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Marine Microbial Pigments as Bioactive Compounds: A Comprehensive Study of Isolation and Characterization

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ABSTRACT
Marine environments harbor a vast diversity of microorganisms capable of producing bioactive pigments with significant industrial and therapeutic potential. This study focuses on the isolation, characterization, and biological evaluation of pigment-producing microorganisms from marine soil samples collected at Kovalam, Besant Nagar, and Marina beaches in Tamil Nadu, India. The research aims to identify potent microbial strains that synthesize pigments with antimicrobial and antioxidant properties, offering eco-friendly alternatives to synthetic dyes. The methodology involved collection of marine soil samples, serial dilution, and spread plate techniques for microbial isolation. Unique colonies were identified, isolated, and subjected to Gram staining, revealing rod-shaped Gram-negative bacteria. The best pigment-producing strain was selected through visual screening and confirmed as Serratia marcescens using 16S rRNA sequencing. The pigment was extracted using acetone and further purified. Bioactivity assays, including the agar well diffusion method, demonstrated antibacterial activity against E. coli and S. aureus, and antifungal activity against Penicillium and Candida species. Analytical techniques such as UV-Vis and FTIR spectroscopy were proposed for pigment characterization, supporting structural and functional insights. The extracted pigments show promising potential for pharmaceutical and industrial applications due to their natural origin, bioactivity, and environmental safety. This work lays the foundation for future exploration of marine microbes as sustainable sources of biologically active pigments with multifunctional applications in medicine, cosmetics, textiles, and food industries. Furthermore, this research emphasizes the importance of marine microbial resources in biotechnology. By optimizing production conditions and exploring mechanisms of action, this study contributes to marine microbiology and natural product research.

Keywords: Isolation, Characterisation, Marine microbes, Pigments

I. INTRODUCTION

Marine microbes are microscopic organisms that inhabit oceanic environments. Some marine bacteria produce bioactive compounds with applications in medicine, such as antibiotics agents. Certain microbes also produce pigments with antimicrobial properties, making them valuable in pharmaceutical and industrial fields. Research on marine microbes provides insights into biotechnology, environmental conservation, and potential climatechange solutions. Their study continues to unlock novel discoveries.

Pigment-producingmarinemicrobesarespecializedmicrobesthatsynthesizebioactive pigmentswithdiverseindustrialsignificance.Thesepigments,includingprodiogsin,violacein, etc. serve as protective agents against environmental stressors like UV radiation, oxidative damage, etc. Isolated from marine environments such as seawater, sediments, etc., Their pigments possess antimicrobial, anticancer properties, etc, making them valuable for pharmaceutical and biotechnological applications. Extraction and characterization of these pigments involve UV-Vis spectroscopy technique to determine their chemical structure and bioactivity.Researchonoptimizingmicrobialgrowthconditionsenhancespigmentproduction, improving industrial scalability. The study of marine microbial pigments contributes to sustainable bioproduct development. Ongoing investigations explore their potential in cosmetics, medical therapeutics, etc.

Marine microbial pigments exhibit significant antioxidant properties, making them highlyvaluablefortherapeuticapplications.Thesepigmentsarenaturalcompoundscapableof neutralizing free radicals, which cause oxidative stress and cellular damage. The antioxidant activity is largely due to the unique chemical structures of these pigments, evolved under extreme marine

conditions. By scavenging harmful reactive oxygen species (ROS), these pigments help in preventing aging-related disorders and degenerative diseases. Studies have shown their potential in protecting cells from oxidative damage in both in vitro and in vivo models. Theyoffer a safer, eco-friendly alternative to synthetic antioxidants used in food and pharmaceutical industries. Marine pigments such as melanin and prodiogsin are particularly notedforthisbioactivity.Thesepropertiesalsoenhance theirstabilityandshelflifeinvarious industrial applications. Continuous research supports their role in enhancing immune defense and reducing inflammation. Thus, marine microbial pigments stand out as promising antioxidant agents for future nutraceutical and biomedical development.

Marine microbial pigments have gained significant attention due to their potent antimicrobial properties. These pigments produced by marine bacteria, fungi, etc., exhibit bioactive compounds that inhibit bacterial and fungal growth. The isolation of such pigments involves culturing marine microbes from sediments under optimized conditions. Characterization technique like UV-Vis spectroscopy help to identify the chemical structure and purity of pigments. Once characterized, these pigments undergo biological evaluation to test their antimicrobial efficacyagainst pathogenic microbes. Studies show that pigments like prodiogsin possess strong antimicrobial effects. Their mode of action includes disrupting microbial cell membranes, interfering with DNA replication, and inhibiting enzyme activity. The natural origin and low toxicity of marine microbial pigments make them promising candidatesforpharmaceuticalapplications.Ongoingresearchfocusesonenhancingpigment yield and optimizing their application in medicine. Overall, marine microbial pigments hold immense potential in developing natural antimicrobial agents.

Isolated pigments from marine microbes offer numerous benefits in various scientific andindustrialfields.Thesepigmentsexhibituniquechemicalproperties,makingthemvaluable for pharmaceutical industries. Their isolation allows researchers to study their structural composition, enhancing our understanding of marine biodiversity. Characterization through advanced analytical techniques like UV- Vis spectroscopy help in determining their stability andsolubility.Manymarinemicrobialpigmentspossessantimicrobialproperties,makingthem suitable for drug development. Additionally, some pigments show UV-absorbing properties, benefiting skincare formulations. The biological evaluation of these pigments helps in assessing their safety, toxicity, and therapeutic potential. Their eco-friendly nature and biodegradability contribute to sustainable industrial practices. Ongoing research aims to enhance their production through biotechnological advancements. Thus, isolated marine microbialpigmentsholdimmensepotentialfordiverse applicationsinmedicine,industry,and environmental sustainability.

II. MATERIALSANDMETHODS

SAMPLECOLLECTION

Sample collection is a crucial step in isolating and characterizing marine microbial pigments.Forthisstudy,sampleswerecollectedfromKovalamBeach(12.7896°N,80.2542° E), Besant Nagar Beach (12.9989° N, 80.2718° E), and Marina Beach (13.0500° N, 80.2824° E). Soil samples were carefully gathered to ensure minimal contamination. Each sample was diluted using a serial dilution technique to obtain different microbial concentrations.

ISOLATIONOFMICROBES

Isolation of microbes is a critical step in studying marine microbial pigments. The collected soil samples from Kovalam, Besant Nagar, and Marina beaches were subjected to serialdilutiontoreduce microbialloadandobtaindistinctcolonies.Thedilutedsampleswere platedonnutrientagarusingthespreadplatemethod.Afterincubation,bacterialcolonieswith uniquemorphologywereidentifiedandselected.Purecolonieswereobtainedthroughquadrant streaking and continuous streaking techniques. The best pigment-producing strains were screened and selected for further study. The isolated strains were then optimized for pigment production under controlled conditions. This structured approach ensures the identification of potential microbial sources for bio-pigment applications.

PROTOCOLFORSERIAL DILUTION

Tensteriletesttubeswerearrangedinatesttubestand,and10mLofsterilesalinewas prepared. A 0.1 g soil sample was accurately weighed and mixed thoroughly with the 10 mL salinetoformtheinitialsuspension.Usingamicropipette,1mLofthissolutionwastransferred into the second test tubecontaining 9 mLof saline, and the process was repeated sequentially up to the ninth test tube to create a series of nine serial dilutions. For

plating, three replicates were prepared for each of the 3 different soil samples, resulting in a total of 9 Petri plates. Nutrientagarwasused astheculturemedium,anditspreparationinvolveddissolving2.925g ofnutrientbrothand4.5gofagarin225mLofdistilledwater,followedbysterilization.After themediumsolidifiedintheplates,thesamplesfromeachdilutionwereinoculatedusingthe spreadplatemethod.Plateswerethenincubatedunders uitableconditionstoallowformicrobial growth and subsequent analysis.

MORPHOLOGICALCHARACTERIZATION OFMICROBES

Morphological characterization of microbes is essential for identifying and differentiatingbacterialstrains.Theisolatedcoloniesfrommarinesoilsampleswereexamined fortheirshape,size,color,elevation,andmarginonnutrientagarplates.Colonycharacteristics such as smoothness, roughness, and texture were recorded. Gram staining was performed to distinguish between Gram-positive and Gram-negative bacteria based on their cell wall composition.Thestainedslideswereobservedundera microscopeusinganoilimmersionlens (100X)toanalyzecellshapeandarrangement.Theresultsrevealedrod-shaped,Gram-negative bacteria among the isolates. The streaking methods, including quadrant and continuous streaking,helpedinobtainingpureculturesfordetailed observation.Microscopicexamination confirmed the structural integrity and purity of selected bacterial strains. The characterized microbes were further subjected to biochemical and molecular identification techniques. This systematicapproachensurestheaccurateidentification ofpigment-producingmicroorganisms for further applications.

QUADRANTSTREAKING

The agar plate was divided into four equal sections to perform quadrant streaking. Using a sterile inoculating loop, the microbial sample was first streaked in the first quadrant. The loop was then flamed to sterilize it before proceeding. From the first quadrant, the loop was dragged into the second quadrant and streaked in a zig-zag pattern to graduallydilute the sample. This process was repeated for the third and fourth quadrants, with the loop being flamed between each quadrant to ensure reduced microbial load and to isolate individual colonies.

GENERALMORPHOLOGY

Afterincubation,eachcolonywascarefullyobservedandmeasuredformorphological characteristics. The colonydiameter was recorded in millimetres using a ruler or scale placed under the Petri dish. Pigmentation was noted, distinguishing between intrinsic pigmentation (colourwithinthecolony)anddiffusiblepigmentation(colourspreadingintotheagar).The colony form, elevation, and margin were described according to standard microbiological referencefigures—commonformsincludedcircular,irregular,orfilamentous;elevationssuch asflat,raised,orconvex;andmarginslikeentire,undulate,orlobate.Surfacetexturewasalso assessed, categorizing colonies as smooth (shiny, glistening surface), rough (dull, bumpy, or matte), or mucoid (slimy or gummy appearance). The opacity was recorded as transparent, translucent, or opaque, and the texture was evaluated using a sterile needle to determine whether the colony felt butyrous (buttery), viscous (gummy), or dry (brittle or powdery). All observations were systematically recorded for comparison and analysis.

PURECOLONY ISOLATION

Pure colony isolation is a crucial step in obtaining single bacterial strains for further study.Thecollectedmarinesoilsamplesunderwentserialdilutionandwereplatedonnutrient agarusingthespreadplatemethod.Afterincubation,distinctbacterialcolonieswereidentified based on their morphology. To ensure purity, selected colonies were streaked onto fresh nutrientagarplatesusingquadrantandcontinuousstreakingmethods.Thistechniqueallowed the isolation of single colonies, minimizing contamination. The isolated colonies were incubated under optimal conditions to promote growth. After incubation, individual colonies were observed and confirmed for purity through repeated streaking. The purified bacterial strains were preserved for further pigment production. This process ensures the successful isolation of pigment-producing microbes for research and industrial applications.

STREAKINGPROTOCOL

King's B medium was prepared and sterilized by autoclaving, then poured into sterile Petriplatesandallowedtosolidifyunderasepticconditions.Asinglecolonywaspickedfrom anexistingplateorbrothcultureusingasterileinoculati

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ngloop.Theloopwasfirstheateduntil red-hotinaBunsenburnerandallowedtocoolbeforecontactingthebacterialculture.Quadrant streaking was performed to isolate the colonies: the agar plate was divided into four sections, andthesamplewasstreakedinthefirstquadrant.Theloopwasthenflamedandcooledbefore draggingfromthefirsttothesecondquadrantinazig-zagpattern.Thisprocedurewasrepeated for the third and fourth quadrants, with flaming between each to ensure proper dilution of the bacterial load. The inoculated plates were then incubated at 30–37°C for 24–48 hours. Following incubation, red or pink pigmented colonies were observed, which is characteristic of Serratia marcescens.

MOLECULARCHARACTERIZATION-16srRNASEQUENCING
DNAISOLATION

1.5 mL of overnight-grown bacterial isolates were centrifuged at5000 rpm for 15 min. After discarding the supernatant, the pellet was mixed with 467 µl of TE buffer, 70 µl of 0.5% lysozyme, 50 µl of 10% SDS, and 5µl of proteinase K. To ensure good mixing, gently invert the tubes several times before incubating at 37 ◦C for 1 h After incubation, add an equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) combination. Gently invert the tubes several times to ensure complete mixing. After centrifugation at 8000 rpm for 10 min, transfer the top aqueous phase to a new tube. Add 1/10th volume of sodium acetate and 0.6 vol of cold isopropanol to precipitate the DNA. After precipitating the DNA, the mixture was centrifuged at 8000 rpm for 10 min. The pellet was washed twice with cooled 70% ethanol and centrifuged again at 5000 rpm for 10 min. After discarding the supernatant, add 30 µL TE buffer to the final DNA pellet. The extracted genomic DNA was examined using agarose gel electrophoresis and stored at 4 ◦C for future use.

AMPLIFICATION

The PCR reaction was run in a gradient thermal cycler. To amplify the 16S rRNA gene fragment, universal primers (forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverseprimer5′-GGTTACCTTGTTACGACTT3′)wereemployed.Thereactionmixtureof 50 µl included 10 ng of genomic DNA, 2.5 units of Taq DNA polymerase, 5 µl of 10X PCR amplification buffer (100 mM Tris–HCl, 500 mM KCl pH-8.3), 200 µM dNTP, 10 per moles of each universal primer, and 1.5 mM MgCl2. The amplification process involved initial denaturationat94◦Cfor3min,followedby40cyclesofdenaturationat94◦Cfor30s.Primers were annealed at 55 ◦C for 30 s and extended at 72 ◦C for 1 min. The last extension was performed at 72 ◦C for 10 min.

16srRNASEQUENCING

The sequences were evaluated with the BLAST query tool (http://blast.ncbi.nlm.nih.gov)toidentifynon-chancesequencesimilarities.BLASTquerieswerelimited tothe16SribosomalRNAdatabase.Thesequencewas examinedusingblast,andthehitwith thelowestexpect value(e-value,indicatingthenumberofnon-chancealignments)waschosen. MEGAsoftwarewas usedtocreatethephylogenetictree, which used aneighbor-joining(NJ) method.

MOLECULARIDENTIFICATIONOFMICROBES

Molecular identification of microbes is a precise technique used to classify and distinguish microorganisms based on their genetic material. This method relies on analyzing specific genesequences,suchasthe16SrRNAgeneforbacteria. PolymeraseChainReaction (PCR) is commonlyemployed to amplifytarget DNA sequences for identification. Advanced techniques like DNA sequencing, quantitative PCR, and metagenomics enable accurate microbial detection. These methods help in diagnosing infections, monitoring environmental microbiomes, and studying microbial evolution. Molecular tools eliminate limitations of traditional culture-based identification, providing faster and more reliable results.

PIGMENT EXTRACTION

Pigment extraction from microbial sources is a key step in obtaining bioactive compounds for various applications. The process begins with harvesting microbial cultures throughcentrifugationtoseparatethebiomassfromthe supernatant.Thepelletcontainingthe pigment is treated with organic solvents like acetone for efficient extraction. The mixture is thenvortexedandallowedtoundergophaseseparationtoisolatethepigment-richfraction.The solvent phase is collected and subjected to evaporation under reduced pressure to concentrate thepigment.Optimizationofsolventchoiceandextractionconditionsensuresmaximumyield and purity. Proper extraction enhances pigment properties such

as color intensity, solubility, and antimicrobial potential. Overall, an optimized extraction process ensures high-quality pigment for sustainable applications.

PIGMENTEXTRACTIONPROTOCOL

Microbial cultures were harvested by centrifugation at 8000 rpm for 10 minutes, resulting in the separation of the pellet and supernatant. The contents of the falcon tube, containing both pellet and supernatant, were then vortexed thoroughly to ensure uniform mixing.Followingthis,20mLofacetonewasaddedtothetubetoaidinextractionorwashing. The mixture was centrifuged again under the same conditions to re-separate the contents. Meanwhile,sterilePetriplateswerewashed,wipedclean,andweighedtorecordtheirempty weight, which would later be used for accurate measurement of any subsequent additions or residues.

OPTIMIZATIONANDESTIMATION

The optimized conditions for prodigiosin production were analyzed under various media conditions to determine their effect on pigment yield. For quantification, bacterial culturesgrowninKing'sBbrothwerefirstmeasuredfor celldensitybyrecordingabsorbance at 620 nm (OD620).The brothwas then centrifuged at 5000 rpmfor 15 minutesto collect the bacterial cellpellet.To extractthepigment,10mLofacetone was addedtothepellet,andthe mixturewascentrifugedagainunderthesameconditions.Acetonewasusedasablankduring absorbance measurements. The acetone extract, containing prodigiosin, was scanned and showedacharacteristicabsorbancepeakat499nm(OD499).Theconcentrationofprodigiosin produced per cell was then calculated using the following formula:

$$\textbf{Prodigiosinunit/cell} = [\textbf{OD499} - (\textbf{1.381} \times \textbf{OD620})] \times \textbf{1000}/\textbf{OD620}$$

Where,OD499–pigmentabsorbance;OD620 – bacterialcellabsorbance;1.381– constant.

BIOACTIVITYOFPIGMENT

The bioactivity of microbial pigments is essential for their potential applications in pharmaceuticals, cosmetics, and food industries. These pigments exhibit strong antibacterial and antifungal properties, making them effective against harmful microbes like E. coli, S. aureus,Penicillium,andCandida.Theagarwelldiffusionmethodiscommonlyusedtoassess antimicrobial activity by measuring zones of inhibition. Pigments also possess antioxidant properties,helpingtoneutralizefreeradicalsandpreventoxidativedamage.Thebioactivityis influenced by factors like concentration, extraction method, and microbial strain. Optimized pigments demonstrate enhanced effectiveness in inhibiting microbial growth and oxidative stress.Furtherstudiesontheirmechanismofactionhelp indevelopingnewtherapeuticagents. Thesebioactivepigmentsprovideeco-friendlyalternativestosyntheticdyeswithaddedhealth benefits. Overall, microbial pigments serve as natural, sustainable compounds with multiple bioactive properties.

Microbial pigments derived from marine sources are gaining attention due to their unique chemical structures and broad spectrum of biological activities. These bioactive compoundsarenaturallyproducedunderextrememarineconditions,contributingtotheir enhancedstability,solubility,andfunctionality.Theynotonlyactasnaturalcolorantsbutalso serve as effective anti-inflammatory, anticancer, and immunomodulatory agents. In addition, theirlowtoxicityandbiodegradabilitymakethemideal forapplicationsingreenchemistryand sustainable product development. Advances in molecular techniques such as 16S rRNA sequencinghelpinidentifyingpotentpigment-producingstrains,whileoptimizedfermentation andextractionprocessesboostpigmentyieldandbioactivity.Asinterestinreplacingsynthetic additives grows, marine microbial pigments stand out as versatile and health-promoting alternatives that align with the global shift toward natural and eco-conscious solutions.

ZOIPROTOCOL

For antimicrobial testing, media preparation was carried out by dissolving 0.75 g of Nutrient Agar (NA) in 50 mL of distilled water for bacterial cultures and 1.2 g of Potato Dextrose(PD)brothin50mLofdistilledwaterforfungalcultures.Themediaweresterilized byautoclavingat121°Cforthreewhistlesandpouredintosterile Petridishestosolidify.Serial dilutionsofthemethanolextractofthepigmentwerepreparedfortesting:500µg/mL(500µL sample+500µLmethanol),250µg/mL(500µLsample+500µLmethanol),and100µg/mL (400 µL sample + 600 µL methanol). For culture preparation, E. coli and S. aureus were

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swabbedontoNAplates,whilePenicilliumandCandidawereswabbedontoPDAplatesusing sterile ear-cleaning cotton sticks for even distribution. Wells were punched in the solidified agarusingasterileagarpuncher,and100µLofeachsampleconcentrationwasloadedintothe respective wells for analysis.

TOTALANTIOXIDANTCAPACITY(TAC)

Thephosphomolybdenummethodwasusedtoassesstheoverallantioxidantcapacity. Variousconcentrations(25,50,100,250,500and1000 µg/ml)ofstandardsolution(Ascorbic acid) and sample (1mg/mL) were prepared. 300µl of standard/extract and 3 ml of phosphomolybdenum reagent solution were mixed (0.6M sulfuric acid, 28mM sodium phosphateand4mMammoniummolybdate).Afterincubatingthereactionmixtureat75°Cfor 90minutesandcoolingit toroomtemperature,theabsorbancewasmeasuredat680nmusing a spectrophotometer against a blank. The blank was made up of reagent (3 ml).

III. RESULTS AND DISCUSSION

SAMPLECOLLECTION

Marinesoilsampleswerecollectedfromthree coastallocationsinTamilNadu,India Kovalam, Besant Nagar, and Marina beaches—with careful attention to minimize contaminationandpreservethemicrobialintegrityofeachsample (Fig 1).Toreducemicrobialdensity and facilitate colony isolation, each sample underwent a serial dilution process. The diluted sampleswerethenculturedonnutrientagarplatesusingthespreadplatemethod,allowingfor even distribution of microorganisms across the agar surface. This approach enabled the isolation of distinct microbial colonies, which were subsequently analyzed for pigment production.

Fig 1 Samples collected from different locations in Tamil Nadu

ISOLATIONOFMICROBES

Soil samples collected from marine sites were subjected to serial dilution to reduce microbial load and facilitate the isolation of individual microbes. The diluted samples were then plated on nutrient agar using the spread plate method (Fig 2-4). Following incubation, colonies exhibiting unique morphological characteristics were selected for further analysis. To obtain pure bacterial isolates, both quadrant and continuous streaking techniques were employed. Among the isolates, the most potent pigment-producing strains were identified and subsequently screened and optimized for enhanced pigment production (Table 1-3).

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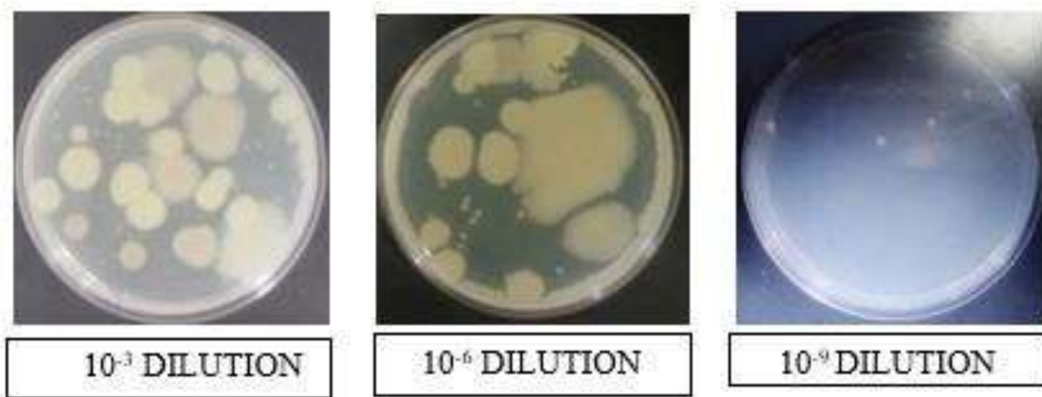

Fig 2SAMPLEA

Table1 TotalnumberofcoloniesinSampleA

SampleA	**10^{-3}**	**10^{-6}**	**10^{-9}**
Totalno.ofcolonies	56	40	5
CFU/ml	$5.6*10^{5}$	$4*10^{8}$	$5*10^{10}$

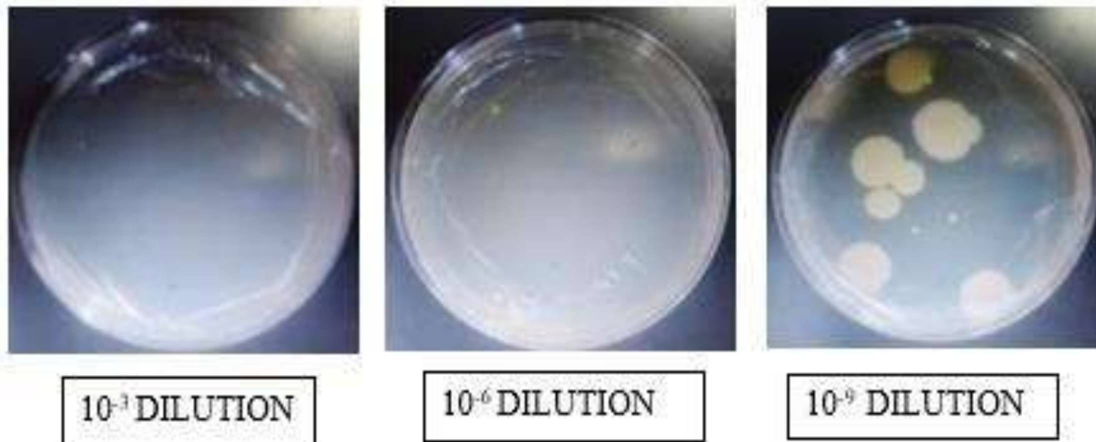

Fig 3SAMPLEB

Table2 TotalnumberofcoloniesinSampleB

Sample	**10^{-3}**	**10^{-6}**	**10^{-9}**
Totalno.ofcolonies	0	24	112
CFU/ml	0	$2.4*10^{8}$	$1.12*10^{12}$

Fig 4SAMPLEC

Table3 TotalnumberofcoloniesinSampleC

SampleC	10^{-3}	10^{-6}	10^{-9}
Totalno.ofcolonies	14	22	5
CFU	$1.4*10^{5}$	$2.2*10^{8}$	$5*10^{10}$

MORPHOLOGICALCHARACTERIZATIONOFMICROBES

Coloniesexhibitinguniquemorphologicalcharacteristics—suchasvariationsincolor, shape, edge definition, and surface texture—were carefully selected and subjected to further isolation through the continuous streaking technique. This method ensured the acquisition of pureand well-isolated colonies forsubsequent analysis.Amongthe isolated colonies, particular attention was given to those displaying distinctive pigmentation, as the presence of vivid or unusual coloration is often indicative of pigment production, which can be of biochemical or industrialsignificance.Onesuchstrain,characterizedbyaclearlyvisibleanduniquecolornot observed in other colonies, was selected for further processing and detailed characterization.

PURECOLONYISOLATION

To ensure the accuracy and reliability of downstream analyses, pure colony isolation was performedusingthestreakplatemethodundersterileconditions.Thistechniquewasemployed to obtain single, geneticallyidentical colonies derived from a single microbial cell or a group of identical cells. The previously selected distinct colony, presumed to exhibit pigment production, was streaked onto fresh agar plates using a sterile inoculation loop. The quadrant streakingmethodwasappliedtoprogressivelydilutethemicrobialpopulationacrossthe surfaceofthemedium,therebyfacilitatingtheemergenceofwell-isolatedcolonies.Plateswere incubated under optimal conditions tailored to the growth requirements of the organism, including appropriate temperature, pH, and atmospheric parameters. After incubation, the plates were examined for colony morphology, uniformity, and absence of contamination. Isolated colonies that retained the unique pigmentation and morphological features of the original strain were further subcultured to confirm purity (Fig 5).

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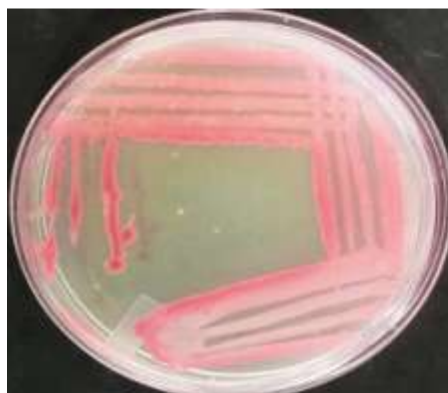

Fig 5 QuadrantStreakingoftheSelected strain

CULTURINGOFISOLATEDCOLONYANDGRAMSTAINING

Theisolatedpurecolony,identifiedbasedonitsdistinctpigmentationandmorphology, was further cultured to obtain sufficient biomass for microscopic and biochemical analysis.A loopful of the pure colony was inoculated into freshly prepared nutrient broth and incubated under optimal conditions—typicallyat 30–37°C for 18–24 hours, depending on the microbial growthrate.Theculturewasmaintainedunderconstantagitationtoensureuniformdistribution ofnutrientsandaeration,promotinghealthybacterialgrowth.Followingincubation,analiquot of the liquid culture was used for Gram staining to determine the bacterial cell wall characteristics and morphology. The observations confirm that the isolated strain belongs to the Gram-negative group of bacteria, with a characteristic rod-shaped morphology (Fig 6). This information provides crucial insights into the bacterial taxonomy and guides further steps in identification,suchasbiochemicaltesting,molecularcharacterization,andpotentialindustrial or pharmaceutical applications.

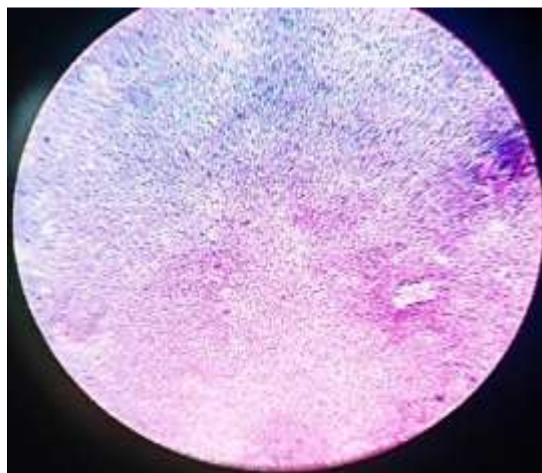

Fig 6 Gram staining of selected strain

BIOCHEMICALANALYSISOFTHESELECTEDSTRAIN

To further characterize the physiological and metabolic traits of the isolated strain, a seriesofstandardbiochemicaltestswereperformed.Theseassayshelpinidentifyingbacterial species by assessing the presence of specific enzymes and the organism's ability to utilize various substrates.Afresh pure culture of theisolatedstrain was inoculated into selective and differential media specific to each biochemical test. All procedures were conducted under sterile conditions and incubated at 35–37°C for 24–48 hours as per standard protocols.

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Table 4 The biochemical profile of the isolated strain

Biochemicaltest	**Inference**
Indole	Negative
Methyred	Negative
Voges Proskauer	Positive
Citrate Utilization	Positive

The biochemical profile of the isolated strain is as follows: Indole: Negative, Methyl Red: Negative, Voges-Proskauer: Positive & Citrate Utilization: Positive (Table 4). This distinct combination of test results is suggestive of organisms typically belonging to the Enterobacter genera. However, further confirmation through molecular techniques such as 16S rRNAgene sequencing is recommended for accurate taxonomic identification (Fig 7).

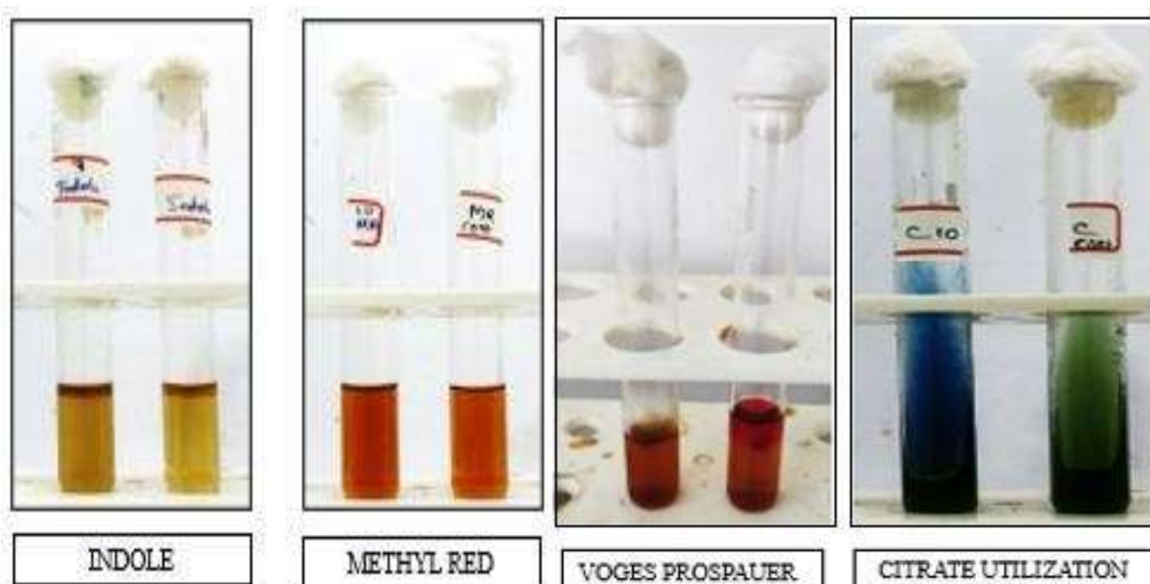

Fig 7 The biochemical profile of the isolated strain

IDENTIFICATIONOFISOLATEDCOLONY DNAISOLATION

1.2mLof overnight-grown bacterial isolates were centrifuged at5000 rpm for 15 min. Afterdiscardingthesupernatant,thepelletwasmixedwith467µlofTEbuffer,70µlof0.5% lysozyme, 50 µl of 10% SDS, and 5µl of proteinase K. To ensure good mixing, gently invert thetubesseveraltimesbeforeincubatingat37◦Cfor1hAfterincubation,addanequalvolume of phenol, chloroform, and isoamyl alcohol (25:24:1) combination. Gently invert the tubes severaltimestoensurecompletemixing.Aftercentrifugationat8000rpmfor10min,transfer thetopaqueousphasetoanewtube.Add1/10thvolumeofsodiumacetateand0.6volofcold isopropanoltoprecipitatetheDNA.AfterprecipitatingtheDNA,themixturewascentrifuged at8000rpmfor10min.Thepelletwaswashedtwicewith cooled70%ethanolandcentrifuged againat5000rpmfor10min.Afterdiscardingthesupernatant,add30µLTEbuffertothefinal DNApellet.TheextractedgenomicDNAwasexaminedusingagarosegelelectrophoresisand stored at 4 ◦C for future use.

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PCRAMPLIFICATION

ThePCRreactionwasruninagradientthermal cycler.Toamplifythe16SrRNAgene fragment, universal primers (forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverseprimer5′-GGTTACCTTGTTACGACTT3′)wereemployed.The reactionmixtureof 50 µl included 10 ng of genomic DNA, 2.5 units of Taq DNApolymerase, 5 µl of 10X PCR amplificationbuffer(100mMTris–HCl,500mMKClpH-8.3),200µMdNTP,10permoles of each universal primer, and 1.5 mM MgCl2. The amplification process involved initial denaturationat94◦Cfor3min,followedby40cyclesofdenaturationat94◦Cfor30s.Primers were annealed at 55 ◦C for 30 s and extended at 72 ◦C for 1 min. The last extension was performed at 72 ◦C for 10 mins (Fig 8).

16srRNASEQUENCING

The sequences were evaluated with the BLAST query tool (http://blast.ncbi.nlm.nih.gov)toidentifynon-chancesequencesimilarities.BLASTquerieswerelimited to the16S ribosomalRNAdatabase.Thesequencewas examined usingblast, and thehit with thelowestexpectvalue(e-value,indicatingthenumberofnon-chancealignments)waschosen. MEGAsoftware was used to createthephylogenetictree, which used aneighbor-joining(NJ) method (Fig 9). The Organism was found to be **Serratia marcescens**.

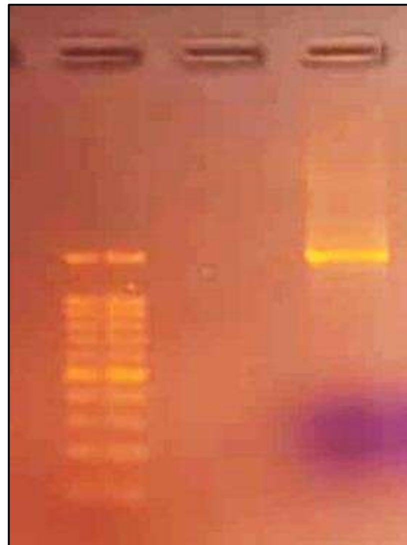

Fig 8 AgaroseGelElectrophoresis–PCRProduct

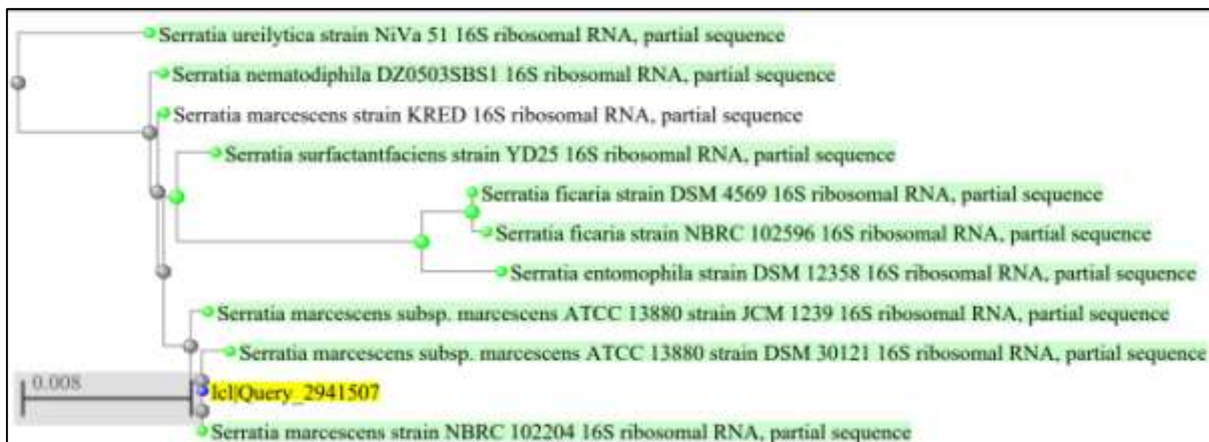

Fig 9 PhylogeneticTree

PIGMENTEXTRACTION

Pigment extraction starts with centrifuging the microbial culture (e.g., Serratia marcescens) at high speed (8000–10000 rpm) to separate the cell pellet from the supernatant. Thecell pellet is treated with acetone, acommon organicsolvent, which helps break open the cells and extract the pigment efficiently.After vortexing and centrifuging again, the pigment- rich solvent phase is collected. This contains the colored compound (like prodigiosin in your case). The acetone extract is then concentrated using a rotary evaporator at around 60°C, resulting in a crude pigment ready for purification. The crude pigment is recrystallized with methanol to enhance its purity and stability, making it suitable for bioactivity tests like antibacterial and antioxidant assays (Fig 10).

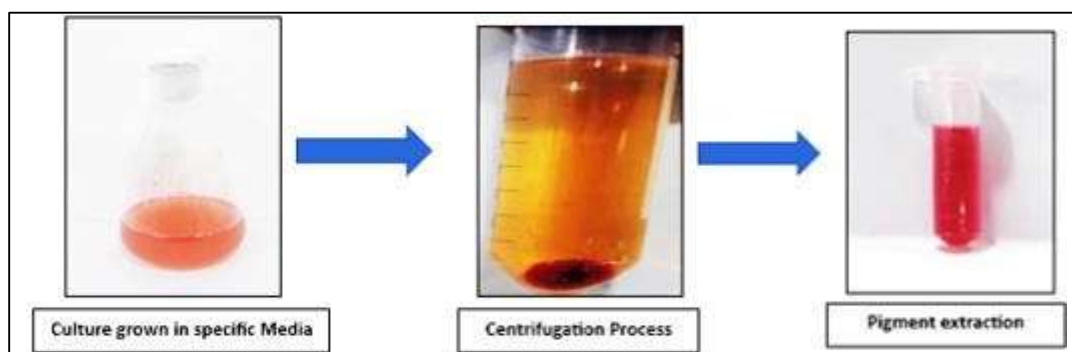

Fig 10 Pigment extraction from crude sample

OPTIMIZATIONOFPIGMENTPRODUCTION ANDESTIMATION

To enhance pigment production by the isolated bacterial strain, a systematic optimizationofgrowthconditionswascarriedout.Thisinvolvedvaryingkeyparameterssuch as **pH**, **temperature**, and **incubation period**, as these environmental factors are known to significantly influence secondary metabolite synthesis, including pigment production.

PIGMENTEXTRACTION

The bacterial strain was cultured in nutrient broth under different environmental conditions to determine the optimal settings for maximum pigment yield. The following parameters were tested individually while keeping other factors constant:

- **pHLevels:** 6.0,7.0, 8.0,9.0, and 10.0
- **TemperatureRanges:**27°C,30°C,34°C,37°C,and 40°C
- **IncubationTimes:** 12hours,24hours,36hours,48hours,and 72hours

Each culture was incubated under shaking conditions to maintain aeration and homogeneity.After the incubation period, the culture broths were subjected to centrifugation toseparatethecellsfromthesupernatant.Thepigmentfromthecellpellet wasextractedwith acetone and the extract was centrifuged at 5000 rpm for 15 minutes. The supernatant was concentrated in Rotary evaporator at 60 °C to obtain crude pigment. The crude concentrated pigment was recrystallized with methanol (Fig 11).

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Fig 11 Pigment extraction under different environmental conditions

OPTIMIZATION-PRODIGIOSINESTIMATION

The pellet obtained after centrifugation was treated with an appropriate solvent commonly acidified methanol or ethanol to extract the intracellular pigment. The mixture was then vortexed and allowed to stand for complete pigment solubilization. Following extraction, the solution was filtered or centrifuged again to remove any cell debris, and the absorbance of the clear pigment-containing solution was measured using a UV-Visible spectrophotometer. The extracted pigment was quantified using the absorbance values at 499 nmand620nm,asmeasuredbythespectrophotometer (Table 5).Thepigmentyieldwascalculatedusing the following formula:

Prodigiosinunit/cell=[OD499 –(1.381xOD620)]x 1000/OD620

Where,OD499–pigmentabsorbance;OD620– bacterialcellabsorbance;1.381–constant

Table 5 Optimization-ProdigiosinEstimation

OptimizationCondition	OD620	OD492	Prodigiosinunit/cell
PH6	0.214	0.396	469.934
PH7	0.160	0.308	542.412
PH8	0.143	0.256	399.403

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PH9	0.156	0.246	192.435
PH10	0.152	0.234	150.066
Temp27°C	0.166	0.249	115.398
Temp30°C	0.228	0.399	368.890
Temp34°C	0.356	0.671	502.375
Temp37°C	0.226	0.469	692.828
Temp40°C	0.228	0.442	560.666
12 hr	0.186	0.265	39.4606
24 hr	0.192	0.308	220.347
36 hr	0.220	0.426	554.059
48 hr	0.186	0.367	586.880
72 hr	0.237	0.482	652.741

Among the various growth parameters tested, the pigment yield was significantly influenced by changes in pH, temperature, and incubation time. Based on the quantitative estimation of pigment using the Prodigiosin unit/cell formula, the following conditions were found to be optimal for maximum pigment production by the isolated strain:

pH:7,Temperature:37°C&IncubationPeriod:72hours

Under these optimized conditions, the strain exhibited the highest pigment output, indicating that neutral pH, physiological temperature, and extended incubation favor the biosynthesisandaccumulationofthepigment.Thesefindingssuggestthattheorganism's metabolic activity related to pigment production is most efficient under these environmental settings (Fig 12).

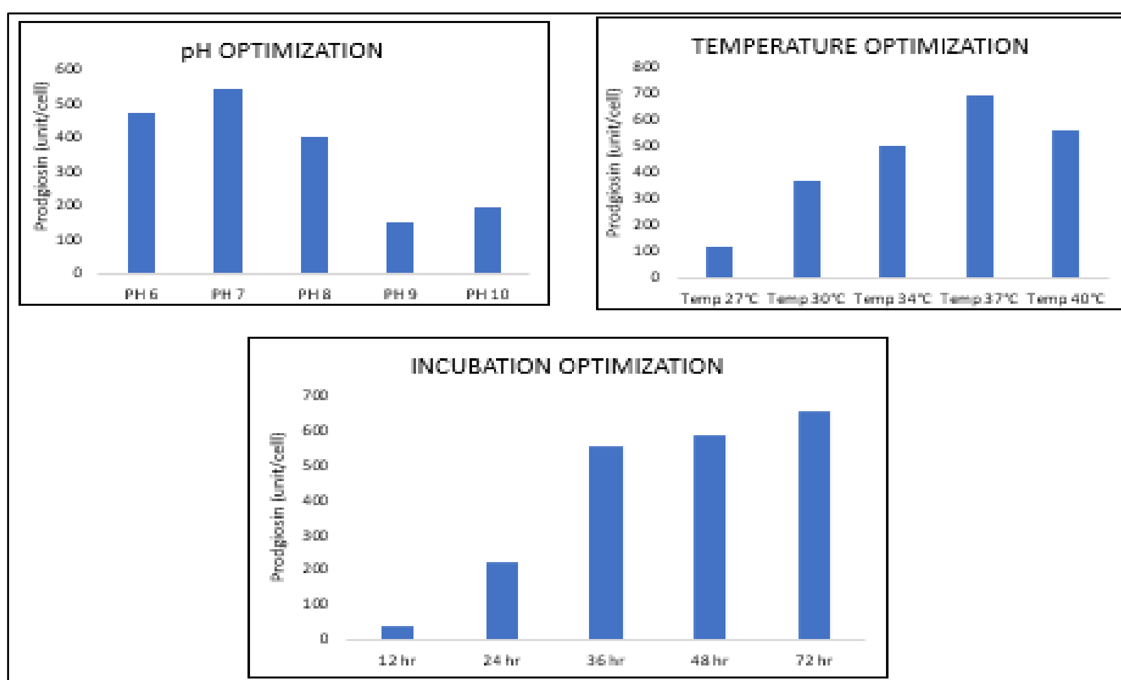

Fig 12 Yield of pigments at different environmental conditions

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CHARACTERISATIONOFPIGMENTUSINGUV-VISSPECTROMETRY

To characterize the optical properties and confirm the presence of the pigment, UV- Visible spectrophotometric analysis was performed. This method provides valuable insights into the absorption maxima (λmax) of the pigment, which is often adistinguishing feature for identifying pigment classes, including prodigiosin and other related compounds. The UV-Vis spectrum of the pigment showed a prominent absorption peak (λmax) at approximately 499 nm,whichischaracteristicofprodigiosin,aredpigment knownforitstripyrrolestructure.This absorption is typically attributed to π→π* transitions within the conjugated chromophore system of the molecule. The absence of significant peaks in the lower UV range and the dominanceofthepeak near499 nm furthersupport thepurityand identityofthepigment as a prodigiosin-like compound (Fig 13). The UV-Visible spectral analysis confirms the presence of a red pigment,withspectralcharacteristicsmatchingthoseof prodigiosin.Thisdata,combinedwith previous biochemical and morphological observations, suggests that the pigment-producing strain is likely associated with prodigiosin biosynthesis pathways.

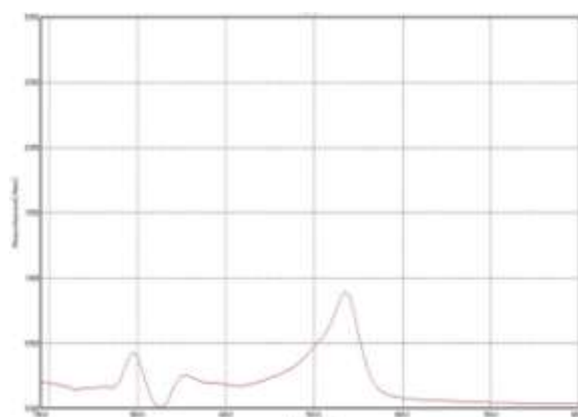

Fig 13 UV-Visible spectrometry

BIOACTIVITYOFPIGMENT
ZONE OFINHIBITION
ANTIBACTERIALPLATES

To explore the potential biomedical applications of the extracted pigment, particularly its antimicrobial efficacy, both antibacterial and antifungalactivities were assessed using the agar well diffusion method. The tests were conducted against a panel of clinically relevant pathogens, including both Gram-positive and Gram-negative bacteria, as well as fungal species.Thezonesofinhibitionweremeasuredinmilli meters(mm)toevaluatetheefficacyof the pigment at various concentrations (Fig 14 and table 6).

E.Coli S.aureus

Fig 14 Zone of inhibition against E. coli and S. aureus

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Table6 Zone of inhibition against E. coli and S. aureus

Organism	Standard (Ciproflaxin)	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml
E.coli	28 mm	15 mm	12 mm	13 mm	12 mm
S.aureus	29 mm	19 mm	15 mm	13 mm	12 mm

The pigment demonstrated significant antibacterial activity, particularly at higher concentrations (1000 µg/ml and 500 µg/ml), with S. aureus showing slightly higher susceptibility compared to E. coli. The dose-dependent inhibitory effect highlights the pigment's potential as a natural antimicrobial agent.

4.11.1.2 ANTIFUNGALPLATES

Theantifungalefficacywasevaluatedagainst Penicilliumsp.andCandidaalbicans, with clotrimazole serving as the standard antifungal control (Fig 15 and Table 7).

Penicillium Sp. Candidaalbicans

Fig 15 Zone of inhibition against Penicillium Sp and Candida albicans

Table 7 Zone of inhibition against Penicillium Sp and Candida albicans

Organism	Standard (Clotrimazole)	1000 µg/ml	500 µg/ml	250 µg/ml	100 µg/ml
Penicillium	25 mm	14 mm	10 mm	12 mm	11mm
Candida	23 mm	15 mm	14 mm	13 mm	11mm

Theextractedpigmentexhibitedmoderateant ifungalactivity,withthehighestzonesof inhibition observed against C. albicans at 1000 µg/ml. Penicillium sp. also showed susceptibility, albeit to a slightly lesser extent. The pigment's broad-spectrum antimicrobial properties underscore its potential for development into natural antimicrobial formulations.

The antimicrobial assays revealed that the pigment possesses both antibacterial and antifungal

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activity, with a clear concentration-dependent effect. These findings suggest its applicability in pharmaceutical or biomedical fields, particularly in the development of alternative therapeutic agents or natural preservatives.

ESTIMATIONOFANTIOXIDANTACTIVITY

To evaluate the antioxidant potential of the extracted pigment, the Total Antioxidant Capacity (TAC) assay was performed using the phosphomolybdenum method. This method provides a quantitative estimate of the total antioxidant effect of a compound by assessing its ability to reduce molybdenum (VI) to molybdenum (V), forming a green phosphate/Mo(V) complex under acidic conditions. 100 mg/ml of the pigment extract was mixed with the phosphomolybdenum reagent and incubated at 95°C for 90 minutes. After cooling, the absorbancewasmeasuredat695nmusingaUV-Visiblespectrophotometer.Ascorbicacidwas usedasthestandardantioxidantforcalibrationandcomparison.Thetotalantioxidantcapacity of the pigment extract was calculated and expressed asAscorbicAcid Equivalents (AAE) (Fig 16).

TotalAntioxidantCapacity:3.988mgAAE/gofextract

Fig 16 TotalAntioxidantcontent

LABELS:-A1-A3-1000 µg/mL;B1-B3-500 µg/mL;C1-C3-250 µg/mL;D1-D3-100 µg/mL; E1-E3-50 µg/mL;F1-F3-25 µg/mL;G1-G3-Control,H1-H3-Sample

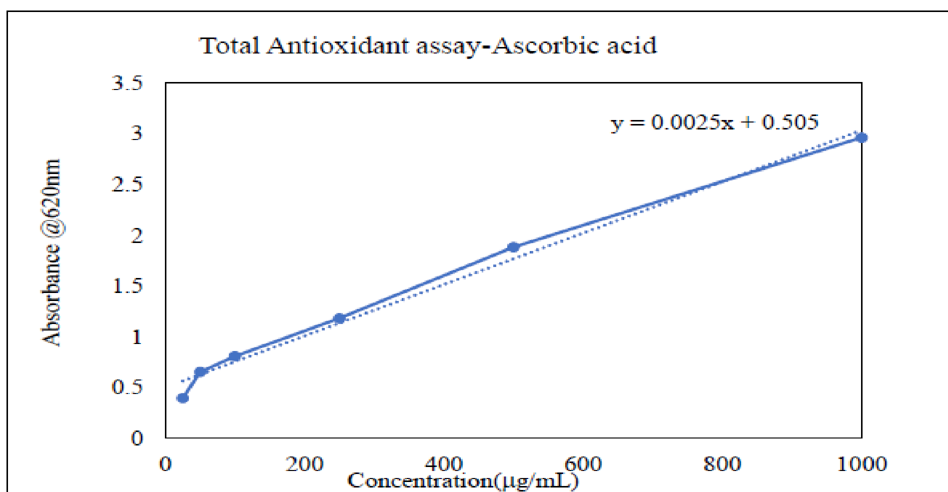

Fig 17 Total antioxidant assay

The pigment demonstrated a measurable antioxidant activity, with an absorbance of 0.6047 and equivalence of 3.988 mg of ascorbic acid per gram of extract (Fig 17). This suggests that the pigment not only has antimicrobial potential but also possesses antioxidant properties, which could further enhance its applicability in pharmaceutical, nutraceutical, and cosmetic formulations.

IV. CONCLUSION

The present study focused on the isolation, optimization, extraction, and characterization of a marine microbial pigment—prodigiosin—produced by Serratia marcescensisolatedfrommarinesoilsamplescollectedacrossthecoastalzonesofTamilNadu, India. Sampling from diverse locations—Kovalam, Besant Nagar, and Marina beaches—ensuredvariabilityinmicrobialcommunitiesandincreasedthelikelihoodofisolatingapotent pigment-producing bacterium. The isolation process utilized serial dilution and spread plate methods, followed by colony selection based on distinct pigmentation, leading to the identification of Serratia marcescens, a gram-negative rod-shaped bacterium known for producingtheredpigmentprodigiosin.Thispigmenthasdrawnattentionforitsbroadspectrum of bioactivities and promising biomedical applications.

Optimization studies were systematically conducted to determine the ideal culture conditions for maximal pigment production. The key influencing parameters investigated included pH, temperature,andincubationtime.Amongthedifferent pHlevelstested,pH7wasfoundtobe most favorable, yielding the highest prodigiosin content (542.41 units/cell). Similarly, a temperature of 37°C promoted the highest pigment yield (692.82 units/cell), and the optimal incubation time was identified as 48 to 72 hours, beyond which a plateau or decline was observed.Thesefindingsemphasizetheimportanceoffinelytuningenvironmentalparameters to enhance metabolite synthesis. The pigment extraction protocol involved solvent extraction using acetone, followed by centrifugation and concentration using a rotary evaporator. The extracted crude pigment was further recrystallized using methanol to ensure purity. Characterization via UV-visible spectrophotometry revealed a strong absorption peak at 533 nm, characteristic of prodigiosin, confirming successful isolation and extraction.

The antimicrobial potential of the extracted pigment was evaluated using the agar well diffusion method, targeting both bacterial and fungal pathogens. The pigment demonstrated significant zone of inhibition (ZOI) against both Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria, with ZOIs measuring up to 19 mm and 15 mm, respectively. Similarly, antifungal activity was evident against Penicillium and Candida species, with inhibition zones reaching up to 14 mm and 15 mm, respectively. These results establish the prodigiosin pigment as an effective antimicrobial agent, with inhibitory effects comparabletostandardantibioticssuchasciprofloxacinandclotrimazole,especiallyathigher concentrations (1000 µg/ml).

In addition to its antimicrobial properties, the pigment extract also exhibited promising antioxidant activity, further reinforcing its potential biomedical utility. The antioxidant capacity, determined through standard assays and expressed as Ascorbic Acid Equivalent (AAE), was 3.988 mg AAE/g, indicating effective free radical scavenging potential. This activity suggests that prodigiosin may protect cells against oxidative damage, making it a potential candidate for combating oxidative stress-related diseases such as cancer, cardiovascular ailments, and neurodegenerative disorders.

The study underscores the biotechnological and pharmaceutical potential of marine-derived pigments,particularlyprodigiosin.Itsbroad-spectrumantimicrobialandantioxidantactivities, coupled with efficient production under optimized lab conditions, make it a promising candidate for development into natural therapeutic agents. Additionally, the eco-friendly production process utilizing marine microbes offers a sustainable and low-cost alternative to synthetic drugs. In conclusion, this research successfully demonstrated the feasibility of harnessing marine microbial biodiversity for the production of high-value bioactive compounds. Serratia marcescens from marine sources proved to be a potent prodigiosin producer, with its pigment exhibiting both high yield and multifunctional bioactivity. Future studies can delve deeper into scaling up production, purifying the compound for pharmacological testing, and exploring its anticancer, immunosuppressive, and anti- inflammatory potentials. This work lays a strong foundation for the continued exploration of marinemicrobesasareservoirofnovelbio compoundswithimpactfulapplicationsinmedicine and industry.

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