

Polyphasic characterization and pigment metabolite profiling of *Penicillium citreosulfuratum* (KUMBASBT-53) from Shivamogga district, Karnataka

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Soil fungi are recognized as prolific producers of secondary metabolites, particularly natural pigments with significant biotechnological potential. In the present study, a pigment-producing fungal strain, designated KUMBASBT-53, was isolated from litter soil and subjected to polyphasic characterization. Morphological observations revealed moderate colony growth with whitish to greenish surfaces, dense sporulation, and vivid yellow pigmentation on the reverse side. Microscopic features, including septate hyphae and brush-like penicilli with chains of smooth-walled conidia, were consistent with the genus *Penicillium*. Molecular identification through ITS-rDNA sequencing confirmed the isolate as *Penicillium citreosulfuratum* (GenBank accession number MW130131), with phylogenetic analysis clustering it within a strongly supported clade of reference strains. Pigment production was optimized under defined physicochemical conditions, showing enhanced yield under favorable nutrient availability. UV-Vis spectral analysis revealed a λ_{max} at 425 nm, indicative of a yellow chromophore. FTIR spectroscopy confirmed the presence of hydroxyl, amine, phosphate, and aromatic functional groups, suggestive of polyketide-derived metabolites. Orbitrap HR-LC/MS analysis identified several pigment-related compounds, including Isochromophilone I and II (azaphilone pigments), 4- and 7-hydroxycoumarins, flavoglaucin, and benzophenone derivatives, alongside lipid and organic acid metabolites. These results correlate with earlier reports of pigmentogenic *Penicillium* species producing structurally diverse polyketides. Collectively, the study highlights *P. citreosulfuratum* KUMBASBT-53 as a stable and promising strain for natural pigment production, offering potential for future industrial applications.

Keywords : FTIR, LC-MS; natural pigments, *Penicillium citreosulfuratum*; Polyphasic characterization, Pigment metabolites

INTRODUCTION

Natural pigments derived from microorganisms have emerged as sustainable alternatives to synthetic colorants, which are often associated with toxicity, environmental pollution, and regulatory concerns (Mapari *et al.* 2005; Dufossé, 2018). Among microbial sources, filamentous fungi are particularly attractive due to their ability to synthesize structurally diverse pigments with applications in food, textile, cosmetics, and pharmaceutical industries (Kalra *et al.* 2020).

In addition to coloration, many fungal pigments possess bioactive properties including antioxidant, antimicrobial, and anticancer

activities, thereby enhancing their industrial relevance (Lin and Xu, 2019).

The genus *Penicillium* is well known for its secondary metabolite diversity, encompassing antibiotics, mycotoxins, and natural colorants (Frisvad and Samson 2004). Several *Penicillium* species have been explored for pigment production; however, relatively less attention has been directed toward rare or underexplored taxa such as *Penicillium citreosulfuratum*. This species, first described as a soil inhabitant, remains poorly studied in terms of its pigment-producing potential and chemical repertoire. Bioprospecting of such unexplored fungal strains from diverse ecological niches may yield novel pigments or bioactive metabolites with potential industrial applications (Pandey *et al.* 2019).

Accurate identification of pigment-producing fungi is critical, as morphological features alone may

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not suffice due to phenotypic plasticity and interspecies similarity. The adoption of a polyphasic characterization approach—integrating morphological, physiological, and molecular analyses—has become the gold standard for robust fungal identification (Samson *et al.* 2014). Metabolite profiling using advanced chromatographic and spectrometric tools provides insights into the chemical diversity of pigments, supporting both functional evaluation and safety assessment (da Costa Souza *et al.* 2016).

In this context, the present study reports the isolation and comprehensive bioprospecting of *Penicillium citreosulfuratum* strain KUMBASBT-53 from litter soil, with emphasis on its pigment-producing ability. A polyphasic characterization strategy was employed for accurate strain identification. Process parameters influencing pigment production were systematically optimized, and metabolite profiling was carried out to elucidate the chemical nature of the pigments. The findings provide a scientific basis for the potential utilization of this underexplored fungus in sustainable pigment production.

MATERIALS AND METHODS

Collection of litter soil sample from forest site

Litter-rich soil was aseptically collected from a forest area near Thammadihalli village, Bhadravati taluk, Shivamogga district, Karnataka, using sterile containers. The exact geographical coordinates of the sampling site were documented. The sample was carefully transported to the research laboratory under aseptic conditions for subsequent fungal isolation and pigment-related studies.

Isolation and screening of pigment-producing fungal strains

Fungal strains were isolated from the collected litter soil using the serial dilution plating technique (Yasanthika *et al.* 2022). Aliquots (100 µL) of 10^{-3} , 10^{-4} , and 10^{-5} dilutions were spread on Potato Dextrose Agar (PDA) plates supplemented with 30 mg/L chloramphenicol to suppress bacterial growth. The plates were incubated at

laboratory temperature ($28 \pm 2^{\circ}\text{C}$) for 5–7 days. Colonies producing bright and intense coloration were selectively transferred to fresh PDA plates for purification and later maintained on PDA slants at 4°C . For extracellular pigment assessment, 5 mm mycelial discs were inoculated into 50 mL Potato Dextrose Broth (PDB) in 100 mL Erlenmeyer flasks and incubated statically at room temperature for 14–21 days. The culture filtrates were collected and visually evaluated for pigment production (Bouhri *et al.* 2020).

Morphological identification of fungal strain KUMBASBT-53

The pigment-producing fungal strain, designated KUMBASBT-19 was initially identified based on colony characteristics such as color, texture, and growth pattern on PDA. Microscopic examination was performed using the lactophenol cotton blue (LPCB) staining technique, observing spore morphology, spore chain arrangement, and hyphal structures under 40X and 100X magnifications. Identification was confirmed by referencing standard mycological manuals including, 'A laboratory guide to common *Penicillium* species' (Pitt, 1985) and 'Handbook of Soil Fungi' (Nagamani *et al.* 2006).

Molecular identification of fungal strain KUMBASBT-53

The fungal strain was cultivated in PDB under static conditions for 7 days at 28°C . Genomic DNA was extracted from freeze-dried mycelia using the cetyl trimethyl-ammonium bromide (CTAB) method (Karthikeyan *et al.* 2010). The ITS region of rDNA was amplified using universal primers ITS-1 (52 -TCCGTAGGTGA-ACATGCGG-32) and ITS-4 (52 TCCTC-CGCTTATTGATATGC-32). Amplicons were purified and sequenced via the Sanger sequencing method. Sequence assembly was performed using FinchTV software, and homology searches were carried out with BLASTn against NCBI GenBank sequences. Phylogenetic analysis was conducted using MEGA 7.0 (ClustalW for multiple sequence alignment), and evolutionary relationships were inferred through RAXML maximum likelihood analysis with 100 bootstrap replications. The resulting phylogenetic

tree was visualized using FigTree v1.4.4 (Mishra *et al.* 2021).

Cultivation and extraction of *P. citreosulfuratum* KUMBASBT-53 pigment metabolites

For large-scale pigment production, the fungus was cultivated in PDB medium, inoculated at a 1:10 (v/v) ratio, and incubated under static conditions for 21 days. Post incubation, biomass was separated from the culture broth via filtration through Whatman No. 1 filter paper. The filtrate was extracted with ethyl acetate and chloroform (1:1 v/v) using a rotary shaker (150 rpm, 30 min), followed by centrifugation at 3000 rpm for 20 min. The organic phase containing pigment was concentrated with a rotary evaporator, lyophilized, and stored in sterile ampoules for subsequent analysis (Aftab *et al.* 2021; Ramesh *et al.* 2022).

Characterization of *P. citreosulfuratum* KUMBASBT-53 pigment metabolites UV-Visible Spectroscopy

Absorption spectra of the pigment (dissolved in methanol) were recorded in the range of 200–800 nm using a PerkinElmer® Lambda 950 UV–VIS spectrophotometer, with a 2 mm optical path length and 0.1 nm resolution (Khan *et al.* 2021).

Fourier Transform Infrared (FTIR) Spectroscopy

Functional groups were analyzed using a PerkinElmer® FT–IR spectrophotometer. Pigment (10 mg) was mixed with KBr (100 mg), pressed into pellets, and scanned across 4000–500 cm⁻¹ (Muhammad *et al.* 2022).

Orbitrap HR–LC/MS Analysis

High-resolution mass profiling of pigment metabolites was performed using a Thermo Fisher Scientific Q Exactive Plus Orbitrap LC–MS equipped with a Hypersil GOLD C18 column (100 × 2.1 mm, 3 μm). Gradient elution was conducted at 0.3 μm/min under 1200 bar for 30 min. Mass spectra were acquired in the m/z range 100–1200. Data were processed using Xcalibur 4.2.28.14 and Compound Discoverer 3.2 software, employing the mzCloud spectral

database for metabolite identification (Nischitha and Shivanna, 2022).

RESULTS AND DISCUSSION

Collection of litter soil sample from forest site

The litter soil sample was collected in a forest area near Thammadihalli village, Bhadravati taluk, Shivamogga district, Karnataka, with geographical coordinates of 13°45'37.0"N, 75°37'51.3"E. The geo-location of the sample collected site is shown in Fig. 1.

Isolation and screening of pigment-producing fungal strains

Soil ecosystems are rich reservoirs of fungal diversity, providing essential micro- and macronutrients that harbor diverse fungi and support their secondary metabolite production. In the present study, a pigment-producing fungal strain (KUMBASBT-53) was isolated on PDA medium at a 10⁻³ dilution. The colony exhibited distinct morphological features: the obverse surface appeared whitish to greyish-green with dense sporulation, while the reverse side displayed a vivid yellow pigmentation diffusing into the surrounding agar (Fig. 2). Successive sub-culturing ensured the purity and morphological stability of the isolate. When cultivated in PDB medium, strain KUMBASBT-53 demonstrated robust extracellular pigment production, imparting an intense yellow coloration to the broth (Fig. 3). These findings corroborate earlier reports that soil-derived fungi are prolific producers of natural pigments and bioactive metabolites (Gill *et al.* 2023; dos Reis Celestino *et al.* 2014).

Morphological identification of fungal strain KUMBASBT-53

The isolated pigment-synthesizing fungal strain KUMBASBT-53 exhibited distinct colony morphology on different agar media (Table 1; Fig. 4). Microscopic examination using lactophenol cotton blue (LPCB) staining revealed septate, branched, hyaline hyphae with characteristic brush-like penicilli bearing divergent whorls of phialides and globose to subglobose, smooth-walled conidia arranged in chains, which are

Table1 : Colony morphology of *P. citreosulfuratum* strain KUMBASBT-53 on different agar media

Medium	Colony characteristics
Potato Dextrose Agar (PDA) HiMedia, India / M096F	Small (~15 mm) dusky green colonies with white margins; velvety compact mycelium; yellow metabolite production; reverse side yellow.
Sabouraud Dextrose Agar (SDA) HiMedia, India / GM063	Light greyish colonies (~5 mm) with white margins; compact, velvety mycelium; reverse yellow.
Malt Extract Agar (MEA) HiMedia, India / M1964	Colonies (~10 mm) with yellow center and green periphery; white margins; velvety compact mycelium; yellow metabolite production; reverse yellow.
CzapekDox Agar (CZA) HiMedia, India / GM075	Buff green raised colonies (8–10 mm) with transparent margins; loosely arranged mycelium; reverse white.
Carrot Agar (CA) HiMedia, India / M1987	Small colonies (3–4 mm), buff green, spreading; feathery mycelium; reverse yellow.
Rose Bengal Agar (RBA) HiMedia, India / M842	Larger colonies (22–25 mm), yellow with transverse lines; thick compact white margins; reverse yellow.

Table 2 : Major pigment-related metabolites identified from *P. citreosulfuratum* strain KUMBASBT-53 by Orbitrap HR–LC/MS

Ion Mode	RT (min)	Compound	Formula	Mol. Wt.	Nature	Reference pigment class
Positive	7.42	3,5-di-tert-butyl-4-hydroxybenzaldehyde	C ₁₈ H ₂₂ O	234.16	Phenolic antioxidant	Phenolic pigment precursor
Positive	14.61	Benzophenone	C ₁₄ H ₁₀ O	182.07	Aromatic compound	Benzophenone pigment
Positive	28.54	Isochromophilone I	C ₂₀ H ₁₆ O ₄	410.46	Azaphilone	Yellow azaphilone pigment
Positive	28.90	Isochromophilone II	C ₂₀ H ₁₆ O ₄	410.46	Azaphilone	Yellow azaphilone pigment
Negative	1.21	4-Hydroxycoumarin	C ₉ H ₆ O	162.03	Polyketide pigment	Coumarin derivative
Negative	2.16	7-Hydroxycoumarin (Umbelliferone)	C ₉ H ₆ O	162.03	Fluorescent pigment	Coumarin derivative
Negative	15.31	Polyphenolic derivative	C ₁₅ H ₁₀ O ₅	258.23	Phenolic pigment	Polyphenolic pigment
Negative	19.25	Phenolic pigment	C ₁₅ H ₁₀ O	214.22	Pigment-related	Phenolic derivative
Negative	28.71	Flavoglauclin	C ₂₀ H ₁₆ O	354.44	Benzophenone pigment	Yellow pigment in <i>Penicillium</i>

consistent with the morphology of the genus *Penicillium* (Fig. 5). These phenotypic features closely resemble earlier descriptions, where *Penicillium* species are characterized by moderately growing colonies with a velvety texture, typically greyish to greenish in color, and conidiophores terminating in brush-like penicilli producing chains of smooth-walled conidia (Pitt and Hocking, 2009; Visagie *et al.* 2014). Strain KUMBASBT-53 also exhibited the production of distinct yellow metabolites, which correlates with previous reports where several *Penicillium* species, including *P. citrinum*, *P. herquei*, and *P. purpurogenum*, were found to synthesize extracellular colored secondary metabolites ranging from yellow to orange and red (Frisvad *et al.* 2015; Fouillaud *et al.* 2017).

Molecular identification of fungal strain KUMBASBT-53

Molecular identification of the pigment-producing fungal strain KUMBASBT-53 was carried out through ITS-rDNA sequencing, and the sequence was deposited in the NCBI GenBank under the accession number MW130131. BLASTn analysis revealed 99% similarity with *Penicillium citreosulfuratum* strains, including strain Z16 (MT406173), confirming its species-level identity. Taxonomic classification based on GenBank data placed the isolate within Kingdom: Fungi; Phylum: Ascomycota; Class: Eurotiomycetes; Order: Eurotiales; Family: Aspergillaceae; Genus: *Penicillium*; Species: *P. citreosulfuratum*. Phylogenetic analysis using the maximum

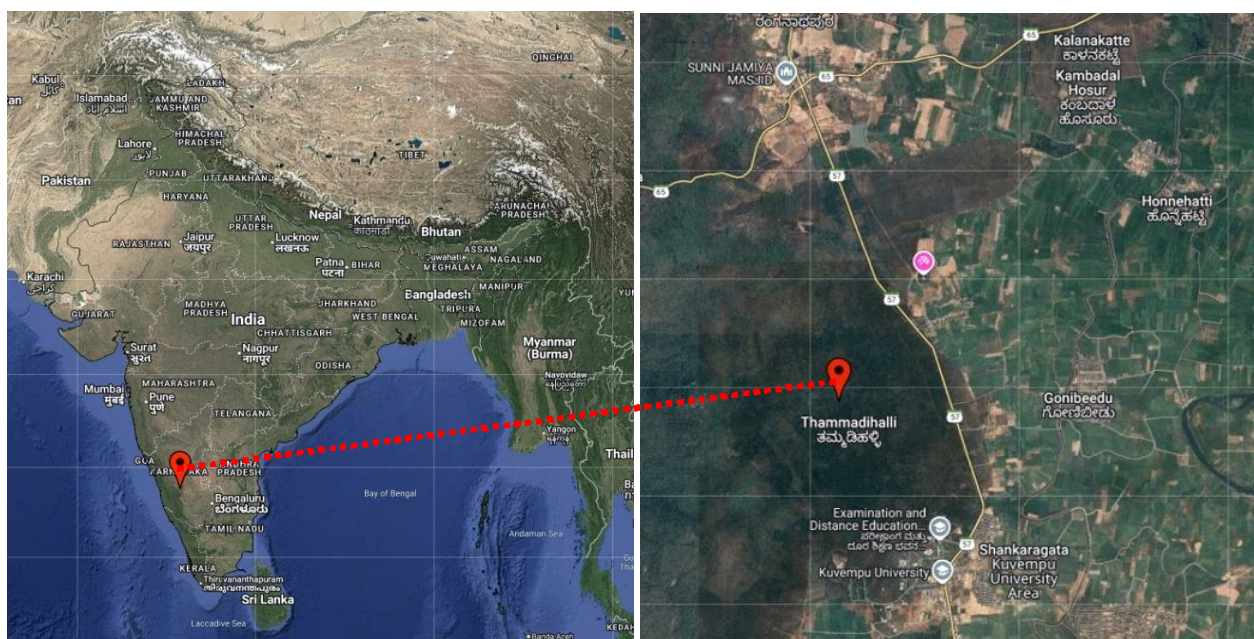


Fig. 1: Geo-location of the sample collected site: Thammadihalli(13°45'37.0"N,

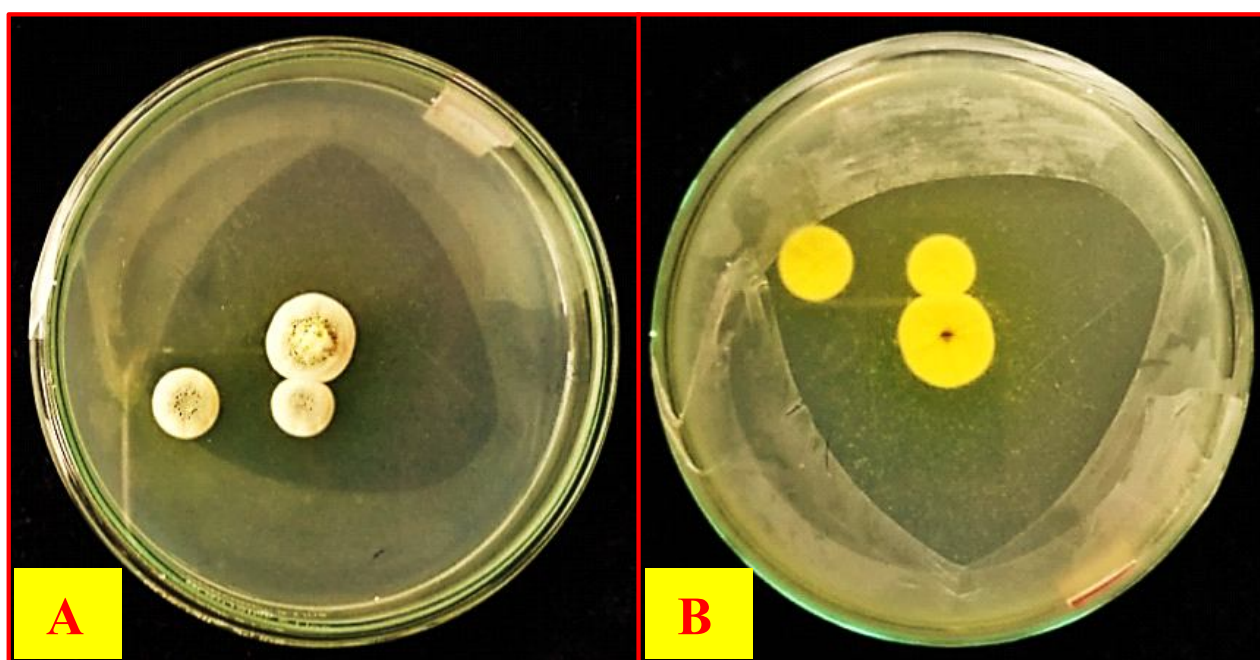


Fig. 2: Culture of *P. citreosulfuratum* strain KUMBASBT-53 on PDA for 7 days (A) Anterior view (B) Converse view

likelihood method further clustered strain KUMBASBT-53 with *P. citreosulfuratum* reference sequences in a strongly supported clade (bootstrap >90%) (Fig.6). The molecular findings were consistent with morphological and microscopic features diagnostic of *Penicillium*. These results underscore the value of a polyphasic approach in fungal taxonomy, as highlighted by Samson *et al.* (2010) and Visagie

et al. (2014), and establish strain KUMBASBT-53 as *P. citreosulfuratum*, a soil-derived fungus with distinct pigment-producing potential.

Characterization of *P. citreosulfuratum* KUMBASBT-53 pigment metabolites UV-Visible Spectroscopy

The UV-Vis absorption spectrum of the pigment extracted from *P. citreosulfuratum* strain

KUMBASBT-53 showed a distinct λ max at 425 nm (Fig. 7), indicating the presence of conjugated chromophores typically associated with yellow fungal metabolites. Similar absorption patterns have been reported in pigments of *Penicillium* species, such as citrinin and other polyketides, which absorb in the 400–450 nm range (Frisvad *et al.* 2004; Mapari *et al.* 2005).

Fourier Transform Infrared (FTIR) Spectroscopy

The FTIR spectrum of the pigment extract from *P. citreosulfuratum* strain KUMBASBT-53 revealed several characteristic absorption bands corresponding to diverse functional groups (Fig. 8). A broad peak at 3269 cm^{-1} indicated the presence of hydroxyl groups due to H-bonded O–H stretching, suggesting the involvement of phenolic or alcoholic compounds. The absorption at 2922 cm^{-1} was attributed to asymmetric and symmetric C–H stretching vibrations of methylene groups, confirming aliphatic moieties in the metabolite structure. A band at 2110 cm^{-1} corresponded to terminal alkyne ($\text{C}\equiv\text{C}$) stretching of monosubstituted compounds, while the absorption at 1633 cm^{-1} reflected N–H bending of secondary amines. Peaks at 1363 cm^{-1} and 1233 cm^{-1} indicated phenolic O–H bending and aromatic phosphate (P–O–C) stretching, respectively, suggesting the presence of phosphate-associated functionalities. Additional bands at 1016 cm^{-1} and 904 cm^{-1} were assigned to aliphatic phosphates and silicate ions, respectively, whereas the band at 509 cm^{-1} represented S–S stretching vibrations of polysulfides. Together, these functional groups highlight the chemically diverse nature of the pigment, which likely contributes to its stability, solubility, and biological activity. The occurrence of hydroxyl, phosphate, and phenolic groups is in line with earlier reports of fungal pigments exhibiting antioxidant and antimicrobial potential (Lin and Xu, 2020; Contreras-Machuca *et al.* 2022).

Orbitrap HR–LC/MS Analysis

The chemical profiling of the pigment extract from *P. citreosulfuratum* strain KUMBASBT-53 by Orbitrap HR–LC/MS revealed a total of 33

metabolites, comprising 17 in positive ion mode and 16 in negative ion mode, reflecting the diverse secondary metabolic repertoire of this fungus (Fig. 9). Among these, several peaks corresponded to known pigment-associated compounds, particularly azaphilones, coumarins, benzophenones, and polyphenolics, which are commonly reported in pigment-producing *Penicillium* species (Mapari *et al.* 2005; Frisvad *et al.*, 2008). In the positive ion mode, pigment-related molecules such as 3,5-di-tert-butyl-4-hydroxybenzaldehyde, benzophenone, and the antioxidant vitamin E acetate were detected, while late-eluting peaks at ~28.5–28.9 min were identified as Isochromophilone I and II, well-characterized azaphilone yellow pigments known for their distinct chromophoric properties (Caro *et al.* 2015). In the negative ion mode, classical polyketide-derived pigments including 4-hydroxycoumarin, 7-hydroxycoumarin (umbelliferone), and flavoglaucon were identified, the latter being a benzophenone-type pigment frequently associated with *Penicillium* spp. such as *P. crustosum* (Lu *et al.* 2024). Alongside these chromophores, additional metabolites such as fatty acid amides, organic acids, and lipid derivatives were recorded, which, although not pigments, may function as stabilizers or cofactors in pigment biosynthesis. The major pigment-related metabolites detected in both ionization modes are summarized in Table 2. Collectively, the metabolite profile strongly supports the yellow pigment-producing capability of *P. citreosulfuratum* strain KUMBASBT-53, with azaphilones, benzophenones, and coumarins forming the core of its pigment chemistry. These findings emphasize the metabolic diversity of soil-derived *Penicillium* species and align with earlier reports on their potential as natural sources of structurally diverse fungal pigments with promising industrial applications.

CONCLUSION

This study demonstrated that *Penicillium citreosulfuratum* strain KUMBASBT-53 is a distinct pigment-producing soil fungus, confirmed through polyphasic characterization integrating morphological, microscopic, and molecular analyses. The strain consistently synthesized extracellular yellow pigments, with spectral and

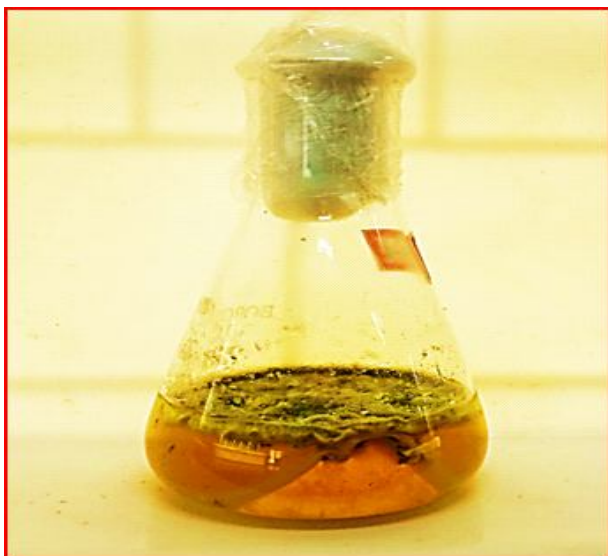


Fig. 3 : Growth of *P. citreosulfuratum* strain KUMBASBT-53 in PDB for 21 days

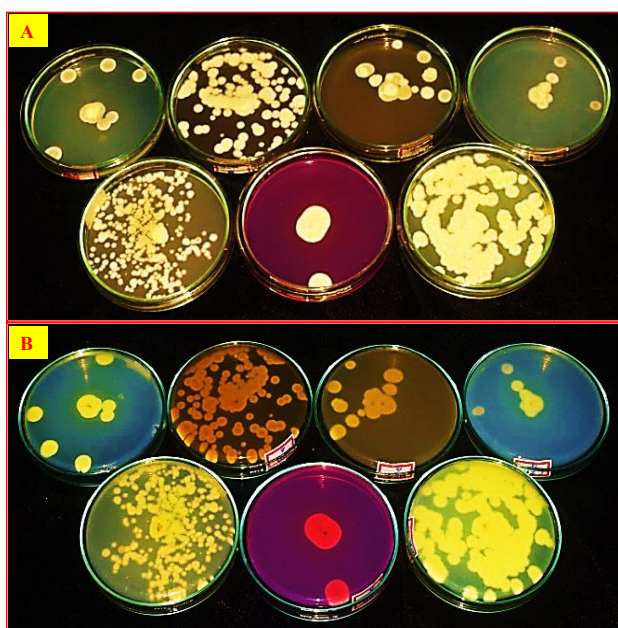


Fig.4 : Colony morphology of *P. citreosulfuratum* strain KUMBASBT-53 on different agar media (PDA, SDA, MEA, CZA, CA, RBA and RSA) (A) Anterior view (B) Converse view

functional group analysis (UV-Vis and FTIR) indicating polyketide-derived compounds. Orbitrap HR-LC/MS further revealed the presence of diverse pigment metabolites, including azaphilones (Isochromophilone I and II), coumarins, flavoglucan, and benzophenone derivatives, which are hallmark metabolites of pigmentogenic *Penicillium* species. The integration of phenotypic, genotypic, and metabolomic evidence not only validates the identity of KUMBASBT-53 but also underscores

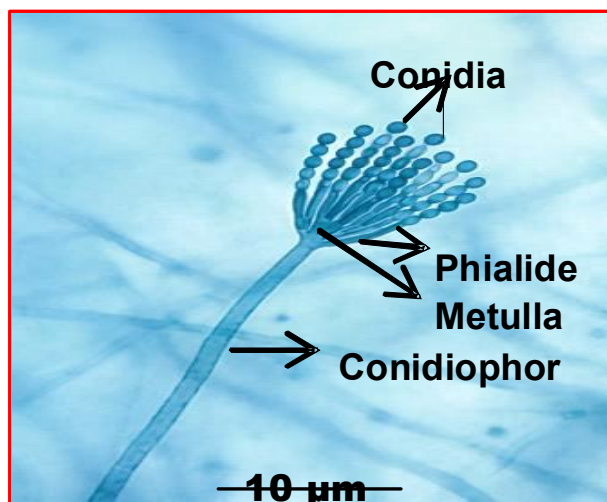


Fig. 5: Morphological features of *P. citreosulfuratum* strain KUMBASBT-53

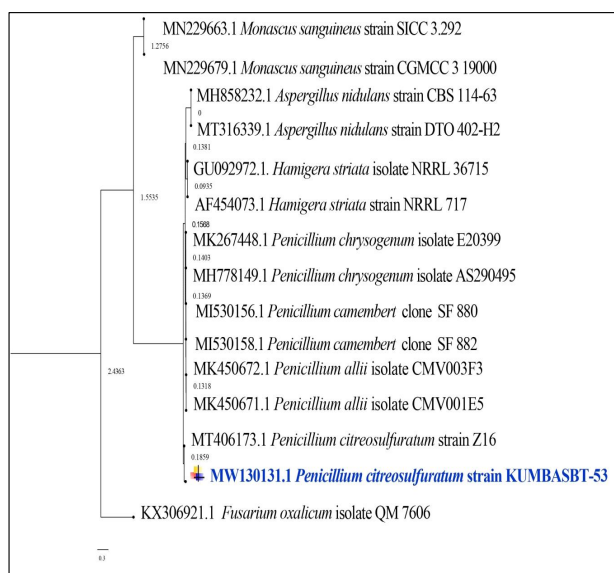


Fig. 6: Phylogenetic analysis of *P. citreosulfuratum* strain KUMBASBT-53 with the reference strains sequence data obtained from NCBI GenBank database

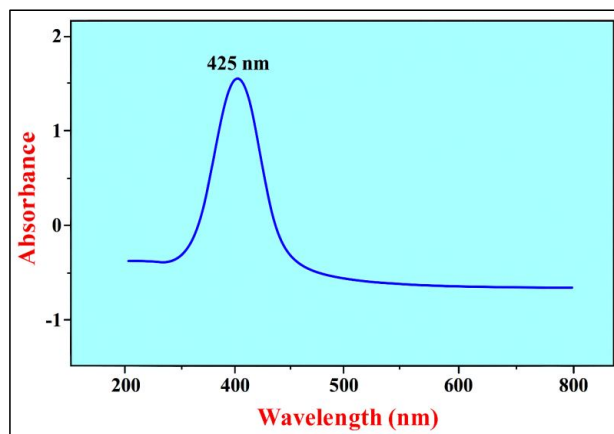


Fig. 7: UV-Vis spectrum of pigment from *P. citreosulfuratum* strain KUMBASBT-53 showing λ max at 425 nm

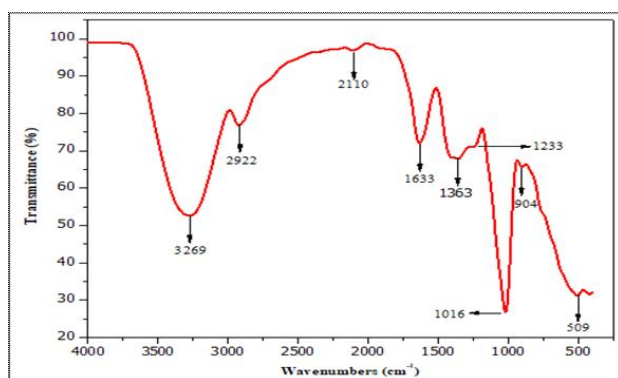


Fig. 8 : FTIR spectrum of pigment from *P. citreosulfuratum* strain KUMBASBT-53 with major absorption bands.

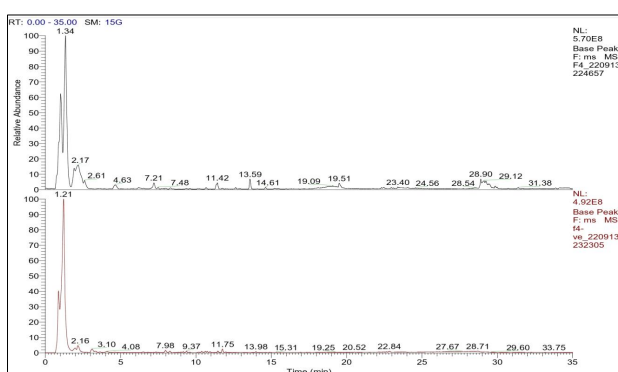


Fig. 9 : Orbitrap HR-LC/MS chromatogram of the pigment extract from *P. citreosulfuratum* strain KUMBASBT-53, showing representative peaks in both positive (top) and negative (bottom) ion modes

its metabolic potential. These findings establish the strain as a reliable candidate for natural pigment biosynthesis, providing a sustainable alternative to synthetic dyes and opening avenues for its application in food, pharmaceutical, and industrial biotechnology.

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DECLARATIONS

Conflict of Interest. Authors declare no conflict of interest

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