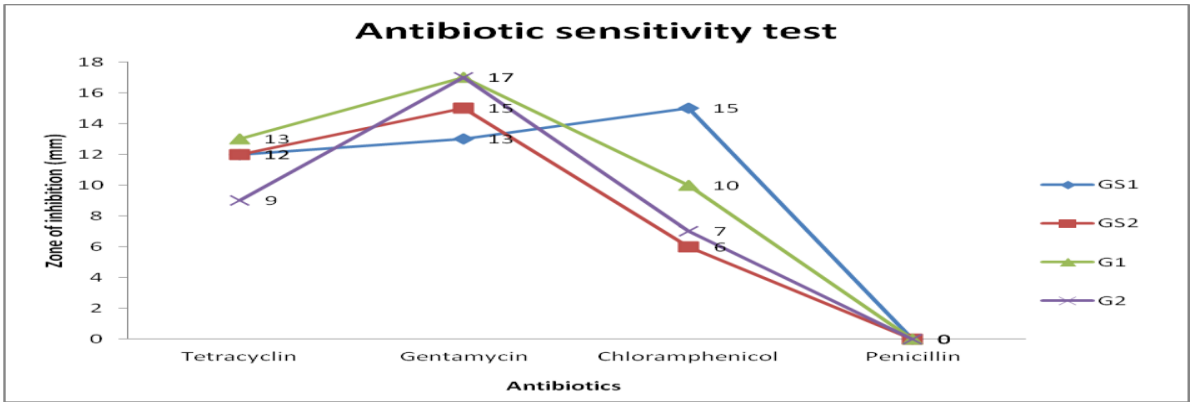
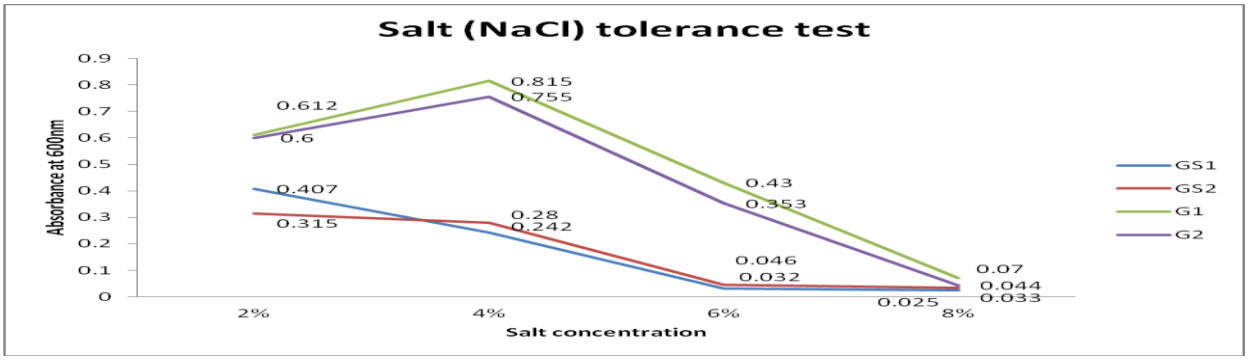
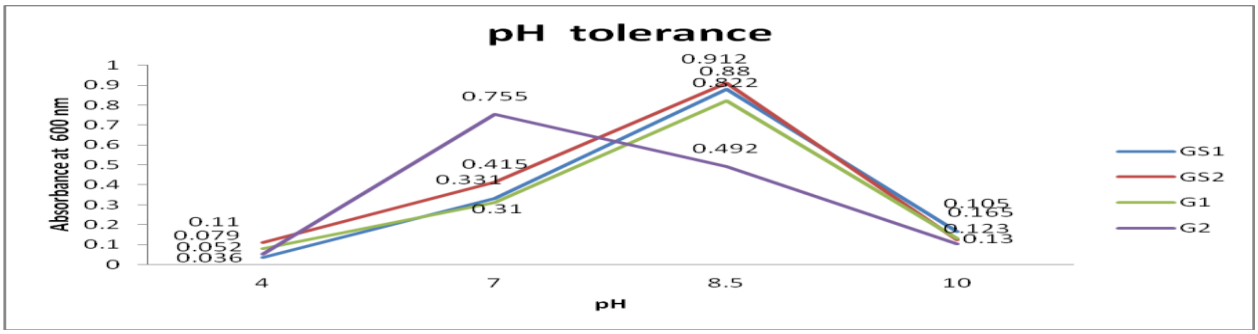


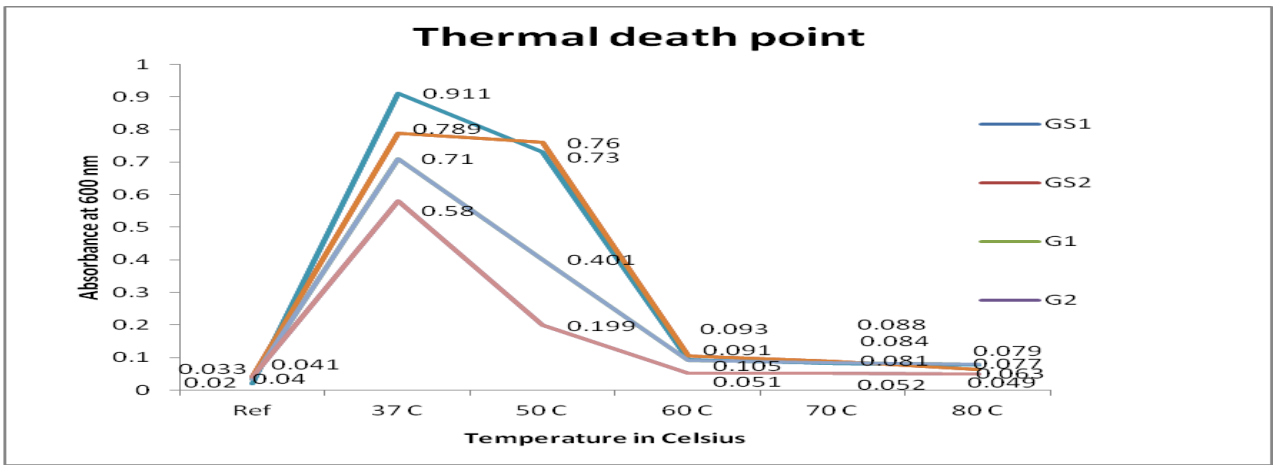
Graph 1 : Showing zone of inhibition for the antibiotics



Graph 2 : Showing Absorbance for different Salt concentration



Graph 3 : Showing Absorbance at different pH



Graph 4: Showing Absorbance observed at varying temperature

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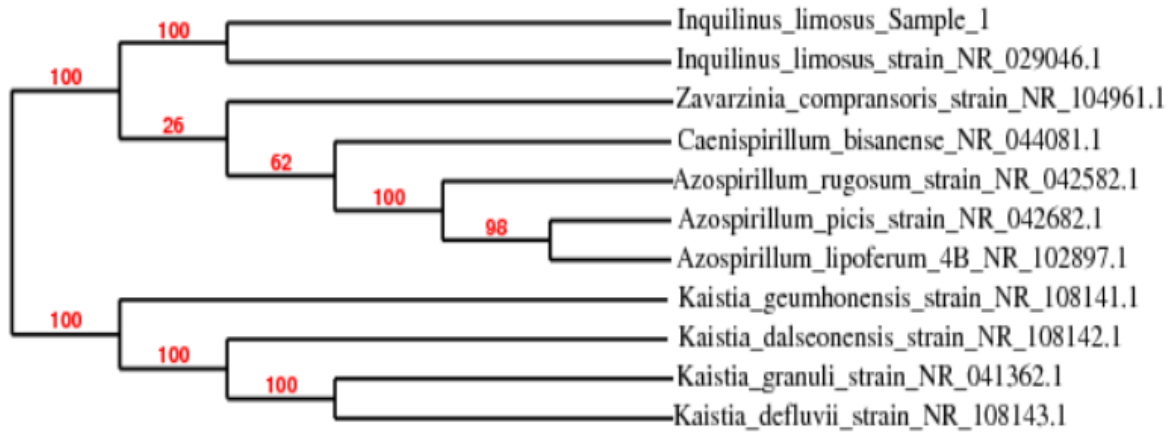

Figure 1: Phylogenetic tree for GS1 and GS2.

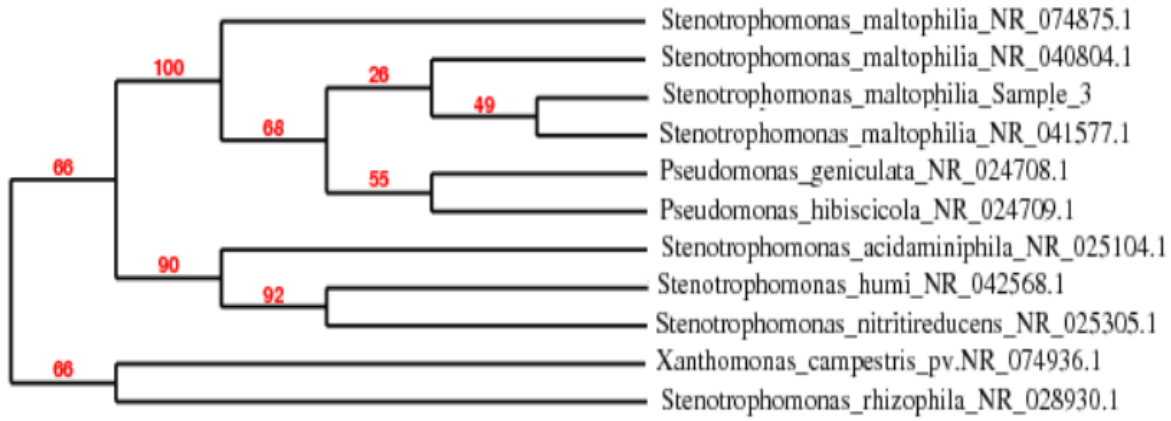

Figure 2: Phylogenetic tree for G1

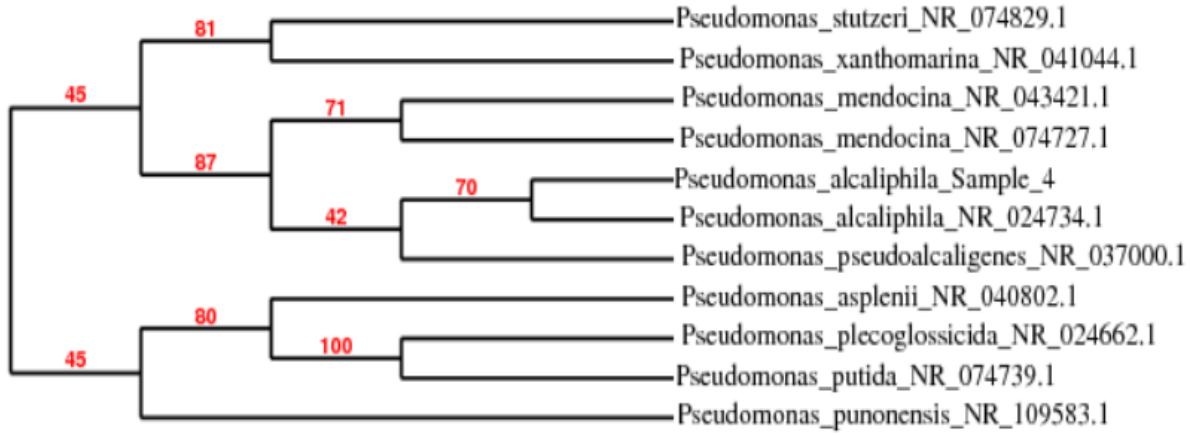

Figure 3: Phylogenetic tree for G2

CONCLUSION AND DISCUSSION

The Goal of isolation and then characterization of bacteria from these four samples has been successfully completed to obtain three disctinct uncommon bacteria in samples of Govind sagar and Goa . For Govind sagar sample 1 and 2, the bacteria found were *Inquilinus limosus.* For Goa sample 1, bacteria was *Stenotrophomonas maltophilia* and for Goa sample 2, it is *Pseudomonas alcaliphila*. These are very rare and uncommon bacteria and hence these are not present in initial edition one of Bergey's manual *Inquilinus*

limosus is bacteria which is isolated from the lungs of cystic fibrosis patient while and *Stenotrophomonas maltophilia* is able to colonize in the breathing tubes and urinary catheters infections. So from this it can be concluded that the bacteria present in the soil of Govind sagar and Goa are pathogenic and it is not safe to work in these areas without proper measures. As different research have already been done on the presence of these bacteria in patients of cystic fibrosis and the infections of breathing and urinary tracks but further research is necessary to check the consequences of presence of such bacteria in these locations which are frequently visited by the people and tourists. So these two geographical locations were selected because people from far off places tend to come to Govind sagar lake bank because there are certain historical temples present in the area which also get submerged each time water level rises. As these temples are of archeological importance, and frequently visited by archaeologists and even by local people who have to cross the region daily, study is conducted to know the pathogenicity of bacteria in soil of the area. Baga beach is also frequently visited by people of varying nationalities annually so these are uncommon bacterium which are pathogenic; hence raise concerns about their impact on health conditions of the animals and people of these two regions. Future study can be explored by whole genome sequencing, proteomics and health associated problems on these bacteria.

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