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Production and partial characterization of the pigment from *Kocuria* sp. ANR with their bioactivities

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Abstract

Bacterial pigments have wide demand in the various industries due to their antimicrobial, antioxidant and anticancer properties, particularly carotenoid provide substitute for natural color for food additives and they are highly biodegradable. This study aims to isolate the pigment producing bacteria and its identification by morphological, biochemical and molecular characteristics. The production of the pigment was optimised by cultural conditions, extraction and further evaluated for antimicrobial, antioxidant, and phytotoxic activities. Based on morphological traits and 16S rRNA sequencing the orange pigmented colony isolated from cow' s milk was identified as *Kocuria* sp and deposited in database with accession number *Kocuria* sp. ANR PZ006117.1. The optimisation studies revealed media pH 7.0 to 8.0., with 0.6% as sucrose concentration & 0.3% beef extract at 48h incubation. Among the different solvent extraction used methanol was better than ethanol, acetone, chloroform and ethylacetate. Moderate antimicrobial, antioxidant activity with nontoxicity to plant cells were observed. The pigment characterisation revealed basically belonged to carotenoid group by TLC, FTIR, LC-ESI-MS/MS spectrophotometers, and UV absorption.

Keywords: Antimicrobial activity, antioxidant activity, bio pigment, *Kocuria sp*

Introduction:

The increasing demand for natural colorants has deepened research interest in microbial pigments, primarily due to the health and environmental concerns associated with synthetic dyes. Conventional synthetic pigments, although widely used, have been reported to pose toxicity, allergenic, and carcinogenic risks to industrial workers and consumers, in addition to causing long-term environmental pollution. Consequently, microbial pigments have emerged as sustainable alternatives owing to their nontoxicity, biodegradability, produced at lesser cost and has diverse biological activities (Masyita et al., 2025).

A wide range of microorganisms such as bacteria, fungi, yeasts, and molds are exploited for pigment production through controlled fermentation processes. These naturally derived pigments are increasingly utilized in food, pharmaceutical, cosmetic, and textile industries. Food-grade pigments such as β-carotene, riboflavin, lycopene, Arpink Red, and Monascus pigments are widely incorporated as natural colorants in food formulations. Similarly, bioactive pigments including prodigiosin, violacein, and anthocyanins have gained attention in pharmaceutical applications due to their therapeutic potential against various diseases (Hakeem et al., 2025).

Bacterial pigments are classified as secondary metabolites and perform vital physiological and ecological functions. These compounds protect microbial cells against ultraviolet radiation, oxidative stress, and extreme environmental conditions, while also contributing to nutrient acquisition and competitive survival. Additionally, certain pigments act as signaling molecules, influencing quorum sensing, virulence expression, and host–microbe interactions. The remarkable structural and functional diversity of bacterial pigments highlights the biosynthetic versatility of microorganisms and underscores their potential for industrial exploitation (Charkoudian et al., 2010; Hakeem et al., 2025).

The production of bacterial pigments is governed by the presence of chromophoric compounds capable of absorbing visible light and inducing electronic excitation, resulting in distinct coloration. Although bacterial pigments are considered non-toxic and environmentally friendly, large-scale application is challenged by issues related to extraction efficiency, pigment purity, and production cost. Optimization of fermentation parameters, along with the development of cost-effective growth media, is therefore essential to enhance pigment yield and commercial feasibility. In this regard, the utilization of agro-industrial wastes as alternative substrates has been widely reported as a sustainable and economical strategy for pigment production (Ahmad et al., 2011; Singh et al., 2022).

Several bacterial species are known for producing pigments with diverse color spectra. For instance, *Serratia marcescens* synthesizes prodigiosin, *Chromobacterium violaceum* produces violacein, and *Streptomyces coelicolor* generates actinorhodin and prodigiosin derivatives. Other pigment-producing bacteria include *Agrobacterium aurantiacum*, *Staphylococcus aureus*, *Bacillus* spp., and *Flavobacterium* spp., producing pigments ranging from red and purple to yellow and creamy shades. Cyanobacteria produce phycobiliproteins, which play a critical role in photosynthesis and exhibit promising commercial potential (Usman et al., 2017; Venil et al., 2020). The genus *Kocuria*, belonging to the family Micrococcaceae, comprises Gram-positive, coccoid actinobacteria that are widely distributed across terrestrial, marine, and food-associated environments. The pigment profile of the genus is diverse, encompassing not only decaprenoxanthin and sarcinaxanthin but also bacterioruberin derivatives and tetrahydrospirilloxanthin, many of which are valued for their commercial potential in the food, cosmetic, and pharmaceutical industries as natural alternatives to synthetic dyes (Ramos et al., 2021; Abdo et al., 2024, Chakraborty et al., 2026).

The present study aims to isolate pigment-producing bacteria from selected food products and to evaluate the bioactivities of the extracted pigment and their characterisation, with particular emphasis on low toxicity, environmental safety, and biodegradability.

MATERIAL AND METHODS

2.1. Isolation of pigment producing bacteria

Different food samples viz., milk (Red Sindhi cow, Tumkur district), cheese, and beetroot juice (Bangalore) were collected in clean and dry container. Milk was stored at room temperature for 4–5 days, while cheese samples were refrigerated at 10 °C for one week prior to analysis Samples were serially diluted under aseptic conditions and plated on nutrient agar by spread technique, followed by incubation at 37 °C for 24–72 h. Orange-pigmented colony was selected based on morphological characteristics and purified by repeated streaking on nutrient agar (Goswami et al., 2010).

2.2. Identification of pigment producing bacteria

2.2.1. Morphological identification

Colony morphology on nutrient agar was described in terms of size, color, shape, margin, elevation, opacity, and consistency, and cells were examined by Gram staining.

2.2.2. Biochemical identification

Standard biochemical tests, including indole, methyl red, Voges–Proskauer, Simmons' citrate, oxidase, catalase, triple sugar iron (TSI), urease, and starch, were conducted following the criteria outlined in *Bergey's Manual of Determinative Bacteriology.*

2.2.3. Molecular Identification

Genomic DNA was extracted from the pigmented bacterial isolate, and the 16S rRNA gene was amplified by polymerase chain reaction (PCR) following the method described by Kavindi et al., (2025). The

PCR reaction mixture (25 µL) comprised template DNA (5 µL), PCR master mix (12.5 µL), ITSF and ITSR primers (5 µM each), and nuclease-free water (2.5 µL). Amplification was performed with an initial denaturation at 94 °C for 10 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 53 °C for 30 s, and extension at 72 °C for 90 s, with a final extension at 72 °C for 10 min.

The amplified products were purified and sequenced using the Sanger dideoxy chain-termination method with universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-AAGGAGGTGATCCAGCC-3′) (Li et al., 2007). Phylogenetic tree was constructed using the Neighbor-Joining method based on 16S rRNA gene sequences. Evolutionary distances were calculated using the Kimura 2-parameter model, and bootstrap values expressed as percentage's of 1000 replicates are shown at the nodes (Felsenstein et al., 1981).

2.3. Screening for pigment production

The orange pigmented bacteria were inoculated on three different viz, Luria Bertani agar, MRS agar, and Nutrient agar. After incubation, growth and pigment production were observed. Among these the pigment was prominent in nutrient agar, hence further studies were executed on Nutrient broth medium.

2.4. Optimization of Cultural Conditions for the pigment production

The effects of key cultural parameters, including pH, incubation temperature, incubation time, and carbon and nitrogen sources, on pigment production were evaluated using a one-factor-at-a-time approach. Experiments were conducted in 150 mL Erlenmeyer flasks containing 20 mL of nutrient broth under optimized growth conditions. The uninoculated flask served as control.

2.4.1. Effect of pH

The effect of pH on pigment production was evaluated by adjusting the nutrient broth pH to values ranging from 5.0 to 9.0. The medium was inoculated with the bacterial isolate and incubated at 37 °C for 24 h and 48 h (Choubey et al., 2021).

2.4.2 Effect of Incubation Temperature

The influence of temperature on pigment production was assessed by incubating the inoculated media at 4 °C, 10 °C, 27 °C, and 34 °C, and was subsequently the growth was analysed for 24 h and 48 h (Goswami et al., 2010).

2.4.3 Effect of Carbon Source

Different carbon sources (1%, w/v), including dextrose, sucrose, starch, and cellulose, were incorporated into the medium to determine their effect on bacterial growth and pigment production (Dutta et al., 2016).

2.4.4. Effect of Concentration of Carbon Source

The optimal carbon source was further evaluated at varying concentrations (0.2%, 0.4%,0.6, and 0.8%, w/v) to determine its effect on pigment yield.

2.4.5. Effect of Nitrogen Source

The impact of nitrogen sources on pigment production was studied by supplementing the medium with sodium nitrate, ammonium chloride, urea, yeast extract, and beef extract at a concentration of 0.3% (w/v).

2.4.6 Effect of NaCl Concentration

The effect of salinity stress on pigment production was examined by supplementing the medium with varying NaCl concentrations at 0.2%, 0.4%, 0.6%, and 0.8% (w/v). (Seo, 2022).

2.5. Harvesting the pigment from the bacteria

The optimised medium for the pigment isolate was cultured in nutrient broth and incubated at 30°C with pH 7.0. for 4-7 days to achieve maximum pigment production (Contreras-Ramos, 2025). Since the pigment was intracellular, the broth media was discarded and cell pellet were used for extraction of the pigment. After incubation cells were harvested by centrifugation at 6000 rpm for 20 min (Bhatt *et al*., 2013).

2.6. Extraction of Pigment

The pigments were extracted by different solvents like methanol, ethanol, acetone, chloroform and ethyl acetate. The pellets were resuspended in different solvents, vortexed, and centrifuged again at 6000 rpm for 10 min. Supernatants containing pigments were filtered through Millipore filters, and solvent was evaporated. The dried pigment residue was dissolved in Dimethyl sulfoxide (DMSO). The extracted pigment was later used for evaluation of bioactivity against human pathogens (Nanaware *et al.,* 2024).

2.7. Biological activities of the crude pigment extract

2.7.1. Antimicrobial activity of pigment extracted from *Kocuria* sp.

The antimicrobial activity of the crude pigment was dissolved in dimethyl sulphoxide (DMSO) at concentration of 20, 60,120 µg/mL respectively and assessed against four food borne pathogens, Viz., *Escherichia coli* (ATCC 25922), *Salmonella enterica* (ATCC 14028), *Staphylococcus aureus* (ATCC 25923), and *Bacillus subtilis* (ATCC 6633) using modified Kirby–Bauer agar well diffusion method (Umadevi & Krishnaveni, 2013). Mueller–Hinton agar plates were swabbed with pregrown bacterial suspensions in Nutrient broth (~10^5 CFU/plate) and wells (6 mm) were aseptically punched using cork borer. Agar wells were filled with different concentrations of crude pigment extract, with positive control tetracycline and DMSO as negative control. All the inoculated plates were incubated at 37 °C for 24 h, and antimicrobial activity was determined by measuring the zone of inhibition (mm).

2.7.2. Antioxidant Activity

The antioxidant potential of the crude pigment was evaluated using a modified DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. The DPPH stock solution (1 mg/mL) was prepared in methanol. In a 96-well microplate, 80 µL of crude pigment extract at different concentrations was mixed with 80 µL of DPPH solution and 80 µL of methanol. The mixtures were incubated at room temperature in the dark, and absorbance was measured at 517 nm using a UV–Vis spectrophotometer. Ascorbic acid was used as the reference standard (Nagajyothi et al., 2015, López-Alarcón & Denicola, 2015).
The percentage of radical scavenging activity was calculated as:

$$\text{Scavenging activity (\%)} = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \times 100$$

2.7. 3. Phytoxicity analysis by seed germination test

The extracted pigment was dissolved in methanol and evaporated to dryness at room temperature. The dried residue was re-dissolved in distilled water and used for the treatment. Green gram (*Vigna radiata*) seeds soaked in distilled water overnight under ambient conditions which served as the control, while another set of seeds soaked in the aqueous pigment solution for 1h constituted the test group. After incubation, seeds were placed equidistantly on pre-soaked blotting paper in sterile Petri dishes and maintained under germination conditions for 7days. Moisture was maintained throughout the experimental period by periodic addition of distilled water to prevent drying of the blotting paper. Germination percentage, radicle length, plumule length, and seedling vigor index were determined according to standard seed testing procedures (Ashraf et al., 2007; ISTA, 2023). Germination percentage was calculated using the following equation:

$$\text{Germination (\%)} = \frac{G}{X} \times 100$$

where G denotes the number of germinated seeds, X represents the total number of seeds sown (excluding empty and infested seeds),

All experiments were performed in triplicate, and results were expressed as mean values. Statistical analysis was carried out using SPSS software (version 20.0), and significance was determined by one-way analysis of variance (ANOVA).

2.8. Characterization of extracted pigment

The extracted pigment was characterized by thin layer chromatography (TLC), liquid chromatography and mass spectroscopy (LC-MS), UV-Vis spectrophotometry and fourier transform infrared (FTIR) spectroscopy technique.

2.8.1. Thin Layer Chromatography (TLC):

Thin-layer chromatography of the pigment extracted from *Kocuria* sp., was developed using different mobile phases such as chloroform:ethyl acetate (4:1, v/v), nhexane:acetone (8:2, v/v), chloroform:acetone (7:3, v/v) among these three mobile phases the bands were observed in chloroform:ethyl acetate (4:1, v/v).

2.8.2. UV-Vis spectrophotometry:

The pigment extract was subjected to scanning spectrophotometry in the range of 200–600 nm, using a Shimadzu UV-mini 1240 spectrophotometer (Shimadzu, Tokyo, Japan), at 5 nm intervals, using methanol as a blank.

2.8.3. FTIR analysis:

Fourier transform infrared (FTIR) spectroscopy of the pigment extract was performed using a Varian 640-IR spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). The dried pigment sample was finely ground with spectroscopic-grade potassium bromide (KBr) and compressed into a pellet prior to analysis. Spectra were recorded over the range of 4000–500 cm^{-1} at a resolution of 4 cm^{-1} (Coates, J. 2000).

2.8.4. Liquid Chromatography Mass Spectroscopy

The LC–MS analysis of the pigment extract was carried out using gradient elution mode. The sample was prepared in a diluent consisting of methanol, acetonitrile, water, and 0.1% formic acid with trifluoroacetic acid (TFA). Chromatographic separation was monitored using a UV–Visible detector prior to mass spectrometric analysis. The mobile phase comprised solvent A (0.1% formic acid in water) and solvent B (MS-grade acetonitrile). The gradient program was set as follows: 85% A and 15% B at 0.01 min, linearly changed to 25% A and 75% B at 6.0 min, maintained up to 8.0 min, returned to 85% A and 15% B at 11.0 min, and held until 15.0 min. These conditions were optimized to ensure efficient separation of pigment constituents prior to MS detection**.**

Mass spectrometric analysis was carried out using a Waters Micromass Quattro Micro triple quadrupole (QqQ) mass spectrometer (Waters Corp., Milford, MA, USA) equipped with an electrospray ionization (ESI) source and controlled by MassLynx software (version 4.1 SCN805). The instrument was operated in positive ion mode for pigment analysis. The capillary voltage was maintained at 3.45 kV, with cone and extractor voltages set at 33 V and 3.0 V, respectively. The source temperature was set at 110 °C, while the desolvation temperature was maintained at 350 °C. Nitrogen was used as both desolvation and cone gas at flow rates of 750 and 50 L h^{-1}, respectively. Mass spectra were acquired over an m/z range of 100–1000 with a scan time of 0.5 s under MS/MS operating conditions.

3.0 RESULTS AND DISCUSSION

3.1. Isolation and Identification of bacteria from food samples:

Bacterial isolates from cow milk, cheese, and beetroot juice exhibited distinct colony morphologies and Gram reaction. Cow milk samples showed heterogeneous populations both Gram-positive cocci and rods across dilutions, off which one of them was orange coloured and the rest displayed creamish white circular colonies displaying entire margins and flat to raised elevations. Though the diversity of colonies were varied in cheese and beetroot juice samples none of them were coloured. The orange-coloured colony from the cow milk sample was subjected for purification and used for further analysis (Timkina et al., 2022).

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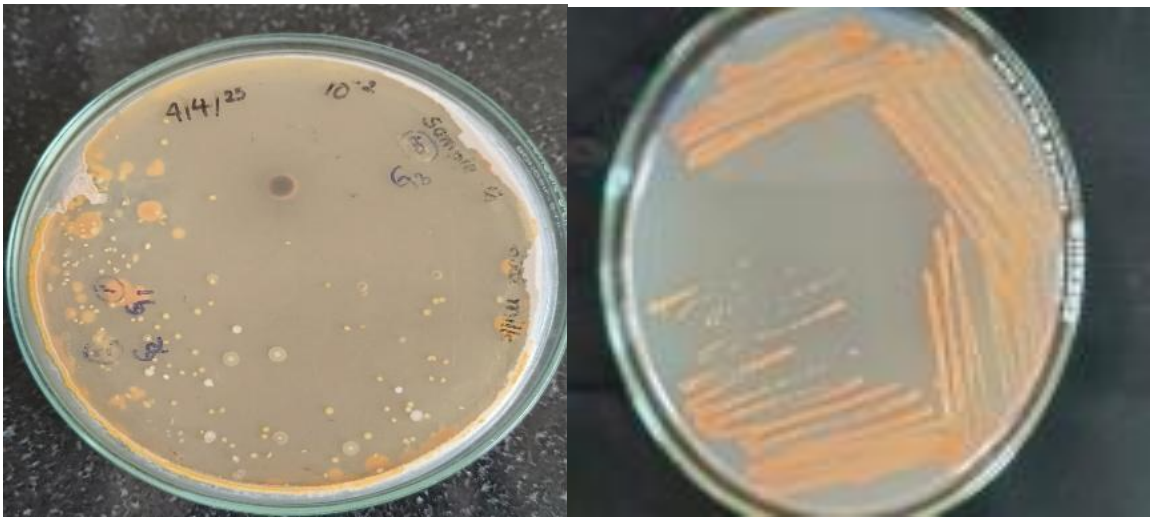


Fig 1:(a) Bacterial isolates from Cow's milk (b) Pure culture of pigmented bacteria

Table1: Isolation and morphological characterization of bacterial colonies from food samples

Sample	Dilution	Colony count (CFU/plate)	Colony morphology	Pigment/colony colour	Gram reaction
Cow milk	10^{-2}	2	Medium, circular, entire, flat	Orange	Gram-positive cocci
Cow milk	10^{-2}	2	Small, circular, entire, flat	Yellow-white	Gram-positive cocci
Cow milk	10^{-2}	1	Small, circular, entire, flat	White	Gram-positive cocci
Cow milk	10^{-3}	1	Small, circular, entire, flat	Creamish white	Gram-negative rods
Cow milk	10^{-3}	1	Small, circular, entire, raised	White	Gram-positive rods
Cheese	10^{-2}	35	Small, circular, entire, raised	White	Gram-negative rods
Cheese	10^{-3}	26	Small, circular, entire, raised	White	Gram-negative rods
Beetroot juice	10^{-2}	39	Small, circular, entire, raised	White	Gram-positive cocci
Beetroot juice	10^{-3}	20	Small, circular, entire, raised	White	Gram-negative cocci

* Colony morphology was assessed based on colony size, shape, margin, and elevation on the nutrient agar medium. Gram reaction and cellular morphology were determined by Gram staining.

3.2. Identification of the pigmented bacteria:

3.2.1. Morphological identification:

The selected orange pigment-producing bacterial isolate was identified based on its morphological characteristics and subjected to biochemical studies.

On nutrient agar colonies was1-2mm in diameter, smooth, round, uniform edged, translucent and orange in colour. On microscopic observation cells were Gram positive cocci of 1 to 1.5 µm in diameter, occurring in pairs, chains and clusters. Endospores are not formed, nonmotile and nonencapsulated.

3.2.2. Biochemical characterisation:

Biochemical analysis of the isolate yielded a profile that aligns well with characteristics documented for *Kocuria* species. The organism exhibited positive oxidase and catalase reactions, confirming an aerobic metabolism. Voges–Proskauer reaction and citrate utilisation tests were also positive. The isolate revealed negative for indole production methyl red and starch hydrolysis tests. Collectively, these biochemical traits correspond closely with previously reported descriptions of *Kocuria* sp., which are characterized as Gram-positive, catalase-positive, non-spore-forming cocci commonly isolated from environmental sources and human skin microbiota (Muneefa et al., 2021; Ziogou et al., 2023).

3.2.3. Molecular identification:

The 16S rRNA gene sequence of the pigmented isolate was compared against the NCBI 16S database (BLAST, Rockville Pike, MD, USA) and deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) with the accession No. SUB16007130. *Kocuria sp*. PZ006117.1and revealed 100% sequence similarity with *Kocuria turfanensis strain.* A tree was generated using the Neighbor-Joining (NJ) method in MEGA software. Evolutionary distances were calculated using the Kimura 2-parameter (K2P) model, and the robustness of the tree topology was tested through bootstrap analysis with 1000 replicates (Felsenstein 1985).

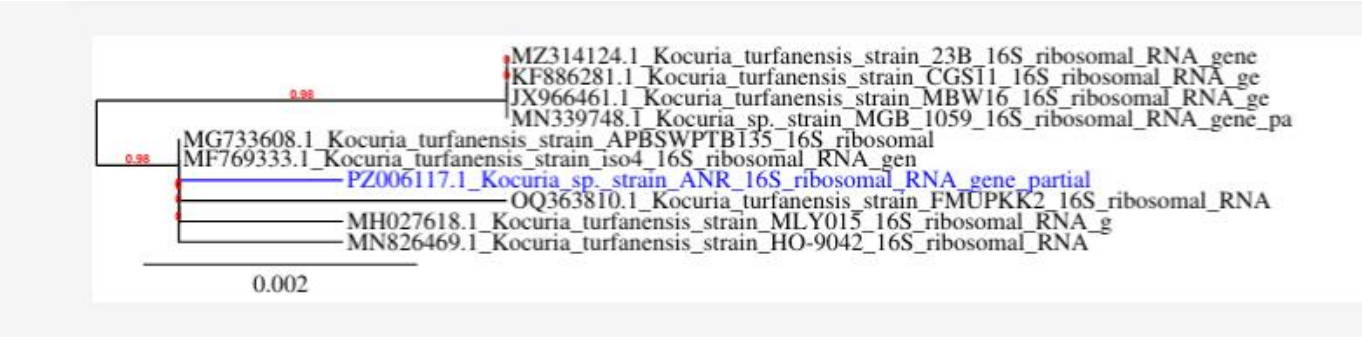
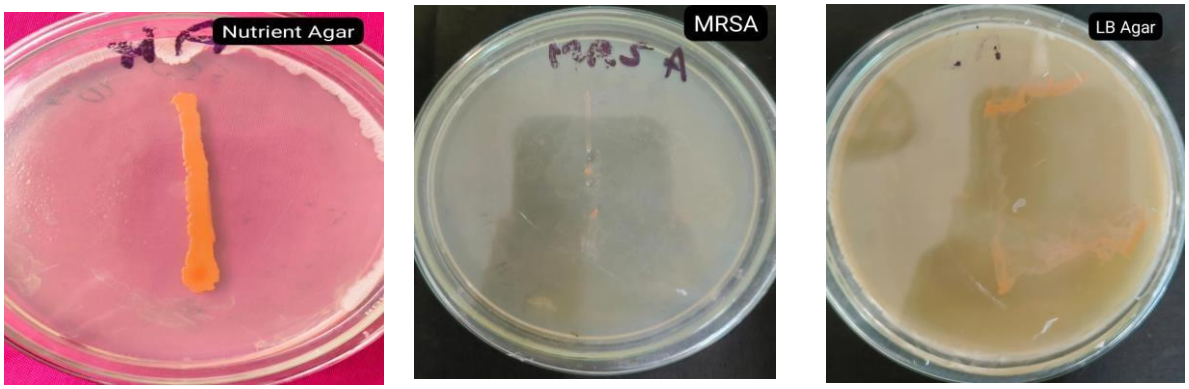


Fig 2. Phylogenetic tree of the *kocuria* sp. based on the 16S rRNA gene sequence.

3.3 Growth of pigmented bacteria on different solid media

Significant growth was observed on nutrient agar compared to reduced growth on LB and MRS agar, likely indicating differences in nutrient composition and media selectivity by the organism. This supports the recent findings that culture media composition markedly influences bacterial growth and pigment yield (Muneefa et al., 2021**)**. Nutrient agar, enriched with peptone and meat extract, supplies readily assimilable carbon and nitrogen along with essential growth factors, making it suitable for enhanced pigment production and thus selected for further optimization studies.



(a) NA (b) MRS Agar
(c) LB Agar

Fig 3 : Growth of pigmented bacteria on different solid agar media

3.4. Optimization of pigment production of *Kocuria* sp.

The growth and pigment synthesis of microorganisms are strongly modulated by environmental parameters, particularly pH and temperature, which affect metabolic activity and enzyme regulation of the pigment production. The production of the from *Kocuria* sp. was evaluated under varying growth conditions to determine the optimum parameters for maximum synthesis of pigment by keeping other parameter constant and included at optimised level in the next experiment.

3.4.1. Optimization of pH for pigment production

The effect of pH on pigment production by *Kocuria* sp. was evaluated over a pH range of 5.0–9.0 at 24 h (Day 1) and 48 h (Day 2) of incubation (Fig.6). Pigment production increased progressively from acidic to alkaline conditions, with significantly higher absorbance values recorded at pH 7.0, 8.0 moderate at pH 9.0 compared to pH 5.0 and 6.0. Maximum pigment production was observed at pH 8.0 on both days, with Day 2 showing a statistically significant increase over Day 1 across all pH levels ($p < 0.05$), indicating the influence of incubation time on pigment synthesis.

The present study confirms that alkaline conditions favour pigment biosynthesis in *Kocuria* sp., emphasizing neutral pH 7.0 to slightly alkaline 8.0 and 48h of incubation time as key parameters for pigment production. These results are consistent with the observations of Pradeep et al. (2020), who reported enhanced carotenoid production in pigmented actinobacteria at alkaline pH, attributing this effect to improved metabolic efficiency and stability of pigment biosynthetic enzymes. Similar findings were reported by John et al. (2019), who observed maximum pigment production in *Kocuria flava* under alkaline pH, whereas acidic conditions resulted in reduced pigment yield due to impaired enzymatic activity.

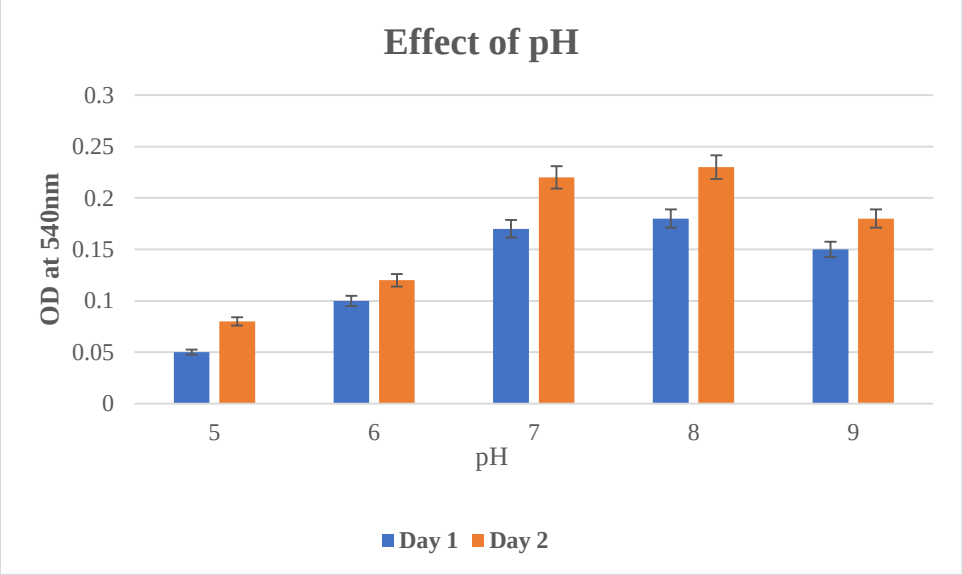


Fig 4 : Effect of pH on pigment production by *Kocuria* sp.

3.4.2. Optimization of temperature for pigment production

The effect of temperature on growth was observed for 24 h and 48 h at 4°C, 20°C, 28°C and 37°C. At 4°C, no growth was recorded in spite of incubating for two days. Whereas the pigment production was maximum in all the other temperatures on the second day compared to first day. Overall maximum growth occurred at 37°C, with significant ($p < 0.05$) increase in the production of the pigment on the 2nd day of incubation. This indicates that production of pigment was temperature-dependent, with growth optimum at 37°C, moderate at 28°C, 20°C, and nil at 4°C and control flask (Fig. 5). The present study indicates that *Kocuria* sp. exhibits mesophilic behaviour, with optimal pigment production at 37 °C. Similar observations were reported by John and Aruna (2019), who documented maximum pigment production by *Kocuria flava* at 30 °C, although the optimum temperature recorded in the present study was slightly higher. Such variation may be attributed to differences in strain characteristics, culture medium composition, and incubation duration in recent studies on microbial pigment optimization (Gharibzahedi & Smith, 2022).

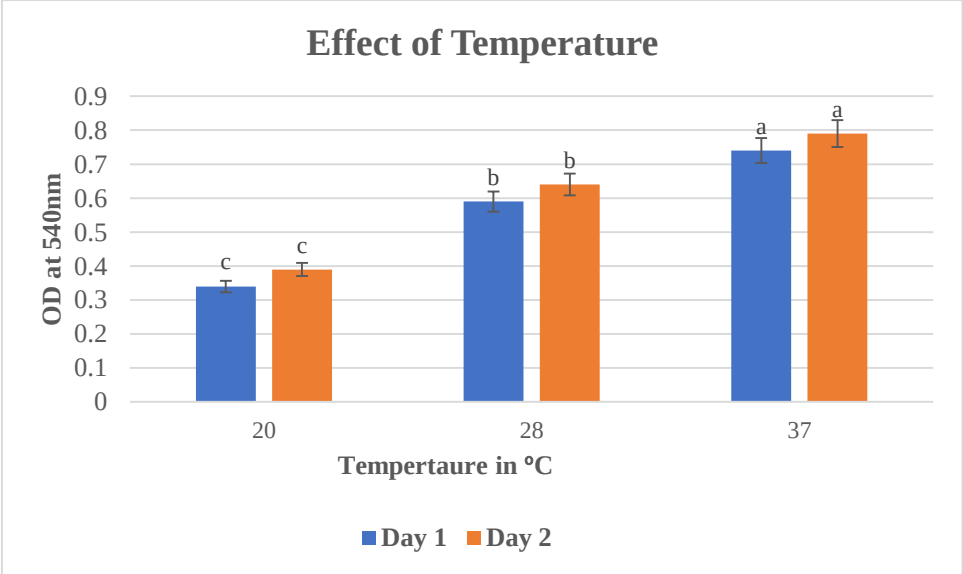


Fig 5: Effect of temperature on pigment production

3.4.3. Optimization of Carbon source for pigment production

Among the different carbon sources evaluated for two days, the yield of pigment was greater after 48 h incubation in tested carbon source. Sucrose supported significantly higher biomass accumulation and pigment yield after 48 h of incubation ($p < 0.05$), indicating that it is the most favourable carbon source for both cellular growth and pigment biosynthesis. Moderate pigment production was observed in case of starch, cellulose and dextrose, least was observed in case of mannitol (Fig: 6). Overall, sucrose was found to be the most effective carbon source for supporting microbial growth, while dextrose showed the moderate pigment production in our studies. This observation corroborates with earlier reports on *Kocuria kristinae* (John & Aruna, 2019) and is further supported by recent studies of Li et al., (2024); Zhang et al., (2025) were enhanced carotenoid production under sucrose-based fermentation systems. These results confirm sucrose as an efficiently utilized carbon source for energy, biomass, and pigment production (Ball et al., 2020)..

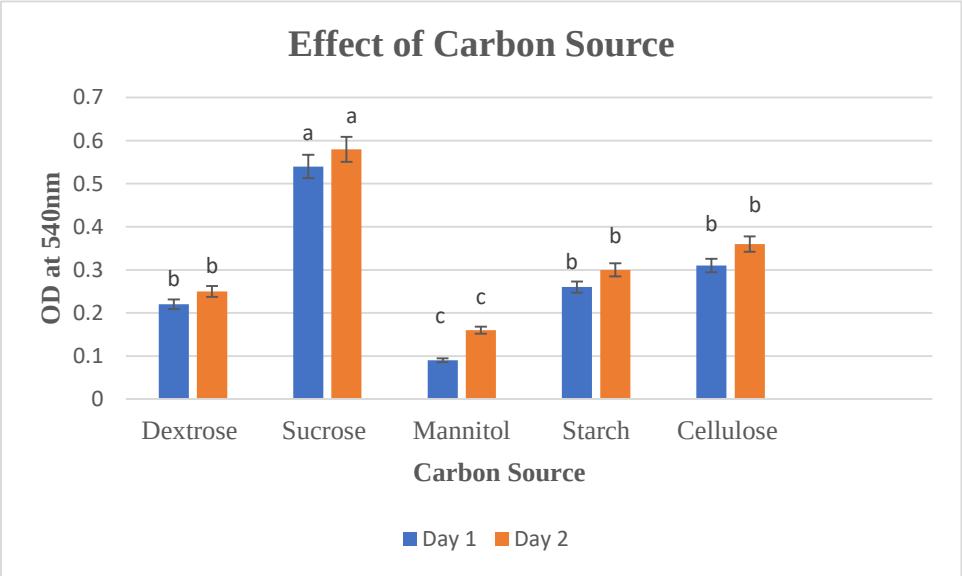


Fig 6: Effect of various Carbon Source on pigment production

3.4.3. Optimization of Nitrogen source for pigment production

The effect of different nitrogen sources on microbial growth was assessed for 24h and 48h. The pigment yield was slightly higher after 48h compared to 24h incubation Growth varied significantly among nitrogen sources ($p < 0.05$), with beef extract supporting the maximum pigment production, followed by yeast extract, peptone, and urea in moderation, ammonium chloride showed very low support for growth, while sodium nitrate showed the least growth (Fig: 7). Similar results were reported by Fatima and Anuradha (2022) with *Micrococcus sp.* producing yellow and orange, *Serratia sp.* producing color pigments red and pink *Salinococcus sp* producing orange color pigment and *Exiguobacterium sp.* producing yellow pigment, respectively, Yeast extract and peptone proved to be good nitrogen sources when supplemented with 0.1 % in growth medium however contrastly low yield was observed in case of beef extract. Whereas Kim and Park (2010) revealed maximum pigment yield with beef extract (1%) as organic nitrogen source followed by tryptone, yeast extract, and peptone from *Kocuria* sp. K70 isolated from marine source with least from inorganic nitrogen sources potassium nitrate, sodium nitrate, and ammonium nitrate which corroborated with our results. Jeong and Park (2008) reported that 1% beef extract supported optimum pigment production in three pigment-producing *Pseudoalteromonas* strains.

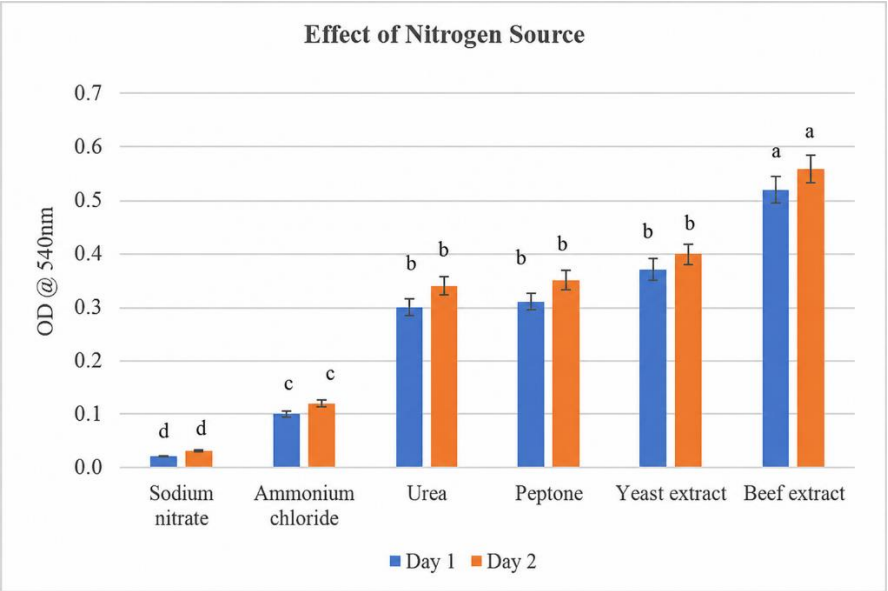


Fig 7: Effect of various nitrogen source on pigment production

3.4.4. Effect of concentration of sucrose on pigment production

The effect of different sucrose concentrations (0.2,0.4, 0.6 ,0.8% and1%) on the growth of the pigment-producing bacterium was evaluated for 48h as from the previous data indicated the production of pigment was consistently high at 48h incubation. No detectable growth was observed in the control. The results revealed that growth increased with sucrose supplementation, reaching a maximum at 0.6%, which was significantly higher than the other concentration tested. Further in 0.8% of sucrose resulted in a slight decline in growth, although it was still above control levels (Fig: 8). John and Aruna (2019) reported that the optimum pigment production was achieved at 0.8% sucrose concentration which was slightly higher when compared to the present finding.

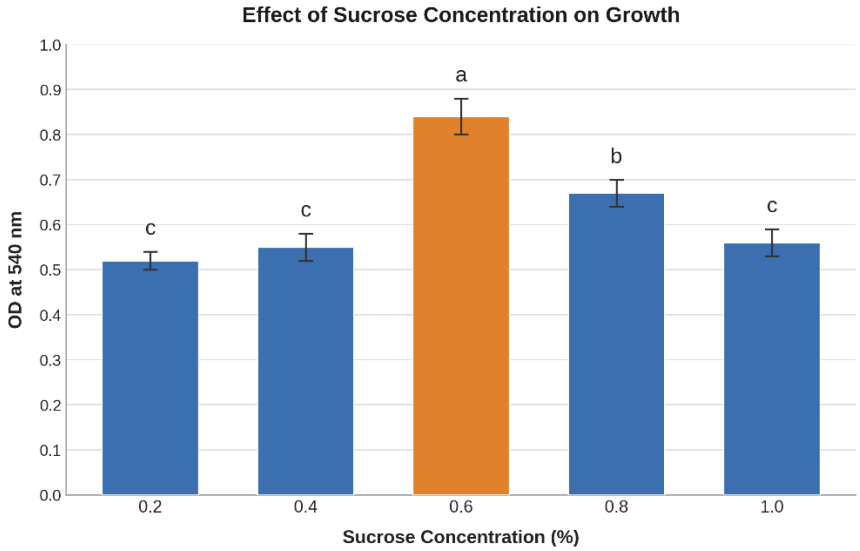


Fig 8: Effect of concentration of sucrose on pigment production

3.4.5. Effect of concentration of salt on pigment production

Salt concentration significantly influenced both pigment and the biomass production of the isolate after 48hrs of incubation. (Fig. 9). Maximum growth and intense pigmentation were observed at 2 % NaCl ($p < 0.05$), indicating optimal physiological conditions for pigment biosynthesis. A progressive decline in growth and pigmentation was recorded at higher salinity levels (4- 8%), likely due to osmotic stress impairing cellular metabolism, while no growth or pigment formation occurred in NaCl-free medium, confirming the salt-dependent and moderately halotolerant nature of the bacterium. Mhaske & Kulkarni, 2026 reported 1to 1.5%

NaCl concentration was optimum for pigment production for the bacterial isolates GLY-7, AGPA-10 and AG-12. These findings are consistent with earlier reports on halotolerant pigment-producing bacteria, where moderate salt levels favoured carotenoid synthesis, whereas excessive salinity suppressed secondary metabolite production (Sharma et al., 2018; Singh et al., 2020; John & Aruna 2019).

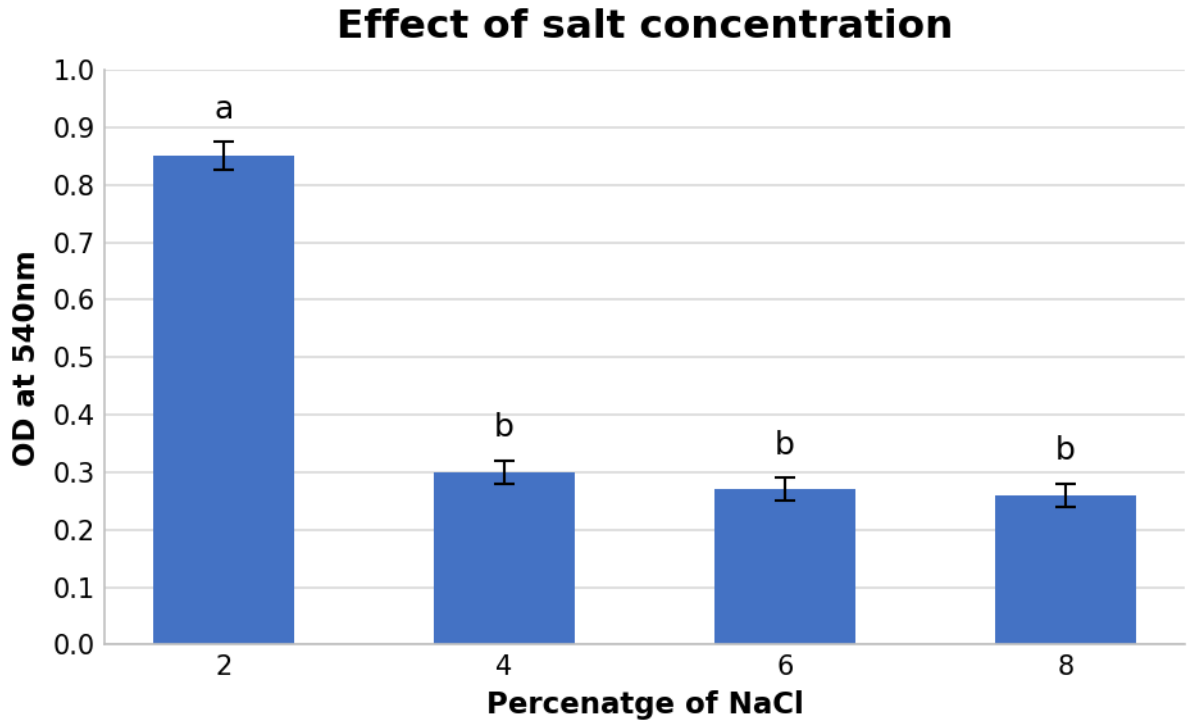


Fig 9: Effect of salt concentration on pigment production

3.5 Mass production and extraction of pigment from *Kocuria*

The *Kocuria* sp. was subjected to mass production of the pigment by using the optimized culture conditions. The pigment was extracted from the nutrient broth through centrifugation at 3000rpm the cell pellets was stored and the supernatant was discarded.

3.6. Extraction of pigment from cultured broth

Equal amount of the cell pellet extract was dissolved in five different solvents, including methanol, ethanol, acetone, ethyl acetate and chloroform. Pigment extraction differed significantly among solvents ($p < 0.05$). Methanol yielded the highest absorbance at 540 nm (0.23), followed by ethanol (0.19) and acetone (0.15), whereas ethyl acetate and chloroform showed no detectable pigment (Table 2). Polar solvents disrupt the hydrogen bonding and electrostatic forces holding the pigment within the cellular matrix thereby enhancing the solubilization of intracellular pigment (Metwally et al., 2025; Weerasinghe et al., 2025).

Table 2: Extraction of pigment with different solvents

Solvent	Absorbance (540 nm)
Methanol	0.23
Ethanol	0.19
Acetone	0.15
Ethyl acetate	0.00
Chloroform	0.00

3.7. Characterization of the extracted pigment:

3.7.1. Thin Layer Chromatography (TLC)

Thin-layer chromatography of the pigment extracted from *Kocuria* sp, developed using different mobile phases such as chloroform:ethyl acetate (4:1, v/v), hexane:acetone (8:2, v/v), chloroform:acetone (7:3, v/v) among these three mobile phases the bands were observed only in chloroform:ethyl acetate (4:1, v/v) **(Fig 10 a)** with three resolved bands at Rf values of 0.68, 0.60, and 0.26, of which the prominent band at Rf 0.60 corresponds to a carotenoid-type pigment which corroborates with earlier reports for *Kocuria* spp. (John & Aruna, 2019). Comparable Rf values for carotenoids have been reported by Weerasinghe et al., 2025 from *Kocuria* sp. RAM1 0.6 and 0.78, similar Rf values were reported by Vora etal., (2015) from *Kocuria* JO1KM216829 and Chaturwedi et al. (2023) from *Staphlococcoccus aureus*, who documented Rf values ranging from 0.56 to 0.70, characteristic of carotenoid compounds in moderately polar solvent systems (Reddy et al., 2013).

(a) Chloroform:Ethyl acetate **(b) n Hexane:Acetone** **(c) Chloroform:Acetone**

Fig 10 : Thin-layer chromatography of the pigment extracted from *Kocuria* sp.

3.7.1. Liquid Chromatography and Mass Spectroscopy

The LC-MS chromatographic analysis of the *Kocuria* sp. extract revealed five distinct peaks, corresponding to a mixture of early metabolites, carotenoid intermediates, and glycosylated pigments **(Fig 11)**. The earliest peaks (<2.0 min) were dominated by low m/z 74, 102, 118 fragments, typically representing polar byproducts and not considered pigment-associated (Metwally *et al.*, 2022). The second peak (RT 9.8-9.9 min) exhibited higher m/z ions (717- 993), characteristic of glycosylated sarcinaxanthin derivatives, including the mono-glucoside (m/z 887) and di-glucoside species, which have been previously reported in *Kocuria palustris* (Mendes-Silva *et al.*, 2021). The major peak at RT 12.8-13.0 min corresponded to sarcinaxanthin (C50 carotenoid), the dominant pigment of *Kocuria* sp, as confirmed by its fragmentation pattern (Mendes-Silva *et al.*, 2021). A minor later peak (RT 13.8-14.3 min) contained m/z fragments associated with decaprenoxanthin-like C50 carotenoids, a chemotaxonomic marker of Micrococcaceae (Scales *et al.*, 2024; Metwally *et al.*, 2022). Collectively, these findings confirm that the isolate predominantly synthesizes C50 carotenoids, particularly sarcinaxanthin and its glycosides, in agreement with previous reports on *Kocuria* sp. (Kesbiç & Gültepe, 2021; Mandelli et al., 2012).

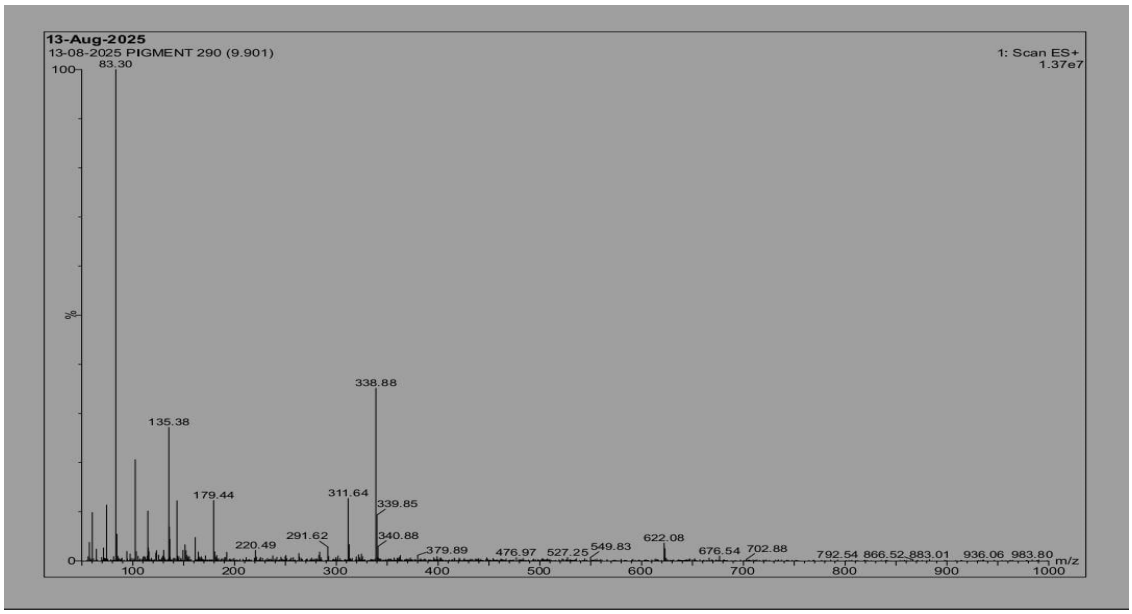


Fig 11 : LCMS graph of the pigment of *Kocuria* sp.

3.7.2. UV-Vis Spectrophotometry

The UV–Vis absorption spectrum of the pigment extract from *Kocuria* sp. shows a strong absorption peak at **440 nm**, and shoulders near 470 and 500 nm (Fig 12). λmax closely matches values reported for the C50 carotenoids sarcinaxanthin and decaprenoxanthin which falls within the characteristic range 450–470 nm reported for bacterial carotenoids, an extensive conjugated polyene chromophore such as sarcinaxanthin (439.7 nm) reported by Mendes-Silva et al., (2021, Netzer, et al. 2010). The observed λmax at 440 nm suggests that the yellow–orange colured pigment is more closely related to oxygenated carotenoid derivatives. Minor spectral variations may be due to solvent effects and mixed carotenoid composition. Similar multi-peak carotenoid absorption patterns have been reported for *Kocuria* spp. (John & Aruna, 2019; Weerasinghe et al., 2025). The secondary small peaks observed around 470 & 500 nm further support the carotenoid nature of the pigment and are characteristic features of conjugated double- bond systems (Asnang et al., 2017)

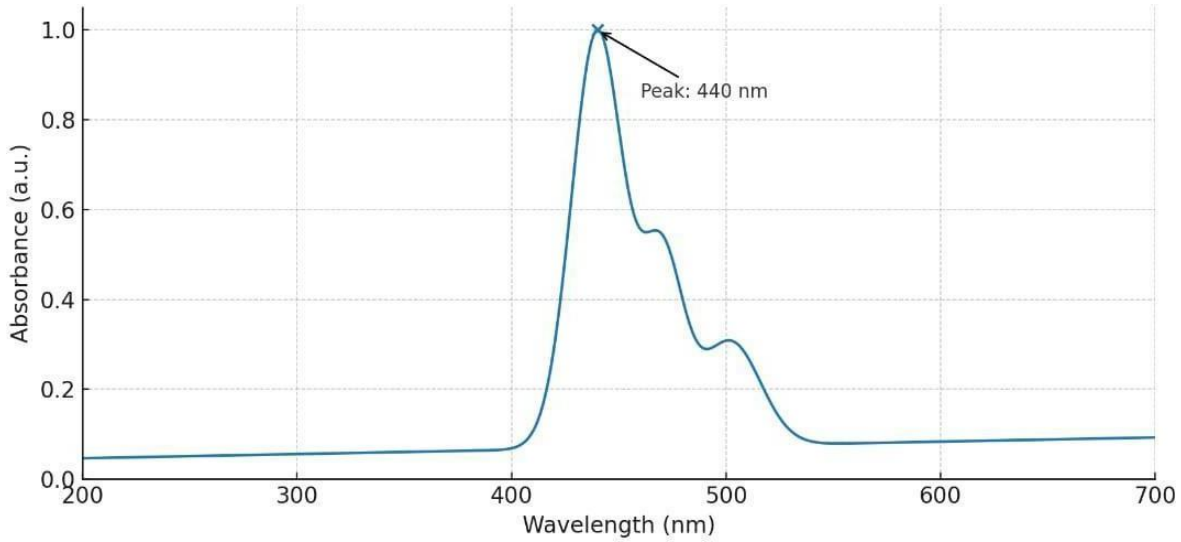


Fig 12 : UV–Vis absorption spectrum of the pigment of *Kocuria* sp.

3.7.3. Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of the pigment extracted from *Kocuria* sp exhibited a broad absorption band at 3346 cm^{-1} corresponding to hydroxyl (O–H) stretching, together with characteristic bands for conjugated C=C (1633 cm^{-1}) and C–O stretching (1223–1061 cm^{-1}), confirming the hydroxylated nature of the pigment (Fig 13). These findings corroborate with the reported structural characteristics of hydroxylated carotenoids from *Kocuria* spp. (; Venil et al., 2020; Mendes-Silva et al., 2021; López-Mora et al., 2025). The absorption at 2950 cm^{-1} reflects aliphatic C–H stretching of $–CH_2/–CH_3$ groups, representing the hydrocarbon backbone of carotenoid pigments similar to the FTIR report of Mal et al., (2020) isolated from *Kocuria flava.*

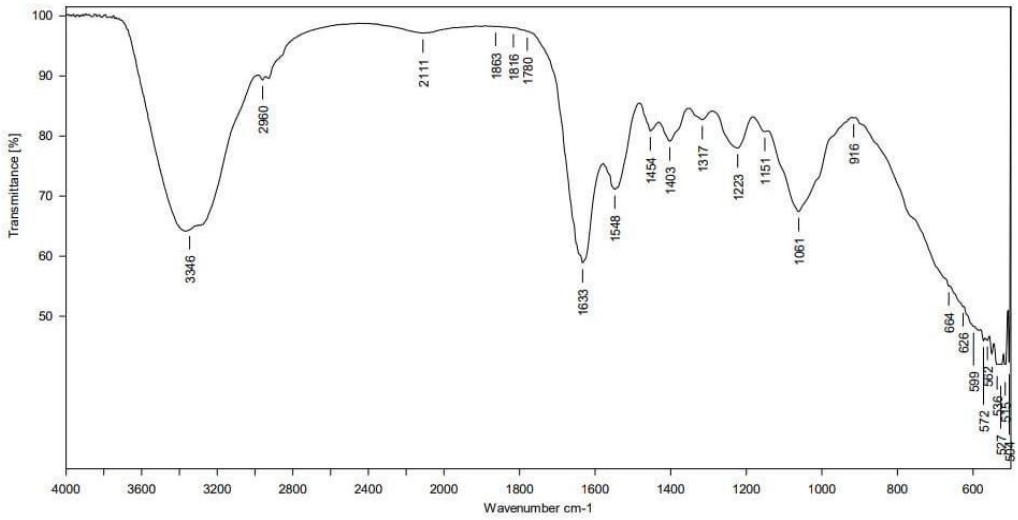


Fig 13 : FTIR analysis of extracted pigment of *Kocuria* sp.

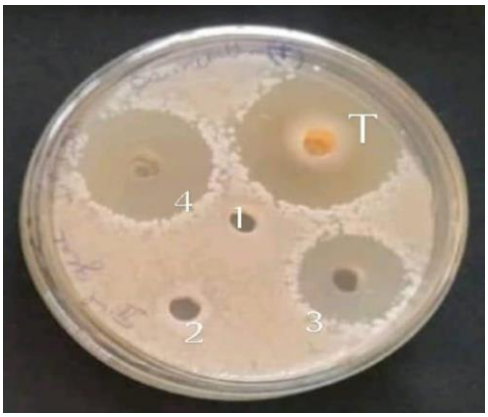
3.8. Biological activities of the crude pigment extract

3.8.1. Antimicrobial activity by agar well diffusion

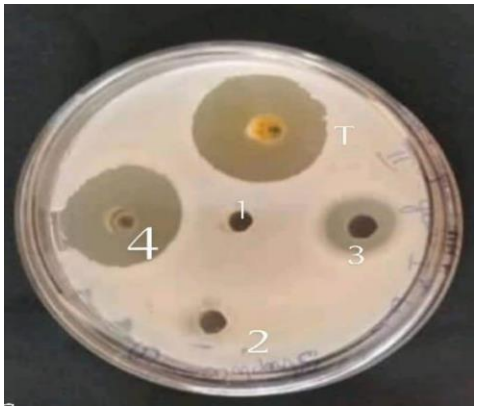
The crude pigment demonstrated dose-dependent antibacterial activity against Gram-positive bacteria *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), while Gram-negative pathogens viz., *Salmonella enterica* (ATCC 14028), and *Escherichia coli* (ATCC 25922 remained unaffected. At 60 and 120 µg/mL, inhibition zones were observed against *Staphylococcus aureus* and *Bacillus subtilis*, with greater efficacy at higher concentrations. The absence of inhibition in negative control DMSO confirmed assay validity, whereas tetracycline exhibited broad-spectrum activity against all tested organisms. (**Table 7, Fig 14)**

Table 7 : Antimicrobial activity of extracted pigment of *Kocuria* sp.

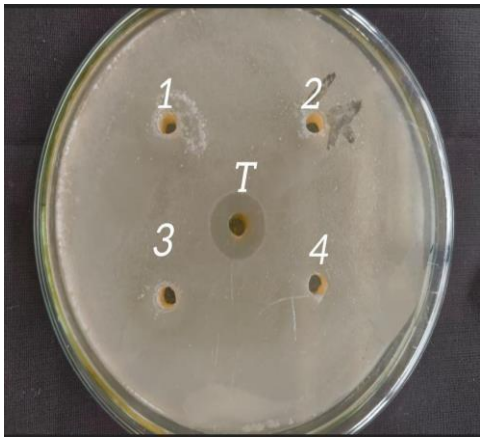
Concentration µg/mL	Bacterial pathogens/Zone of inhibition in mm			
	Sa	***Bs***	***St***	***Ec***
DMSO 100µL	0	0	0	0
30 µg/mL	2	2	0	0
60 µg/mL	8	6	0	0
120 µg/mL	10	8	0	0
Tetracycline 50 µg/mL	15	12	6	6



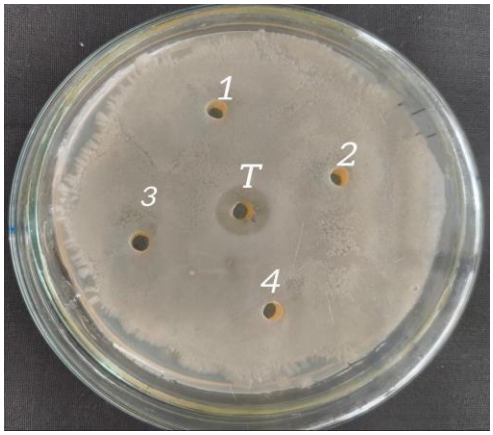
(a) *Staphylococcus aureus*



(b) *Bacillus subtilis*



c) *E.coli*



d) *Salmonella enterica*

Fig 14 : Antimicrobial activity of extracted pigment by agar well diffusion

1. Negative control (DMSO); T: Positive control Tetracycline (50 µg/mL)
Concentration of the pigment extract 2. 30 µg/mL; 3. 60 µg/mL; 4. 120 µg/mL,

The selective activity of the pigment is consistent with previous studies showing that Gram-positive bacteria are generally more susceptible to carotenoids than Gram-negative strains (Kumar et al., 2018). This is attributed to membrane permeability alteration that affects microbial virulence characteristics of Gram-negative bacteria (Miller, 2016). Similar patterns have been observed in carotenoids from *Micrococcus* spp. and other microbial sources, which inhibited Gram-positive bacteria like *S. aureus* and *B. subtilis* but showed no effect on Gram-negative organisms (Asker et al., 2009; Weerasinghe et al., 2025).

3.8.2 Antioxidant activity by DPPH assay

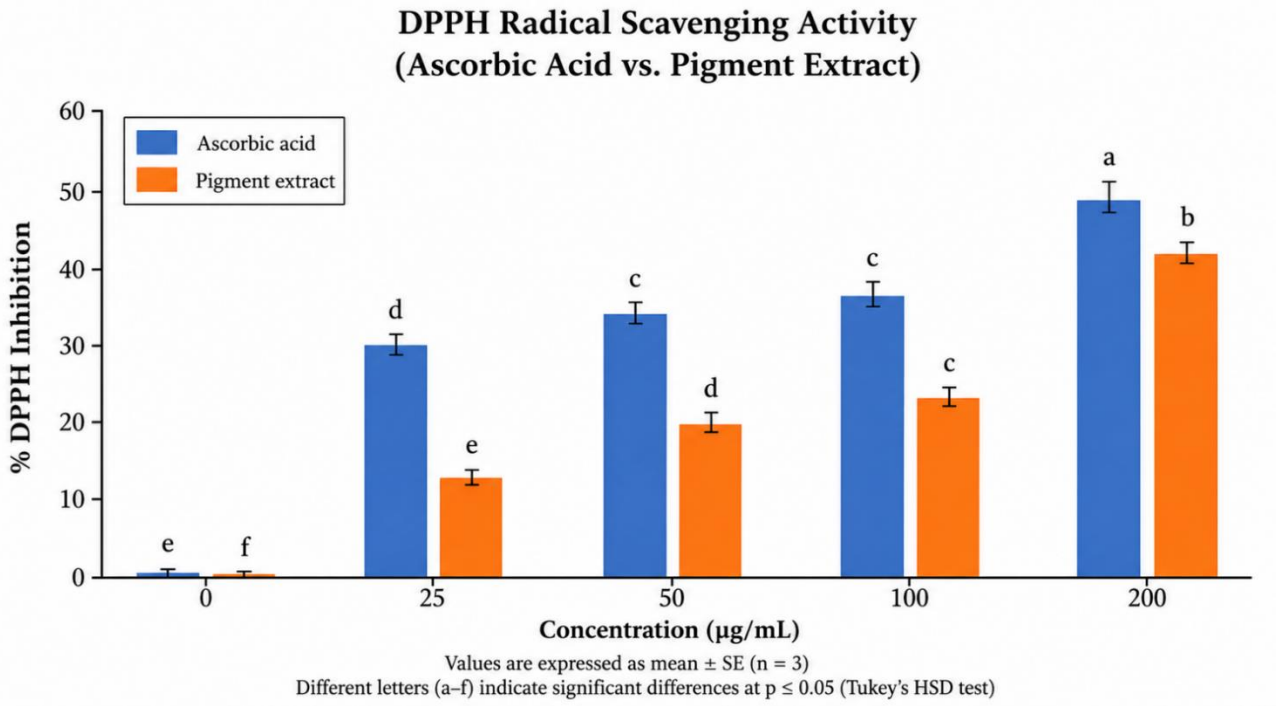


Fig 15 : Antioxidant activity of extracted pigment of *kocuria* sp.

The antioxidant activity of the *Kocuria* sp. pigment was evaluated using the DPPH radical scavenging assay with ascorbic acid as the standard. Ascorbic acid exhibited a strong scavenging activity, showing 27.86% inhibition at 25 µg/mL and almost 50% activity at 200
µg/mL In contrast, the pigment extract derived from *Kocuria* showed a relatively lower but clear dose-dependent antioxidant activity, with **12.43%** inhibition at 25 µg/mL, **20.12%** at 50 µg/mL,**23.66%** at 100 µg/mL, **26.63%** at 150 µg/mL, and a maximum of **42.01%** at 200 µg/mL(**Fig 15**). Similar studies on carotenoid extracts from *Kocuria marina* have reported higher antioxidant activity, with DPPH inhibition reaching approximately 76.9% at around 53.8 µg/mL (Samanta et al., 2016), whereas other strains like *Kocuria flava PUTS1_3* demonstrated IC50 value of 181.95 ± 4.57 µg/mL(Weerasinghe et al., 2025).

3.8.3. Phytotoxicity test by seed germination method:

Seed germination bioassay is a simple and effective method to evaluate the effect of toxicity of the extracted pigment on plant cells. The pigment extract from *Kocuria* sp. enhanced seed germination and seedling development compared with the control. Germination percentage increased from **65% (control)** to **75% (treated)**, while the average root length improved from **2.44 cm to 3.15 cm** and shoot length from **5.95 cm to 8.75 cm**.

(a) Control seeds soaked in sterile water **(b) Germination of control seeds**

(c) Seeds soaked overnight in pigment **(d) Germination of seeds in pigment extract**

Fig 16 : Phytotoxicity test by seed germination assay

Table 8: Seed germination assay of green gram

Note: Values represent mean ± standard deviation of germinated seedlings (n = 30). Different superscript letters within a column indicate statistically significant differences according to ANOVA followed by Tukey's post hoc test ($p < 0.05$).

Treatment	Root length (cm, mean ±SD)	Shoot length (cm, mean ± SD)	Germination (%)
Control	2.44 ± 0.97^{a}	5.95 ± 3.65^{a}	65
Pigment extract	3.15 ± 1.52^{b}	8.75 ± 3.27^{b}	75

Banerjee et al., 2025 reported the pigment extract of *Rhodotorula* sp. KSB1 was not toxic to *Vigna mungo* seeds. When compared with previous studies, similar stimulatory effects of microbial pigments on seed germination have been reported. For instance, carotenoid pigments from *Kocuria flava* increased germination to 72% in legumes (Mercy & Aruna, 2019), while yellow pigments from *Kocuria* sp. promoted 78% germination in cereal seeds (Ibrahim *et al.*, 2024). Phenolic pigments from *Streptomyces* sp. resulted in 74% germination in rice seedlings (Venil *et al*., 2020). Goswami et al., (2014) has reported *Kocuria turfanensis* as an incredible plant growth-promoting bacteria isolated from saline desert.

These observations indicate that the germination-promoting effect of *Kocuria* sp. pigments is consistent with previously reported microbial pigments, with variations likely arising from differences in pigment structure (e.g., conjugated double bonds, hydroxyl groups), concentration, extraction methodology, and plant species sensitivity. Overall, the findings support the potential application of *Kocuria* sp. pigments as natural bio-stimulants for enhancing seedling establishment and early growth.

Conclusion:

This study demonstrates that *Kocuria sp.* isolated from the cow milk sample had the ability to produce pigment. Hence culture was confirmed by morphological, biochemical and 16s rRNA sequencing confirmed *Kocuria turfanensis*. The optimization studies revealed the pigment production was maximum at 48h incubation with pH of media around 7 to 8, with 0.6% Sucrose as carbon source,0.3% beef extract as nitrogen source and 2% sodium chloride. Supplementary LCMS analysis, UV spectrum and FTIR spectrum evidence supports tentative identification of the pigment a belongs to glycosylated C50 carotenoid of the sarcinaxanthin/decaprenoxanthin family, definitive identification of the individual carotenoid(s) requires HPLC purification of the compound LC–MS and other structural analyses. The pigment revealed moderate antibacterial activity against Gram positive bacteria with mild antioxidant activity with no phytoxicity indicating it can find is application as natural pigment in food industries. This study also revealed that it promoted the growth of the green gram indicating it can used in agricultural field to promote the growth of the crops. Nevertheless, further investigations are necessary to assess the functionality and scalability of the pigment under industrial processing conditions, as well as to establish its feasibility for potential applications in the food, textile, cosmetic, and pharmaceutical sectors.

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