Original Article

ISOLATION AND PLASMID PROFILING OF PIGMENT PRODUCING BACTERIA FROM SALT PAN ECOSYSTEM

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Abstract

Soil sample was collected from the salt pan of Tuticorin,Tamilnadu. From the collected sample, bacteria were isolated through enrichment plating technique. Five morphologically different colonies were selected from a5% salt concentration agar plate. Among the isolated bacteria, one bacterium was found to produce the pink pigment colonies.Among the 5 isolates which were Gram stained; most of the isolates were Gram negative rod shaped except TSP3 which is found to be Gram positive and pleomorphic. Among all the isolates, TSP1, 2, 4 and 5 were found to be Gram negative rod shaped. The bacteria were identified as Halobacterium sp., Pseudomonas sp., Bacillus sp., Escherichia coli, and Micrococcus sp.Plasmids were isolated from all the cultures and directly from samples. Under submerged LB broth the pH supports the growth and determines pigmentation on liquid medium. Out of 5 pH only 6 and 8 have concentrated pigmentation. Both the intracellular and extracellular pigments purified and maximum recovery of pigment was noted on the intracellular. Both extracted pigments have antibacterial effects on P.aeruginosa but activity varies among them due to concentration. Pigments are characterized by ninhydrin which denotes the presence of primary amines on the pigment and further confirmed by the FTIR spectrum. The pigment FTIR reflects prodigiosin like characters. The pigment incorporated gels have significant anti-oxidant and anti-inflammatory effects equal to standard drugs. Presence of 12 Kbp size plasmid was detected from the sample and not found in any one of the culturable isolates. Further, plasmid profiling among isolates and Metaplasmid shows the presence of different sizes of plasmid which are absent in culturable bacteria.PCR amplicon of Chromosomal DNA of pigmented bacteria reveals the culturable bacteria belong to Halobacterium sp. The present study concludes that the Halobacterium sp produced pigment has potent medicinal value which can act as antibacterial, antioxidant and anti-inflammatory agent followed by toxicity evaluation.

Keywords: Salt tolerance, Plasmid profiling, Metagenome, Halobacteriumsp.

1. INTRODUCTION

Extremophiles research is crucial for improving the extraction of novel chemicals and exploring potential biomedical applications. The hyper saline habitats will favor the microbial species which are complex in their composition and nature. Microbes living at high salt concentrations are extremely diverse. Halophiles are present in diverse habitats including the most noxious environments on the planet. Halophilic microbes flourish in generally shifting centralizations of NaCl going from (0.2 – 5.1M or 2 – 30%).

Based on this shifting NaCl focus, halophiles are classified as slight, moderate and outrageous halophiles. Halophilic microorganisms play a vital role in the production of many enzymes and bioactive compounds.

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Halophilic bacteria are a potential source of awide range of secondary metabolites and extracellular polysaccharides, enzymes etc [1]. Salt pans are large ponds filled with saltwater from the marine or another source and less explored among pharmaceutical industries [2]. The salinity of the water gradually increases as water evaporates until it reaches saturation (26% at 20°C).Ninety-nine percent of all microorganisms in almost every environment on earth remain, as yet, uncultured. Metagenomeis the approach where the isolation and characterization of the microbes can be done from the sample of hyper saline environments [3]. Metagenome sequencing revealed the functions like biogeochemical cycling and tolerance of soil microbes. A current challenge is to go beyond predictive understanding of gene function based on the genome/Metagenome to understanding of actual functions carried out by the soil micro biome in situ to construct a DNA library on sequences of interest.

Soil borne microorganisms are one of the earth's greatest sources of bio diversity with estimates ranging between 3000 and 11,000 microbial genomes per gram of soil. Moreover, nearly 140 mega bases of sequence taken from Minnesota farmland soil contained <1% of sequence with any overlaps and formed no contains demonstrating that complete sequencing of highly diverse environments is virtually impossible with current technologies[4].However, because of the huge diversity of soil and its history as a source of commercially important molecules in agriculture, chemical, industrial and pharmaceutical industries, it remains the most common target for studies of functional metagenomics. The soil composition plays the major role in the diversity of the microbe and its capability to produce bioactive compounds, many studies has been focused on sediment types with unique features characteristic of extreme environments. Microorganism Spongiococcum excentricum produce Lutein (yellow) as cancer prevention agent. Monascus sp. Showed antimicrobial anthraquinoid and monascorubramin (red) creation. Pyocyanin showed potent activity against urinary plot microorganisms like S. aureus, S. saprophyticus, S. epidermidis, E. coli and C. freundii [5].

2. Materials and Methods

2.1. Sample collection:

From the keela arasadi salt pan which is located in Tuticorin as shown in image 1,the soil samples were collected in the aseptic box and transported to the laboratory for the further process.

Image 1: Study area

2.2. Isolation and characterization of bacteria from soil sample:

The collected soil sample was plated by pour plate technique followed by serial dilution. One ml of 107was plated on LB agar enriched with different salt concentration(3,5,10 and 15 %). Plates incubated at 35 ±2°C for 24-48 h and colony forming units were recorded. Colonies with pigments are selected and subcultured. Isolated salt tolerant bacterial colonies were subjected to Biochemical tests IMViC, catalase and Oxidase.Cell morphology was studied by by gram staining

2.3. Acid tolerance test

Isolates of HSB 1 were propagated twice in nutrient broth for 20 h at 37 °C before experimental use. The cells from fresh culture were suspended in (1/10) cultivation volume of nutrient broth at pH of 2-5. Flask was kept in incubation 12 h shaking and 12 h static condition. OD was taken to record the growth under different hydrogen ion concentration

2.4. Effect of medium pH on pigment production

Nutrient broth was prepared at different pH by adjusting medium pH with 1N HCL then autoclaved at 121ºC for 15 min, cooled, inoculated with 1 mL of culture of bacterium and incubated at 37°C for 48h.pigment was extracted from broth after centrifugation at 7,000 rpm. The quantitative determination of the red pigment was done by measuring the absorbance at 600 nm using a double beam UV-Visible spectrophotometer as suggested by Chen et al.

2.5. Solid state pigment production: Nutrient agar, Yeast glucose agar, LB agar plates were prepared and lawn culture of strain were prepared. After appropriate incubation 25 ml of ethanol was added and pigment was excluded .Cell weight was calculated followed by taking of pre weight of ethanol in a flask. The mixture was vigorously vortexes for 2 min. The solution was then centrifuged for 10 min at 6000 rpm and eluted with a silica 120 mesh column. The OD of eluted pigment was taken at 600 nm.

OD x (dilution factor) x (total volume of pigment) / Weight of substrate

2.6. Characterization of pigment

Extracted pigment purified with a silica column with methanol solvent and purity detected by TLC using chloroform acetone and methanol as mobile phase (5:2:1). The nature of pigment such as presence of amine group were detected by ninhydrin test and subjected to FTIR

characterization

2.7. Antibacterial activity

Screening of antimicrobial activity of crude pigment extract was determined by agar well diffusion method. About 100 µl of 24h MDR P.aeruginosa bacterium was inoculated on Petri dishes containing Nutrient agar medium under aseptic conditions. Known volume (100 µL) of crude pigment loaded on sterile disc. Methanol alone is applied as negative control. The plates were incubated at 37°C for 24 hours. After incubation for 24 hours at 37oc the zone of inhibition was measured with the help of standard scale.

2.8. Synergy test

The stock solutions and twofold serial dilutions of each drug to at least double the MIC were prepared according to NCCLS. A total of 1000 µl of Mueller-Hinton broth was taken into each 1 ml tube. The first antibiotic of the combination amoxicillin was serially diluted along the ordinate, while the second drug pigment was added at different concentrations to the rows. An inoculum equal to a 0.5 McFarland turbidity standard was prepared from P.aeruginosa. Each tube was inoculated with 100 µl of a bacterial inoculum of 5 × 105 CFU/ml, and the plates were incubated at 35°C for 48 h under aerobic conditions. The resulting checkerboard contains each combination of two antibiotics, with tubes that contain the highest concentration of each antibiotic at opposite corners. According to the NCCLS guidelines for broth micro dilution, the MIC was defined as the lowest concentration of antibiotic that completely inhibited the growth of the organism as detected with the naked eye. Synergy is more likely to be expressed when the ratio of the concentration of each antibiotic to the MIC of that antibiotic was the same for all components of the mixture. The combination is considered synergistic when the ΣFIC is $\leq$0.5, indifferent when the ΣFIC is >0.5 to <2, and antagonistic when the ΣFIC is $\geq$2.

- The ΣFICs were calculated as follows: ΣFIC = FIC A + FIC B,
- where FIC A is the MIC of drug A in the combination/MIC of drug A alone,
- And FIC B is the MIC of drug B in the combination/MIC of drug B alone.

2.9. Antioxidant activity

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) of the extracted intra- and extracellular pigment incorporating gel 5g/ mL was taken to determine antioxidant activity. The standards used in this test were ascorbic acid at 100 µg concentration. The final concentration pigment used was 1 mg/ml. 2 mL of ethanol solution was taken in a test tube and pigment gel at concentration of 1mg was added with 0.2 ml of DPPH reagent. Then, the tube was wrapped in aluminum foil and incubated on the shaker at room temperature for 20 minutes. The absorbance was recorded at 517 nm.

C-T/CX100

2.10. HRBC anti-inflammatory activity

Blood was obtained from healthy volunteers and mixed with equal volume of sterilized PBS solution. This blood solution was centrifuged at 3 000 rpm and the packed cells were separated. The packed cells were washed with isosaline (0.85% NaCl) solution and a 10% v/v suspension was made with isosaline. This HRBC suspension was used for the estimation of anti-inflammatory properties. Different concentrations of pigment, standard diclofenac and control were separately mixed with 1mL of phosphate buffer, 2 mL of hypo saline (0.36% NaCl) and 0.5 mL of HRBC suspension. All the assay mixtures were incubated at 37 °C for 30 minutes and centrifuged at 3000 rpm. The supernatant liquid was decanted and the hemoglobin content was estimated by a spectrophotometer at 560 nm. The percentage hemolysis was estimated by assuming the hemolysis produced in the control as 100%.

Percentage protection= 100- (OD sample/ OD control) × 100

2.11. The anti-inflammatory protein denaturation

Anti-inflammatory activity of pigment was studied by using inhibition of protein denaturation method. The reaction mixture (5ml) consists of 0.2 ml of egg albumin (hen's egg), 2.8 ml phosphate buffered saline (pH: 6.4) and 2 ml of varying concentration of plant extracts. Similar volume of double distilled water served as control. Then the mixtures were incubated at 37±2 ℃ in an incubator for 15 minutes and then heated at 70 ºC for 5 minutes. After cooling, their absorbance was measured at 660nm by using the vehicle as blank. Diclofenac was used as a reference drug and treated similarly for determination of absorbance. The Percentage of inhibition of protein denaturation was calculated as follows

ODC-ODS/ODC X 100

2.12. Plasmid Isolation

1.5 ml of isolated culture was taken and cells are harvested by centrifugation for 10 minutes and 100 µl of GTE buffer was added to the cell pellet and the cells are suspended in the buffer by gentle mixing then 100µl of lysis solution was added to the content and gently mixed and 150µl of neutralization solution is added to the contents, mixed well and kept at ice cold condition for 10 minutes and then the contents are centrifuged for 7000 rpm for 10 minutes supernatant was transferred to a fresh tube and equal volume of isopropanol were added and the contents are centrifuged at 1000 rpm for 10 minutes pellets was washed twice with 70% ethanol and suspended in 50µl TE buffer.Isolated plasmid profile was studied by AGE

2.13. Chromosome DNA

2ml of culture and collected soil filtrate sample was taken and centrifuged at 6000 rpm for 15 minutes and the pellet was resuspended with 600µl of TE buffer. 30µl of 10% SDS, 20µl of lysozyme in the concentration of 10ml 100µl of Nacl was added and then incubated at room temperature for an hours.Then the mixture was treated with 100µl of

CTAB and incubated at 35˚c for 10 minutes. After incubation, the mixture was treated with chloroform and isoamyl alcohol in the ratio of 24:1 and centrifuged at 5000 rpm for 15 minutes. Then the supernatant was collected and mixed with chloroform phenol and isoamyl alcohol in the ratio of 24:25:1 and centrifuged.An equal volume of isopropanol was added to the supernatant and centrifuged.10 ml of 75% ethanol and 1ml of 3m sodium acetate was added to the precipitate and centrifuged at 5000 rpm for 10 minutes and the pellet was resuspended with TE buffer.

2.14. PCR amplification:

Tubes were taken and a Hi-chrom PCR mix was added which is a premixed solution containing Taq DNA polymerase, dnTPs, MgCl2, and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR. Upstream and downstream primer 12.5µl was(5′ - ATYCCGGTTGATCCTAC −3′) and D56 (5′ - GYTACCTTGTTACGAC-3′)added and downstream primer 12.5µl was added and DNA sample was added about 5µl and molecular biology grade water for PCR 25µl was added and sample mixed tubes were placed in thermocycler initial denaturation was 94˚c for 10 minutes, denaturation was 94˚C for 50 seconds, annealing was 60˚c for 35 seconds, extension was 75˚C for 35 seconds and final extension was 72˚C for 10 minutes and PCR started to run for about 30 cycles. Amplicons were electrophoresed and the sequences were BLAST at NCBI.

3. Results and Discussion

3.1. Isolation of Bacteria:

Totally 23X 107 bacterial colonies were isolated by enriched isolation procedure (plate Different shades of red, yellow and white coloured colonies were observed. The results showed bacterial colonies were observedonlyon 3 and 5% NaCl concentration. Nocolonies were recorded among 1 and 2%NaCl. The observed colonies were subcultured individually on enriched nutrient agar. From the present investigation, only 5 morphologically diverse isolates were subjected to further works. Morphologically 5distinct colonies were designated as TSP1-5. Colonies were irregular rhizoidal, small circular opaque, filamentous, smooth pigmented (TSP1), opaque and translucent in nature. The Gram staining showed that most of the isolates were Gram negative rod shaped bacilli. Interestingly, TSP3 were found to be Gram positive and irregular in shape (pleomorphic). Among all the isolates, TSP1, 2, 4 and 5 were found to be Gram negative rod shaped. Another study revealed the predominance of Gram negative rod and cocci shaped bacteria among salt pan[6], [7] .The results of biochemical analysis of isolated bacteria as predicted in table 1 which reveals that most of the isolates showed negative for Indole, Methyl red, Vogues Proskauer and catalase, oxidase tests. Based on biochemical features, isolates TSP1-5 was identified as Halobacterium sp, Pseudomonassp, Bacillussp, E.coli and Micrococcus sp. Isolates TSP1 and 4 were found to be MR positive. Methyl red test showed positive by isolated strain which indicates the bacterium undergoes carbohydrate metabolism leading to the formation of acidic end products like organic acid.

Table 1: Biochemical Results for Halophilic Bacterial Isolates

Culture code	Gram's stain	Indole	MR	VP	Citrate	Catalase	Oxidase	Possible Genus
TSP1	Negative	Negative	Positive	Negative	Positive	Negative	Positive	*Halobacterium* sp
TSP2	Negative	Negative	Negative	Negative	Negative	Positive	Negative	*Pseudomonas sp*
TSP3	Positive	Negative	Negative	Negative	Positive	Negative	Positive	*Bacillus* sp,
TSP4	Negative	Negative	Positive	Negative	Negative	Negative	Negative	*E.coli*
TSP5	Negative	Negative	Negative	Negative	Positive	Negative	Negative	*Micrococcus sp*

The pigment from isolate grown on selected agar plates such as Nutrient agar, yeast glucose agar, LB agar and pigment was separated by solvent extraction. The coloured crude supernatant was collected and the process was repeated until the pellet turned white and purified with column. Production of pigment extracellularly under nutrient broth with different pH (plate 1b) was also quantified. The figure 1 represents the yield of intracellular pigment at unit level shows maximum pigmentation at LB medium (595U) followed by nutrient agar. Less significant pigmentation was quantified from YG agar. Likewise the quantity of pigment from submerged broth was found best at pH 6 and less significant among other tested pH (Figure 2). But the maximum turbidity of cell suspension was found on pH 8 and the high pigmentation was recorded at pH 6. Both 6-8

pH does not affect the bacterial growth but showed variation on pigmentation. According to Imhoff, (2005) pigment producing bacteria like serratia sp capable to grow well on pH values ranging from 6.0 - 8. Further previous reports state that production of prodigiosin is largely associated with the pH and biomass [8].

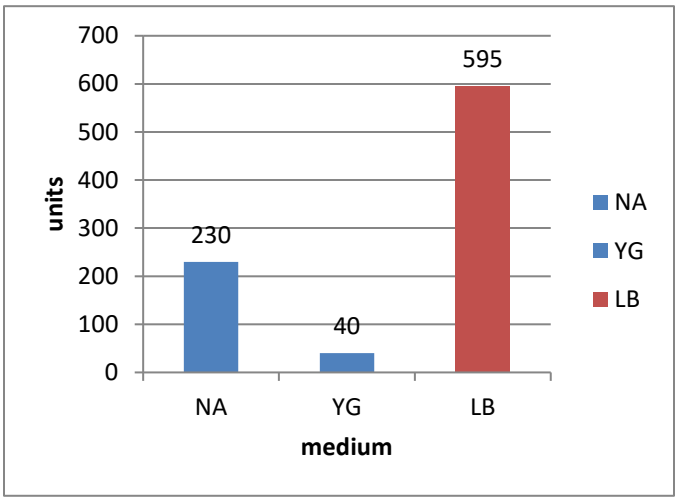

Figure 1. Unit levels of yield Intracellular pigment

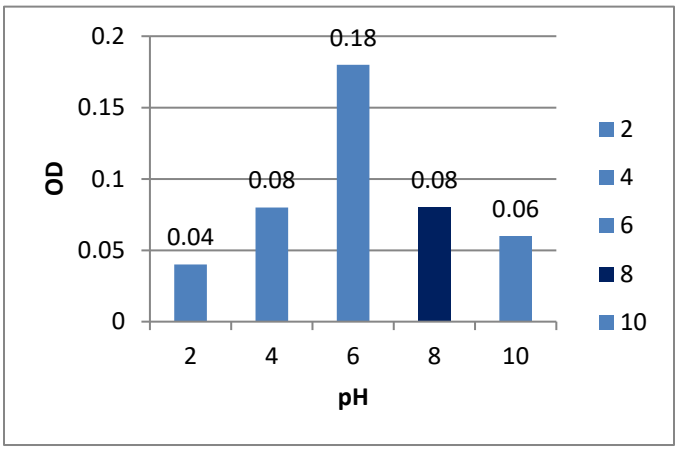

Figure 2. Quantity of pigments from submerged broth

3.2. Characterization of pigment

Crude pigment extracted from isolates was purified using column chromatography with silica gel as an absorbent with ethanol as solvent systems. The crude pigment extract reveals presence of amino acid and absence of sugar moiety. TLC analysis of pure pigments reveals presence of single fraction on both intra and extracellular The pigment of crude and purified reveals both crude and purified shows 0.38 Rf and no changes were observed in retention factor. The FTIR spectrum of column pured pigment is given in Figure 3 has 11 vibrations. Data reveal that the extracted pigments have strong and broad absorptions at 3389 cm-1, 2,925 and 2857 cm-1 are related to N-H stretch, C-H and C=O stretches. Absorption at 1640 cm-1 corresponding to C=N stretching of imine / oxime group. Furthermore the weak absorption at 1,404 cm-1 indicated C-F stretching, and 1,021 cm-1 and 1,324 cm-1 showed the C-N bend amines and C-O carboxylic stretch, while the 711 cm-1 indicated the C-H phenyl ring bend. All the N-H group and phenyl rings recorded are fingerprint regions, which was characterized by the weak intensity. Previous studies revealed that prodigiosin pigment exhibits similar absorptions between 1600-800 cm-1[9]. Prodigiosin is a kind of pigment synthesized by different genera including Bacillus sp, Streptomyces and Serratia marcescens. In spite of the fact that prodigiosin has an indistinct part in the physiology of creating strains, its production is related with extracellular or the intracellular granules is largely regulated.

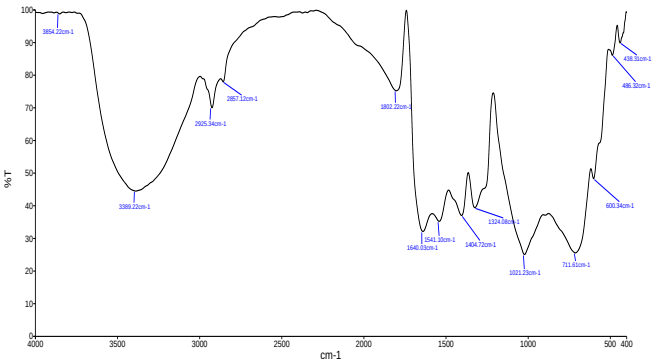

Figure 3. FTIR spectrum of pure pigment

3.3. Antibacterial activity of pigment

The results of pigment from intracellular and extracellular pigment against MDR P.aeruginosa demonstrated. The antibacterial properties imply that the higher concentration ofintracellular pigment has good antibacterial at 100 µL and no activity at 50µL. Likewise, extracellular pigment at different pH reveals that pigmentation only at 6 and 8 has moderate antibacterial activity with a 12 mm zone of inhibition against test pathogens. Activity of many pigments against gram-negative bacteria depends on yield of pigment due to the permeability of cells, whereas gram positive does not require high concentration of prodigiosin [10].Prodigiosin produced from bacteria are found to be good antifungal, antibacterial, antiprotozoal, antimalarial, cytotoxic and immunosuppressive activities [11].The pigment showed good antibacterial activity and showed a maximum zone of inhibition 16 mm against P.aeruginosa. The crude extracellular pigment has no more inhibitory activity at disc and well diffusion method. In addition microbial pigments have a synergy effect with amoxicillin and activity against MDR P.aeruginosa. Further the MIC of tested pigment is estimated as 100 µg and the MIC of amoxicillin was 50µg against P.aeruginosa. This results reveals the checkerboard assay has given fractional inhibitory 0.25 and 0.125 respectively for pigment and antibiotic has given 0.37 ΣFICs denotes that both are having synergistic effect.

3.4. Antioxidant assay

Antioxidant properties of extracellular, intracellular pigment and standard ascorbic acid have a promising antioxidant potential and the percentage of free radical scavenging property as shown in figure 4. Nutrient broth extracted pigment showed 48% antioxidant activity and intracellular

pigment from LB agar had 68% at 1000 µL. The activity was close to the activity of standard ascorbic acid (72%). In vitro study using carotenoid pigment extracted from the Sporobolomyces sp exhibited antioxidant and antimicrobial property [12].

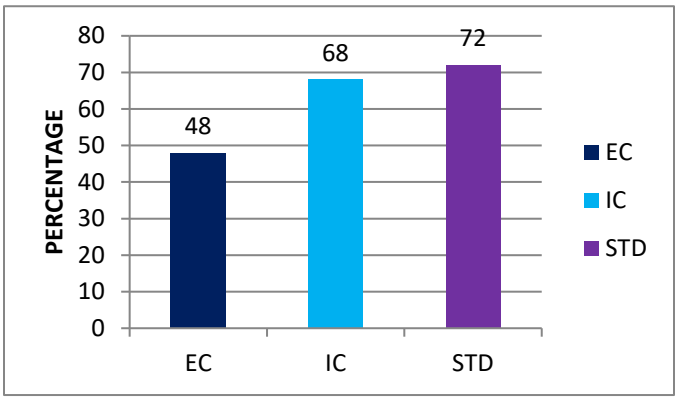

Figure 4. Percentage of free radical scavenging activity

3.5. Anti-inflammatory assay

In vitro anti-inflammatory activity of formulated pigment gel evaluated using albumin denaturation, membrane stabilization assay. Diclofenac was used as a standard drug for the study of anti-inflammatory activity.Pigment showed in vitro anti-inflammatory activity by inhibiting the temperature elevated albumin coagulation found to be 95% at 500 µg/mL and minimum inhibition rate 36% at 62.5µg/ml. Likewise HRBC assay on red blood cells membrane stabilization with 74% at 500 µg/mL and 84% respectively at 62.5µg/ml. In both case diclofenac exhibited 92% activity. Data reveals that pigment have anti-inflammatory potential equivalent to standard. Pigment based gel showed concentration dependent albumin denaturation inhibition activity and hemolysis of HRBCs, showed maximum 26% have had highest stabilization 74 %. The HRBC data confirm increases in concentration decreases stabilization of RBC membrane in test as well as standard (table 2).Orange pigment derivatives from Monascus have reported as good anti-inflammatory activities [13].

Table 2 Percentage of anti-inflammatory activity of pigment

Concentration of pigment	**Inhibition of Albumin denaturation**	**% of RBC lysis**	**Stabilization**
500	95	26	74
250	70	20	80
125	52	21	79
62.5	36	16	84
Diclofenac			
500	92	18	82
250	89	16	84
125	87	12	88
62.5	84	8	92

Extremely halophilic Archaea are able to produce red pigments called carotenoids which gives red, yellow and orange color to the organism and also to tolerate the high salt and intense UV radiation. Studies of the different halophilic microorganisms, and its limitations of life at high salt concentrations, encourage their potential for biological application [14]. Different genera of halophiles have evolved multiple mechanisms favoring their survival in the noxious environments. Some microorganisms when propagated in culture media give distinctive colors of their colonies. Such pigmentation plays an important role in laboratory diagnosis for the identification of isolates. These microbial pigments also play a role in disease pathogenesis by interfering with host immune clearance mechanisms or by exhibiting pro-inflammatory cytotoxic properties [15].The pigmentation in serratia marcescens is by production of prodigiosin [16]. The red to pink pigment produced by Serratia marcescens was prodigiosin isolated and reported as anticancer agent [17].

Similarly, A novel Halophilic strain was isolated and characterized morphologically as well as bio chemically under aerobic conditions from the sample [18].Hyper saline habitats favor microbial species having complexity in their composition and nature. Even with recent advancements in technology in molecular science, the fraction of discovered microorganisms is minor and exploration is still needed [19]. The novel halophilic bacteria, Salinibacter sp. isolated from most of the salt pans and most isolates were able to grow from 0.5 to 20% NaCl and at temperatures as low as 0 to 5°C were reported [20].

3.6. Molecular Identification of the Isolates:

The introduction of 16SrRNA sequence analysis is considered to be a useful tool for identifying bacterial species. In order to identify the halophilic bacteria, 16SrRNA was amplified using specific primers. A PCR product of around 1400bp was detected in the isolates TSP1 (Figure 5.). The amplified sequenced was subjected to BLAST analysis for sequence similarity. The phylogenetic and evolutionary analysis showed in figure 6. The similarity matrix for these comparisons is 98% and divergence is 0.006. Thephylogram reveals that the isolate is closely related to Halobacterium salinarum. Further plasmid profiling among isolates and Metaplasmid given in Plate 1b shows that the presence of different size of plasmid Compared with 25kbp marker DNA. From direct sampling isolation one band with 12 kbp in size was isolated and characterized. It was found that isolates TSP1 showed presence of 3 different plasmid ranged between size of 1-5 kbp.TSP2-4 showed single plasmid band with molecular weight 5kbp. No plasmid was isolated in TSP5 (Lane 8). The plasmid size of isolated bacterial colonies are1-8Kbp ranges only. Microorganisms from samples collected from the various sites of salt mining sites were found highly diverse group of halo tolerant and halophilic microorganisms with different morphological characteristics [21].Several studies have been conducted on the ecology, taxonomy, and phylogeny of halophilic bacteria as well as their biochemical properties. Recent studies based on 16S rRNA sequence analysis have permitted a determination of

the phylogenetic position of most moderately halophilic bacteria and metagenomics a versatile tool permit to find out metabolomics of unculturable bacteria [22] .In addition, several studies have identified the phylogenetic position of most gram-negative moderately halophilic aerobic species[23] .

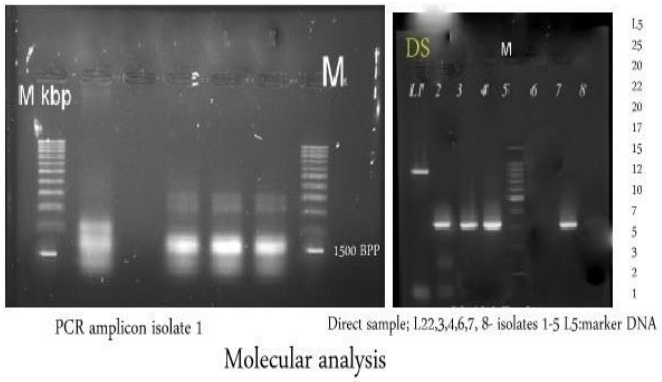

Figure 5: PCR amplification and Plasmid profile

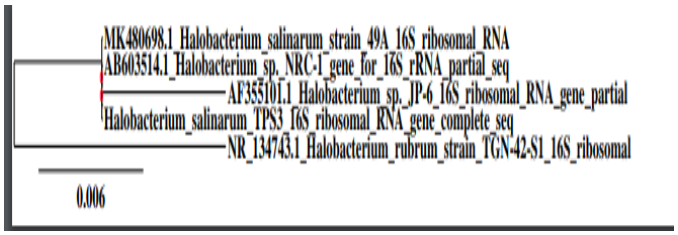

Figure 6. Phylogenetic relatedness of pigmented halophile isolate

4. Conclusion

This research findings shows the presence of non culturable large plasmid carrying bacterial strain and culturable pigment producing Halobacterium sp. Thus the bacteria Halobacterium sp produced pigment found to have good antibacterial, antioxidant and anti-inflammatory properties. This research will be useful for further in vivo pharmaceutical studies.

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Conflict of interest

No conflict of interest.

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