RESEARCH PAPER

Isolation, Characterization, and Bioactive Potential of a Red Pigment Producing *Bacillus licheniformis* Strain from the Gulf of Mannar

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Abstract
Marine ecosystems represent a valuable reservoir of metabolically diverse microorganisms with significant biotechnological potential. In the present study, a red pigment–producing bacterium was isolated from seawater samples collected from the Gulf of Mannar and evaluated for its antimicrobial and antioxidant properties. Among isolates, strain KW-1 exhibited intense red pigmentation and was selected for detailed characterization through the morphological, biochemical, and 16S rRNA gene sequencing analysis (99% similarity; GenBank Acc. No. PQ008550) the isolates was identified the isolate as *Bacillus licheniformis*. Optimization studies revealed maximum pigment production in medium supplemented with 5% starch and 5% yeast extract. The methanolic pigment extract demonstrated selective antibacterial activity, with the highest inhibition observed against *Escherichia coli* (1.07 ± 0.05 cm) and *Helicobacter pylori* (1.27±0.05 cm). Antioxidant potential was confirmed through DPPH and ABTS radical scavenging assays, showing concentration-dependent activity with IC_{50} values of ~63 µg/mL (DPPH) and ~82.9 µg/mL (ABTS), indicating moderate to strong antioxidant capacity. TLC (Rf = 0.5), FTIR, and GC–MS analysis revealed the predominance of cyclic dipeptides (diketopiperazines), while LC–MS confirmed the presence of Pulcherriminic acid (m/z 257.150 $[M+H]^+$), the precursor of the iron-chelating pigment pulcherrimin. Collectively, these findings establish *B. licheniformis* KW-1 as a promising marine-derived source of bioactive pigments with antimicrobial and antioxidant potential suitable for biotechnological applications.

Keywords: *Bacillus licheniformis*, pulcherrimin, Pulcherriminic acid, antimicrobial activity, antioxidant activity, cyclic dipeptides, marine bacteria

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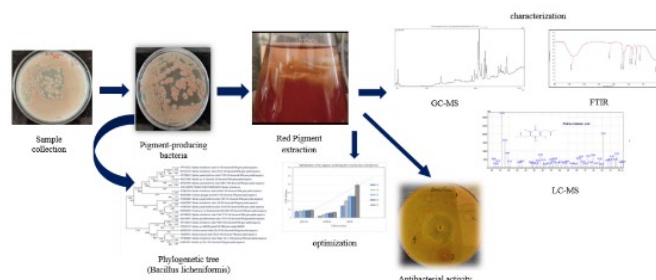

Fig 1: Schematic diagram of Pulcherriminic acid production and its bioactive potential

1.INTRODUCTION

Microorganism-derived pigments have attracted considerable scientific and industrial interest due to their natural origin, eco-friendly production processes, scalability under controlled fermentation, and remarkable structural diversity. Unlike synthetic dyes, microbial pigments are biodegradable, often non-toxic, and can be produced independently of seasonal or geographical constraints, ensuring consistent yield and quality. These pigments are increasingly explored for

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applications in food, feed, dairy, textiles, pharmaceuticals, nutraceuticals, and cosmetics (Tiago *et al.,* 2021). In addition to their colouring properties, microbial pigments are recognized as multifunctional bioactive metabolites exhibiting antimicrobial, antioxidant, anticancer, anti-inflammatory, photoprotective, and biodegradable characteristics (Narsing Rao *et al*., 2017; Venil *et al*., 2020a; Ramesh *et al*., 2019; Paillié-Jiménez *et al*., 2020). Their antioxidant capacity is particularly significant, as oxidative stress is implicated in aging, carcinogenesis, neurodegenerative disorders, and metabolic diseases. Many microbial pigments contain conjugated π-electron systems and redox-active moieties that facilitate electron or hydrogen atom transfer, enabling effective scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Consequently, these natural pigments represent promising alternatives to synthetic antioxidants such as BHT and BHA, aligning with the growing consumer demand for clean-label and sustainable products (Novoveská *et al*., 2019).

Among pigment-producing bacteria, *Bacillus licheniformis* has emerged as a microorganism of considerable biotechnological importance. This Gram-positive, endospore-forming, mesophilic bacterium is widely distributed in soil, dairy environments, agricultural settings, and marine habitats, and is occasionally associated with food spoilage (Krawczyk *et al*., 2016). Owing to its robust growth, genetic tractability, and Generally Recognized as Safe (GRAS)-related status of certain strains, *B. licheniformis* is extensively utilized for enzyme production, bioactive metabolite synthesis, bioremediation, and probiotic formulations (Muras *et al*., 2021). Some strains demonstrate adaptability to the gastrointestinal tract, supporting their application in human, veterinary, and aquaculture systems. Nevertheless, the detection of antibiotic resistance genes in specific isolates necessitates comprehensive genomic and safety evaluation before industrial deployment (Muras *et al.,* 2021).

A prominent red pigment associated with *B. licheniformis* is pulcherrimin, an iron-chelating compound formed extracellularly through the oxidation and complexation of Pulcherriminic acid. Functional studies have demonstrated that pulcherrimin contributes to antimicrobial activity primarily via iron sequestration, limiting pathogen growth through nutrient competition (Wang *et al*., 2020). From a biosynthetic perspective, pulcherrimin production is metabolically efficient, requiring only three enzymatic steps, in contrast to the multi-step chemical synthesis from L-leucine (rose Aligué *et al*., 2024). Although pulcherrimin was initially described in the yeast *Metschnikowia pulcherrima* (Kluyver *et al.,* 1953), its genetic regulation, biosynthetic pathway, and ecological role have been extensively characterized in *Bacillus subtilis* and *B. licheniformis* (Randazzo *et al*., 2016; Gore-Lloyd *et al*., 2019). Importantly, iron chelation by pulcherrimin may also mitigate iron-catalysed Fenton reactions, thereby indirectly reducing oxidative stress and contributing to antioxidant defense mechanisms (Gore-Lloyd *et al*., 2019).

In addition to pulcherrimin, *B. licheniformis* produces cyclic dipeptides (CDPs), also known as diketopiperazines (DKPs), which constitute a structurally stable and pharmacologically versatile class of secondary metabolites. DKPs exhibit diverse biological activities including antibacterial, antifungal, antiviral, antitumor, immunomodulatory, neuroprotective, and antioxidant properties (Kamal *et al*., 2015). Their heterocyclic frameworks and resonance-stabilized structures enhance radical scavenging capacity and redox stability, further supporting their therapeutic and industrial relevance.

Given the increasing demand for natural bioactive compounds with combined antimicrobial and antioxidant properties, systematic investigation of pigment-producing *B. licheniformis* strains is warranted. The present study therefore explores the genomic and metabolic potential of a red pigment–producing *Bacillus licheniformis* strain. By integrating molecular identification, metabolite profiling, antimicrobial assessment, and antioxidant evaluation, this research aims to strengthen the scientific basis for industrial exploitation of pulcherrimin-producing *B.*

licheniformis in food preservation, agriculture, pharmaceuticals, and nutraceutical applications.

2.Materials and methods

2.1. Collection of samples and Isolation of marine bacteria

Marine water samples were collected from the Keelakaveri coastal site in the Gulf of Mannar and transported to the laboratory under chilled conditions. The samples were stored at 4 °C until further analysis. Serially diluted samples were spread onto Zobell marine agar plates and incubated for 24–48 h to isolate marine bacteria. Pigment-producing colonies were selected based on colony morphology and color and purified by repeated streaking. Preliminary identification was carried out using Gram staining, followed by standard biochemical tests. The purified isolates were preserved for further studies (Usman *et al*., 2018).

2.2. Extraction of Genomic DNA and Sequencing of the 16S rRNA Gene

Genomic DNA was extracted from the bacterial isolate using the NucleoSpin® Tissue Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. Briefly, the bacterial culture was lysed using T1 buffer and Proteinase K at 56 °C, followed by RNase A treatment. After lysis, DNA was purified through a silica membrane column and eluted in BE buffer. The quality and integrity of the extracted DNA were assessed by 0.8% agarose gel electrophoresis.

The 16S rRNA gene was amplified by PCR using the universal primers 16S-RS-F (CAGGCCTAACACATGCAAGTC) and 16S-RS-R (GGGCGGWGTGTACAAGGC). PCR amplification was performed in a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 40 s, and extension at 72 °C for 60 s, with a final extension at 72 °C for 7 min. The amplified PCR products were confirmed by 1.2% agarose gel electrophoresis.

PCR products were purified using ExoSAP-IT (USB Corporation) to remove excess primers and nucleotides. Sequencing reactions were carried out using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and analyzed on an ABI 3500 Genetic Analyzer following the Sanger sequencing method. The obtained sequences were checked for quality using Sequence Scanner Software v1, and sequence editing and alignment were performed using Geneious Pro v5.1(Zhang *et al.,*2025).

2.3 Phylogenetic analysis

The phylogenetic analysis of the aligned file was conducted using MEGA software. The MEGA program was employed with an aligned file comprising the top 15 highly similar sequences. The phylogenetic tree was constructed utilizing the Neighbor-Joining (NJ) method (Madden.,2013).

2.4. Extraction of pigment

The pigment-producing bacterial isolate was cultured in Zobell Marine Broth and incubated at 37 °C with shaking (100–150 rpm) for 24 h. The culture was centrifuged at 5000 rpm for 10 min to obtain the bacterial pellet. The pellet was extracted with methanol and centrifuged at 10,000 rpm for 10 min to recover the pigment-containing supernatant. The extraction was repeated sequentially using methanol until the pellet became colourless. The collected supernatants were pooled and filtered using Whatman filter paper. The solvent was evaporated at 80 °C to obtain the dried pigment, which was stored at 4 °C for further analysis (kumar.,2018).

2.5. pharmacological screening of pigment isolated from *bacillus licheniformis*

2.5.1. Antibacterial Activity by Agar Well Diffusion Method

Sterile aqueous pigment solutions were prepared by dissolving the pigment in sterile distilled water and filtering through a 0.2 µm membrane filter. Antibacterial activity was assessed using the agar well diffusion method. Briefly, a lawn of each test microorganism was prepared by spreading a standardized

suspension onto the surface of Mueller-Hinton Agar (MHA) for bacteria *Bacillus subtilis* (MTCC 441), *Escherichia coli (*MTCC 443),*Staphylococcus aureus (*MTCC 737), *Streptococcus mutans(* MTCC 497), *Helicobacter pylori* (MTCC 11637) obtained from the microbial type culture collection . Wells (6 mm diameter) were aseptically punched into the agar, and 100 µL of the pigment extract (40 mg/mL) was added to each well. Plates were incubated at 37°C for 24 hrs. Antibacterial activity was evaluated by measuring the diameter of the zones of inhibition (in mm) surrounding the wells (Mounyr *et al*., 2016).

2.5.2. Antioxidant activity

a) DPPH Assay

The antioxidant activity of the pigment sample was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay according to Sarker and Oba, 2019b. DPPH is a stable nitrogen-centred free radical that undergoes discoloration upon reduction by antioxidant compounds. The pigment was prepared at concentrations ranging from 10 to 100 µg/mL in dimethyl sulfoxide (DMSO). Each concentration was mixed with 150 µL of freshly prepared 0.1 mM DPPH solution and incubated in the dark at room temperature for 20 min. Absorbance was measured at 517 nm using a UV–Visible spectrophotometer. A control containing DPPH solution without sample was used. Antioxidant activity was expressed as the percentage of DPPH radical scavenging relative to the control. The free radical scavenging activity was calculated using the following formula

% Inhibition = ((A control - (A sample - A blank)) / A control) × 100

b) ABTS Radical scavenging assay

The ABTS radical scavenging activity was evaluated following the method described by (Sarker *et al*., 2018). The ABTS radical cation was generated by reacting 20 mM ABTS solution with 70 mM potassium persulfate and incubating the mixture in the dark at room temperature for 24 h. For the assay, 0.6 mL of the sample extract at concentrations ranging from 10 to 100 µg/mL was mixed with 0.45 mL of the ABTS reagent and incubated for 10 min. Absorbance was measured at 734 nm using a UV–Visible spectrophotometer. A control without sample was included, and antioxidant activity was expressed as percentage radical scavenging. ABTS radical scavenging assay was calculated using the following formula.

ABTS radical cation scavenging assay (%) = [(AB-AA) / AB] x 100 : Where, AB - absorbance of control, AA- absorbance of test sample.

2.6. Characterization of red pigment extracted from *Bacillus licheniformis*

2.6.1 Gas chromatography-Mass Spectroscopy

GC-MS analysis separation was achieved on a TG-5MS capillary column (30 m × 0.25 mm ID, 0.25 µm film thickness) with helium as the carrier gas at a constant flow rate of 1 mL/min. The injector temperature was set to 300°C. Constituents were identified by comparing their mass spectra and relative retention times to those of known compounds in the NIST mass spectral library (Hamad *et al*.,2020 & mounyr *et al*.,2016).

2.6.2 Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis

LC-MS analysis was performed on an Agilent 6200 Series TOF (or 6500 Series Q-TOF) mass spectrometer (B.09.00 software) using electrospray ionization in positive mode. Chromatographic separation was carried out on a C18 column (1.7 µm, 1 × 50 mm) maintained at 45°C. A gradient elution was employed using acetonitrile as the mobile phase. An injection volume of 10 µL was used and the pigment was identified by comparing its retention time and mass spectral data to those of a pyocyanin standard, (DeBritto *et al*.,2020).

2.6.3 Fourier Transform Infrared (FTIR) Spectroscopy

The pigments isolated with methanol were analysed in liquid form within the 4000–400 cm−1 frequency range using the attenuated total reflectance (ATR) technique with an FTIR spectrometer (Shimadzu IRTracer-100) at room temperature (Hamad *et al*.,2020).

3. Results and Discussion

3.1 Isolation and Identification of a Pigment-Producing Bacterium

Marine water sample was collected from the Gulf of Mannar at Keelakaveri site to isolate pigment-producing bacteria. Out of 55 bacterial isolates , one isolate was designated as KW-1 exhibited the most pronounced pigment production and was selected for detailed characterization (fig 2). Morphological analysis revealed that isolate KW-1 is a Gram-positive, rod-shaped bacterium forming distinct pigmented colonies on solid media. Its Gram-positive nature suggests the presence of a thick peptidoglycan cell wall, consistent with typical *Bacillus* morphology. These results are presented in Table 1 and Figure 3.

The identification was subsequently confirmed by biochemical characterization KW-1 possessed negative responses for Voges–Proskauer, indole production, urease, oxidase, citrate utilisation, and H_2S production, but good results for methyl red, nitrate reduction, catalase, triple sugar iron test, starch hydrolysis, casein hydrolysis, and CO_2 fermentation (table 2) and the isolate was initially identified by comparing its phenotypic profile with the conventional taxonomic keys described in Bergey's Manual of Systematic Bacteriology (choo, 2005)

3.2 Identification of pigment-producing bacteria through 16S rRNA gene sequencing and phylogenetic analysis

Molecular identification based on 16S ribosomal RNA (rRNA) gene sequencing has become a well-established method for the taxonomic classification of bacteria, particularly within the *Bacillus* genus where numerous species exhibit overlapping phenotypic characteristics (Johnson *et al*., 2019). In the present study, isolate KW-1 was conclusively identified as *Bacillus licheniformis* based on 99% 16S rRNA gene sequence similarity to established type strains—a threshold widely recognized as sufficient for species-level assignment (choi *et al*., 2019). This finding aligns with several previous studies that have successfully employed 16S rRNA sequencing to characterize pigment-producing *Bacillus* species. For example, Tofick *et al*. (2022) isolated a red-pigment-producing *B. licheniformis* strain from marine sediments, with definitive identification achieved through 16S rRNA analysis supported by biochemical profiling.

The high degree of genetic similarity between KW-1 and *B. licheniformis* type strains corroborates its classification within this species, which is renowned for its metabolic versatility and significant biotechnological potential. Nevertheless, it is important to acknowledge that while 16S rRNA sequencing is highly reliable for initial classification, it possesses limitations in resolving closely related species such as *B. licheniformis* and *B. paralicheniformis*, which share substantial sequence homology (rahman *et al*., 2022). Despite these considerations, the 99% sequence identity observed here provides strong support for the designation of KW-1 as *B. licheniformis*, consistent with current practices in microbial systematics. The public deposition of the 16S rRNA sequence in the NCBI GenBank database (Accession No. PQ008550) ensures the reproducibility of these findings and facilitates comparative genomic studies. This robust molecular identification establishes a solid foundation for further investigation into the functional properties and applicative potential of the pigment produced by *B. licheniformis* strain KW-1(table 3 & fig 4).

3.3 Pigment production and optimization of the pigment producing bacteria *Bacillus lechiniformis*

In this study, red pigment-producing bacteria (*Bacillus licheniformis*) was isolated from water samples which showed luxuriant growth and high color intensity on glucose media for 24 to 48 hrs. After 3 days of bacterial incubation, the pigments were extracted using methanol. The extracted pigments were

concentrated by evaporation (Fig. 5,a) and then dried at 60 °C. The Rf value for the kw-1 red-pigmented isolates was 0.5 according to the TLC method used to characterize the isolated pigments(fig.5b). Similarly, Goswami *et al*. (2014) reported red pigment production by *Bacillus spp*., initiating after 72 hours and peaking on the sixth day. Pigment extraction with methanol and subsequent UV-Vis analysis showed a characteristic absorbance peak at 600 nm, consistent with Pulcherriminic acid. The decline in pigment yield after day 6 likely reflects the onset of stationary or death phase, where metabolic activity diminishes.

The effect of different carbon and nitrogen sources on Pulcherriminic acid production was significant, as presented in (Table 4). Among carbon sources tested, starch emerged as the most effective, yielding 0.391 ± 0.021 mg/mL at 5% concentration (Fig 6a). A positive correlation between starch concentration and pigment yield suggests that complex carbohydrates favor Pulcherriminic acid biosynthesis. In contrast, simple sugars such as glucose and maltose supported minimal pigment production (<0.1 mg/mL), indicating a possible occurrence of catabolite repression. Fatima *et al.* (2022) optimized pigment production in *Serratia* using glucose (1%) and yeast extract (0.1%) as ideal carbon and nitrogen sources, respectively. Although their glucose concentration was lower, the trend supports the advantage of organic nitrogen sources like yeast extract. This mechanism, widely reported in microbial secondary metabolism, downregulates pigment biosynthetic pathways in the presence of readily utilizable carbon sources, thereby limiting pigment accumulation. Nitrogen source selection also played a pivotal role. Yeast extract yielded the highest production of pulcherriminic acid (1.483 ± 0.037 mg/mL at 5%), showing a dose-dependent response(table 4 & fig 6b). Its effectiveness may be attributed to its complex composition, including amino acids, peptides, and vitamins, which potentially serve as biosynthetic precursors or enzymatic cofactors. Interestingly, ammonium sulfate supported moderate production (0.85 ± 0.095 mg/mL) at 3%, but yields declined significantly at higher concentrations. This suggests an inhibitory effect, possibly due to ammonium-mediated repression of secondary metabolite genes, a phenomenon previously documented in pigment and antibiotic biosynthesis in various bacterial species (Arora *et al*., 2021).Mantri Sai Ram *et al*. (2017) further demonstrated the importance of nitrogen sources in pigment synthesis, finding peptone (1%) to be most effective, supporting both maximum growth and pigment production (1.30 units). In contrast, tryptone yielded significantly lower pigment levels (0.16 units), and no pigmentation was observed with inorganic nitrogen sources such as sodium nitrate, sodium nitrite, and ammonium chloride. These findings reinforce the idea that complex organic nitrogen sources are superior for pigment biosynthesis in many bacterial systems.

3.4 Antibacterial activity of selected methanol extract of red pigment pulcherrimin

The pigment extract demonstrated selective antibacterial activity against the tested organisms. The most pronounced inhibition was observed against *Escherichia coli* (1.07 ± 0.05 cm), though this was notably weaker than the ampicillin positive control (1.86 ± 0.05 cm). Moderate inhibitory effects were detected against *Bacillus subtilis* (0.87 ± 0.12 cm) and *Staphylococcus aureus* (0.54 ± 0.05 cm), while *Streptococcus sp.* (0.37 ± 0.05 cm) and *Helicobacter pylori* (1.27±0.05 cm) exhibited minimal sensitivity (Table 5). The zone fraction inhibited the growth of *E. coli, Shigella flexneri, Micrococcus lutea, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Vibrio cholerae, and Proteus vulgaris*, while DMSO control showed no inhibition (Mantri Sai Ram *et al*., 2017).Comparable results were reported by Miraj *et al*. (2020), who demonstrated that ethanol-extracted pigments of *Micrococcus sp.* were effective against *Bacillus sp.* (5 mm), *Staphylococcus aureus* (5 mm), *Escherichia coli* (6 mm), *Pseudomonas aeruginosa* (5 mm), and *Klebsiella pneumoniae* (6 mm). Similarly, additional pigment-producing isolates such as *Salinococcus sp., Serratia sp.,* and *Exiguobacterium sp.* exhibited notable antibacterial activity. The present findings are also in associated with Sinha *et al*.

(2017), who showed that pigments derived from *Micrococcus sp*. inhibited the growth of several Gram-positive bacteria. Likewise, the red yeast *Rhodotorula glutinis* pigment investigated by Yolmeh and Mahmoud (2016) demonstrated broad-spectrum antibacterial effects, with susceptibility ranging from low inhibition against *Bacillus sp.*

3.5 Antioxidant activity of red pigment pulcherrimin

3.5.1 DPPH Assay

The antioxidant potential of the methanolic extract of pulcherrimin pigment was evaluated using the DPPH free radical scavenging assay. The extract exhibited a clear concentration-dependent increase in radical scavenging activity, with %RSA increasing from 9.3% at 10 µg/mL to 82.2% at 100 µg/mL, accompanied by a progressive decrease in absorbance. This trend indicates effective reduction of DPPH radicals and confirms the free-radical scavenging capability of the extract.

The IC_{50} value, defined as the concentration required to scavenge 50% of DPPH radicals, was calculated by linear interpolation between 60 µg/mL (47.6% RSA) and 70 µg/mL (55.1% RSA), yielding an IC_{50} of approximately 63 µg/mL. This value suggests moderate to strong antioxidant activity and falls within the range commonly reported for natural extracts with notable antioxidant potential. A marked increase in scavenging activity beyond 50–60 µg/mL further indicates enhanced hydrogen-donating ability at higher concentrations (showed in table 6 and fig 7). Pulcherrimin protective role against ROS in *Bacillus subtilis* biofilms, via iron sequestration and DNA damage mitigation, supports its efficacy here, as reported by Kregiel *et al.,* (2022) who linked it to downregulated oxidative stress genes. Similarly, isolated pulcherrimin showed DPPH scavenging in yeast studies.

Mechanistically, the observed activity can be attributed to the ability of bioactive compounds to donate hydrogen atoms to stabilize the DPPH radical, resulting in a measurable decrease in absorbance. This mechanism has been well described by olofinsan *et al.* (2022), who emphasized the importance of accurate IC_{50} determination for reliable comparison across antioxidant studies. Moreover, recent evidence indicates that phenolic acids and flavonoids play a dominant role in in vitro antioxidant activity due to their redox properties and structural features, including hydroxyl group distribution and conjugation (Khan *et al.,* 2025).

3.5.2 ABTS Radical scavenging assay

The ABTS radical cation decolorization assay confirmed that the red pigment exhibits concentration-dependent antioxidant activity, with %RSA increasing from 5.87% at 10 µg/mL to 61.75% at 100 µg/mL. Although the standard showed higher inhibition (74.25%), the pigment demonstrated substantial radical scavenging ability. The IC_{50} value (~82.9 µg/mL) indicates moderate antioxidant potency, comparable to several marine microbial pigments. The strong linear correlation ($y = 6.7226x - 5.0076$; $R^2 = 0.9849$) further validates the reliability and reproducibility of the assay. (showed in table 7 and fig 8)

Marine microorganisms are well-known producers of antioxidant red pigments such as prodigiosin and carotenoids. Kuncham *et al.* 2017harl reported stronger activity (IC_{50} = 65 µg/mL) for prodigiosin from marine *Serratia marcescens*, attributed to its tripyrrole structure and efficient electron delocalization. Aruldass *et al.* (2014) documented a comparable IC_{50} (78 µg/mL) from marine Vibrio sp., emphasizing the role of extraction and purity in antioxidant performance. Similar inhibition (~60% at 100 µg/mL) was observed by Priyadharshini *et al.* (2024) in marine Streptomyces sp., closely matching the present findings. El-Bondkly *et al.* (2021) reported IC_{50} values of 70–120 µg/mL for marine fungal pigments, placing the current pigment within the effective antioxidant range. Overall, the pigment demonstrates biologically relevant antioxidant potential comparable to previously reported marine red pigments.

3.6 Characterization of pulcherrimin pigment (GC-MS, LCMS, FTIR)

3.6.1 Gas chromatography-Mass spectrometry

The GC–MS analysis of the pigment extract revealed nine major compounds, with cyclic dipeptides (diketopiperazines) representing the predominant class of metabolites (Table 8; Fig. 9). Among these, Cyclo(L-prolyl-L-valine) (Peak 5: 19.31% area) was identified as a bioactive cyclic dipeptide with reported antimicrobial and antioxidant properties. Similarly, Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione (Peaks 6 and 7: 18.78% and 11.74% area, respectively) was detected, another diketopiperazine derivative frequently associated with microbial secondary metabolism. A third abundant compound, 3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione (Peak 4: 19.10% area), further highlights the dominance of nitrogen-rich heterocyclic scaffolds. Collectively, Peaks 4–7 and 9 (~70% of the total chromatogram area) were assigned to pyrrolopyrazine-dione structures, which are well recognized for their antimicrobial, antifungal, and cytotoxic activities. Additionally, Acetic acid ester (Peak 3) was identified, possibly reflecting fatty acid metabolism pathways commonly associated with *Bacillus licheniformis*. Comparable results have been reported in related studies. Fakorede *et al*. (2020) identified ten major compounds in the yellow pigment of isolate G152, with octadecanoic acid (20.40%), n-hexadecanoic acid (20.03%), cis-vaccenic acid, and pyrrolo(1,2-a)pyrazine-1,4-dione (14.02%) as the most abundant. Eugenol and phenol were also present in smaller but significant proportions (4.32%), both detected in high purity (98% and 96%, respectively). Their analysis of the green pigment from isolate G153 revealed 18 compounds, including 2-furancarboxaldehyde (26.41% and 23.59%), octadecanoic acid (11.98%), and 1-hydroxyphenazine (retention time 20.170 min, purity 95%).The detection of phenazine derivatives is consistent with earlier findings by Mostafa *et al*. (2025), who confirmed pyocyanin production by *Pseudomonas aeruginosa* via GC–MS. Such compounds are widely studied for their antibacterial and cytotoxic properties, further supporting the bioactive potential of pigments derived from marine and soil bacteria. The predominance of diketopiperazines in the current analysis agrees with prior reports highlighting them as key microbial metabolites with diverse biological activities, including quorum-sensing regulation, antifungal activity, and cytotoxicity against cancer cell lines (Shen *et al*., 2015).

3.6.2 Liquid Chromatography-Mass Spectrometry

In liquid chromatography-mass spectrometry (LC-MS) analysis, cyclo(Leu-Leu) was detected with a characteristic mass-to-charge ratio (m/z) of 227.176 MH]+. The iron-chelating metabolite Pulcherriminic acid showed a molecular ion peak at m/z 257.150 MH]+(Fig:10). The detection of these peaks confirms the presence of diketopiperazine intermediates and Pulcherriminic acid-related metabolites in the pigment extract. Cyclo(Leu-Leu), also known as cyclodileucine, is a cyclic dipeptide derived from leucine. It plays a key role in the biosynthetic pathway that leads to Pulcherriminic acid formation. This compound chelates ferric ions (Fe^{3+}) to produce the insoluble red pigment pulcherrimin (Shiyi Wang *et al*., 2020).

Richard Gore-Lloyd *et al*. (2019) reported similar findings. They found a peak at about m/z 227.18 related to cyclo(Leu-Leu) in the LC-MS spectra from pulcherrimin-producing microorganisms. Their study showed that cyclodileucine serves as a precursor for Pulcherriminic acid biosynthesis. It is important for pigment production and antimicrobial activity (Alicia *et al*., 2025).Further evidence supporting this pathway came from Shiyi Wang *et al*. (2020). They reported that Pulcherriminic acid is made from cyclo(L-Leu-L-Leu) in *Bacillus licheniformis*. This process involves enzymatic reactions with the yvmC-cypX gene cluster. Their metabolic engineering studies confirmed that increasing intracellular leucine availability significantly boosts Pulcherriminic acid production. This finding highlights the central role of leucine-derived diketopiperazines in the biosynthetic pathway. The identification of Pulcherriminic acid in this study supports previous reports. It indicates that pulcherrimin-producing microorganisms mainly exert antimicrobial activity through iron

sequestration mechanisms. Pulcherriminic acid binds to ferric ions to form the insoluble pigment pulcherrimin. This process limits iron availability to competing microorganisms and hinders their growth.

The detection of cyclo(Leu-Leu) and Pulcherriminic acid in the LC-MS profile confirms the presence of the pulcherrimin biosynthetic pathway in the marine isolate *Bacillus licheniformis* KW-1. These findings align with earlier studies and further support the idea that the antimicrobial activity observed in this study may be partly linked to iron-chelating metabolites involved in pulcherrimin formation.

3.6.3 Fourier transform infrared spectroscopy

The spectrum exhibits the characteristic vibrational signatures expected for the pulcherriminic acid ligand. The broad absorption band observed at 3378 cm^{-1} is indicative of N-H stretching vibrations, a finding consistent with the presence of primary amines as reported by Parker (2012) for pulcherrimin. The most diagnostic feature for the core structure is the strong amide I band observed at 1650 cm^{-1}, assigned to the carbonyl (C=O) stretching vibration of the cyclic dipeptide. This assignment is further supported by a corresponding peak at 1645 cm^{-1}, which Parker (2012) attributes to ketonic linkages within the pulcherrimin complex. The presence of alkyl chains is confirmed by C-H stretching vibrations (likely present between 2800-3000 cm^{-1}) and C-H bending modes between 1366-1421 cm^{-1}. Furthermore, the peak at 1233 cm^{-1} aligns with the C-N stretching vibration noted by Mukundan *et al*. (2025), confirming the nitrogenous connections within the structure (Table 9, fig 11)

Critically, the spectrum confirms the central metallo-organic nature of the compound. The series of absorptions in the fingerprint region, specifically the C-O stretching vibrations observed at 1093 cm^{-1} and others in the 1000-1100 cm^{-1} range (Mukundan *et al*., 2025), are indicative of the ether or ester linkages inherent to the molecule. The most definitive evidence for successful iron chelation is the prominent low-frequency absorption at 636 cm^{-1}. This peak is unequivocally assigned to Fe-O stretching vibrations, a result that aligns with the findings of Griesiute *et al*. (2021), who reported Fe-O bonds at 580 cm^{-1} and 536 cm^{-1}, respectively. This signal provides incontrovertible proof of the iron-oxygen coordination bonds that are fundamental to the pulcherrimin complex.

Table 1: Morphological and Characterization of KW-1 strain

Sl.no	Morphological Characterisation	Observation
1	Gram stain	+
2	Cell shape	Rod (bacilli)
3	Colony pigmentation	Red
4	Colony morphology	circular, irregular, raised, flat)

Table 2: Biochemical Characterization of Kw-1 **Strain**

Sl. No.	Biochemical Test	Result
1	Methyl Red	+
2	Voges Proskauer	-
3	Indole Production	-
4	Nitrate Reduction	+
5	Urease	-

6	Catalase	+
7	Oxidase	-
8	Triple Sugar Iron	+
9	Simmons Citrate	-
10	Starch Hydrolysis	+
11	Casein Hydrolysis	+
12	CO_2 Production from Glucose	+
13	H_2S Production	-

Note: +, positive reaction; -, negative reaction.

Table 3: Identification of pigment-producing bacteria through 16S rRNA gene sequencing

Isolation code	Identification	Gen Bank number	Similarity rate (%)	Base length (nucleotide)
KW-1	*Bacillus licheniformis*	PQ008550	99%	1185

Table 4: Optimization of the pigment producing bacteria KW-1

source		Concentration (mg/ml)				
		1% Mean ± SD	2% Mean ± SD	3% Mean ± SD	4% Mean ± SD	5% Mean ± SD
Carbon source	Glucose	0.076±0.0016	0.081±0.003	0.088±0.0009	0.088±0.004	0.093±0.0029
	Maltose	0.021±0.0009	0.037±0.001	0.053±0.0037	0.059±0.001	0.068±0.0028
	Starch	0.144±0.0411	0.203±0.006	0.258±0.0057	0.258±0.04	0.391±0.021
Nitrogen source	Peptone	0.066±0.005	0.066±0.005	0.066±0.005	0.270±0.026	0.33±0.020
	Yeast	0.629±0.060	0.794±0.040	0.918±0.049	1.20±0.058	1.483±0.037
	Ammonium sulphate	0.277±0.045	0.421±0.070	0.85±0.095	0.180±0.034	0.277±0.031

Table 5: Antibacterial activity of methanol extract of red pigment pulcherrimin

Test organism	Zone of inhibition (cm)	
	Methanol extract	Positive control(ampicillin)
Bacillus subtilis	0.87 ± 0.12	-
Escherichia coli	1.07 ±0.05	1.86±0.05
Staphylococcus aureus	0.54±0.05	-
Streptococcus sp	0.37±0.05	0.24±0.05
Helicobacter pylori	1.27±0.05	1.75±0.05

Table 6: Antioxidant activity of methanol extract of red pigment pulcherrimin using DPPH assay

Concentration µg/ml	Mean ± SD	%RSA
10 µg/ml	0.097±0.0024	9.3%
20 µg/ml	0.091±0.0032	14.9%
30 µg/ml	0.082±0.0057	23.3%
40 µg/ml	0.073±0.0057	31.75%

50 µg/ml	0.067±0.0032	37.3%
60 µg/ml	0.056±0.0008	47.6%
70 µg/ml	0.048±0.0035	55.1%
80 µg/ml	0.036±0.0021	66.3%
90 µg/ml	0.023±0.0049	78.5%
100 µg/ml	0.019±0.0024	82.2%
		IC 50 = ~63.2µg/mL

Table 7: Antioxidant activity of methanol extract of red pigment pulcherrimin using ABTS Assay

Concentration µg/ml	Mean ± SD	%RSA
10 µg/ml	0.753±0.0098	5.8%
20 µg/ml	0.734±0.0008	8.5%
30 µg/ml	0.687±0.0080	14.1%
40 µg/ml	0.618±0.0080	22.7%
50 µg/ml	0.577±0.0082	27.8%
60 µg/ml	0.542±0.0078	32.25%
70 µg/ml	0.497±0.0061	37.87%
80 µg/ml	0.419±0.0072	47.62%
90 µg/ml	0.354±0.0008	55.75%
100 µg/ml	0.306±0.0037	61.25%
		IC 50 = ~82.9µg/ml

Table 8: Gas chromatography-Mass Spectrometry analysis of bioactive compound present in KW-1

Peak	Retention Time (min)	Area	Area %	Height	Height %	Name
1	4.118	19,674,360	13.65%	5,579,844	17.92%	1-Propanol, 2,2-dimethyl-, acetate
2	12.894	6,967,682	4.83%	1,427,272	4.58%	2-Butanone, 4-phenyl-
3	19.326	2,387,613	1.66%	1,108,389	3.56%	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester
4	19.817	27,527,976	19.10%	3,537,658	11.36%	3-Methyl-2,3,6,7,8,8a-hexahydropyrrolo[1,2-a]pyrazine-1,4-dione
5	21.199	27,843,494	19.31%	4,711,469	15.13%	Cyclo(L-prolyl-L-valine)
6	21.828	27,071,722	18.78%	8,079,902	25.94%	Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione
7	22.237	16,927,236	11.74%	2,797,042	8.98%	Hexahydro-3-(1-methylpropyl)pyrrolo[1,2-a]pyrazine-1,4-dione
8	24.715	9,595,103	6.66%	2,051,726	6.59%	Octahydroquinoline- 9-hydroxyperoxide

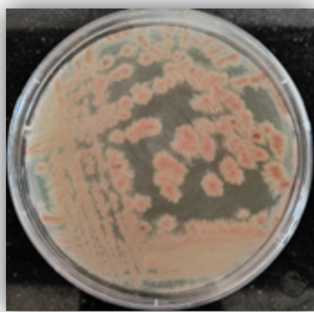
9	25.212	6,161,585	4.27%	1,852,722	5.95%	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-
Total		**144,156,771**	**100.00%**	**31,146,024**	**100.00%**	

Table 9: Fourier transform infrared spectroscopy-functional group present in KW-1 **Strain**

Wavenumber (cm^{-1})	Intensity	Functional Group	Vibration Mode
3378.39	Strong, Broad	N-H stretch (1˚, 2˚ amine/amide) or O-H stretch (carboxylic acid)	Stretching
2009.17	Weak	C≡C stretch (alkyne) or C≡N stretch (nitrile)	Stretching
1697.67	Medium	C=O stretch (carbonyl - ester, carboxylic acid)	Stretching
1650.77	Strong	C=O stretch (amide I band) / C=C stretch (alkene)	Stretching
1421.93	Medium	C-H bend (CH_2 scissoring)	Bending
1366.79	Medium	C-H bend (CH_3 symmetric bending)	Bending
1233.17	Strong	C-N stretch (amine) or C-O stretch (ester, ether)	Stretching
1093.04	Strong	C-O stretch (secondary alcohol, ether)	Stretching
636.43	Strong	N-H wag (out-of-plane bend, amide)	Bending

Isolation and Identification of a Pigment-Producing Bacteria

Fig 2: Isolation of a Pigment-Producing Bacteria

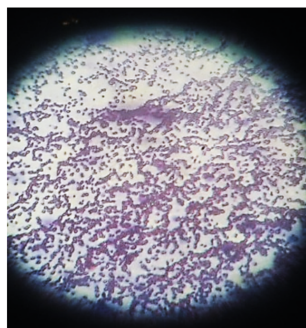


(a)

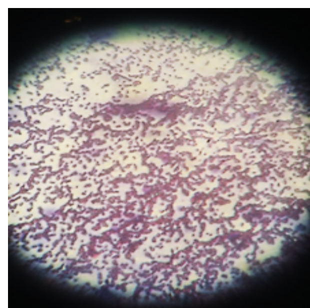
(b)

a & b – Red pigment producing bacteria (KW1)

Fig 3: Morphological Identification of a Pigment-Producing Bacteria



(a)



(b)

a & b –Gram positive bacteria

Fig 4:Identification of pigment-producing bacteria through 16S rRNA gene sequencing and phylogenetic analysis

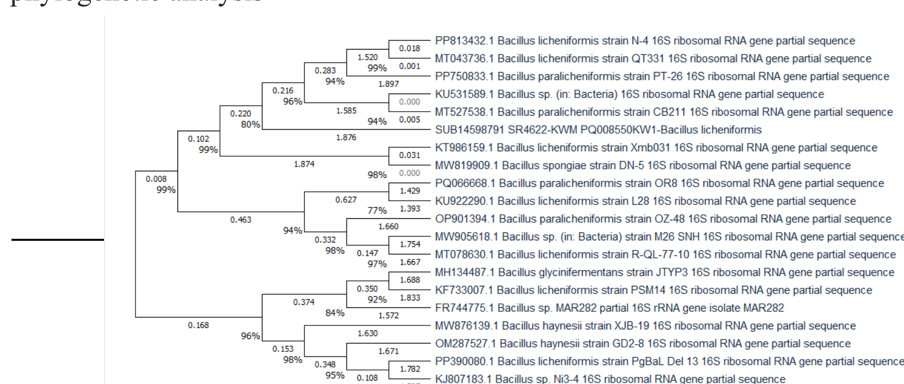


Fig 4 (a): A phylogenetic tree was reconstructed from 16S rRNA gene sequences using the Neighbour-Joining method in MEGA 11.

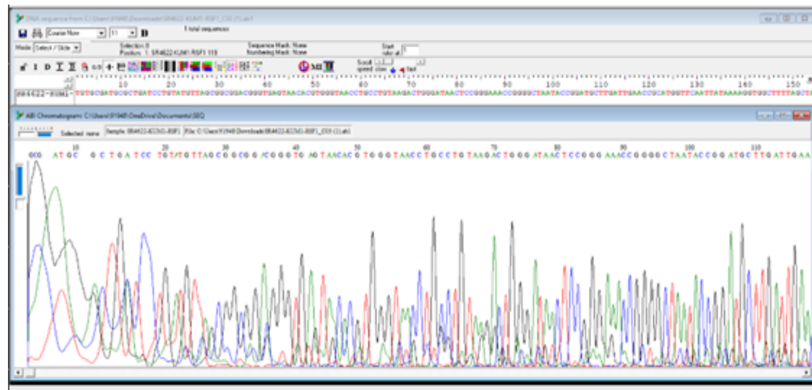


Fig 4(b):Chromatogram output of 16S rRNA gene sequencing showing nucleotide peaks and base-calling analysis.

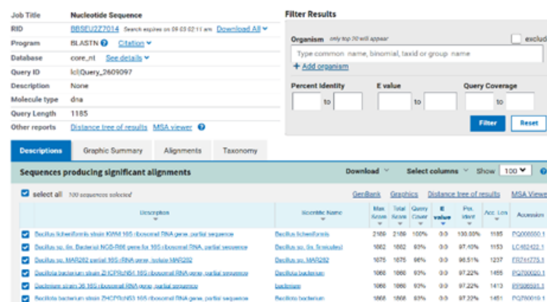


Fig 4(c): “BLASTn alignment results of 16S rRNA gene sequence showing significant similarity with *Bacillus licheniformis* and related species.

>SUB14598791 SR4622-KWM PQ008550KW1-Bacillus licheniformis

TGTGCGATGCGCTGATCCTGTATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGG
AAACCGGGGCTAATACCGGATGCTTGATTGAACCGCATGGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCG
CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGA
CTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGT
GAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCT
AACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAG
CGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCA
GAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC
TGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAA
GTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTTCGAAGCACGCGAAAACCTTACCAGGTCTTGACCTC
CTCTGAACACCCCTAGAGATAAGGGCTTCCCCTTTCGGGGGCAGAGTGAACAGGTGGTGCATGTTGTCGTCAGCTCGTGTCTGAAT
GTTGGGGTAAATCCGCACGAGCGCACCTTGATCTAGTGGCAGCATTCACTGGGCACTCCTAAGTGACTGGCGGTGACAAGCGAGG
AAAGTGGGCATTACTCCATCATTCTTGGCGTTGACTGGCGTAGCACTTGGCTACACTGGCGTGAATCAAACGGACGA

Fig 4(d):Nucleotide sequence of the 16S rRNA gene from the isolated *Bacillus licheniformis* strain.

Pigment production and optimization of the pigment producing bacteria *Bacillus licheniformis*

Fig 5(a): Red Pigment production from *Bacillus lechiniformis* Fig 5(b): TLC analysis of the red pigment

Optimization of the pigment producing bacteria *Bacillus licheniformis*

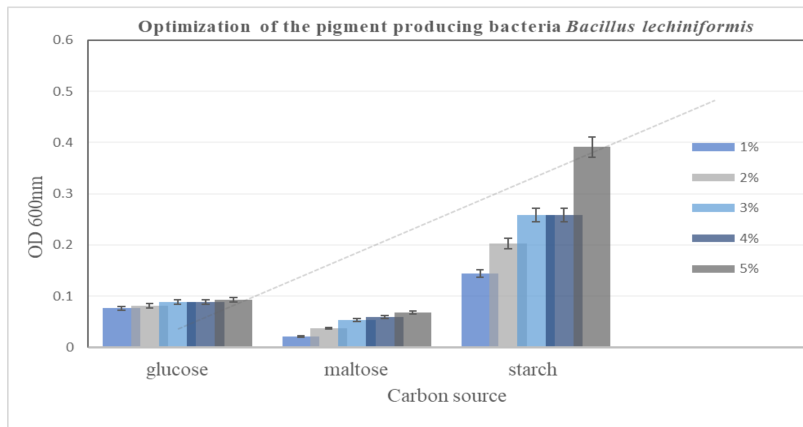


Fig 6(a) Optimization of the pigment producing bacteria *Bacillus licheniformis* by using carbon source

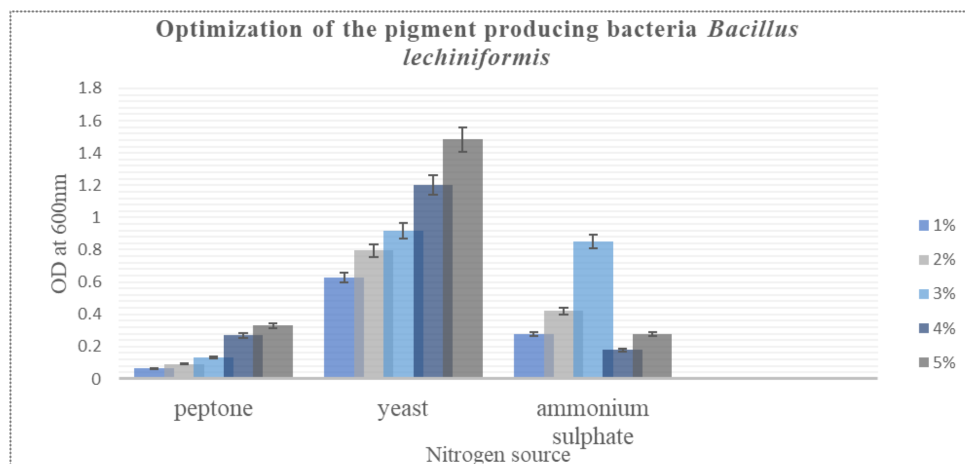


Fig 6(b) Optimization of the pigment producing bacteria *Bacillus licheniformis* by using nitrogen source

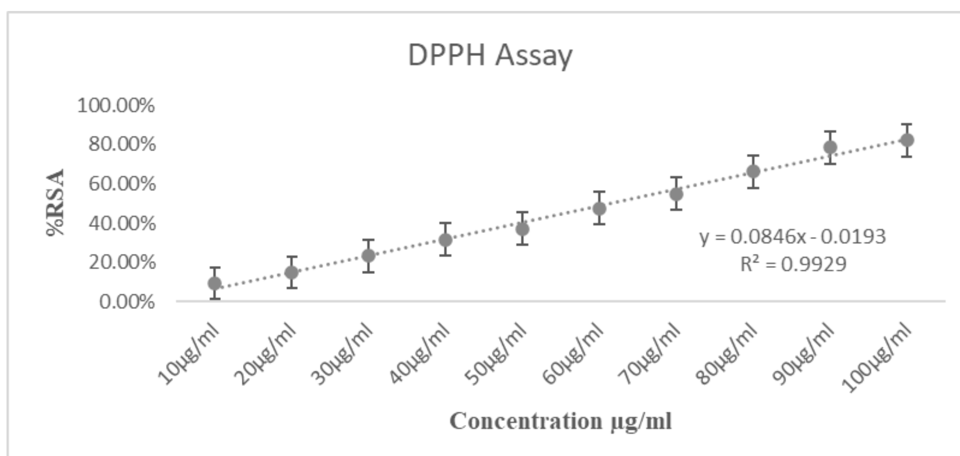


Fig 7 : Antioxidant activity of methanol extract of red pigment pulcherrimin using DPPH Assay

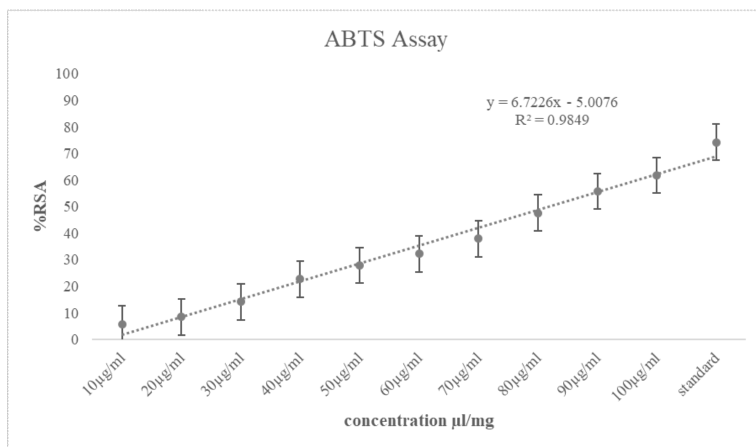


Fig:8 Antioxidant activity of methanol extract of red pigment pulcherrimin using ABTS Assay

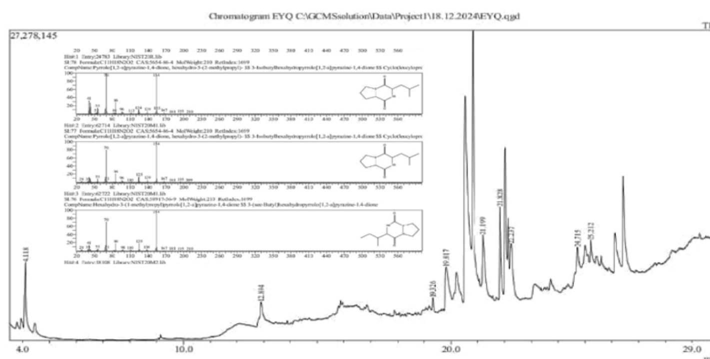


Fig 9: GC–MS analysis of the pigment extract revealed nine major compounds, with cyclic dipeptides (diketopiperazines) representing the predominant class of metabolites.

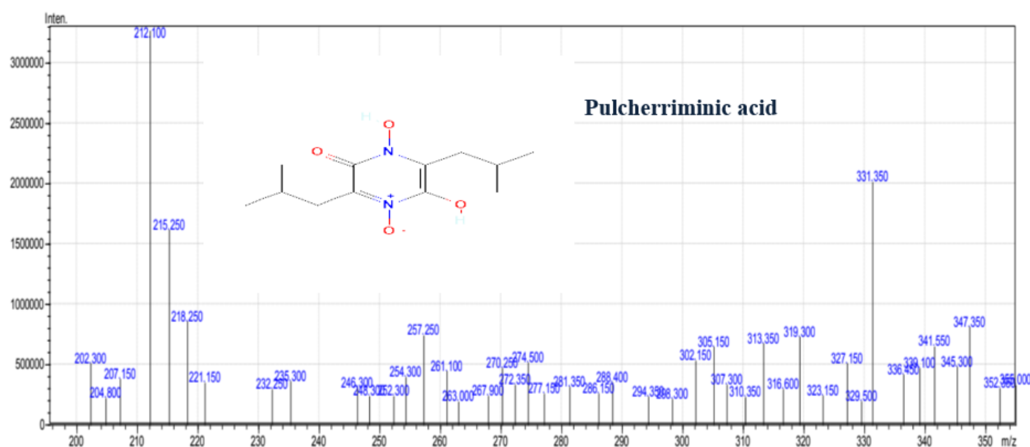


Fig 10: Liquid Chromatography-Mass Spectrometry of Pulcherriminic acid

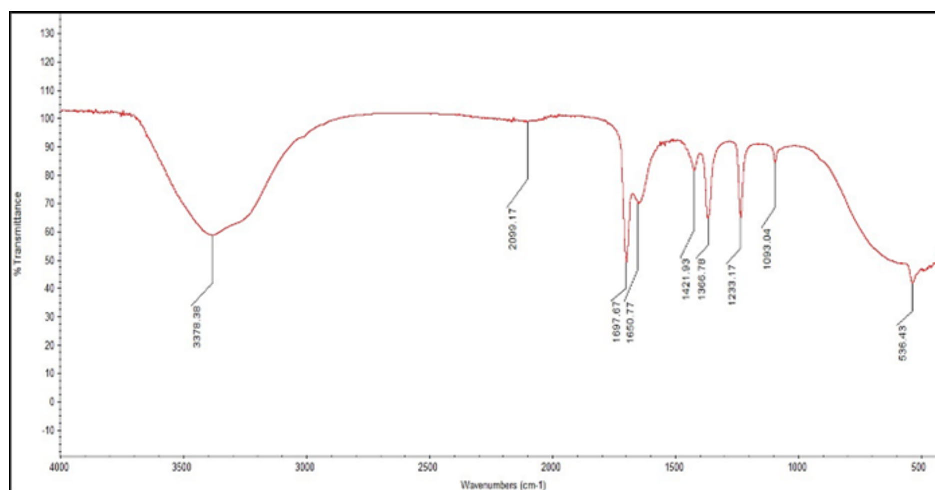


Fig 11: Fourier transform infrared spectroscopy of Pulcherriminic acid

4. Conclusion

The current study isolated and characterized a red pigment-producing marine bacterium named *Bacillus licheniformis* strain KW-1 from the Gulf of Mannar. Molecular identification through 16S rRNA gene sequencing confirmed its classification with high sequence similarity. Optimization experiments showed that starch and yeast extract significantly boosted pigment production, which highlights how nutrient composition affects secondary metabolite production in this marine isolate.

Chemical characterization using TLC, FTIR, GC-MS, and LC-MS analyses revealed several bioactive compounds, including cyclic dipeptides (diketopiperazines) and the iron-chelating compound Pulcherriminic acid as main components of the pigment extract. FTIR analysis confirmed the presence of specific functional groups like hydroxyl, amide, and carbonyl groups, supporting the existence of peptide-derived compounds. The methanolic pigment extract showed strong antibacterial activity against various pathogenic bacteria, with significant inhibition noted against *Escherichia coli* and *Helicobacter pylori*. Additionally, antioxidant tests showed strong free-radical scavenging activity. DPPH and ABTS assays indicated concentration-dependent inhibition with moderate IC_{50} values, suggesting the pigment's ability to neutralize reactive oxygen species.

Overall, these findings emphasize the multifunctional bioactivity of the pigment produced by *B. licheniformis* KW-1 and point to marine microorganisms as valuable sources of natural bioactive compounds. The detection of Pulcherriminic acid-linked metabolites alongside diketopiperazines indicates that this marine isolate has notable antimicrobial and antioxidant capabilities. Future research should focus on purifying individual metabolites, uncovering their molecular mechanisms, assessing toxicity, and developing large-scale fermentation methods to enhance their practical uses in pharmaceuticals, nutraceuticals, food preservation, and aquaculture.

This study provides one of the first detailed reports on the production, chemical characterization, and bioactive potential of Pulcherriminic acid-associated red pigment from a marine-derived *Bacillus licheniformis* isolated from the Gulf of Mannar. It highlights the potential of this compound as a promising source of natural therapeutic agents.

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6. Authors contribution

Briscilla C – Original work done & writing

W.A Manjusha- Supervision & editing

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