ORIGINAL RESEARCH PAPER

Microbiology

KEY WORDS: Strain, Ultra Violet, alkaline tolerant and Heat shock

BIOCHEMICAL CHARACTERIZATION AND BACTERIAL SENSITIVITY RESPONSE TOWARDS ULTRA VIOLET RAYS OF AN ESTUARINE - MULTIPLE STRESS TOLERANT NOVAL *BACILLUS SP.* G3A

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ABSTRACT

The present research is centered on the microorganism associated with aquatic nearby resources of Bay of Bengal. Different level of stress tolerance was studied for the isolated bacterial samples from Gostani River, Visakhapatnam, Andhra Pradesh, India. Multiple stress tolerant studies were studied on G3A strain.The isolate were found to be gram positive rod shaped spore forming motile Bacteria.The Biochemical analysis was prepared to recongnize the qualitative potential by the following conventional tests like Indole test,methyl red.voges proskauer,citrate utilization,Nitrate reduction,catalase,oxidase,and carbohydrate fermentation test.The G3A bacterial isolates were tested for their tolerance to different concentrations of salt in solid media(1-12%).In their natural environment ,bacteria most often,live in nutrient limiting conditions and are exposed to various stress such as Ultra Violet radiation was identified, Alkaline tolerant studies and heat shock treatment studies were conducted and the organisam was capable to sustain in the multiple stress tolerent levels.

INTRODUCTION:

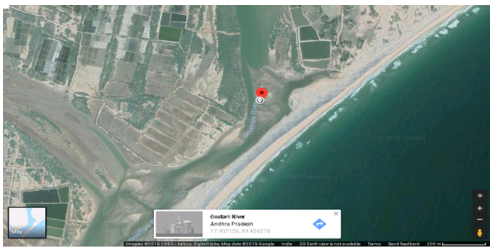
Adaptation is an evolutionary process through which living organisms develop to live in its changed habitats. Lower to higher living organisms are influenced but developed to adjust with different abiotic stresses i.e. changes in salinity of soil and water, temperature, pH, atmospheric humidity, air circulation and radiation (Mendpara et.al.2013). Inspite of the fact that much of life on Earth is directly or indirectly based on solar energy, radiation in the ultraviolet (UV) range is damaging to DNA and thus often lethal to organisms. Surface microorganisms have developed a number of mechanisms to protect themselves from the destructive effects of UV, probably very early in the history of the Earth (Walter 1983; Yasue and McCready, 1998). spores and vegetative forms are more resistant when heated in substantially dry materials than when heated in dilute aqueous solutions. In most of the studies the moisture status of the substrate was not controlled, and the results did not provide quantitative evidence of the change in heat resistance with change in water content or water activity (Murrel and Scott et.al.1966)

Accurate data on the effect of UV on aerosolized bacteria under controlled conditions exists for only a limited number of microbial species. Even fewer data is available for human pathogens and still even fewer for microbes of interest in bio defense. (Bardley et.al.2011) Bernard and Morgan, 1903 determined the lethal action of ultraviolet on bacteria lies between the region 3287 A and 2265 A. The optimum wave length probably varies with the type of organism (Duggar and Hollaender, 1934). Aquatic organisms living near the water surface are in constant risk of exposure to UV-B radiation. Several studies of the impact of UV-B radiation on the aquatic microbial food web have confirmed significant effects on bacterial assemblages at UV-B levels representative of the top layer of ocean waters. Inhibition of bacterial production (Bailey et al., 1983; Jeffrey et al., 1996; Aas et al., 1996; Pakulski et al., 1998), bacterial biomass (Sieracki and Sieburth, 1986; Helbling et al., 1995) and metabolically-important enzymes (Herndl et al., 1993; Müller-Niklas et al., 1995; Garde and Gustavson, 1999) has been reported. Present investigation was carried out on multiple stress tolerant bacteria, isolated from the near by coastline area of The Bay of Bengal, Visakhapatnam.

MATERIALS AND METHODS:

Sample Collection:

Studies were carried out in esturina river Gostani at Bhimili region, Visakhapatnam district, Andhra Pradesh, India. Sampling procedure was done as per Gayatri Devi and DurgaPrasad. 2018.



Satilite view of Gostani River Longitude 17.907126 Latitude 83.459278

Isolation and purification of bacterial isolates:

Isolation of bacteria was performed on SCDA medium by using spread plate technique. The SCDA plates were incubated for 24h at 37°C after inoculation with 1ml of the sample and subsequently observed for bacterial growth and isolation.Differential colony morphology were selected by the all the isolates. The isolates were purified and characterized by Gram stain examination.

Gram staining

24 hours old, loopful of culture was removed from the broth and made a bacterial smear on clean microscope slide with a small drop of distilled water by mounted. Air dried the thinly spread bacterial film. Fix the bacterial smear on the slide, by passing it two times on sprit lamp. Wash the slide with few drops of crystal violet solution for 1 minutes. Rinse the slide with distilled water and allow it for few seconds for the removal of excess water by air. Use few drops of grams iodine solution for rinse the slide for one minute. After that it was decolorized with 95% ethanol or decolorizing solution for 30 seconds and again rinsed with distilled water Counter stained the slide with 0.5% safranine for 10 seconds at last. It was rinsed under running tap water for a few seconds and excess water was removed by air. Then the glass slide was examined at 40x and 100x magnification using a drop of oil immersion microscope. (Tanni et.al. 2019)

Characterization of growth: Overnight cultures were prepared by inoculating a single colony of each strain into 125 mL Erlenmeyer flask containing 30 mL LB media. The flasks were incubated overnight at 37°C in a shaking incubator at 200 rpm. The following day, 5 mL of each overnight culture was diluted 1-in-10 in LB broth in a 250 mL Erlenmeyer flask and

incubated in a 37°C shaking incubator at 200 rpm. Using the Spectronic-20D spectrophotometer (Milton Roy), turbidity measurements were taken from culture samples at 600 nm (OD600) every 15 minutes for the initial 90 minutes, and subsequently every 30 minutes thereafter. For OD600 readings above 0.60, appropriate dilutions using fresh LB broth were made to adjust the turbidity to fall between 0.10 and 0.60. LB broth was used as blank. Turbidity measurements were stopped when cultures entered stationary phase and the OD readings remained constant for at least three successive data points.

Alkaline Tolerance Test:

Tolerance for alkalinity for Bacillus sp. G3A were studied by using 2 to 12 % Nacl by culturing the bacterial cell cultures on Starch casein digest agar buffered at pH 8.0. Incubation temperature was maintained at 35°C, 120 RPM for 24 hours..

UV treatment study:

Stationary phase bacterial culture G3A were obtained following the characterization of growth procedure. The suitable volume of bacterial culture to yield a final bottle concentration of 5 x 10^5 bacterial cells/mL was centrifuged at 8000 x g for 10 minutes at room temperature. Pellets were resuspended in 1 mL of sterile distilled water and transferred to 50 mL Test tubes with 10 mL of sterile distilled water. The samples were subjected to UV irradiation with a wavelength of 254 nm for 0 minutes to 60 minutes. All tubes were laid horizontally to provide even light distribution. Controls were set up identical to UV irradiated tubes except they were covered in aluminium foil and kept in a dark cupboard. Samples were taken from control and UV irradiated tubes at 0 and 60 minutes. 50 L of each sample was plated on LB agar plates at final dilutions of 10-2, 10-3, and 10-4. The dilutions are chosen to ensure that the number of colonies was within the statistically acceptable colony range of 30-300. Plates were incubated at 37° C for overnight. Colony counts were performed the following day and survival frequencies were determined by comparing viable concentrations at 120 minutes compared to time 0 minutes for the identical treatment.

Heat shock Treatment:

Heat shock treatment was given to *Bacillus sp.* G3A strain for 30°C to 100°C for 60 minutes in water bath.

Biochemical characterization:

Indole Production Test: Indole production test was used to whether the organism can oxidize tryphtophan resulting in the formation of indole, pyruvic acid and ammonia. Tryptone broth was inoculated with the isolate and one tube was kept as an un inoculated comparative control, incubate tubes for 24-48 hrs at 37°C. The indole production during the reaction was detected by adding Kovac's reagent (dimethyl aminobenzaldehyde) which produces a cherry-red layer in the top of the test tube was indicates positive. There was no development of a cherry (deep) red colour in the top layer of the tube indicates negative reaction. (Cappuccino and Sherman, 2002).

Methyl Red test: The methyl red test was employed to detect the ability of microorganisms to oxidize glucose with the production of high concentration of Acid and acid products. MR-VP broth tubes were inoculated with the isolate. One tube was kept as un inoculated comparative control. Incubate tubes for 24-48 hrs at 37°C. After 48 hrs of incubation, 5 drops of methyl red indicator was added, the colour of methyl red turned to red indicates positive test. The colour of methyl red turned to yellow indicates negative test. (Cappuccino and Sherman, 2002).

Voges-Proskaur test: Voges-Proskaur test was used to differentiate the microrganisms to produce some non acidic or neutral end product such as acetyl methyl carbinol and 2, 3 butanediol. The isolate was inoculated with test tubes containing MR-VP broth. One tube was kept as control. Incubate tubes for 24 hrs at 37°C. Development of deep red colour in the inoculated tubes 15 minutes after addition of Barrit's reagent was the indication of positive the absence of red colouration indicates negative. (Cappuccino and Sherman, 2002).

Citrate utilization test:

Citrate test was used to differentiate bacteria on the basis of their ability to utilize citrate as the sole carbon source. The utilization of citrate depends on the presence of an enzyme citrase produced by the organism that breaks down the citrate to oxaloacetic acid and acetic acid. Simmon's Citrate agar slants were inoculated with the selected isolate. An uninoculated Simmon's Citrate agar slant was kept as control. Then these tubes were incubated at 37°C for 24-48 hrs. Development of green to blue color and this constitutes a positive test. No change in the colour of the medium indicates negative. (Cappuccino and Sherman, 2002).

Catalase test

A Loop ful of the bacterial culture were placed on the glass slide for the identification of the catalase test. 3% hydrogen peroxide was used for the mixing of the culture and an immediate bubbling indicated a positive catalase test.

Oxidase test

Nutrient broth was used for this experiment. Each strain was grown on 10 ml nutrient broth and kept for 24h of incubation, each test tube was added with ready to use oxidase discs (Himedia, India) and observed for color changes. Strains which produce oxidase, made the disc color changes to blue within 15 to 30 sec. Strains were delayed oxidase positive, when the color changes to purple within 2 to 3 min. Microorganisms were oxidase negative if the color did not change. (Gayatri Devi and Durgaprasad. 2018)

Carbohydrate utilization test

About 2 or 3 loopful of 18-24 hrs old broth culture of isolate was inoculated into the glucose or sucrose broth medium and incubated for 24 and 48 hrs at 30 °C. The collection of gas inside the Durham's tube was found positive for gas production, and the change in colour of the medium to yellow indicated acid production. No gas and colour change was not indicates negative (Sharma, 2002).

RESULTS AND CONCLUSION:

Isolated bacterial strain from biofilms of gostani river was identified by the biochemical characterization and compared the results with bergy's manual of determinative bacteriology, confirmed as Bacillus sp. Among 310 isolates of the study, G3A strain shows luxuriant growth different stess tolerant levels. The bactericidal mechanism of UV light is based on DNA absorption of light, causing cross-linking between neighbouring pyrimidine nucleotide bases (thymineand cytosine) in the same DNA strand. This, in turn,impairs formation of hydrogen bonds to the purinebases on the opposite strand. DNA transcription and replication is thereby blocked, compromising cellularfunctions and eventually leading to cell death (Harm1984, Durbeej & Eriksson 2003). To cause this damage, the UV light must first be absorbed by the bacterial DNA. UV light absorption by DNA peaks at awavelength of 254 nm, which explains the excellent bactericidal effect of this specific wavelength (Bolton1999).

Strain	G3A Strain
Indole production test	Negative
Methyl red test	Negative
Voges proskaur test	Positive
Citrate utilization test	Positive
Gram Staining	Gram positive
Oxidase test	Negative
Catalase test	Positive
Carbohydrate utilization	Positive

Strain shows luxuriant growth at 8% NaCl. Bacillus sp. G3A strain grows well in 2% - 10% NaCl then the cells was found to decrease drastically. No growth was observed in 12% NaCl medium.

NaCl Tolerence Test	G3A Strain
1 %	Growth Observed
2 %	Growth Observed
4 %	Growth Observed
8 %	Growth Observed
10 %	Growth Observed
12 %	No Growth Observed

The wavelength for UV processing ranges from 200 to 280 nm, called the germicidal range, since it effectively inactivates the microorganisms. The germicidal properties of UV irradiation are mainly due to DNa mutations induced through absorption of UV light by DNa molecules (Setlow and Carrier. 1966, Wang et.al. 2005). The bacterial spores are 10 to 50 times more resistant to UV at 254 nm than vegetative cells (Nicholson, 2000, Nicholson, 2003). During germination, light-independent repair occurs by lyase activation of the spore photoproduct and nucleotide excision repair, restoring the two thymines. Variations in resistance to UV may be attributed to differences in sporulation conditions, such as the availability of metal ions present during sporulation, or germination conditions (Rose and Connell, 2009). Bacillus sp. G3A shows luxuriant growth after 90 min exposure of the ultra violet rays.

UV Exposure Time at (250 -350nm)	G3A Strain
15 min	Growth Observed
30 min	Growth Observed
45 min	Growth Observed
60 min	Growth Observed
75 min	Growth Observed
90 min	Growth Observed
105 min	No growth Observed
120 min	No growth Observed

Certain microbes like Bacillus species are seen to be more tolerant to heat, given to their spore forming property. Endospores can survive without nutrients. They are resistant to ultraviolet radiation, desiccation, high temperature, extreme freezing and chemical disinfectants. Endospore formation is usually triggered by a lack of nutrients, and usually occurs in Gram-positive bacteria. Whereas, Gram-negative bacteria do not undergo sporulation and are therefore, more heat labile compared to spore forming bacteria. It has been known for years that, bacterial spores are more resistant to biocides than non-sporulating bacteria (Priyanka et.al. 2014). The G3A strain shows growth after given heat shock treatment for 30°C to 100°C for 60 minutes.

Heat Shock Treatment	G3A
30°C	Growth Observed
40°C	Growth Observed
50°C	Growth Observed
60°C	Growth Observed
70°C	Growth Observed
80°C	Growth Observed
90°C	Growth Observed
100°C	Growth Observed

The average sensitivity varies with the incubation time and the rate of bacterial growth. Stimulation of growth was not observed to result from the activation of ultraviolet light on bacteria. Bacillus sp. G3A was grown in multiple stress level conditions.

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