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Optimization of Prodigiosin Production using RSM from Moderate Halophile Inhabiting Solar Salterns

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Abstract: Solar salterns samples, such as solar salts and sun-dried salted fish, were used in this study to isolate a pigmented halophilic bacterial strain of *Bacillus aquimaris* (MP-1). The physicochemical and morphological features of twenty-one pigment-producing bacterial species were used to identify them. Among these, depending upon the salt tolerance ability, three bacterial species (MP-1, MP-3, and MP-4) were identified as moderately halophilic bacteria, and one (MP-2) was identified as an extreme halophile. Out of four, the crimson-coloured halophilic bacterial isolate (MP-1) was chosen for further research. Standard techniques were used to characterise the phenotypic and biochemical properties of the selected pigmented, moderately halophilic bacterial strain. In this study, the prime factors that strikes the expansion of the pigmented halophilic bacterial strain MP-1 were screened based on Plackett-Burman design, and the optimised values were determined by optimization of the screened standards using Box Behnken Design (BBD) in response surface methodology. Using 16S rRNA homology, a potent NaCl-tolerant halophilic bacterial isolate was identified and named *Bacillus aquimaris* (MP-1). The phylogenetic analysis of the strain MP-1 revealed 100% homology with *Bacillus aquimaris* (GenBank accession number MN865170). The crimson-coloured strain of *Bacillus aquimaris* (MP-1) displayed potential for multifaceted applications, such as antimicrobials, heavy metal tolerance, solvent tolerance, biofilm formation, and antagonistic activities. The aim of this project was to isolate, identify, and characterise the halophilic bacterial strains from solar salterns as well as extract, purify, and characterise their potential prodigiosin pigments using various chromatographic techniques (TLC, HPLC, and GC-MS). To characterise the obtained pigment, spectroscopic methods focused on UV-VIS spectra and FTIR. The antioxidative capacity of prodigiosin pigment was confirmed using the DPPH assay.

Keywords: Halophilic bacteria, *Bacillus aquimaris*, Plackett-Burman design, Prodigiosin pigment, Response Surface Methodology, Solar salterns, Box Behnken Design

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Introduction

Salt-loving microorganisms of halophiles thrive in solar salterns such as evaporation ponds or salt lakes, salt marshes, salt pans and salt-rich foods such as salt pork and salted sun-dried fish etc., (Baati *et al.*, 2010). Saltpans in environments with high salinity have a diverse range of microbial diversity, particularly halophilic bacteria, which are by far the most dominant and successful group of microbes. However, the microbial diversity present in south Tamil Nadu, India particularly in Marakkanam, Ramanathapuram, Tuticorin, and, Vedaranyam saltpans, remains poorly understood. According to their NaCl requirements, halophiles are classified as mild, moderate, and extreme halophiles (Kushner, 1993). The tolerance and salt requirements of halophilic microorganisms are dependent on different parameters, such as pH, growth medium and temperature. The solitude and characterization of these halophilic micro-organisms is critical because they are capable of producing compounds with diverse industrial, pharmaceutical, biomedical, and photoelectrical potentials. The majority of halophilic bacteria contain a high amount of carotenoid pigments in their membranes. Prodigiosin, Bacterioruberin, Bacteriorhodopsin (Margesin and Schinner, 2001), and β-carotene are the naval pigments of the halo-bacteria species. In the recent years, colourants have been extensively utilized in various forms in many industries. Focusing on the development of alternative sources for the manufacture of natural pigment is vital to prevent the endless use of synthetic pigment, which is dangerous and destructive to human health, particularly pigments of microbial origin. Hence, special attention has been paid to pigmented *Bacillus aquimaris,* which is a nontoxic, moderate halophilic bacterium that has been extensively utilised as a natural colourant in many industries (food, textile, and pharma). It is a very foremost to study the outcome of the prodigiosin pigment produced by *Bacillus aquimaris* (MP-1) under different culture conditions (salt, pH, and temperature), for successful and safe application in the pharmaceutical and food industries (Liu *et al.*, 2012). This is the first study at the process of obtaining the pigment from a moderate halophilic bacterial isolate of *Bacillus aquimais* (MP-1).

Materials and Methods

(i) *Procurement of solar saltern samples and bacteriological analysis of collected samples*

The naturally sun-dried salted fish samples were procured from nearby fish drying yards in Vellore, Tamil Nadu, India while the salt samples were obtained from various locations inside the saltern in the Marakkanam region of the Villupuram District. On the same day, the gathered specimens were meticulously ensconced within aseptic polyethylene enclosures and transported to the laboratory in a thermally regulated icebox, maintaining a controlled temperature of approximately 8°C. Subsequently, these acquired samples underwent immediate processing procedures for comprehensive bacteriological scrutiny.

(ii) *Primary identification of the chromogenic halophilic bacterial isolate*

Zobell Marine Agar medium was used for the identification of chromogenic halophilic bacterial isolates. The bacterial colony isolated from the nutrient agar plate was inoculated in Zobell Marine Broth supplemented with 5 % NaCl present in a flask. The vessels imbued with bacterial cultures were carefully housed within a Rotary shaker incubator, where they underwent a meticulous incubation period spanning 3 to 4 days, sustained at a temperature of 37°C and agitated at a rate of 100 revolutions per minute. This process was undertaken with the explicit aim of facilitating the extraction of bacterial pigments. Subsequently, a calibrated loopful of bacterial inoculum sourced from Zobell Marine Broth was delicately dispensed onto Zobell Marine Agar plates. These meticulously inoculated plates were then left to incubate for a duration ranging from 24 to 48 h, maintaining a constant temperature of 37°C. After incubation, morphologically different isolates of bacteria were selected based on the

pigment colour and it was then subcultured on Zobell's Marine Agar plates in order to get a pure culture of the isolates (Rahul *et al.*, 2017).

(a) *Phenotypic characterization of a pigmented bacterial isolate of MP-1*

The bacterial isolate was checked for morphology of the colony and cells. Pigmentation, shape, size, and texture were the main emphasis of colony morphology. Out of 21 bacterial isolates, the crimson-coloured pigmented bacterial strain MP-1 was chosen for biochemical characterization. The biochemical characterization of the selected isolate was assessed using standard methods.

(b) *Effect of salinity on the chromogenic halophilic bacterial isolates*

The upshot of different concentrations of NaCl was investigated on the expansion of chromogenic bacterial strains (MP-1, MP-2, MP-3 and MP-4). Zobell Marine Agar medium supplemented with 0 %, 2.5 %, 5 % 7.5 %, 10 % 15 % and 20 % NaCl was used to investigate the salinity requirement of the bacterial isolates. According to their salinity requirements, Kushner (1993) classified halophilic bacteria into slight (tolerance to < 5 % salt), moderate (tolerance to > 5- 20 % salt) and extreme (tolerance to > 20 % salt), respectively.

(iii) *16S rRNA gene sequencing of an isolated pigment-producing halophilic bacteria strain of MP-1*

The pigment-producing halophilic bacterial strain MP-1 was identified using 16S rRNA gene sequencing (Vora *et al.*, 2014). By Utilising the Microbial DNA Isolation Kit the genomic DNA was isolated from microbial samples using the technique outlined by Trivedi *et al*. (2011). PCR employs primers and the extremely special enzyme DNA polymerase to amplify particular cloned or genomic DNA sequences. From PCR products using a PCR Clean-up Kit (Millipore) the dNTPs and unincorporated PCR primers are eliminated. The primers 27F and 1492R were used to sequence the PCR result. ABI Sequencing procedures of isolated pigment-producing halophilic bacteria strains of MP-1 are processed using Applied Biosystems' PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS Enzyme).

(a) *Bioinformatics protocol of an isolated pigment-producing halophilic bacterial strain of MP-1*

Employing the esteemed BLAST search software, accessible via the distinguished repository of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), the sequence procured from the 16S rRNA gene underwent rigorous analysis via the BLAST program. This analysis entailed a meticulous comparison with sequences catalogued within publicly available databases. Subsequent to the discerning phylogenetic scrutiny of both the query sequence and its closely analogous counterparts derived from the BLAST results, a series of meticulous multiple sequence alignments were diligently executed. For multiple sequence alignments, the MUSCLE 3.7 software was employed (Edgar, 2004). Using the Gblocks 0.91b tool, the obtained aligned sequences of the isolated halophilic bacterial species of MP-1 were treated. These Gblocks remove divergent sections and poorly aligned locations (removing alignment noise) (Talavera and Castresana, 2007). Ultimately, HKY85 was employed as a replacement model and PhyML 3.0 aLRT was utilized for phylogenetic analysis. TreeDyn Software (version 198.3) was used to analyse a phylogenetic tree.

(b) *Prediction of the DNA structure of Bacillus aquimaris* (*MP-1*)

In the study, the Snap gene algorithm was applied to predict the structure of isolated DNA from MP-1 along with their enzymatic systems. This algorithm provides in-depth information about isolated DNA from selected strains (structural types, base pairs, molecular weight, G+C values, enzymes involved, and their appropriate regions).

(iv) *Experimental Design and statistical data analysis for screening and optimization of significant variables that influence the biomass production of pigmented halophilic bacterial isolate* (*MP-1*)

The Plackett-Burman experiment design (PBD) was used to screen the notable variables that influence the growth of *Bacillus aquimaris* (MP-1). A total of eleven variables, including peptone, yeast extract, Na_2SO_4, KCl_2, $NaCl_2$, $CaCl_2$, pH, incubation period, temperature, agitation, and media volume, were tested at high (+) and low levels (-) in a total of twelve runs. After the assortment of all responses calculated of all the runs, finally the computation of regression coefficients was started. Using the first-degree fractional factorial model PBDs results are interpreted using the following equation.

$$Y=A_0 + A_1X_1+ A_2X_2 + \_\_\_\_\_ + A_{11} X_{11}$$

Where "y" represents the predicted response, "X_1" to "X_{11}" stand for the selected factors, "A_1" to "A_{11}" are the specific coefficients, and "A_0" means the intercept of the mean. Following the estimation of regression coefficients (factors), an ANOVA was carried out to determine which of the factors significantly influenced the dependent variable of interest (response). Through the utilization of a normal probability plot in conjunction with a Pareto chart, discerning attention was directed towards identifying the preeminent variables, distinguished by their pronounced positive impact, amidst a spectrum of variables exhibiting negligible influence on the response level. These discerned variables were judiciously earmarked for subsequent optimazation endeavours. The analytical framework of the Plackett-Burman experimental design was meticulously scrutinized utilizing the sophisticated Design Expert® software, specifically version 8.0.7.1, facilitating comprehensive analysis and elucidation of pertinent insights.

Utilizing the sophisticated Response Surface Methodology (RSM) in tandem with the Box-Behnken Design (BBD), an endeavour was undertaken to augment the identified variables, thereby striving to maximize the pigment yield of the pigmented halophilic bacterial isolate denoted as MP-1. The delineated variables underwent meticulous examination across three distinct levels, denoted as (-1), (0), and (+1), signifying low, intermediate, and high levels, respectively. The experimental framework and subsequent analysis of acquired data were rigorously conducted employing the cutting-edge Design Expert® software, version 8.0.7.1.

Each factor's significance, along with their potential interactions, was meticulously scrutinized through an in-depth ANOVA analysis. Subsequently, a quadratic polynomial model was derived to ascertain the optimal points of the selected variables, leveraging the principles of the multiple regression method. The data gleaned from this comprehensive approach were further refined through fitting a second-order polynomial equation, ensuring a precise alignment with empirical observations.

$$Y = \beta_0 +\beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2+ \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2+ \beta_{12} X_1 X_2 +\beta_{13} X_1 X_3+ \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4$$

Within this framework, the focal variable of interest, denoted as biomass and symbolized by Y, is subject to the influence of several independent variables, specifically designated as X1, X2, X3, and X4, which represent the meticulously screened parameters. The linear impact of these variables is encapsulated by coefficients β1, β2, β3, and β4, while the nuanced interactions between them are elucidated through second-order coefficients, namely β12, β13, β14, β23, β24, and β34. Additionally, the quadratic coefficients β11, β22, β33, and β44 provide insights into the nonlinear effects within the model. To comprehensively visualize the intricate interplay among these physicochemical factors, three-dimensional response surface curves were meticulously crafted utilizing the advanced Design Expert® software, version 8.0.7.1. This facilitated a nuanced exploration of the dynamic relationships between the dependent and independent variables, offering invaluable insights into the complex system under scrutiny.

(v) *Characterization of a pigmented halophilic bacterial isolate Bacillus aquimaris* (*MP-1*)

(a) *Antibiotic sensitivity test of pigmented halophilicBacillus aquimaris* (*MP-1*)

The antibiotic susceptibility assay was meticulously executed employing the disc diffusion technique, meticulously adhering to the stringent standards delineated in the 2012 guidelines set forth by the esteemed Clinical and Laboratory Standards Institute (CLSI). Minor modifications were applied to the growth medium to ensure optimal conditions for the halophilic bacteria under scrutiny.

Instead of the conventional Muelle-Hinton Agar (MHA), the growth substrate employed was the Zobell Marine Agar medium, enriched with a 15% concentration of sodium chloride, as procured from Himedia, India. This deviation from the norm was necessitated by the inadequacy of MHA in supporting the proliferation of a diverse array of moderate halophiles, thus warranting a tailored approach to cultivate and assess their antibiotic sensitivity profile. The microbial inoculum was prepared as per the guidelines of CLSI (2012). In brief, the exponential phase culture's turbidity was tuned to match the turbidity of 0.5 McFarland standards, which corresponds to 10^8 cells/ml. The incubation temperature was 37°C –40°C for 24 h or till growth appeared. The antibiotics used were Kanamycin (30 µg), Streptomycin (10 µg), Tetracyclin (30 µg), Amoxyclin (10 µg), Erythromycin (15 µg), Amikacin (30 µg), Vancomycin (30 µg), Nystatin (100 µg), Carbencillin (100 µg), Cefuroxime (30 µg), Azithromycin (30 µg), Ticarcillin (75 µg), Colistin (10 µg), Piperacillin (10 µg), Cephalexin (30 µg), Ofloxacin (30 µg), Co-Trimoxazole (25 µg), Gentamycin (10 µg), Ketoconazole (10 µg), and Sulphamathizole (25 µg).

(b) *Heavy metal tolerance assay of pigmented halophilic Bacillus aquimaris* (*MP-1*)

Utilizing an agar dilution method with a multiple inoculator system, tolerance to heavy metals was determined (Halder *et al.*, 2016). Tubes containing 20 ml of melted standard Zobell Marine Agar supplemented with varying concentrations (0.5, 0.1, 0.15, and 2.0 µg/ml for Cd, Mn, Cr, Cu, Co, Ni, and Zn; and 0.05, 0.1, 0.2, and 0.4 µg/ml for Hg and Pb) of metals were poured onto plates on the day of the experiment. The plates were prepared in triplicate and incubated at 37 °C for 24-48 h.

(c) *Solvent tolerance assay*:

The solvent tolerance assay (Wang *et al.*, 2008**)** was used to assess tolerance to acetone, methanol, xylene, benzene, heptane, cyclohexane, and toluene. A fraction of 3 µl of inoculum was dappled on Zobell agar plates and allowed to dry. The surface was covered by solvents to a depth of 2–3 mm. The plates were covered to avoid vaporization. After 48 h of incubation at 30°C, bacterial growth was recorded.

(d) *Biofilm formation assay of pigmented halophilic Bacillus aquimaris* (*MP-1*)

A well-established biofilm is one of the most significant factors in efficient electron transfer, and it is also used in microbial fuel cell modelling to treat waste sewage and water purification, where it can be used for organic nutrient trapping (Kumar *et al.*, 1998). A PVC pipe measuring 7 cm in length and 4 cm in width was utilized to foster biofilm formation. The process involved preparing glass slides (75 mm in height, 25 mm in width, and 1 mm in thickness) by precleaning them with 1N HCl, treating them with a 10 mg/l sodium hypochlorite solution for 24 h, rinsing them with sterile distilled water, and then inserting them into a PVC chamber. The chamber was periodically irrigated at a flow rate of 10 to 20 drops per minute over the course of a month to facilitate bacterial adherence for the development of a biofilm from the MP-1 microbial culture (Lehtola *et al.*, 2002).

(e) *Polar lipid extraction from halophilic Bacillus aquimaris* (*MP-1*)

Experimental setup (*Litchfield et al., 2000*)

A volumetric measure of 10 ml from the culture underwent a meticulous centrifugation process, operating at an accelerated speed of 10,000 revolutions per minute (rpm) for a duration of 10 min. This precise manipulation resulted in the formation of discernible pellets, which were subsequently reconstituted in a precisely

measured volume of 1 ml of distilled water. These reconstituted pellets were delicately transferred into glass tubes, whereupon they were subjected to the addition of 3.75 ml of a meticulously prepared methanol-chloroform solution, adhering strictly to the prescribed ratio of 2:1 (v/v). Following an incubation period of approximately 4 h, the composite mixture underwent a secondary centrifugation process, operating at a reduced speed of 5,000 rpm for a duration of 5 min.

The resultant supernatant, having undergone meticulous separation, was gently transferred into fresh glass receptacles containing a solution composed of chloroform and distilled water in equal parts (1:1). Subsequent to a further round of centrifugation, operating at the same reduced speed and duration, the lower phase of the separated solution was carefully collected. This collected phase was then subjected to a controlled desiccation process under vacuum conditions until complete dryness was achieved. The desiccated material was then carefully stored within a refrigerated environment, awaiting subsequent analysis.

The polar lipids thus extracted were dissolved with precision in a limited quantity of chloroform, subsequently introduced into an environment maintained by a meticulously crafted solvent system. This system comprised a solution characterized by a delicate balance of chloroform, methanol, acetic acid, and water, with volumetric ratios of 85:22.5:10:4, respectively. The dissolution process was allowed to proceed for an approximate duration of 2 h, ensuring thorough integration within the solvent environment. For the subsequent two-dimensional phase system, the thin-layer chromatography (TLC) plates were methodically immersed in a solution consisting of chloroform, methanol, and water, adhering to a volumetric ratio of 65:25:10, and allowed to incubate for a duration of 2 h. Following this initial incubation, the plates were carefully reoriented to a 90-degree angle and subjected to a further incubation period of approximately 2 h within a solution comprising chloroform, methanol, acetic acid, and water, meticulously balanced at ratios of 80:12:15:4, respectively. Subsequent to this meticulous process, the TLC plates were allowed to air dry, ensuring the preservation of the extracted compounds for subsequent analysis.. To detect glycolipids, plates were sprayed with solution 1 (0.5% (w/v) α-naphthol in methanol/ H_2O solution (1:1, v/v), followed by solution 2 (5 ml H_2SO_4, 95 ml ethanol) and incubate at 150°C until lipids could be visualized. Using Molybdenum Blue Spray Reagent (1.3%) phospholipids were detected, whereas total polar lipids were detected using Orcinol Ferric Chloride Spray Reagent. Using the Ninhydrin Spray Reagent, lipids containing amino acids was found.

(f) *Screening for the study of the antagonistic activity of Bacillus aquimaris* (*MP-1*)

As stated by the Clinical and Laboratory Standards Institute, the disc diffusion method was used to screen the antagonistic activity between *Bacillus aquimaris* isolates (MP-1), tested bacteria (MDR) *of Salmonella sp.*, *Pseudomonas sp.*, *Klebsiella pneumonia*, *Proteus sp.*, *Escherichia coli*, *Staphylococcus aureus*, *Vibrio sp.*, *Bacillus sp.*, and fungi (*Aspergillus niger*, *Aspergillus flavus*, and *Rhizopus* sp.). Circular discs with a 6 ml diameter were fashioned from stacks of three Whatman No. 1 filter papers, and then subjected to sterilization at 121°C for 15 min and subsequent drying. These discs were positioned onto Zobell Marine Agar plates that had been previously swabbed with the desired bacterial isolates. Fractions of the MP-1, MP-3, and MP-4 cultures in Zobell Marine Broth (incubated for 5 days at 28°C) were pipetted onto the discs in 20 µl quantities. Before incubation, the Petri plates were refrigerated for 1 h to facilitate the diffusion of antibacterial compounds. The diameter of inhibition zones was assessed following incubation at 37°C for 24 h for bacteria and at 28°C for 72 h for fungi. Fresh cultures of both the pathogen and the bacterial antagonists were employed for each individual experiment.

(vi) *Extraction of pigment produced from moderate halophilic Bacillus aquimaris* (*MP-1*) (*Elevi et al., 2007b*)

A volume of 100 ml from a mature culture underwent centrifugation for 20 min at 7,500 revolutions per minute (rpm). Following the removal of the supernatant, 1 ml of an acetone-methanol solution (in a ratio of 7 parts acetone to 2 parts methanol, v/v) was added to the pellet. Before that, different solvents like ethyl acetate, acetone, hexane, and methanol were used to check the solubility of pigment. The solvent was selected by checking the maximum solubility of the pigment in it. After 1 h of incubation in the dark, the extract was centrifuged for 3 min at 7,500 rpm. The supernatant underwent filtration using a sterile membrane with a pore size of 0.45 micrometres. Subsequently, all extractions were stored at -20°C in complete darkness, shielded by aluminium foil. The pigment obtained through liquid-liquid extraction was subjected to overnight drying in a vacuum oven set at 50°C. Following drying, the pigment was dissolved in methanol or chloroform through repeated iterations (2-3 times) to attain its pure form. The confirmation of type and name of the pure pigment obtained from pigmented moderate halophilic *Bacillus aquimaris* (MP-1) was done by further studies.

(viii) *Quantification of prodigiosin pigment produced from moderate halophilic bacterial isolates of Bacillus aquimaris* (*MP-1*)

A 1 ml culture broth of halophilic *Bacillus aquimaris* (MP-1) was vigorously mixed with 3 ml of methanol using a vortex mixer, followed by centrifugation at 5000 revolutions per minute (rpm) for 5 min. Subsequently, 0.8 ml of supernatant extract from the centrifuged suspension was combined with 0.2 ml of acidified methanol. The optical density of the resulting solution was determined according to the method described by Wei and Chen (2005). For the quantification of prodigiosin pigment produced from halophilic *Bacillus aquimaris* (MP-1) in Zobell Marine Broth, it was measured using the following equation (Mekhael and Yousif, 2009):

$$\text{Prodgiosin unit/cell} = \frac{OD536 - (1.381 \times OD620)) \times 1000}{OD620}$$

Where, OD 536 = Pigment absorbance; OD 620 = Bacterial cell absorbance, 1.381 = Constant

(viii) *Purification and characterization of Prodigiosin*

(a) *Thin-Layer Chromatography* (*TLC*)

The initial raw carotenoid extract derived from the halophilic *Bacillus aquimaris* strain, denoted as MP-1, underwent a meticulous purification process employing thin-layer chromatography (TLC) conducted on aluminum sheets coated with a fine layer of silica gel. Employing a fine-tipped capillary tube, with a calibrated volume typically ranging between 2 to 5 µl, the crude extract was meticulously applied along a predefined line etched onto the surface of the silica-coated plates, subsequently allowing for controlled air drying. Following this meticulous application, the TLC plate was immersed within a solvent solution for a duration of precisely 20 min, ensuring submersion just below the demarcated line indicative of sample loading. A comprehensive exploration of diverse solvent systems, as delineated within Morgan's authoritative thesis, was undertaken to refine and optimize the chemical separation process to its utmost efficacy. For the specific aims of this investigation, the acetone-methanol extract was meticulously introduced onto a TLC silica gel plate, with subsequent development achieved through the application of a solvent mixture comprising hexane and acetone in a precise ratio of 7:3. After a precisely timed interval of 45 min, the TLC plate underwent meticulous scrutiny, and upon the manifestation of the discernible red-colored band, indicative of carotenoid compounds, the plate was delicately withdrawn from the development chamber. Subsequent determination of the Retention factor (Rf) value, a crucial parameter for assessing compound mobility within the chromatogram, was meticulously computed utilizing the prescribed equation, thereby providing invaluable insights into the separation dynamics achieved.

Rf =Distance travelled by the compound/Distance travelled by the solvent front

To detect the average Rf value, triplicate experiments were conducted and compared with the values obtained by other researchers (Song *et al.*, 2006; Davaraj *et al.*, 2009; Samrot *et al.*, 2011).

(b) *Absorption of UV-Visible Spectra*

The dried pigment extracted from MP-1 using the aforementioned method and dissolved in methanol underwent spectral analysis. An examination of the extract's spectrum was performed using a UV-visible spectrophotometer, scanning the extract from 400 to 800 nm to determine the maximum absorption spectra. Methanol served as the blank in this analysis.

(c) *Reversed-Phase High-Performance Liquid Chromatography* (*HPLC*)

The dried carotenoid extract was mixed with a small quantity of chloroform-methanol solution (in a ratio of 3:1) and subsequently subjected to analysis using HPLC equipment. Reverse-phase high-performance liquid chromatography (HPLC) stands out as the preferred analytical method for separating, quantifying, and characterizing the structure of naturally occurring and synthesized carotenoids, along with their principal metabolic byproducts (Rodriguez-Amaya, 2015). Furthermore, the detection of carotenoids done with the aid of photodiode array detector following HPLC separation utilizing spectral features (Schoefs *et al.*, 1995). Peaks were detected using retention durations and absorption spectra that were compared to previous investigations. The extracted pigments were separated into several fractions by HPLC.

(d) *GC/MS analysis*

The analytical endeavor was conducted utilizing the Clarus 680 Gas Chromatography (GC) system, distinguished by its utilization of a high-grade fused silica column meticulously packed with Elite-5MS stationary phase. This column, characterized by a composition of 5% biphenyl and 95% dimethyl polysiloxane, boasted dimensions of 30 meters in length, 0.25 millimeters in inner diameter, and a film thickness of 250 micrometers. Helium gas, esteemed for its inert nature, was judiciously employed as the carrier gas, maintaining a precise and consistent flow rate of 1 milliliter per minute, thus facilitating the requisite separation of individual components within the sample matrix.

The parameters of the mass detector were meticulously configured to ensure optimal performance. These settings encompassed a transfer line temperature and an ion source temperature, both set at a precisely calibrated 230°C. Employing the electron impact ionization mode operating at 70 electron volts (eV), the detector conducted scans with a duration of 0.2 sec, with intervals of 0.1 sec, thus ensuring a comprehensive assessment of the sample constituents. Subsequent to data acquisition, the spectra of individual components were subjected to meticulous comparison with the comprehensive repository of known component spectra housed within the GC-MS NIST (2008) library. This exhaustive reference database facilitated the identification and characterization of the sample constituents, ensuring a rigorous and comprehensive analysis of the chromatographic data.

(ix) *Application of prodigiosin*

(a) *Evaluation of antioxidant activity* (*DPPH*)

DPPH quenching ability in methanol extracts of carotenoid derivative such as prodigiosin, extracted from halophilic bacterial isolates MP-1 was measured according to Harbone and Baxter (1995). The extracted dry pigment was dissolved in 1mg/1ml was used to determine their antioxidant activity. The prodigiosin percentage of scavenging activity on the DPPH radical was calculated as a percentage (%) inhibition of DPPH (I %) using the following formula:

$$\text{Inhibition \%} = \frac{(Ao-As) \times 100}{(Ao)}$$

Where, Ao = Absorption of control; As = Absorption of tested compound

Antioxidant activity of the extracted dry pigment from MP-1 was tested at five dissimilar

concentrations (50 µg, 100 µg, 150 µg, 200 µg, 250 µg). Using a linear regression method the IC_{50} value (half maximal inhibitory concentration) for the concentration of the sample that scavenged 50% of the DPPH free radical was calculated.

(b) *Additive to Commercial Sunscreens*

We bought two different commercial sunscreens from the nearby Vellore market, labeled SPF 15 and SPF 24. We bought ethanol from real chemicals in India. Ten grams of cucumber fruit and ten grams of Aloe Vera leaf were macerated separately and filtered via muslin fabric to create a plant extract, which was then utilized for more research.

- *Sunscreen Protection Factors (*SPFs*) Determination*

Spectrophotometric analysis was used to assess the SPFs of sunscreens enhanced with prodigiosin pigments as well as commercial sunscreens (SPF-15 and SPF-24) *in vitro*.

- *Sample preparation*

Each weighed sample, comprising one gram, underwent dissolution in ethanol, followed by a meticulous degassing process conducted within an ultrasonic bath for a duration of 5 min. Subsequent to this preparatory step, the solution underwent filtration through filter paper, with the initial 10 ml being meticulously discarded to ensure the elimination of any impurities or particulate matter. Following this meticulous filtration, a precisely measured aliquot of 5 ml was meticulously transferred into a volumetric flask with a capacity of 50 ml, subsequently being diluted to volume utilizing ethanol. Another aliquot of identical volume, precisely 5 ml, was meticulously transferred into a volumetric flask with a capacity of 25 ml, and was likewise topped up with ethanol to achieve the desired volume. Subsequent to these meticulous preparation steps, the absorption spectra of the resulting solutions were meticulously recorded within the spectral range of 290 to 450 nm, employing a quartz cell with a path length of 1 cm. Ethanol was judiciously employed as the reference blank for these spectral measurements. Following each measurement, the Sun Protection Factor (SPF) values were meticulously determined utilizing the well-established Mansur mathematical equation (Mansur *et al.*, 1986), ensuring precise quantification of the sample's photoprotective efficacy.

$$SPF_{\text{spectrophotometric}} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where, CF denotes the correction factor (= 10); EE is the Erythemal Effect spectrum; I is the solar intensity spectrum; Abs is the absorbance of the sunscreen product.

The values of EE × I are constants, Abs 290 = 0.0150 (EE I), and Abs 320 = 0.0180 (EE I) were determined (Ashawat *et al.*, 2006).

(c) *Textile dyes*

Tests were carried out using concentrations of 100 g/ml solutions of prodigiosin to determine its dyeing properties on cotton and polyiso-prenematerials. Cotton and latex materials were heavily washed for several hours with hot water and detergents in order to evaluate the dyeing efficiency of prodigiosin.

(d) *Prodigiosin pigments in candle preparation*

In a container, 50 g of commercially manufactured transparent gel wax was melted using a water bath. Then, 2 ml of a methanolic pigment extract at a concentration of 4 mg/ml were added and thoroughly blended. Subsequently, the mixture was poured into the mold and left to cool for 2 h at room temperature (Mehta and Shah, 2015).

Results and Discussion

Isolation of halophilic bacterial strains from saline ecosystems in Villupuram and Vellore District in Tamil Nadu, India

The chosen sampling locations were illustrated on a satellite map as shown in Figure 1. One of the selected sampling sites, the Marakkanam salt pans (Fig. 2), situated in Tamil Nadu along the Bay of Bengal on the East coast of India, are characterized by thalassohaline properties similar to salterns found worldwide, where sodium and

Fig. 1: Satellite map showing the sampling areas in Marakkanam salt pans and Vellore fish yard, Tamil Nadu, India.

Fig. 2: Salt sample collected from Marakkanam salt pan.

Fig. 3: Sun dried salted fish samples collected from Vellore fish yard.

chloride ions predominate in the water. Additionally, sun-dried salted fish samples were collected from the Vellore fish drying yard (Fig. 3). The samples that were obtained had neutral pH values between 7.0 and 7.5 and salinities that varied from 20% to salt saturation (> 35% w/v). It

Fig. 4: Halophilic bacterial colonies isolated from solar saltern samples.

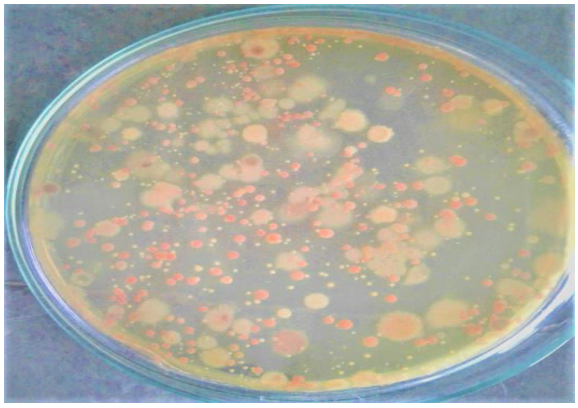


Fig. 5: Halophilic bacterial colonies isolated from sun dried salted fish samples.

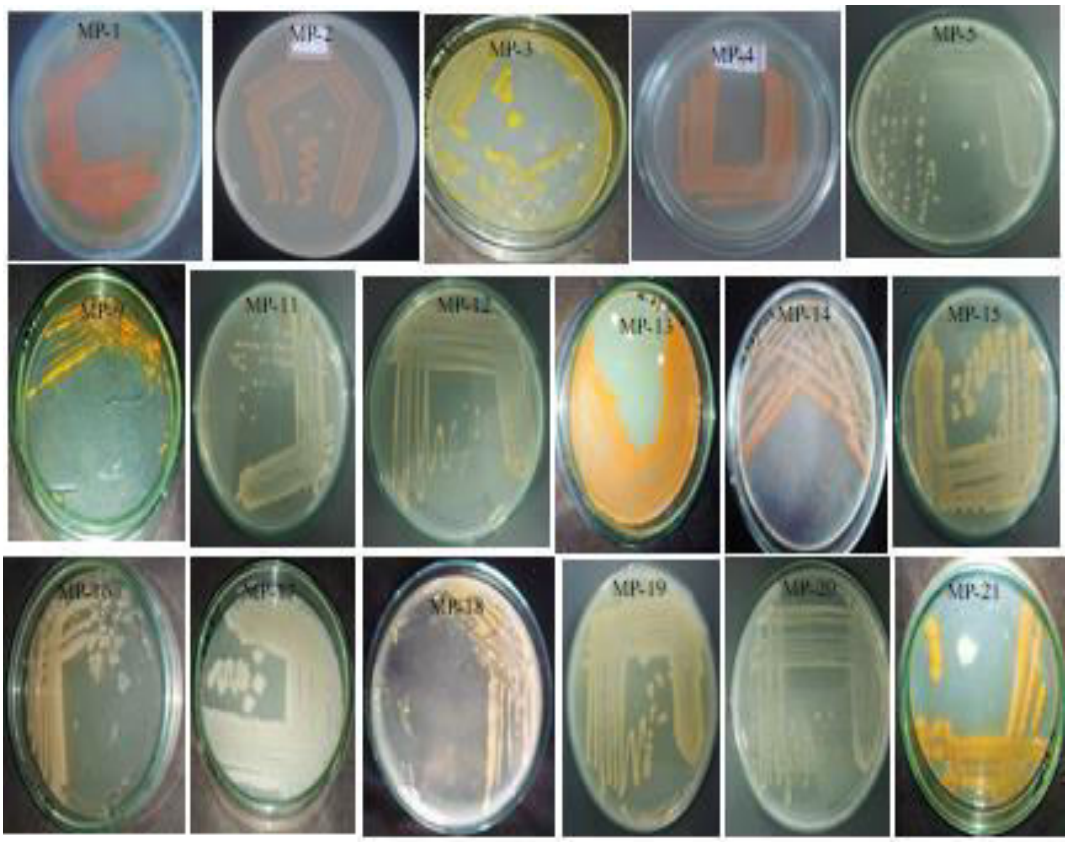


Fig. 6: Pigment producing (chromogenic) halophilic bacterialisolates MP-1 toMP-21.

was discovered that halophilic bacteria, which can flourish in a broad range of NaCl and MgSO4, reside in the solar salterns. For the solar salt sample, the number of colonies ranged from 9.8 x 10^3 to 4.0 x 10^4 CFU /ml, whereas for the sun-dried salted fish sample, the range was 2.8 x 10^2 to 5.1 x 10^4 CFU /ml (Figs. 4, 5). Twenty-one pigmented bacterial colonies, designated MP-1 through MP-21, were extracted from sun salterns in total (Fig. 6). A unique code, consisting of the prefix "MP" (Microbial Pigments) and integers ranging from 1 to 21 and preserved at 4 °C for

further studies.

Phenotypic characterization of pigment producing bacterial isolates

Primary characterization of morphological, cultural, and biochemical tests of pigment-producing bacterial isolates was carried out by referring to Bergey's Manual of Systematic Bacteriology. Most of the isolates were crimson, red, yellow, white, pink, and orange in colour, and the surface of some colonies was smooth, mucoid in nature, and the shape of some colonies was circular or irregular. All 21 isolates were subjected to gram staining. Table 1 shows the overall cell morphology of pigmented bacterial isolates.

In order to test the salt tolerance ability solar salt and naturally Sun-dried salted fish samples were grown on Zobell Marine Agar plates supplemented with NaCl. Zobell Marine Agar containing 2.5%, 5%, 7.5%, 10%, and 20% NaCl, the growth of bacteria occurs within 48 h at 37°C. While, the Zobell Marine Agar containing 15%, and 20% NaCl concentration, growth requires 72 h. Table 2 indicates that as the concentration of NaCl rises, the growth of the isolates steadily declines. The salt tolerance capacity of bacterial isolates is evaluated based on their growth performance at different salt concentrations. With increasing NaCl concentration, the growth of the isolate gradually diminishes. The isolate MP-1, known for producing pigments and classified as a halophilic bacterium, demonstrated the ability to grow in salt concentrations of at least 5% (w/v) NaCl, with fair growth observed up to 20% NaCl. Hence, it was categorized as a moderate halophile and chosen for further investigation. The optimal growth conditions for the halophilic bacterial isolate MP-1 were assessed by varying parameters.

Out of twenty-one, only the crimson-coloured bacterial isolate MP-1 was chosen based on unique colonial characteristics, and their various bio-chemical parameters such as Indol, MP-VP, Catalase, Oxidase, Protease, Geletinase, Nitrate reductase, Urease, Amylase, Esterase, Simmons citrate, Casin hydrolysis, Starch hydrolysis, Tween 80 hydrolysis, Aesculin hydrolysis, Acid production, and Endospore formation were studied, and their results are detailed in Table 3. The primary fatty acids found in MP-1 were iso C15:0 and anteiso C15:0, while the prevailing menaquinone type was an unsaturated menaquinone consisting of seven isoprene units (MK-7).

Screening of significant factors by Plackett-Burman (PB) design

This investigation utilized the Plackett-Burman experimental framework to meticulously sift through and discern the pivotal factors impacting the production of bacterial biomass. Table 4 outlines the distinct factors at two levels (-1 denoting low and +1 indicating high) that were examined within the Plackett-Burman design. Moreover, Table 5 presents the Plackett-Burman Design matrix, constructed to evaluate the chosen variables in relation to the pigmented halophilic bacterial isolate MP-1. The graphical representations in the form of the Pareto chart (Fig. 7) and the normal probability plot (Fig. 8), generated by the Plackett-Burman (PB) design, vividly depict the nuanced effects, both positive and negative, of the selected variables on the growth dynamics of the pigmented halophilic bacterial isolate MP-1.

Final equations in terms of coded factors are:

$$Y_{1(MP-1)} = 6.62 + 0.046\ C - 0.15\ D + 0.18\ E - 0.22\ F + 0.050\ G - 0.083\ H + 0.055\ J - 0.14\ K$$

The model F-value, as indicated by the value of 129.73 in Table 6, suggests the significance of the model. There exists only a 0.10% probability that such a large "Model F-value" could arise due to random variation. "Prob > F" values below 0.05 signify the significance of model terms. Within this study, parameters C, E, G, and J demonstrate positive significance, while D, F, H, K, and L exhibit negative model terms. The "pred R2" of 95.39% aligns reasonably well with the "adj R2" of 98.94%. A ratio exceeding 4, such as the calculated ratio of 30.97, indicates an adequate signal. Following the Plackett-Burman design conducted the most influential parameters with positive effects were identified for further optimization studies using Response Surface Methodology

Table 1: Colony Morphology of isolated Bacterial Species from Solar salts and Dry fish

Isolates	Size	Shape	Colour	Texture	Opacity	Elevation	Margin	Grams nature	Motility
MP-1	2-3	Circular	Crimson	Smooth	Opaque	Convex	Entire	Variable	Motile
MP-2	0.5-2	Rod	Red	Smooth	Translucent	Convex	Entire	-Ve	Motile
MP-3	0.5-1.0	Circular	Yellow	Smooth	Opaque	Convex	Entire	+Ve	Non motile
MP-4	1-2.5	Spherical	Pinkish red	Smooth	Opaque	Convex	Entire	+Ve	Non motile
MP-5	2-3	Circular	Craemy white	Smooth	Mucoid	Elevated	Entire	+Ve	Non motile
MP-6	2-3	Circular	White	Dry	Opaque	Flat	Entire	+Ve	Non motile
MP-7	1-3	Irregular	Dirty white	Smooth center and dry margin	Opaque	Flat	Wavy	+Ve	Non motile
MP-8	0.5-1	Circular	Shiny white	Smooth	Opaque	Elevated	Entire	-Ve	Non motile
MP-9	1-2	Circular	Dark yellow	Butyrous	Opaque	Convex	Entire	-Ve	Non motile
MP-10	0.5-2.5	Circular	Cream	Smooth	Mucoid	Elevated	Entire	+Ve	Motile
MP-11	1-3	Circular	Pale yellow	Smooth	Opaque	Convex	Entire	-Ve	Non motile
MP-12	2-2.5	Circular	Light yellow	Smooth	Opaque	Flat	Entire	-Ve	Non motile
MP-13	0.5-2.0	Circular	Pale orange	Smooth	Opaque	Convex	Entire	-Ve	Non motile
MP-14	0.5-2.5	Circular	Light red	Smooth	Opaque	Convex	Entire	-Ve	Non motile
MP-15	0.5-1.5	Circular	Creamy yellow	Smooth	Opaque	Sticky	Irregular	-Ve	Motile
MP-16	2-2.5	Circular	Cream	Smooth	Opaque	Flat	Entire	-Ve	Motile
MP-17	1-3	Circular	White	Dry	Mucoid	Elevated	Irregular	+Ve	Non motile
MP-18	0.5-3	Circular	Creamy pink	Smooth	Opaque	Shiny	Entire	+Ve	Motile
MP-19	0.5-1	Circular	Yellow	Smooth	Opaque	Sticky	Entire	-Ve	Non motile
MP-20	2-4	Circular	Translucent cream	Smooth	Mucoid	Flat	Entire	+Ve	Non motile
MP-21	1-3	Circular	Glossy yellow	Dry	Opaque	Flat	Entire	+Ve	Non motile

Table 2: Detection of Salt tolerance ability of selected bacterial isolates based on growth expressed as optical density at different salt concentration

Bacterial isolates	0%	2.5%	5%	7.5%	10%	15%	20%
MP-1	0.05	0.43	0.55	0.61	0.48	0.19	0.00
MP-2	0.03	0.41	0.53	0.71	0.81	0.92	1.16
MP-3	0.01	0.38	0.49	0.52	0.19	0.02	0.00
MP-4	0.10	0.39	0.49	0.34	0.32	0.18	0.04

(RSM) employing the Box-Behnken design. Four crucial variables were selected and assessed across twenty-nine trials at three equidistant levels (1, 0, and +1), as detailed in Table 7. Multiple regression analyses were then applied to the experimental data to derive the second-order polynomial equation:

$$Y_{(MP-1)} = 1.17 + 0.075\,A - 0.026\,B + 0.037\,C + 0.12\,D - 0.005\,AB - 0.035\,AC - 0.03\,A - 0.0025\,BC - 0.065\,BD - 0.0075\,CD - 0.19\,A2 - 0.17\,B2 - 0.14\,C2 - 0.13\,D2$$

Table 3: Biochemical characteristics of the selected bacterial isolate of MP-1

S. No.	Biochemical Test	MP-1
1.	Indol	-Ve
2.	Methyl red	-Ve
3.	Voges-Proskauer	-Ve
4.	Catalase	+Ve
5.	Oxidase	-Ve
6.	Protease	+Ve
7.	Geletinase	+Ve
8.	Nitrate reductase	-Ve
9.	Urease	-Ve
10.	Amylase	+Ve
11.	Esterase	+Ve
12.	Simmons citrate	Ve
	Production of hydrolases from	
13.	Casin hydrolysis	+Ve
14.	Starch hydrolysis	+Ve
15.	Tween 80 hydrolysis	+Ve
16.	Aesculin hydrolysis	-Ve
Production of acid from		
18	D.Mannose	-Ve
19	D.Manitol	-Ve
20	D.Maltose	+Ve
21	D.Glucose	+Ve
22	L.Arabinose	+Ve
23	Endospore forming	Endospore forming
24	G+C content (mol %)	54%
25	Respiratory Menaquinone	MK 7
26	Major fatty acids	iso C15:0 *and*anteiso C15:0

Table 4: Two level factors of selected variables studied in the Plackett-Burman Design

S. No.	Factors	Name	Units	-1	1
1.	A	Peptone	g/l	2	18
2.	B	Yeast extract	g/l	2	18
3.	C	Na2So4	w/v	2	18
4.	D	KCl2	w/v	1	9
5.	E	NaCl2	w/v	1	9
6.	F	CaCL2	w/v	1	9
7.	G	G.pH	-	5	7
8.	H	Incubation period	hrs	24	48
9.	J	Temperature	C	25	37
10.	K	Agitation	rpm	100	150
11.	L	Media volume	ml	50	200

Table 5: Plackett-Burman Design (PBD) Matrix for screening of selected variables

Std	Run	Factors											Response-1
		1	2	3	4	5	6	7	8	9	10	11	
		A	B	C	D	E	F	G	H	J	K	L	MP-1 biomass
		g/l	g/l	w/v	w/v	w/v	w/v	-	h	^{0}C	rpm	ml	g/l
1	12	1	1	-1	1	1	1	-1	-1	-1	1	-1	0.04
2	8	-1	1	1	-1	1	1	1	-1	-1	-1	1	1.05
3	11	1	-1	1	1	-1	1	1	1	-1	-1	-1	0.02
4	3	-1	1	-1	1	1	-1	1	1	1	-1	-1	0.94
5	10	-1	-1	1	-1	1	1	-1	1	1	1	-1	0.31
6	5	-1	-1	-1	1	-1	1	1	-1	1	1	1	0.01
7	7	1	-1	-1	-1	1	-1	1	1	-1	1	1	0.82
8	9	1	1	-1	-1	-1	1	-1	1	1	-1	1	0.16
9	1	1	1	1	-1	-1	-1	1	-1	1	1	-1	0.76
10	6	-1	1	1	1	-1	-1	-1	1	-1	1	1	0.06
11	4	1	-1	1	1	1	-1	-1	-1	1	-1	1	1.32
12	2	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	0.75

A: Peptone, B: Yeast extract, C: Na_2So_4, D: KCl_2, E: $NaCl_2$, F: $CaCl_2$, G: pH, H: Incubation period, J: Temperature, K: Agitation, L: Media volume

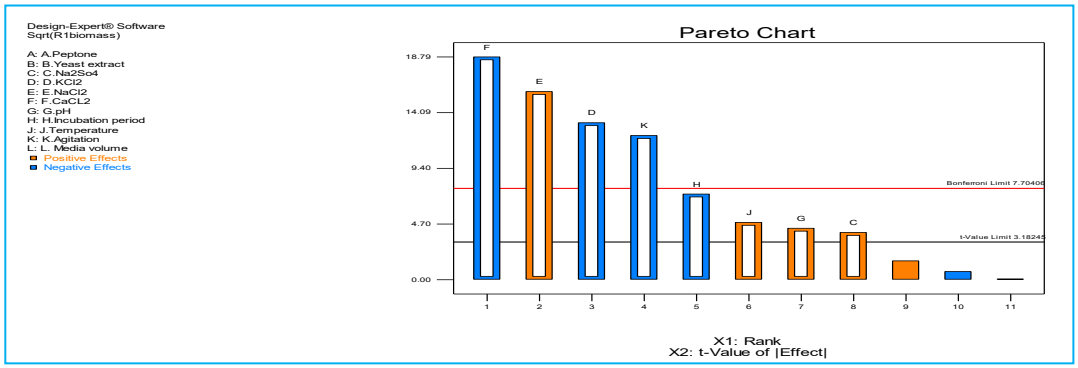


Fig. 7: Pareto chart for biomass (g/l) production of pigmented moderately halophilic bacterial isolates (MP-1) showing positive and negative effect of selected factors.

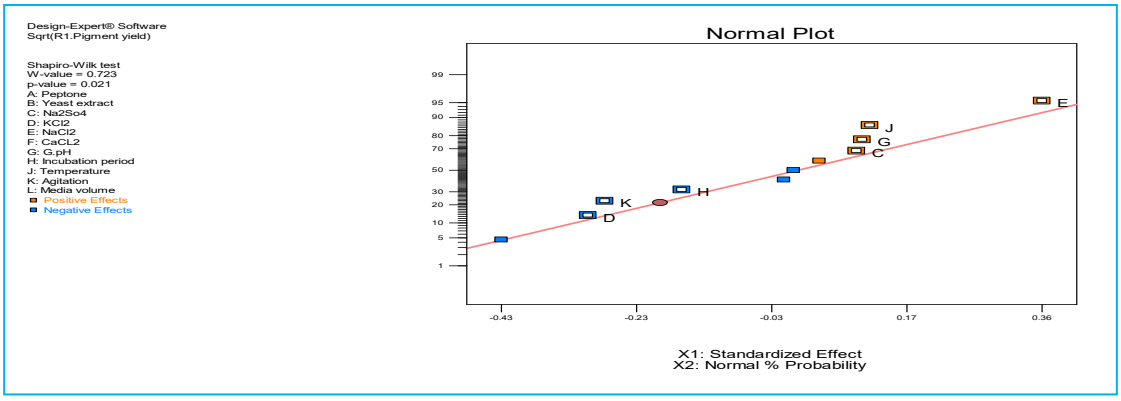


Fig. 8: Normal probability plot generated by Plackett-Burman (PB) design of Design expert software shows the positive and negative effects of selected variables on the growth of pigmented halophilic bacterial isolates (MP-1).

Table 6: ANOVA for selected factorial model in Plackett Burman design for MP-1

Response1 - MP-1 Pigment yield						
Transform:	**Square Root**	**Constant: 0**				
ANOVA for selected factorial model						
Analysis of variance table (Partial sum of squares - Type III)						
Source	**Sum of Squares**	**df**	**Mean Square**	**F Value**	**p-value Prob > F**	
Model	1.64	8	0.20	129.73	0.001	**Significant**
C-Na_2SO_4	0.025	1	0.025	15.85	0.028	
D-KCl_2	0.28	1	0.28	175.18	0.0009	
E-$NaCl_2$	0.40	1	0.40	251.64	0.0005	
F-$CaCl_2$	0.56	1	0.56	353.09	0.0003	
G-pH	0.030	1	0.030	18.806	0.0226	
H-Incubation period	0.08	1	0.08	52.17	0.0055	
J-Temperature	0.037	1	0.037	23.34	0.0169	
K-Agitation	0.23	1	0.23	147.77	0.0012	
Residual	0.005	3	0.0016			
Cor Total	1.64	11				
Adj R-Squared	98.94 %					
Pred R-Squared	95.39 %					

The Model F-value of 129.73implies the model is significant. There is only a 0.10% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case C, D, E, F, G, H, J, K are significant model terms.The "Pred R^2 " of 0.9539 is in reasonable agreement with the "Adj R^2" of 0.9894."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Ratio of 30.97 indicates an adequate signal. This model can be used to navigate the design space.

In the context of this study, Y represents the maximum yield of pigment (g/l) by MP-1, while A, B, C, and D denote the coded values of NaCl2, pH, temperature, and Na_2SO_4, respectively. The detailed summary provided by BBD in Design-Expert software is presented in Table 8. The "Model Summary Statistics" primarily focus on maximizing the "adjusted R-Squared" and the "predicted R-Squared." The variance of independent variables was analyzed using ANOVA in a response surface quadratic model, with the results displayed in Table 9. A P-value of < 0.0001 was considered significant. The model's significance is indicated by the F-value of 1233.02, with such a significant "Model F-value" being 0.01% likely to result from noise. Values of "Prob > F" less than 0.05 often denote the significance of the model variables. In this case, important model terms include A, B, C, D, AC, AD, BD, CD, A2, B2, C2, and D2. The "Lack of Fit F-value" of 0.44 suggests that pure error is more relevant than Lack of Fit, and a "Lack of Fit F-value" of this magnitude might be attributed to noise 86.64% of the time, indicating a favorable non-significant mismatch.

The fitness of the quadratic polynomial regression model can be assessed by the coefficient of determination (R2) predicted by Design-Expert software. Typically, the R2 value ranges between 0 and 1, with values closer to 1 indicating better and stronger response prediction. In the findings generated using BBD, the R2 (predicted and nearby) ranged between 99.70% and 99.84%, indicating that the model

Table 7: Factors to be Screened in Plackett-Burman Design (Screening Test) and their Coded and Actual values for the Three Levels of the Selected Variables in
Box-Behnken Design (BBD)

Factors	Name	Units	Minimum value (-1)	Center value (0)	Maximum value (+1)
A	Salt	%	4.5	10	15.5
B	Temperature	C	20	35	50
C	pH	-	4	7	10
D	Na_2SO_4	%	2	5.5	9

Table 8: Box-Behnken design matrix for 4 variables at 3 levels and 29 runs for optimization of pigment production from moderate halophilic bacterial isolates of MP-1using Polynomial Quadratic Model Analysis in RSM

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1
Run	A:Salt	B:Temperature	C:pH	D:Na2SO4	R1-Pigment yield of MP-1
	%	C		%	g/L
12	4.5	20	7	5.5	0.76
14	15.5	20	7	5.5	0.92
5	4.5	50	7	5.5	0.71
21	15.5	50	7	5.5	0.85
17	10	35	4	2	0.74
20	10	35	10	2	0.82
27	10	35	4	9	0.99
6	10	35	10	9	1.04
29	4.5	35	7	2	0.62
25	15.5	35	7	2	0.83
16	4.5	35	7	9	0.92
28	15.5	35	7	9	1.01
4	10	20	4	5.5	0.84
23	10	50	4	5.5	0.8
11	10	20	10	5.5	0.92
18	10	50	10	5.5	0.87
15	4.5	35	4	5.5	0.69
19	15.5	35	4	5.5	0.91
13	4.5	35	10	5.5	0.84
9	15.5	35	10	5.5	0.92
3	10	20	7	2	0.71
2	10	50	7	2	0.79
10	10	20	7	9	1.08
1	10	50	7	9	0.9
7	10	35	7	5.5	1.17
8	10	35	7	5.5	1.18
24	10	35	7	5.5	1.16
22	10	35	7	5.5	1.17
26	10	35	7	5.5	1.18

could explain these percentages of response variability. There is considerable agreement between the "adjusted R-squared" of 0.9984 and the "predicted R-squared" of 0.9970. The signal-to-noise ratio in this design is measured by the "Adeq Precision," with a ratio exceeding 4 preferred in RSM. With our ratio of 117.77 indicating an appropriate signal, we can explore the design space with this model. The residual plot points for a response form a straight line, as observed in Figure 9, and the normal probability plot appears normally distributed.

Predicted values closely match actual values, as shown in Figure 10. Box-Cox plots for the power transforms of variables are depicted in Figure 11. Three-dimensional response surface

Table 9: The ANOVA analysis ofoptimizationexperimental design for pigment production from moderate halophilic bacterial isolates MP-1

Response1(R1)-Pigment yield of MP-1						
ANOVA for Response Surface Quadratic Model						
Source	**Sum of Squares of MP-1**	**df**	**Mean Square**	**F-Value**	**p-value Prob > F**	
Model	0.73	14	0.052	1233.02	< 0.0001	**Significant**
A-Salt	0.068	1	0.068	1606.23	< 0.0001	
B-Temperature	0.008	1	0.008	190.567	< 0.0001	
C-pH	0.016	1	0.016	383.909	< 0.0001	
D-Na2SO4	0.17	1	0.170	4055.04	< 0.0001	
AB	0.00001	1	0.00001	2.3796	0.1452	
AC	0.0049	1	0.0049	116.601	< 0.0001	
AD	0.0036	1	0.0036	85.6657	< 0.0001	
BC	0.00003	1	0.00003	0.5949	0.4534	
BD	0.017	1	0.0169	402.153	< 0.0001	
CD	0.00023	1	0.00023	5.35411	0.0364	
A^2	0.24	1	0.2387	5680.18	< 0.0001	
B^2	0.19	1	0.18875	4491.46	< 0.0001	
C^2	0.13	1	0.13049	3105.07	< 0.0001	
D^2	0.115	1	0.11488	2733.77	< 0.0001	
Residual	0.00059	14	0.00004			
Lack of Fit	0.00031	10	3.1E-05	0.44048	0.8664	not significant
Pure Error	0.00028	4	0.00007			
Cor Total	0.73	28				
Adj R-Squared	0.9984					
Pred R-Squared	0.9970					

The model has an F-value of 1233.02, indicating that it is significant. There is only a 0.01 percent chance that a "Model F-Value" this large could occur due to noise. In this case A, B, C, D, AC, AD, BD, CD, A^2, B^2, C^2, D^2 are significant model terms.Values >0.1 indicate the model terms are not significant. The "Lack of Fit F-value" of 0.44 implies the Lack of Fit is not significant relative to the pure error. There is a 86.64% chance that a "Lack of Fit F-value" this large could occur due to noise.A non-significant lack of fit is desirable because we want the model to fit.The "Pred R-Squared" of 0.9970 is reasonably close to the "Adj R-Squared" of 0.9984. The signal-to-noise ratio is measured by "Adeq Precision." A ratio greater than 4 is preferred. Our signal-to-noise ratio of 117.773 indicates a sufficient signal. This model can help to find a way around the design space.

graphs were plotted to assess the interaction between variables and determine the optimum concentration of each factor for maximum pigment production. Fig. 11 displays the 3-D graphs and contour plots predicted by RSM, with the peaks and curvature indicating the maximum production of pigments in the response surface plots. The shapes of the three-dimensional response surfaces, whether circular or elliptical, suggest significant interaction between the

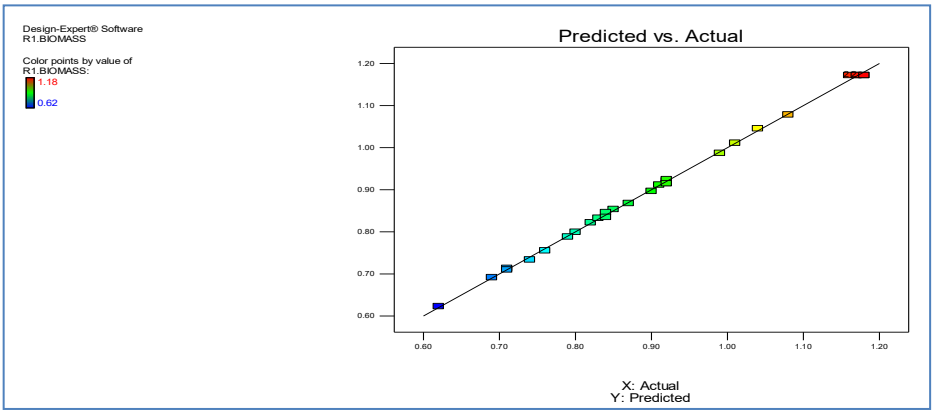


Fig. 9: Diagnostic Plot for the Box–Behnken Model Adequacy of the Tested Variables of MP-1.

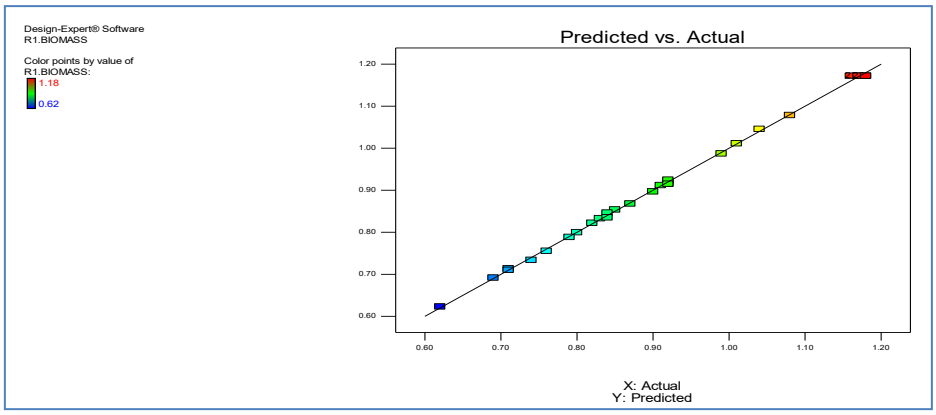


Fig. 10: Predicted Vs Actual values plot for MP-1.

independent variables. Table 10 presents the optimum conditions predicted by statistical calculations after conducting the Box-Behnken design, along with the actual results obtained in laboratory work (Ryazantseva *et al.*, 2012).

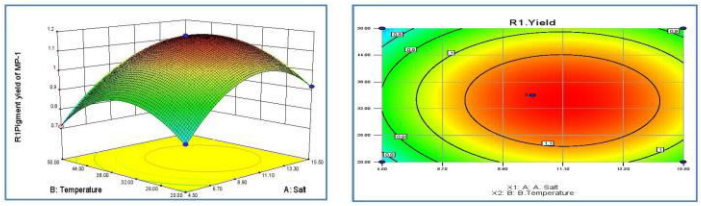
Molecular characterization of an isolated pigmented halophilic bacterial strain, MP-1

The isolated halophilic bacterial strain MP-1, obtained from solar salterns, was identified using the 16S rRNA sequencing molecular method. Through analysis of the 16S rRNA sequence, the isolate MP-1 was determined to be *Bacillus aquimaris* (MP-1). Utilizing the BLAST program for sequence alignment, it was revealed that strain MP-1 exhibited 100% identity with *Bacillus aquimaris*. A phylogenetic relationship tree (Fig. 12) for the halophilic bacterial isolate MP-1 strain was constructed using the neighbour-joining tree method. Subsequently, the MP-1 sequence was deposited into the NCBI Gene Bank database, with the assigned GenBank accession number for its nucleotide sequences being MN865170. The range of similarity in terms of percentage and the accession number for sequence as given by NCBI are shown in Table 11. Using the Snap gene algorithm, the DNA isolated from MP-1 was identified as double-stranded linear DNA, with 1302 bp and a MW of 807,835 Da, containing 54% G + C values and 46% A+T values. The DNA structure of MP-1, along with their enzymatic system and agarose gel electrophoresis pictures are depicted in Figure 13 and 14.

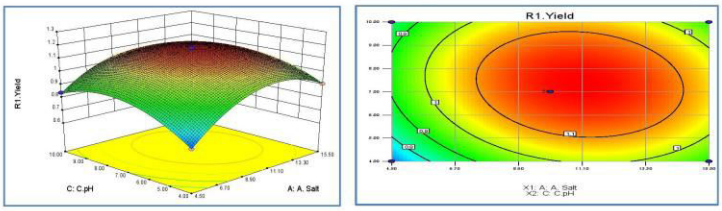
Characterization of pigmented halophilic Bacillus aquimaris (*MP-1*)

(a) *The antibiotic sensitivity test*

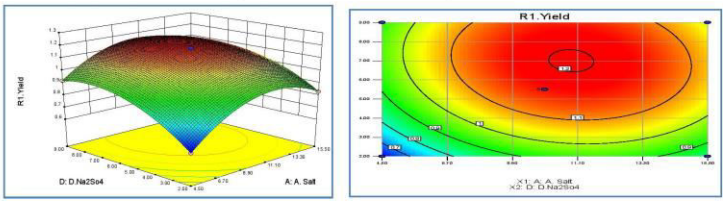
Generally, due to their high salinity, moderate



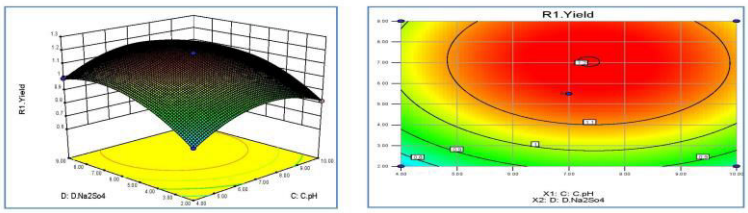
a) Interaction between temperature and salt



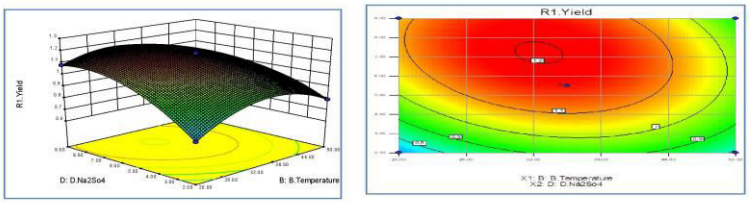
b) Interaction between pH and salt



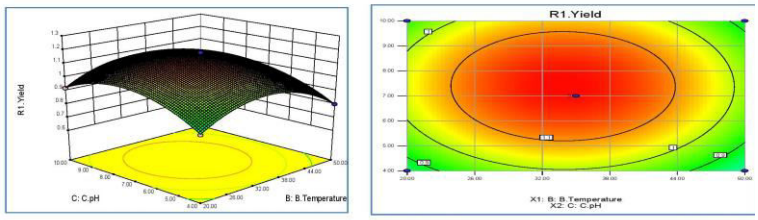
c) Interaction between Na_2SO_4 andsalt



d) Interaction between Na_2SO_4 and pH



e) Interaction between $Na_2SO_4$and temperature



f) Interaction between pH and temperature

Fig. 11: Response surfaces (3-D graph and 2-D contour plot) obtained through the Box-Behnken Design (BBD) for maximize the yield of pigment extracted from MP-1.

Table 10: The actual and predicted optimal values for MP-1

Optimum values predicted by statistical calculations after performing the Box-Behnken design					
	Salt (%)	**Temperature (^{0}C)**	**pH**	**Na_2SO_4 (%)**	**R1-Pigment yield (g/L)**
MP-1	9.27	30.28	7.25	6.46	1.181
Actual values predicted in lab work					
MP-1	9.0	37	7	6	1.175

Table 11: The range of similarity in terms of percentage and the accession number for sequence as given by NCBI

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession Number
Bacillus aquimaris strain MP-1 16S ribosomal RNA gene, partial sequence	*Rossellomorea aquimaris*	2405	2405	100%	0	100	1302	MN865170.1
Bacillus sp. (in: Bacteria) strain C10-711 16S ribosomal RNA gene, partial sequence	*Bacillus sp.* (in: Bacteria)	2303	2303	100%	0	98.62	1453	MG757517.1
Bacillus aquimaris strain TCCC11049 16S ribosomal RNA gene, partial sequence	*Rossellomorea aquimaris*	2300	2300	100%	0	98.54	1546	EU231632.1

Table12: List of Antibiotics used for the Antibiograms

S. No.	Name of Antibiotic	*Bacillus aquimaris* (MP1)	Zone of inhibition(mm)
			MP1
1.	Kanamycin	Sensitive	28
2.	Streptomycin	Sensitive	30
3.	Tetracyclin	Sensitive	24
4.	Amoxicilin	Resistance	13
5.	Erythromycin	Intermediate	20
6.	Amikacin	Sensitive	26
7.	Vancomycin	Sensitive	22
8.	Nystatin	Resistance	-
9.	Carbencillin	Resistance	12
10.	Cefuroxime	Resistance	-
11.	Azithromycin	Sensitive	20
12.	Ticarcillin	Resistance	-
13.	Colistin	Sensitive	17
14.	Piperacillin	Resistance	14
15.	Cephalexin	Resistance	-
16.	Ofloxacin	Sensitive	33
17.	Co-Trimoxazole	Sensitive	40
18.	Gentamycin	Sensitive	37
19.	Ketoconazole	Resistance	11
20.	Suphamathizole	Sensitive	32

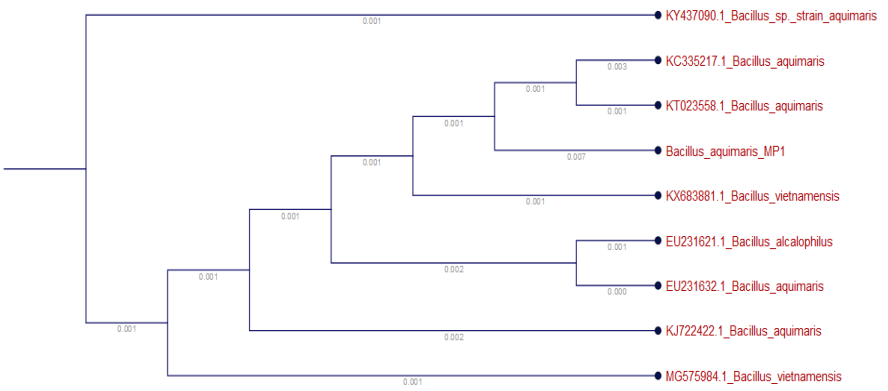


Fig. 12: Pylogenetic relationship tree of halophilic bacterial isolate *Bacillus aquimaris*(MP-1) constructed by neighbour-joining method using16s rRNA gene sequencing.

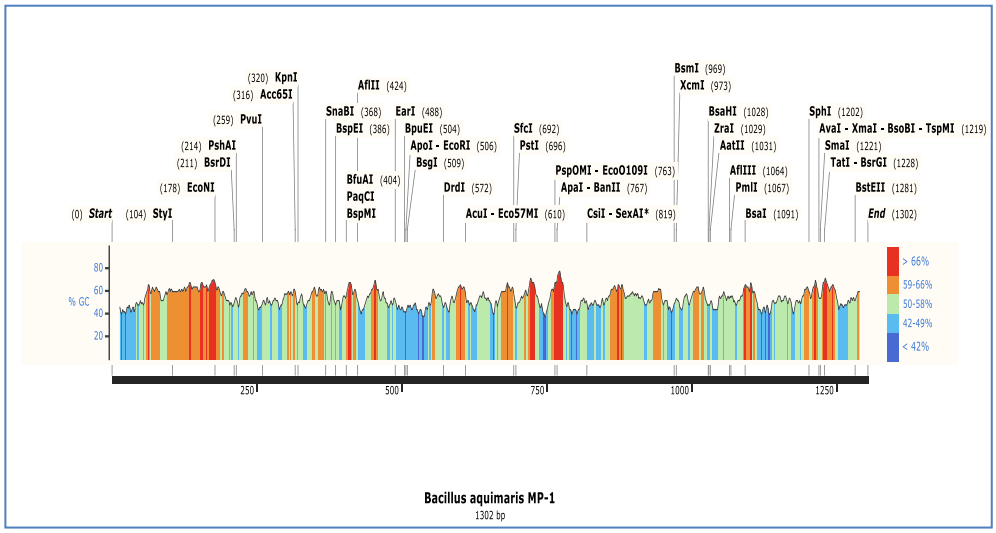


Fig. 13: The Structure of DNA of *Bacillus aquimaris* (MP-1)along with their Enzymatic Systems and GC values Predicted by Snap gene algorithm.

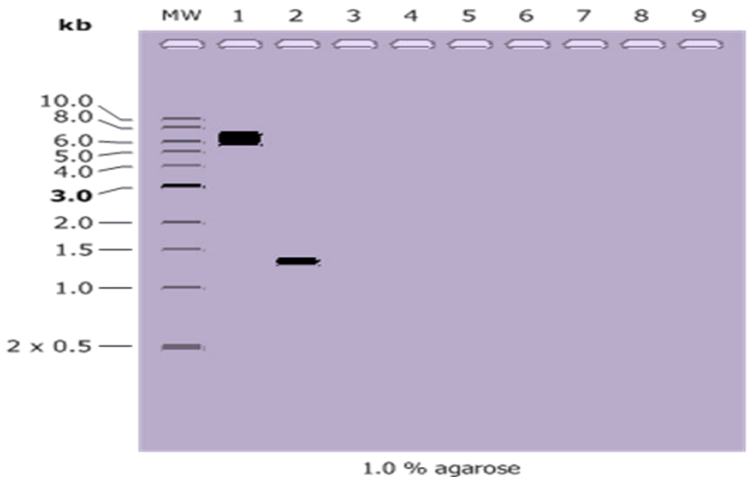


Fig. 14: Agarose gel electrophoresis of 16S rRNA of *Bacillus aquimaris* (MP-1).

Table 12: List of Antibiotics used for the Antibiograms

S. No.	Name of Antibiotic	*Bacillus aquimaris* (MP1)	Zone of inhibition(mm)
			MP1
1.	Kanamycin	Sensitive	28
2.	Streptomycin	Sensitive	30
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4.	Amoxicilin	Resistance	13
5.	Erythromycin	Intermediate	20
6.	Amikacin	Sensitive	26
7.	Vancomycin	Sensitive	22
8.	Nystatin	Resistance	-
9.	Carbencillin	Resistance	12
10.	Cefuroxime	Resistance	-
11.	Azithromycin	Sensitive	20
12.	Ticarcillin	Resistance	-
13.	Colistin	Sensitive	17
14.	Piperacillin	Resistance	14
15.	Cephalexin	Resistance	-
16.	Ofloxacin	Sensitive	33
17.	Co-Trimoxazole	Sensitive	40
18.	Gentamycin	Sensitive	37
19.	Ketoconazole	Resistance	11
20.	Suphamathizole	Sensitive	32

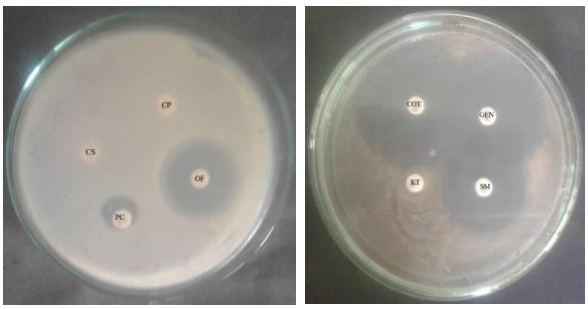


Fig. 15: Antibiotic sensitivity test of isolated halophilic bacterial strains of *Bacillus aquimaris* (MP-1).

halophiles tolerate high concentrations of most antimicrobial agents. As observed from Table 12 and Figure 15, the halophilic bacterial isolate MP-1 was resistant to Nystatin, Ticarcillin, Cephalexin, Ketoconazole, Amoxicillin, Carbencillin, Cefuroxime, and Piperacillin but highly sensitive to Kanamycin, Streptomycin, Azithromycin, Colistin, Tetracyclin, Amikacin, Vancomycin, Ofloxacin, Co-Trimoxazole, Gentamycin and shows their intermediate response to Erythromycin.

(b) *Heavy metal tolerance assay and solvent tolerance assay*

The halophilic *Bacillus aquimaris* (MP-1) isolate utilized in this study exhibited a promising heavy metal tolerance profile, suggesting its potential application as a biomedical tool for the removal of metal pollutants from contaminated sites. The results are detailed in Table 13. Due to its capability to thrive in varying salinity levels or hypersaline conditions, this halophilic bacterial strain of *Bacillus aquimaris* (MP-1) demonstrated exceptional metal tolerance against zinc (Zn), copper (Cu), manganese (Mn) at concentrations ranging from 0.05 mg to 0.2 mg, and lead (Pb) at

Table13: Heavy metal tolerance assay of halophilic *Bacillus aquimaris* (MP-1)

Isolate	Heavy metals	Concentration(mg/ml)			
		0.05 mg	0.1 mg	0.15 mg	0.2 mg
Bacillus aquimaris(MP-1)	Cadmium	++	-	-	-
	Cobalt	+	+	-	+
	Nickel	+	-	-	-
	Lead	++++	++++	++++	++++
	Zinc	++++	++++	++++	++++
	Copper	++++	++++	++++	++++
	Mercury	+++	+	+	+
	Manganese	++++	++++	++++	++++

Highest growth (++++), higher growth (+++), Fair growth (++), lower growth (+), Nil (-)

Table14: Solvent tolerance assay of selected halophilic bacterial isolates of MP-1

S. No.	Solvents	*Bacillus aquimaris* (MP-1)
1	Xylene	++++
2	Hexane	+++
3	Cyclohexane	+++
4	Toluene	++++
5	Acetone	++++
6	Methanol	++++
7	Benzene	++++
8	Heptane	++++

Higher growth rate (++++), Moderate growth rate (+++), Fair growth rate(++)

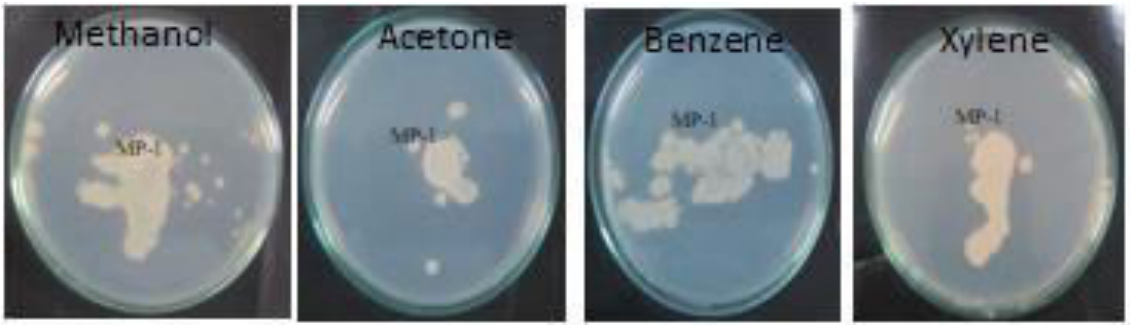


Fig. 16: Solvent tolerance assay of isolated halophilic bacterial isolates MP-1.

levels from 0.05 mg to 0.2 mg. Recent observations have highlighted the solvent stability of halophilic bacterial species as a noteworthy trait. The *Bacillus aquimaris* (MP-1) isolates exhibited remarkably robust resistance to various solvents such as acetone, methanol, xylene, benzene, heptane, cyclohexane, and toluene. Their solvent tolerance ranked as follows: methanol > acetone > benzene > xylene > heptane > toluene > ethyl acetate > cyclohexane (Table 14; Fig. 16).

(c) *Biofilm formation assay*

The biofilm formation ability of halophilic bacterial isolates plays a significant role in wastewater treatment processes using microbial fuel cells (MFCs). Anode biofilm is a crucial

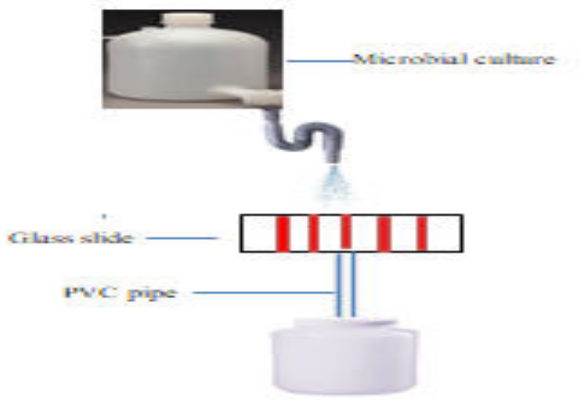


Fig.17: Biofilm formation experiment of *Bacillus aquimaris* (MP-1).

Table 15: Chromatography values (Rf) of Polar lipid extracted from pigmented halophilic *Bacillus aquimaris* (MP-1)

S. No.	Polar lipid	Standard Rf value	Rf value obtained (cm)
			MP-1
Phospholipids			
1	Diphosphatidylglycerol(DPG)	0.71-0.98	0.84
2	Phosphatidyl glycerol(PG)	0.60-0.91	0.64
3	Phosphatidyl ethanolamine(PE)	0.48-0.50	0.49
4	Glycerophospholipids(GL)	0.45	-
5	phosphatidylinositol (PI)	0.39	-
6	Unknown	-	-

component for electrogenesis in microbial fuel cells (MFCs). It is thought that better understanding of the biofilm formation process on the electrode surface can enhance MFC Performance (Fig. 17).

(d) *Chromatography analysis of Polar lipid extracted from pigmented halophilic Bacillus aquimaris* (*MP-1*)

The extracted polar lipids were subjected to thin layer chromatographic analysis, and the obtained band was calculated using the following formula:

Rf value (cm) = Distance moved by the solute (cm)/Distance moved by the solvent (cm)

Another polar lipid was seen as a yellow-brown mark, while glycolipids showed up as dark pink or blue-purple dots. Compounds that include phosphorus will show up as blue dots on a white backdrop. The polar lipids detected are shown in Figure 18 and Table 15. There were three major phospholipids (DPG, PG, and PE) and one unidentified phospholipids (Pl) and one unknown lipid that were comparable to other studies (Daroonpunt *et al.*, 2019).

(e) *Screening of antagonistic activity*

A halophilic isolate of *Bacillus aquimaris* (MP-1) did not produce an inhibition zone against *E.coli*. *Salmonella* sp., *Vibrio* sp., *K. pneumoniae*, *Pseudomonas* sp., and *Rhizobium* sp. showed inhibition zones against the halophilic bacterial isolate *Bacillus aquimaris* (MP-1). The antagonistic activity of *S. aureus* test bacteria against *Bacillus aquimaris* (MP-1) was found to be greater when compared to others (Table 16).

Quantification of prodigiosin

The crimson-colored supernatant obtained from the culture broth, indicating the presence of prodigiosin, was evaporated using a rotary

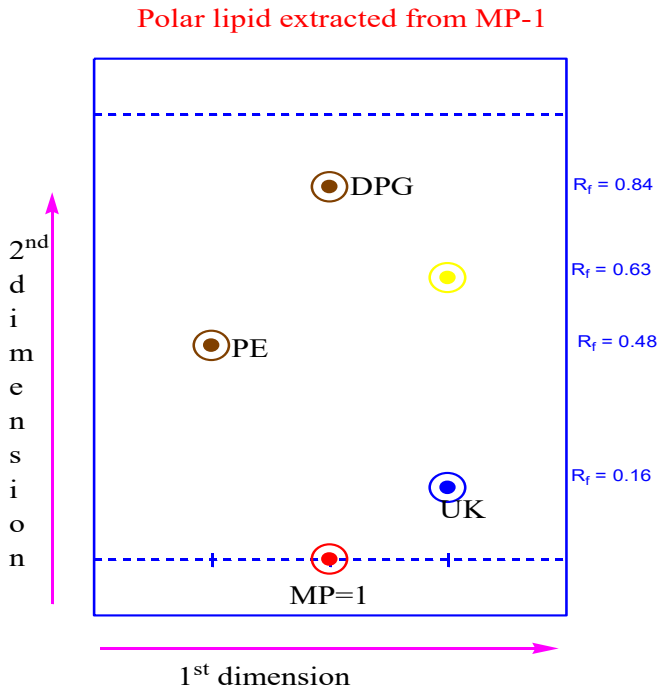


Fig. 18: Polar Lipids detected from chromogenic halophilic bacterial isolate MP-1.

Table 16: Screening of Antagonistic Activity of Halophilic bacterial isolate of MP-1

S. No.	Test pathogens	Growth	Zone of inhibition (mm)
			***Bacillus aquimaris* (MP1)**
1	*E. coli*	-	-
2	*S. aureus*	±	>10
3	*Bacillus sp.*	±	-
4	*Salmonella sp.*	+	<10
5	*Vibrio sp.*	+	<10
6	*K. pneumoniae*	+	>10
7	*Pseudomonas sp*	+	<10
8	*Proteus sp*	±	-
9	*A. niger*	±	-
10	*A. flavus*	±	-
11	*Rhizobium sp*	+	>10

Inhibited by isolates (+), Not inhibited by isolates (-), Inhibited and Non inhibited by isolates (±)

evaporator and subsequently measured for further analysis. The maximum quantity of prodigiosin obtained was approximately 47.5 mg/ml.

Conformation of prodigiosin pigments

Acetone and methanol demonstrated the ability to extract pigment from the isolated halophilic bacterial strain. However, the most significant pigment extraction from MP-1 was observed with methanol. Following extraction, various techniques were employed to confirm that the extracted product is carotenoid derivatives. Initially, TLC was conducted, and upon comparison with known values from other studies, it exhibited considerable similarity. Then UV/VIS spectrophotometry and FTIR were performed on the TLC-purified sample. Finally, the pigment produced by the halophilic bacterial isolate was identified and characterised using HPLC and their molecular mass were calculated by GC/MS. In this study, we used different solvent

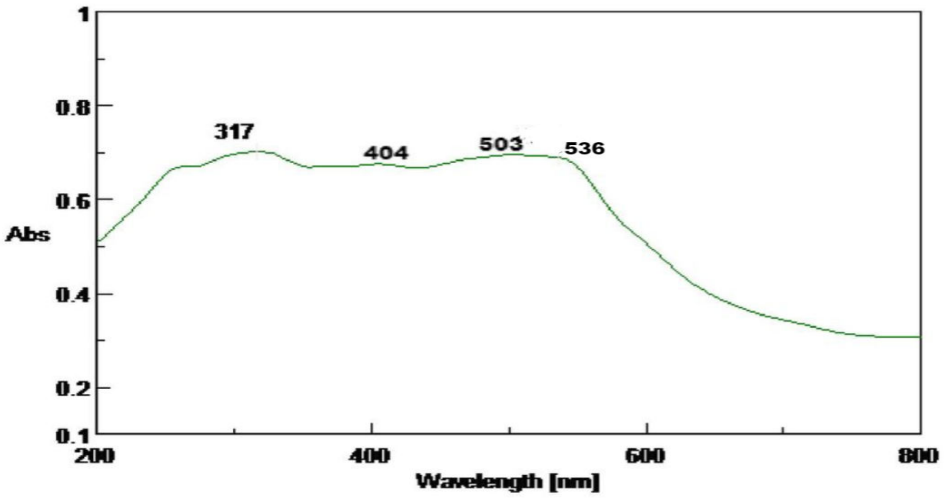


Fig. 19: UV-Vis. absorption spectra of halophilic bacterial pigments of MP-1

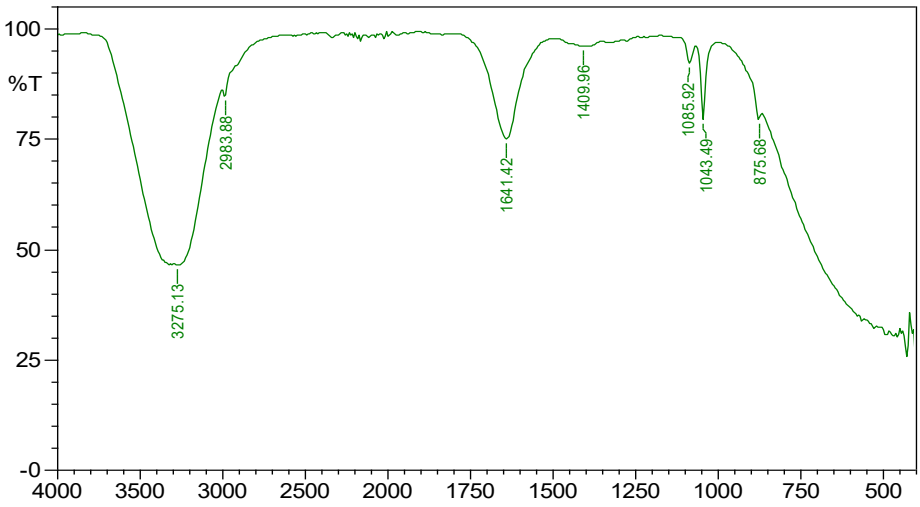


Fig. 20: FT-IR Spectrum of Halophilic Bacterial Pigment Isolated from MP-1.

systems ranging from low polarity to high polarity to test for the effective separation of carotenoid pigment compounds. Purification of the extracted pigment from *Bacillus aquimaris* (MP-1) was carried out, the Rf value was calculated, and the obtained result of about 0.87 was compatible with the result of Vora *et al.* (2014), who pointed out the Rf value found to be in the range of 0.875, due to using other solvents (ethanol) in the purification. Samrot *et al.* (2011) discovered that when the isolated red pigment prodigiosin was subjected to TLC, the Rf value obtained was 0.87.

The crimson coloured pigments in the extract solution showed absorption peaks at 404 nm, 503 nm, and 536 nm (Fig. 19), indicating prodigiosin (for MP-1). The maximum absorbance of 536 nm of crimson coloured pigment extracted from MP-1 was matched with Ahmad *et al.*'s (2012) result. In other studies done by Hines *et al.* (1988) and Monreal *et al.* (1969), the maximum absorbance spectrum was 535 and 536.6, respectively. The obtained results also agreed with those of a previous study published by Patil *et al.* (2011).

The FT-IR spectrum of the extracted pigment from halophilic *Bacillus aquimaris* (MP-1) shows similarity with carotenoid indicating that the extracted pigment is a carotenoid derivative like prodigiosin (MP-1) (Fig. 20).

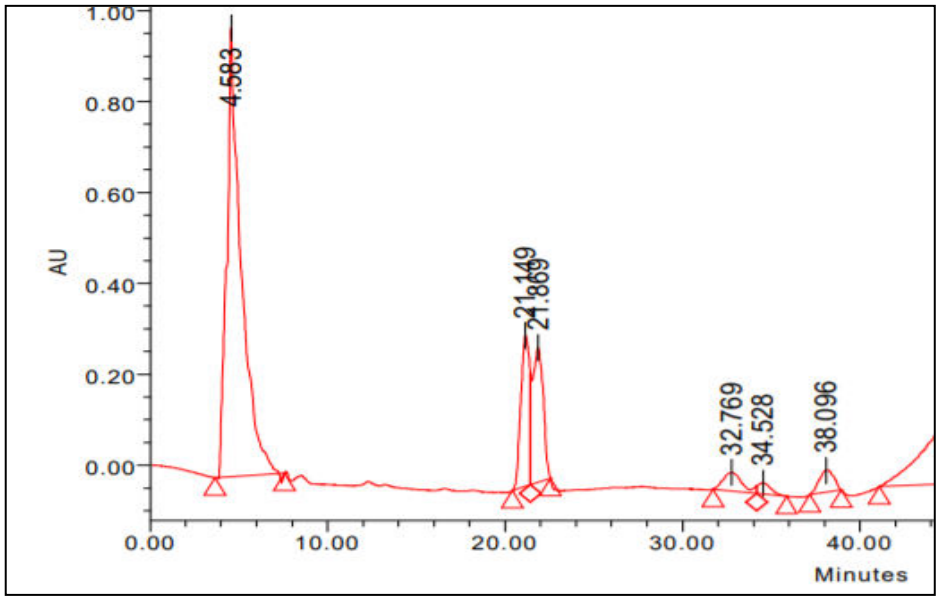
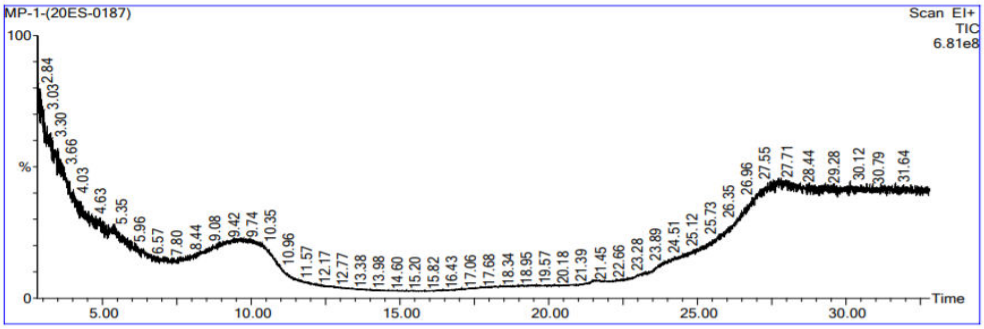


Fig. 21: HPLC analysis of Prodigiosin Pigment Extracted from Moderate Halophilic Bacterial isolate MP-1.

The crimson hue of the pigment extract was conclusively identified as prodigiosin, a determination rooted in spectroscopic analysis bearing striking resemblances to known prodigiosin spectra (Sumathi *et al.*, 2014). Within the crimson pigment extracts of MP-1, infrared peaks of notable breadth, reminiscent of prodigiosin, were discerned within the frequency spectrum ranging from 3273.20 to 449.41 cm^{-1}. Notably, the discernment of a sharp peak at 3273 cm^{-1}, commonly attributed to N–H stretching, suggests a structural pattern akin to that observed in prodigiosin carotenoids (Patil *et al.*, 2011). Furthermore, peaks resonating at 2916 cm^{-1} and 1631.78 cm^{-1} were ascribed to the symmetrical stretching of the methylene group and the presence of –NH bending and C=C stretching, respectively. The presence of C-H bending was corroborated by the peak at 1550.77 cm^{-1}, while the bands at 1401.18 cm^{-1} and 1107.14 cm^{-1} corresponded to C-O stretching. These distinctive infrared peaks align closely with those reported in previous studies on prodigiosin (3105 cm^{-1}, 2928 cm^{-1}, 1644 cm^{-1}, 1602 cm^{-1}, and 1125 cm^{-1}). Song *et al.* (2006) notably underscored the distinctiveness of Serratia sp. KH95, characterized by robust bands at 2928 cm^{-1} (CH stretching) and 1602 cm^{-1} (aromatic C = C).

Our HPLC analysis of MP-1 unequivocally revealed that the predominant constituent eluted at 4.583 min (Fig. 21), confirming its identity as prodigiosin, a consistency noted in prior inquiries (Patil *et al.*, 2011). The mobile phase, comprising acetonitrile/methanol/dichloromethane (in ratio of 75:20:5), was rigorously maintained at a steady temperature of 30°C.

Subsequently, the purified pigment sample derived from MP-1 underwent comprehensive Gas Chromatography-Mass-Spectrometry scrutiny. From the resultant spectra, the molecular weight of the extracted pigment was precisely determined, with residual impurities in the sample discerned through the ensuing chromatograms. Moreover, as illustrated in Figures 22 and 23, prodigiosin exhibited a distinct molecular ion peak M + H at m/z 323.53, precisely matching the molecular weight of the compound (C20H25N3O, 323 kD). Earlier research by Yang *et al.* (2013) and Silva *et al.* (2012) substantiated that prodigiosin produced by *S. marcescens* and *Microcystis aeruginosa* exhibited a molecular weight of 323



#	RT	Scan	Height	Area	Area %	Norm %
1	2.818	4	681,151,104	282,733,696.0	21.213	44.26
2	3.499	140	385,602,304	638,821,824.0	47.930	100.00
3	7.345	909	89,274,576	27,790,912.0	2.085	4.35
4	9.646	1369	133,161,896	383,462,912.0	28.771	60.03

Fig. 22: GC-MS analysis of prodigiosin pigment extracted from moderate halophilic bacterial isolate *Bacillus aquimaris* of MP-1.

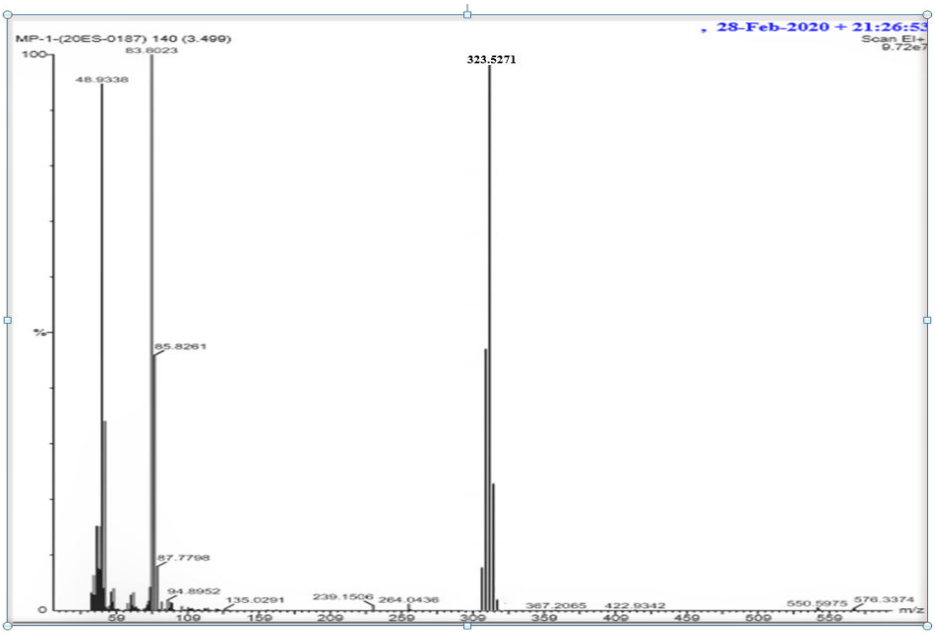


Fig. 23: Mass spectra of prodigiosin pigment extracted from moderate halophilic bacterial isolates of *Bacillus aquimaris* (MP-1).

m/z. These collective findings strongly suggest that the prodigiosin isolated from *Bacillus aquimaris* (MP-1) closely mirrors the standard pigment in terms of both purity and quality.

Application of prodigiosin pigment extracted from Bacillus aquimaris (MP-1)

(a) *Antioxidant activity of prodigiosin pigment*

The prodigiosin pigments extracted from *Bacillus aquimaris* (MP-1) demonstrate promising antioxidant capabilities through their ability to diminish the DPPH radical. DPPH, a stable free radical characterized by its violet hue, serves as a widely employed benchmark for assessing the efficacy of natural antioxidants in neutralizing free radicals. Upon interaction with an antioxidant compound capable of providing hydrogen or an electron, DPPH transforms into yellow-colored diphenylpicrylhyfrazine. The results indicate that increasing the prodigiosin level up to a maximum

Table 17: Antioxidant Activity of Prodigiosin Extracted from *Bacillus aquimaris* (MP-1)

Concentrations	Standard	Prodigiosin	% RSA	IC_{50}
50µg	87.37	55.51	57.40	11.90
100µg	90.5	55.62	62.71	
150µg	91.17	55.75	63.53	
200µg	92.53	56.39	64.09	
250µg	93.27	56.47	65.17	

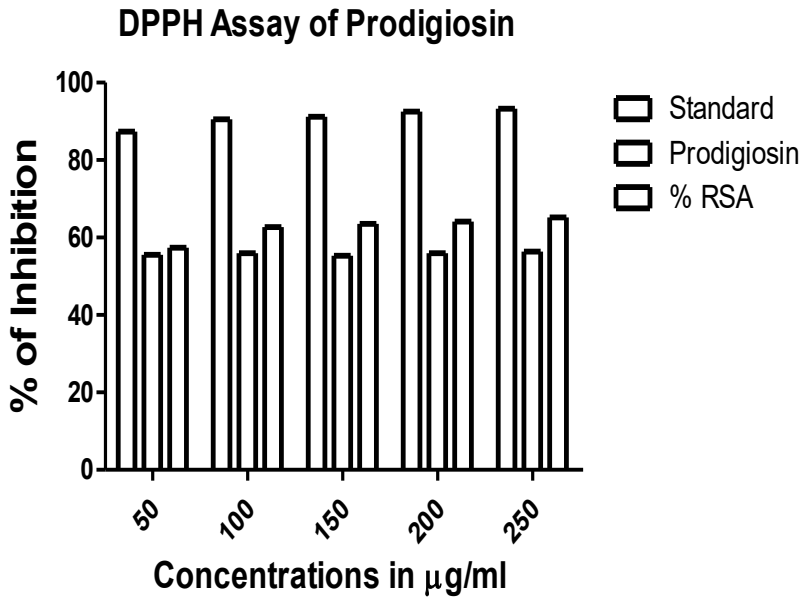


Fig. 24: Antioxidant activity (DPPH assay) of halophilic bacterial pigments isolated from MP-1.

of 250 g enhances the DPPH scavenging values of all tested samples, as presented in Table 17 and Figure 24. However, the findings suggest that prodigiosin exhibits lower antioxidant activity compared to standard ascorbic acid. To determine the sample concentration required to inhibit 50% of radicals, the IC_{50} value was calculated. Samples with higher antioxidant activity typically exhibit lower IC_{50} values. The IC_{50} value of the prodigiosin pigment obtained was 11.90, as indicated in Table 17. According to Phongpaichit *et al.* (2007), extracts with IC_{50} ranging between 50 and 100 are considered intermediate antioxidants. Meanwhile, extracts with IC_{50} values falling between 10 and 50 are deemed to possess high antioxidant activity (Harman *et al.*, 2016). In this study, prodigiosin pigment demonstrated high antioxidant activity.

(b) *Prodigiosin, a Sunblock Enhancer for Commercial Sunscreens*

Prodigiosin was combined with commercial sunscreen formulations and extracts from *Cucumis sativus* (cucumber) fruit and Aloe vera gel, both of which exhibit photoprotective properties. The Mansur equation and the UV spectrophotometric technique were utilized to determine the SPF values for each sample. The findings are displayed in Table 18. According to empirical results, two commercial sunscreens (A and B) with claimed SPF values of 15 and 24 had SPF values of 15.26 ± 0.45 and 23.42 ± 1.06. Whereas, in combinational

Table 18: Prodigiosin a Sunblock Enhancer for Commercial Sunscreens

S. No.	Sample	Labeled SPF	Found SPF Mean (± SD*)
1	Commercial Sunscreen A	15	15.26 ± 0.45 %
2	Commercial Sunscreen B	24	23.42 ± 1.06%
3	Commercial SunscreenA + Aloe Vera gel extract+cucumber fruitextract + prodigiosin (8% w/w	15	23.09 ± 1.23%
4	Commercial SunscreenB + Aloe Vera gel extract+cucumber fruitextract + prodigiosin (8% w/w)	24	30.26 ± 0.78%

Sunscreen Protection Factors (SPF)

studies, prodigiosin pigment (8% w/w) combined with sunscreens with SPFs of 15 and 24 showed 23.09 ± 1.23% and 30.26 ± 0.78% increases, respectively. Suryawanshi *et al.* (2015) demonstrated the use of prodigiosin as a sunscreen enhancer. Prodigiosin was found to enhance the sun protection factors (SPF) of commercial sunscreens.

(c) *Prodigiosin's dyeing capability*

The dyeing ability of prodigiosin pigment on cotton fabrics has been previously established. In light of this, the extracted pigment was evaluated on both cotton and polyisoprene fabrics using a concentration of 100 g/ml for staining. Despite undergoing thorough washing treatments involving hot water and detergents, the red pigment persisted in the polyisoprene material. prodigiosin's effectiveness has been demonstrated in dyeing polyolefin and polyethylene, indicating its potential as a colouring agent for non-fabric materials.

(d) *Prodigiosin pigments in candle preparation*

The hot, melted transparent gel wax was thoroughly mixed with the extracted prodigiosin pigment, which markedly coloured the candle wax. In a similar study, Shaikh (2016), and Mehta and Shah (2015) discovered that the candle can be successfully coloured using a prodigious red pigment derived from *Serratia marcescens.*

Conclusion

There is a high demand in various industries for the isolation of new organisms, innovative methods for characterization, and thoroughly mixed pigment extracts. Microbially extracted pigments find extensive applications in industries such as pharmaceuticals, cosmetics, food (as antioxidants and natural colorants), animal husbandry (as animal feed), among others. Initially, the study aimed to isolate, identify, and characterize a pigment-producing halophilic bacterial strain from solar salterns. Next, this study was continued in order to optimise the important physico-chemical parameters to maximise the yield of pigment. According to thr result in the present study, a crimson-colored halophilic bacterial isolate named *Bacillus aquimaris* (MP-1) was successfully identified and characterized. Antibiotic responses, heavy metal tolerance, and biofilm formation of the halophilic *Bacillus aquimaris* (MP-1) showed varying responses. Further use of these moderate pigmented halophilic bacterial strains could lead to the degradation of organic and inorganic waste, pigment formation, and potential industrial uses. Recently, many researchers have investigated how to isolate BTEX (benzene, toluene, ethylbenzene, and xylene) degraders from terrestrial

environments, to treat areas contaminated with highly toxic monocyclic aromatic hydrocarbons. When separated from sun salterns, *B. aquimaris* develops adaptable defense against the entrance of organic solvents, including vesicles and rigidification. Their organic solvent tolerance and metal resistance make them suitable for application in a variety of biotransformation processes, including bioremediation, of industrial effluents.

In this study, the Plackett-Burman design, a statistical tool, widely used in microbiology and biotechnology studies, was employed to screen various physico-chemical parameters that contribute to the biomass production of a pigmented halophilic bacterial isolate, *Bacillus aquimaris* (MP-1). This method proved effective in swiftly assessing the significance of numerous factors in a single experiment. Subsequently, only the most influential variables exhibiting positive significance were chosen for further optimization studies, while those demonstrating a significant negative impact on pigment yield were excluded from subsequent experiments. The selected key variables were further optimized in the second phase using the Box-Behnken design within the Response Surface Methodology framework to maximize pigment yield. The Box-Behnken design aids in predicting the optimal conditions for maximum production by examining the effects of factor interactions. To confirm and characterize the extracted pigment from *Bacillus aquimaris* (MP-1) as prodigiosin pigment, a range of analytical techniques including TLC, UV/VIS spectrophotometry, FTIR, HPLC, and GC/MS analyses were employed. This versatile pigment exhibits promising applications such as antioxidant activity, enhancement of commercial sunscreens as a sunblock enhancer, colouring agents for both fabric and non-fabric materials, and as a colouring agent in candle waxes.

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Conflict of Interest

The authors declare no conflicts of interest.

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