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Carotenoid pigments of *Kocuria flava* PUTS1_3 isolated from sediments of Puttalam lagoon mangrove ecosystem, Sri Lanka exhibit bioactive properties

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Microorganisms, inhabiting various ecological niches, exhibit a capacity to produce a diverse array of pigments with different shades. These colorful microbial pigments may also potentially possess beneficial bioactivities. This dual functionality together with the ease of mass production and downstream processing has shifted the global attention towards the use of microbially-derived pigments as bioactive colorants in different industries. Therefore, the present study was conducted with the aim of characterizing the pigments from *Kocuria flava* and identifying their potential biotechnological applications. The bacterium, PUTS1_3, was isolated using the surface sediment samples from the Puttalam mangrove ecosystem, Sri Lanka and it was identified as *Kocuria flava* using 16S rRNA gene sequencing. The yellow, intracellular pigment of PUTS1_3 was obtained by treating the cell pellet with methanol. Characterization of the pigment extract using UV-visible spectroscopy, TLC, and HPLC confirmed the presence of three carotenoid compounds, including β-carotene. The pigment extract also demonstrated antibacterial activity, against Gram positive bacteria tested. Antioxidant properties were observed with an IC_{50} value of 181.95 ± 4.57 µg/ml in the DPPH free radical scavenging assay. Although its sun protection factor was comparatively low (SPF 7.69 ± 0.01), the pigment showed promising results as a textile dye demonstrating good color performance and stability in washing and pH stability tests. Moreover, fabrics dyed with the pigment extract displayed antibacterial activity against *Staphylococcus aureus* (ATCC 25923). These findings suggest the potential use of the yellow pigments of *K. flava* PUTS1_3 for various biotechnological applications.

Keywords Antibacterial activity, Antioxidant activity, *Kocuria flava*, Mangrove bacteria, Microbial pigments, Textile dye

The application of colorants is a prevalent practice across numerous sectors, including food, cosmetics, pharmaceuticals, and textiles, for the purpose of optimizing the aesthetic appeal of finished goods[1,2]. Natural dyes, primarily derived from different plant sources, were extensively employed in various applications prior to the invention of synthetic pigments[3]. But, as synthetic dyes became more widely available and more affordable, the emphasis shifted away from natural colorants[4]. However, the increased consumer awareness regarding the detrimental effects associated with many of these synthetic colorants has re-shifted the global focus towards the utilization of natural colorants[3,5]. Among different sources of natural colorants, microorganisms, particularly those that live in marginal and challenging environments, stand out due to several advantages. The availability of relatively cost-effective production and extraction processes for microbial pigments, as well as the adaptability of microbial sources to be cultivated under controlled conditions using cheap raw materials have made microbial pigments a preferred candidate over other available natural pigments[6]. Moreover, microbial

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growth is independent of seasonal and geographical variations, ensuring the consistent availability of microbial pigments[2,6].

Isolated from various environmental niches, bacteria and fungi are widely known for their ability to create a diverse range of secondary metabolites including different types of pigments, such as carotenoids[7,8], melanin[9], quinones[10], violacein[11,12], and prodigiosin[13,14]. In addition to their aesthetically pleasing colors, many of these microbial pigments possess noteworthy bioactive traits that make them useful in a variety of industrial applications[3,15]. Pigments derived from *Monascus* species, which range in color from yellow to red, have been used for centuries as food colorants[16]. These *Monascus* pigments exhibit bioactive properties, including anticarcinogenic, antibacterial, and anti-obesity effects[17,18]. Similarly, the red-colored prodigiosin pigment, primarily produced by *Serratia marcescens*, is well-known for its wide range of biological activities. These include antimicrobial[19], antiparasitic[20], anticancer[21,22], and antimalarial activities[23]. To date, various microbial pigments have been evaluated for their bioactive potentials, revealing that many of these pigments possess significant bioactive properties.

Carotenoids that represent a compelling subset of these microbial pigments exhibit shades that range from yellow to red[24]. Beyond their pigmentation properties, carotenoids are known to play important biological roles in protecting microbial cells from UV irradiation and also by exhibiting inhibitory effects on competing microorganisms[25,26]. Furthermore, they participate in photosynthesis in some algae and bacteria[27], and most importantly, they are well-acknowledged scavengers of reactive oxygen species[28,29]. β-carotene-producing *Rhodotorula glutinis*[30], *Blakeslea trispora*[31], and *Phycomyces blakesleeanus*[32], as well as astaxanthin-producing *Haematococcus pluvialis*[33], are few of the well-studied microbial carotenoid-producers that are of significant commercial interest, especially as food colorants. Moreover, the proven provitamin A activity of carotenoids has made them be used as dietary supplements in some foods[24,34].

Kocuria flava is also a bacterium having the ability to produce bright yellow colored, intracellular pigment that is reported to have carotenoid-like properties[35]. Previous research has highlighted the antioxidant and antibacterial activities of this pigment derived from an airborne *K. flava*[35]. The yellow pigment produced by the soil-derived strain *K. flava* HO-9041 has demonstrated substantial dye uptake in wool and silk fabrics, with a notable antibacterial activity against *Bacillus subtilis*[36]. While these pigments have shown some promising traits to become potential coloring agents in industrial applications, they remain relatively understudied, warranting further investigation to fully understand their properties and possible uses.

Mangrove ecosystems are ecologically diverse, yet challenging environments, distributed throughout the intertidal zones of tropical and subtropical coastlines[37]. As a tropical island, Sri Lankan mangroves provide ideal reservoirs for a vast and diverse array of microorganisms with immense biotechnological potential. In the present study, sediment samples from the Puttalam lagoon mangrove ecosystem in Sri Lanka were used to isolate the yellow-pigmented *K. flava* PUTS1_3. Furthermore, the carotenoid pigments produced by the bacterium were analyzed to understand its potential to be used as a functional coloring agent with dual functionalities in future biotechnological applications.

Methodology

Isolation of the pigment-producing bacteria

Pigment-producing bacteria were isolated from top-most sediment samples collected from the sampling site in the mangrove ecosystem in Puttalam Lagoon, Sri Lanka (7°97′94.5″N 79°82′17.6″E) using standard plating techniques. Composite sediment samples were used to prepare a series of decimal dilutions. The initial suspension was made by suspending 10 g of sediment sample in a suspension medium (100 ml) prepared with sterile distilled water and sea water (1:1 v/v). Using sterile distilled water and sea water (1:1 v/v) solution as the diluent, a series of dilutions was prepared. Aliquots (0.1 ml) of diluted samples were plated using sea water – nutrient agar medium (sea water 50% v/v). Plates were incubated at 30 °C for 24–48 h, and selected colonies were further purified with three successive single colony isolations. Among the pigment-producing bacteria isolated, a yellow-pigmented bacterium labeled as PUTS1_3 was selected for further studies.

The preliminary characterization of PUTS1_3 was performed using standard morphological and biochemical characterization methods. The ability of PUTS1_3 to produce oxidase, catalase, and indole, as well as its ability to utilize glucose, citrate, and gelatin, was assessed using respective standard microbiological methods.

16S rDNA sequencing, and phylogenetic analysis of the bacterium

The genomic DNA of the bacterial isolate PUTS1_3 was extracted using Wizard® genomic purification kit (Promega, USA) as per the manufacturer's guidelines. PCR amplification of the 16S rDNA was done using fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') universal primer pair[38]. The PCR reaction mixture contained the template DNA (5 µl), PCR master mix (Promega, USA) (12.5 µl), fD1 and rD1 primes (5 µM of each), and nuclease-free water (2.5 µl). The amplification profile consisted of an initial denaturation at 94℃ for 10 min, 30 cycles of denaturation (94℃ for 30 s), annealing (53℃ for 30 s), and extension (72℃ for 90 s) followed by a final extension at 72℃ for 10 min. The purified PCR product was sequenced using Sanger dideoxy-chain termination method with universal primer pair, 27 F (5'-AGAGTTT GATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') at Macrogen, Korea. BioEdit V 7.2.5 was used for the sequence alignment[39] (http://bioedit.software.informer.com/). The resulting 16 S rDNA sequence was compared with available sequences in the GenBank sequence repository (https://www.ncbi.nlm.nih.gov/genbank) through BLAST search. Neighbour-joining algorithm with 1000 re-sampling bootstrap test was selected to construct a phylogenetic tree using MEGA-X (Version 10.2.6) software[40].

Extraction of the yellow pigment

A seed culture of PUTS1_3 was prepared by inoculating a nutrient broth supplemented with 1.5% (w/v) D-glucose and incubating overnight on a rotatory shaker at 100 rpm. After the incubation, an aliquot (10 ml) of the overnight-grown seed culture was transferred into a sterile nutrient broth (150 ml) supplemented with 1.5% (w/v) D-glucose. Flasks were incubated at 30℃ on a rotary shaker operated at 100 rpm for 5–7 days[41].

Bacterial cells with intracellular pigments were separated by centrifugation (11,000 g for 20 min) followed by the removal of the supernatant. Cell pellets were extracted using six different solvents, including methanol, ethanol, acetone, dichloromethane, chloroform, and hexane. Based on the extraction efficiencies, methanol was selected as the solvent for the subsequent pigment extractions. To optimize the extraction process, flasks were incubated in a water bath at 60 °C overnight[42]. Pigment was separated from cellular debris by centrifuging at 11, 000 g for 10 min. The extracted pigment was collected into a clean vial and filtered through a 0.45 µm millipore membrane to remove the remaining cell debris after the centrifugation. The methanol was evaporated by rotary evaporation and the dried pigment was stored at -20℃ until further analysis.

Purification and characterization of the yellow pigment

UV-visible spectroscopy

UV-visible spectrophotometric analysis was carried out to identify the characteristic features of absorption spectrum of the yellow pigment extract. The pigment was dissolved in methanol, and absorption spectrum was recorded using a UV-visible spectrophotometer (Thermo Scientific™ Multiskan™ GO Microplate Spectrophotometer) within the wavelength range of 400 nm to 700 nm.

Thin layer chromatography (TLC)

The concentrated pigment was applied onto a TLC plate (silica gel 60 F_{254}), and the plate was placed in a pre-saturated chamber with a solvent system of butanol: ethanol: water at a ratio of 9:1:2. The developed TLC plate was illuminated under a UV lamp at wavelengths of 254 nm and 356 nm. The retention factor (R_f) value was calculated using the equation R_f = d/D (where 'd' is the distance traveled by the spot, and 'D' is the distance traveled by the solvent front).

High- performance liquid chromatography (HPLC)

The pigment extract was analysed employing high-performance liquid chromatographic (HPLC) separation, utilizing a reversed-phase column (C18 4.6 × 150 mm × 3.5 µm) from Agilent Technologies, USA. The separation was conducted at an oven temperature of 35 °C, employing a mobile phase comprising acetonitrile: water (90:10), and eluted at a flow rate of 0.6 ml/min. Detection was performed using a PDA detector with a detection wavelength of λ = 450 nm[43].

Isolation, purification, and identification of pigment compounds

To purify the pigment extract, and isolate its constituents, column chromatographic analysis was performed using silica as the stationary phase, featuring 60 Å pore size and 200 mesh size. The concentrated sample was loaded onto a column and subjected to elution using a solvent gradient of increasing polarity, progressing from hexane, through dichloromethane, to methanol. A total of 290 fractions (1 ml each) were collected into clean glass tubes at a rate of 1 ml/min, and each colored fraction was analysed using thin layer chromatography to confirm its purity. Fractions with identical R_f values were combined. The relatively non-polar compound was then analysed using the same HPLC protocol previously described. The resulting chromatogram was compared with the HPLC chromatogram of standard β-carotene (Sigma-Aldrich, USA).

Determination of the biological activities of the yellow pigment

Antimicrobial activity

The agar well diffusion method was employed to determine the antibacterial activity of methanolic pigment extract against six bacterial test cultures, namely *Staphylococcus aureus* (ATCC 25923), *Bacillus subtilis* (ATCC 6633), *Listeria monocytogenes* (NCTC 11994), *Salmonella enterica* (ATCC 14028), *Escherichia coli* (ATCC 25922), and *Pseudomonas aeruginosa* (ATCC 27853), as well as against three fungal cultures, *Candida albicans* (ATCC 10231), *Aspergillus niger* and *Sclerotinia sclerotiorum*[44] on Mueller-Hinton agar and potato dextrose agar respectively. *A. niger* and *S. sclerotiorum* used in this study are local isolates that were isolated in previous studies. *S. sclerotiorum* was previously studied and characterized[44]. *A. niger* was isolated from onions infected with black mold. The isolate is well-characterized in terms of morphology, and multi locus phylogeny and conformed to be *A. niger*. Further the isolate is fully characterized using whole genome sequence data of an on-going study (Attanayake et al., Unpublished data).

Briefly, using a sterile corkborer, uniform wells (~ 8 mm) were prepared on agar plates that were inoculated separately with standardized test bacterial cultures (~ 1×10^8 CFU/ml; $OD_{600} \approx 0.4$). For antifungal assay, wells were prepared on plates inoculated with *C. albicans* (~ 1×10^6 CFU/ml; $OD_{600} \approx 0.2$) and *A. niger* spore suspension (~ 1×10^6 spores/ml) separately. For *S. sclerotiorum*, a mycelial disk (6 mm in diameter) was aseptically placed at the center of the plate. Wells were filled with 100 µl of methanolic extract of the pigment (4000 µg/ml). Chloramphenicol (20 µg/ml) and carbendazim (100 µg/ml)[45] were used as the positive control for bacteria and fungi respectively, while methanol was used as the negative control. Antibacterial activity of the pigment extract was determined by measuring the zone of inhibition against each test bacterial culture after 24–48 h of incubation at 30℃. The zone of inhibition against fungal cultures was measured after incubating the plates at 25℃ for 3–5 days[46].

Antioxidant activity

Antioxidant activity of the pigment extract was analyzed by the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging assay using butylated hydroxytoluene (BHT) as the reference. The pigment was dissolved in methanol and 160 µl of different concentrations of the sample (1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1024 µg/ml) were mixed with 40 µl of methanolic DPPH solution. After incubating the mixture for 30 min in the dark, absorbance was measured at 517 nm[47]. The percentage inhibition was calculated as per the equation given below.

Percent inhibition = $((A_0 - A_1)/A_0) \times 100$; where A_0 = absorbance of the control at 517 nm and A_1 = Absorbance of the sample at 517 nm.

The concentration that provides 50% inhibition (IC_{50}) was determined using the plot of percentage inhibition versus concentrations of the samples[48,49].

Determination of the biotechnological potentials of the pigment

Sun protection factor (SPF)

The pigment extract was dissolved in methanol (2 mg/ml) for spectral analysis. Absorbance spectra were measured using a UV-Vis spectrophotometer within the range of 290–320 nm (UV-B region) at 5 nm intervals. A commercially available sunscreen product was used as the reference sample. In vitro sun protection factor (SPF) was calculated using the Mansur mathematical equation[50,51]:

$$\mathrm{SPF} = CF \times \sum_{320}^{290} \mathrm{EE}(\lambda) \times \mathrm{I}(\lambda) \times \mathrm{Abs}(\lambda)$$

Where, CF = correction factor (10), EE (λ) = erythmogenic effect of radiation with wavelength λ, Abs (λ) = spectrophotometric absorbance values at wavelength λ. The values of EE x λ are constants[50,51].

Application as a fabric colorant

To assess the suitability of the yellow pigment extract as a fabric colorant, ten different fabric types, namely poplin, satin, tetron cotton, polyester, challenger, muslin cotton, grey cloth, koshibo, raw silk, and georgette, were selected. Prior to dyeing, all fabrics were treated with a solution of sodium carbonate (0.5% w/v) and soap water (2 g/l) to remove impurities on fabrics. It was kept at 60 °C for 1 h (scouring process). Subsequently, the fabrics were thoroughly rinsed with water at room temperature to remove any residual scouring agents[52]. To optimize the dye uptake and color durability, fabric-specific pre-mordanting was used. Wool, silk, and other protein fibers were treated with a pre-mordant solution containing alum (15% WOF (Weight of Fibre); i.e., 15 g of alum was used for every 100 g of fabric dyed.)[53] and cream of tartar (6% WOF). Fabrics were immersed in a mordanting bath at 85 °C and it was kept at that temperature for 1 h. For cotton and other cellulose fibers, only an alum solution (15% WOF) was used. Fabrics were soaked in the mordanting bath at 87–93 °C and kept for 45 min. Both sets of fabrics were allowed to cool in the mordanting bath before dyeing. Subsequently, fabrics were dyed with the pigment extract as per the method described by Sastrawidana et al.[54]. with some modifications. Fabrics were immersed in the dye bath at a 1:20 material-to-liquor ratio (MLR) and kept in the dye bath at 70–80 °C for 1 h. The unbound pigment was removed by washing with water at room temperature. Finally, the fabrics were air-dried[54].

Colorfastness properties of dyed fabrics

pH stability The resistance of the dyed fabrics to varying pH environments was evaluated by treating them with buffer solutions spanning the range of approximately pH 1 to ~14. Fabrics were immersed in the buffer solutions for 5 min, and visual changes of the color were recorded.

Washing Stability Washing durability of dyed fabrics was assessed following the method described by Yang et al. (2018). Fabrics were treated with a soap solution containing 5 g/l soap powder at a 1:50 material-to-liquor ratio (MLR) and the soap bath containing fabrics was maintained at 60 °C for 30 min[55].

Light stability Light fastness property of dyed fabrics was tested by exposing them to direct sunlight for 7 days. Visual assessment was used to detect any color changes.

Antibacterial activity of dyed fabrics

The antibacterial efficacy of dyed fabrics against *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) was assessed following the method described in ISO 20645:2004 standard[56,57]. Briefly, 25 ± 5 mm diameter circular samples of the dyed fabrics were placed on double-layer agar plates. The bottom layer contained tryptone soy agar (TSA) (10 ± 0.1 ml), while the top layer contained TSA (5 ± 0.1 ml) inoculated with the test bacterium at a concentration of $1–5 \times 10^8$ CFU/ml. Plates were incubated at 37 °C for 18–24 h[56,57]. The level of antibacterial activity was assessed as per the following formula, H = (D- d)/2; where, H = inhibition zone in mm, D = total diameter of fabric and inhibition zone in mm and d = diameter of the fabric in mm. The results were interpreted in accordance with the guidelines outlined in the ISO 20645:2004 standard (Table 1).

Results and discussion

Isolation and characterization of yellow-pigmented bacterium

Sediment samples obtained from the Puttalam mangrove ecosystem in Sri Lanka were used to isolate pigmented bacteria. Bacterial colonies with different morphologies were observed on inoculated sea water-nutrient agar plates. Among the different bacterial isolates, a yellow-colored colony with an irregular shape, and entire margins, was selected and further purified using the single colony streak isolation method for future analysis

Average diameter of the inhibition zone (mm)	Growth underneath the cloth	Description	Assessment
> 1	None	Inhibition exceeding 1 mm, no growth underneath the cloth	Good effect
1 – 0	None	Inhibition zone up to 1 mm, no growth underneath the cloth	
0	None	No inhibition zone, no growth underneath the cloth	
0	Slight	No inhibition zone, only some restricted colonies, growth nearly totally suppressed	Limited effect
0	Moderate	No inhibition zone, compared to control growth reduced to half	Insufficient effect
0	Heavy	No inhibition zone, compared to the control no growth reduction or only slightly reduced growth	

Table 1. Interpretation of results of the test for the antibacterial activity of fabrics according to the ISO 20645:2004 standard.

(Fig. 1A). The isolate was labeled as PUTS1_3 and was identified as a Gram positive coccus arranged in clusters using Gram's staining. PUTS1_3 exhibited the ability to produce catalase, while it was unable to utilize citrate or gelatin (Table 2). No acid and gas production were observed during glucose utilization. Furthermore, PUTS1_3 lacks the ability to produce cytochrome C oxidase and was unable to convert tryptophan into indole.

Molecular identification of PUTS1_3 was done using partial 16S rRNA gene sequencing. The 16S rRNA sequence of PUTS1_3 can be accessed under the GenBank accession number OQ442354.1. According to the BLAST searches, PUTS1_3 exhibited the highest sequence similarity to *Kocuria flava* 1WO (identity%– 98.26%, query coverage– 100%, E-value– 0). Further, the 16S rRNA gene sequence of the PUTS1_3 was compared with available 16S rRNA gene sequences of different *Kocuria* spp., and their phylogenetic relationship was inferred using a neighbor-joining phylogenetic tree (Fig. 1B). Based on the database searches and phylogenetic analysis, the isolate PUTS1_3 was identified as *Kocuria flava*. The genus *Kocuria*, belonging to the family Micrococcaceae, is composed of Gram positive, non-endospore-forming, catalase positive, cocci bacteria. *K. flava* has been found in various ecological niches, including marine ecosystems[58–61] and recognized for their versatile metabolic functions such as thermotolerant lipase production[62], inhibition of aquaculture pathogens[59], anti-fouling activity[63] and for plant growth promotion ability[64]. However, studies related to *K. flava* pigments, and their applications are scarce.

Extraction of yellow pigment of PUTS1_3

Yellow pigment production was facilitated by culturing them in nutrient broth supplemented with an additional 1.5% (w/v) D-glucose and was incubated under shaking conditions for 5–7 days to enhance the pigment yield. D-glucose is an important carbon source that was found to have a significant effect on biomass and pigment production in microorganisms[65]. A substantial increase in the zeaxanthin pigment yield by *Arthrobacter gandavensis* MTCC 25325 was observed when supplemented with 1.5% (w/v) glucose[41]. Therefore, in the present study, culture broths were additionally supplemented with 1.5% (w/v) D-glucose to enhance the pigment production. The cell pellet with intracellular pigments resulted after the centrifugation of the bacterial culture was treated with different organic solvents separately to identify the suitable solvent with maximum pigment extraction efficacy. The maximum pigment extraction was observed with the methanol among the tested solvents (methanol, ethanol, acetone, dichloromethane, chloroform, and hexane), suggesting that the target pigments possess polar characteristics. For example, zeaxanthin, a well-known polar carotenoid, exhibits a high affinity for polar solvents like methanol[66]. Therefore, the preferential extraction of the yellow pigment of PUTS1_3 in methanol aligns with this observation, indicating the possible presence of polar carotenoids in the pigment extract.

Purification and characterization of the yellow pigment

Thin-layer chromatography (TLC) analysis of the pigment extract revealed three distinct spots with corresponding R_f values of 0.88, 0.78, and 0.61 (Fig. 2A). The result suggests the presence of multiple components within the pigment extract, each exhibiting different mobilities on the chromatographic plate due to their varied polarities. The three compounds with R_f values of 0.88, 0.78, and 0.61 were labeled as compound 1 (C1), compound 2 (C2) and compound 3 (C3) respectively. Aligning with the results obtained in this study, the orange-colored pigment extract of marine *Kocuria* sp. RAM1 was reported to contain three compounds with 0.4, 0.6, and 0.73 R_f values[67].

The UV- visible spectrum of the pigment extract in the methanol solvent exhibited a shoulder-like peak pattern with three broad bands at 409 nm, 437 nm, and 466 nm (Fig. 2B). This "three-finger" shape spectrum is a characteristic feature of carotenoid-like pigments due to the presence of long chains of conjugated double bonds[68,69]. Consistent with our findings, a strain of *K. flava* isolated from Saudi Arabia exhibited comparable absorption patterns, with a maximum absorption peak at 437 nm[35]. Yellow pigment extracted from *K. palustris* isolated from Northeastern Brazil Caatinga soil also exhibited shoulder peaks with three absorption maxima at 415.5 nm, 439.7 nm, and 468.9 nm[43].

The HPLC chromatogram of the pigment extract contained three notable peaks at specific retention times, specifically at 1.69 min, 3.23 min, and 3.47 min for C3, C2, and C1 compounds respectively (Fig. 2C). This confirmed the presence of three distinct compounds with different polarities within the analyzed pigment extract, each exhibiting a unique elution time during the chromatographic separation. In agreement with the above observation, three individual compounds were isolated from the pigment mixture using silica gel column

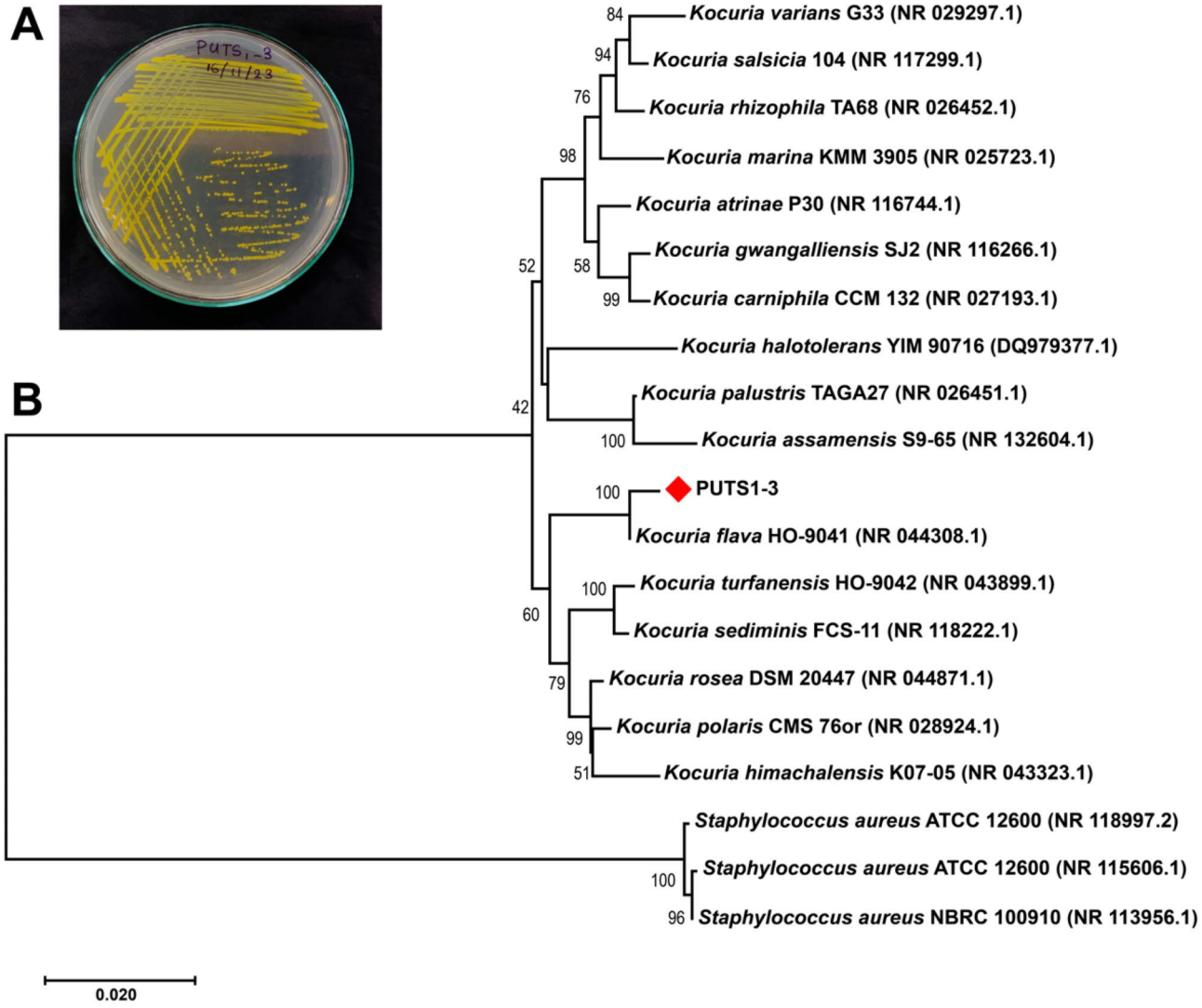

Fig. 1. Colony characteristics and phylogenetic relationship of PUTS1_3.
Colony morphology of PUTS1_3 on nutrient agar after 24 h of incubation at 30 ℃ (**A**). The evolutionary relationship of bacterial isolate PUTS1_3 was inferred using a neighbor-joining method based on the partial 16S rRNA gene sequences. PUTS1_3 used in this study is indicated with a diamond (♦). Bootstrap support values from 1,000 replicates are shown next to the branches. *Staphylococcus aureus* was used as the out-group of the analysis. The scale bar represents 0.02 nucleotide substitutions per character (**B**).

Biochemical/ Morphological Characteristic	Result
Gram's reaction	+
Cell shape	Coccus
Motility	–
Presence of oxidase	–
Presence of catalase	+
Indole production	–
Glucose utilization	–
Oxidation fermentation	Oxidative
Citrate utilization	–
Ability to liquefy gelatin	–

Table 2. Morphological and biochemical characteristics of PUTS1_3. (+) Positive result (–) Negative result.

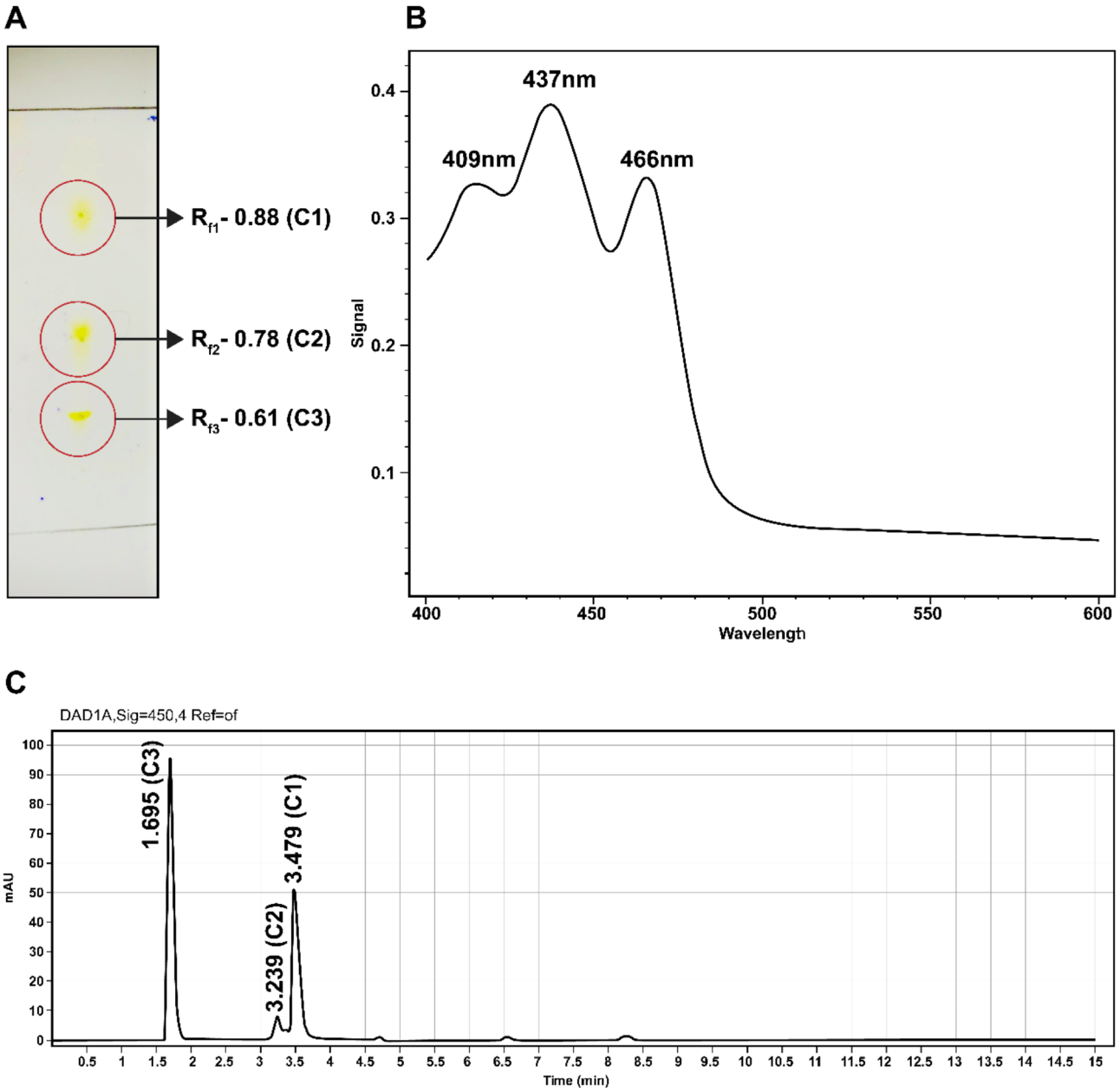

Fig. 2. Characterization of the yellow pigment extracted from PUTS1_3.
Three separate spots were observed on the TLC plate using a butanol: ethanol: water (9:1:2) solvent system as the mobile phase (**A**), while three distinct absorption maxima were observed in the UV spectrum at 409 nm, 437 nm, and 466 nm wavelengths (**B**). Three distinct peaks were observed on the HPLC chromatogram (**C**).

chromatography. The separated fractions with similar R_f values were pooled and analyzed on a TLC plate alongside the crude pigment extract.

Carotenoids can be categorized into two main groups: carotenes, which are composed of long hydrocarbon chains, and xanthophylls, which are more polar than carotenes due to the presence of oxygen atoms in their structure[70]. α-carotene, β-carotene, lycopene, and phytoene are the main carotenes, while fucoxanthin, astaxanthin, lutein, zeaxanthin, and β-cryptoxanthin represent the most abundant xanthophylls[27]. A symmetric C_{40} phytoene backbone forms the basis of more than 95% of all naturally occurring carotenoids, with very few C_{30} and even fewer C_{50} carotenoids known to exist[71]. *Kocuria* sp. RAM1 was reported to produce three carotenoids, and two of them were identified as C_{50} carotenoids, characterized by 13 conjugated double bonds and the presence of a hydroxyl group. In nature, C_{50} carotenoids are primarily known to be produced by bacteria belonging to the order *Actimomycetales*, such as sarcinaxanthin, a C_{50} carotenoid is produced by *Micrococcus lutes*[71]. Sarcinaxanthin is identified as a relatively polar xanthophyll pigment. Further, *K. palustris*, isolated from Northeastern Brazil Caatinga soil, also expressed the sarcinaxanthin pigment[43]. Based on the literature, it can be suggested that the two relatively polar compounds present in the pigment mixture of *K. flava* PUTS1_3

(C2 and C3), may possibly belong to the C_{50} carotenoid group. Apart from these two compounds, a relatively non-polar compound (C1) was also present in the pigment extract of PUTS1_3. The HPLC chromatogram of this compound was compared with those of a β-carotene standard and a purified C1 spiked with β-carotene standard (Fig. 3). The overlapping chromatograms confirmed the identity of the relatively non-polar compound C1 present in the pigment extract as β-carotene. While a previous study has reported the β-carotene production by *K. carniphila* MY and *K. polaris* MO[72], this is the first to identify β-carotene in the pigment extract of *K. flava*. In addition, other microorganisms, such as *Blakeslea trispora*[73,74], *Citricoccus parietis*[75], and *Paracoccus homiensis*[76], are known to produce β-carotene.

Determination of biological activities of the crude pigment extract

Antimicrobial activity

The antimicrobial activity of the yellow pigment extract of PUTS1_3 was analyzed using the standard well-diffusion method against 6 bacterial strains and 3 fungal strains (Fig. 4). All Gram positive bacterial strains used in the analysis were inhibited by the pigment extract, while the highest antibacterial activity was observed against *L. monocytogens* with 17.00 ± 00 mm inhibition zone followed by *S. aureus* (15.55 ± 0.32 mm) and *B. subtilits* (14.89 ± 0.16 mm). However, no inhibition zones were observed for Gram negative bacterial strains used in the study for the concentration of pigment tested (4000 µg/ml). Further, the pigment extract failed to exhibit any anti-fungal effect against tested fungal strains at the given pigment concentration. Some researchers have reported a higher sensitivity of Gram positive bacteria to carotenoid pigments compared to Gram negative bacteria[76]. This result may be due to the challenges in penetrating the lipopolysaccharide cell membranes of Gram negative bacteria. Usually, lipopolysaccharide membranes can selectively prevent the entrance of active compounds such as antibiotics[77]. Therefore, the reduced sensitivity of Gram negative bacteria to carotenoids may be due to the difficulties faced by the pigments in passing through the cell membranes. Accordingly, a carotenoid pigment isolated from *Micrococcus* spp. has demonstrated antimicrobial activity against *Staphylococcus aureus* and *Streptococcus faecalis* but lacks the efficacy against *Escherichia coli*[25].

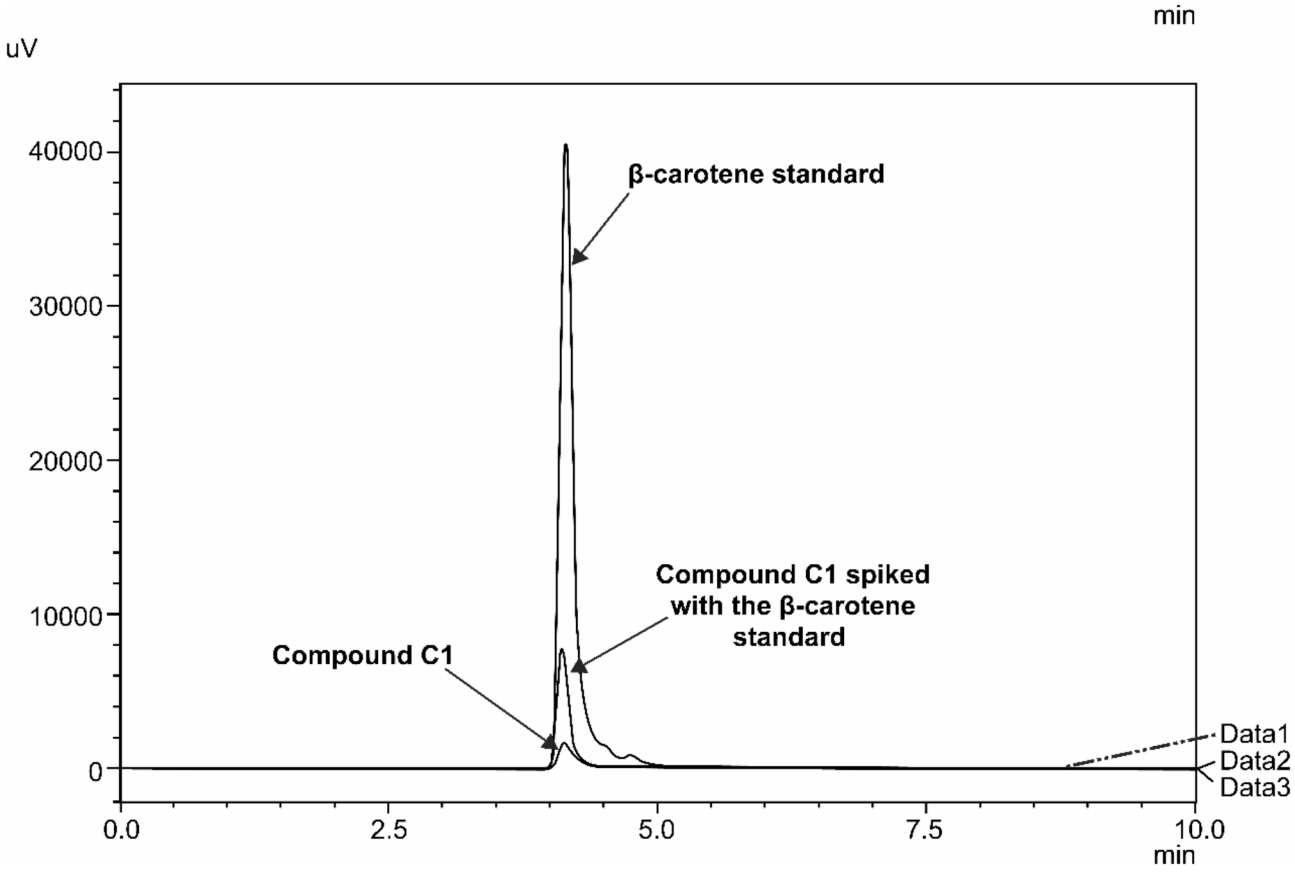

Fig. 3. Comparison of HPLC chromatograms of the relatively non-polar compound C1 from the pigment extract, β-carotene standard, and spiked sample.
The HPLC chromatograms of the non-polar compound C1 from the pigment extract, the β-carotene standard, and the spiked sample show overlapping peaks with retention times of 4.124, 4.139, and 4.110 min, respectively.

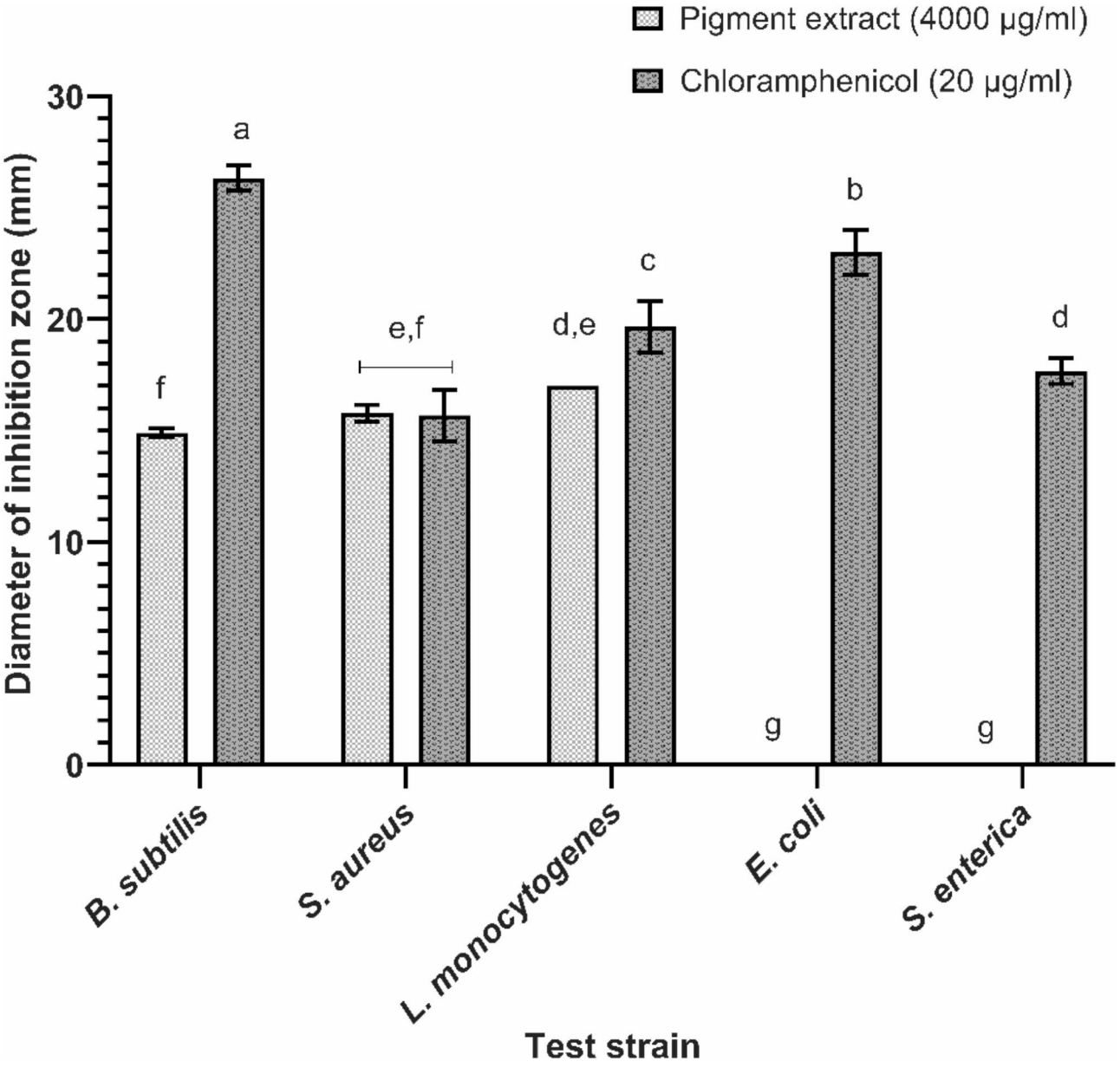

Fig. 4. Antibacterial activity of the pigment extract of PUTS1_3.
The graph shows the average inhibition zone diameters (mm). Inhibition zones of pigment extract (4000 µg/ml) were observed against *S. aureus*, *L. monocytogenes* and *B. subtilis*. Chloramphenicol (20 µg/ml) was used as the positive control, while methanol was used as the negative control. Error bars represent standard error of mean ($n = 3$). The bars that do not share same lower case letter are significantly different from each other (Tukey's test < 0.05).

Antioxidant activity

Yellow pigments, particularly carotenoids, are well-known for their antioxidant activity. Antioxidant carotenoids, like astaxanthin and lycopene are useful in the food industry, not only to impart an appealing color to foods but also to revolutionize the food preservation procedures by extending the shelf life of these foods[78,79]. Additionally, anti-inflammatory properties of these carotenoids hold a promise for developing natural alternatives to synthetic preservatives in cosmetics[80]. Therefore, the antioxidant potential of the yellow pigment extract was evaluated using the DPPH free radical scavenging assay using BHT as the reference. The DPPH free radical scavenging activity of the pigment extract increased gradually in a dose-dependent manner and reached a plateau with 80% inhibition around 600 µg/ml (Fig. 5A). However, BHT reference exhibited a rapid increase in percentage inhibition with the increasing concentration, reaching a plateau around 200 µg/ml. The IC_{50} values for the yellow pigment extract and BHT were calculated as 181.95 ± 4.57 µg/ml and 14.91 ± 0.16 µg/ml, respectively.

In a previous study, the IC_{50} value for the pigment produced by *K. marina* DAGII was found to be 53.76 µg/ml, which was notably lower than the 146.56 µg/ml value of β-carotene, a well-known antioxidant, measured in

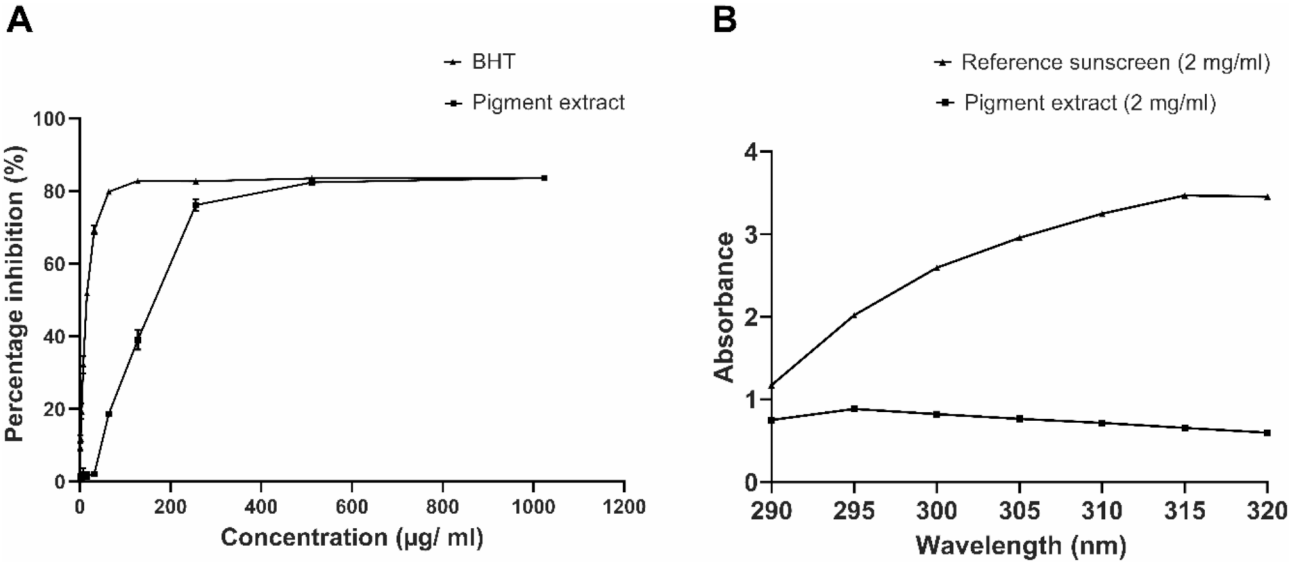

Fig. 5. Bioactivities of pigment extract of PUTS1_3.
DPPH free radical scavenging activity of pigment extract and BHT control (**A**). Absorption values of the pigment extract ((2 mg/ml) and reference sunscreen (2 mg/ml) at different wavelengths at UV-B region (**B**). Data in all tests are expressed as mean ± SD ($n = 3$).

the same study suggesting the efficient free radical scavenging activity of *K. marina* pigment than the β-carotene standard[81]. The β-carotene pigment isolated from the novel yeast strain *Sporidiobolus pararoseus* DAGIII was also found to possess a higher IC_{50} value of 449.11 µg/ml[82]. However, the observed antioxidant activities of pigments can be influenced by various factors beyond their inherent structure. These include the source of the pigment, the extraction protocol employed, the choice of solvent, and any downstream processing steps that have a huge impact on pigment deterioration and thereby the bioactive potential[83,84].

Determination of biotechnological potentials of the pigment extract

Sun protection factor (SPF)
Harmful solar radiation comes mainly from ultraviolet (UV) rays, divided into UV-A, UV-B, and UV-C. While UV-B causes sunburns, UV-A penetrates deeper, promoting skin aging and contributing to skin cancer development[50]. While individual preferences for sun protection may vary, sunscreen is a widely recommended and accessible method used to protect the human skin from damages caused by UV rays[85]. The efficacy of sunscreens is measured by Sun Protection Factor (SPF) value. SPF is defined as the UV energy required to cause a minimal erythema dose (MED) on protected skin, divided by the UV energy required to produce a MED on unprotected skin[51]. Proper application of SPF 15 sunscreen offers 93% protection, while SPF 30 sunscreen offers 97%, and SPF 50 sunscreen offers 98% protection against harmful UV radiation[86]. According to the European Commission's Recommendation 2006/647/EC, sunscreen products can be categorized into four categories based on their SPF, which measures how well they block UV-B rays. As per the recommendations, sunscreens with measured SPF values of 6-14.9 offer low protection, 15-29.9 offer medium protection, 30-59.9 offer high protection, and 60 ≤ offer very high protection[87,88].

The applicability of the yellow pigment extract of PUTS1_3 in the cosmetic industry to formulate sunscreens was calculated according to the Mansur mathematical equation. The absorbance values of the pigment extract and the reference sunscreen were recorded in the UV-B region. The UV spectrum of the crude pigment revealed the increased absorption for the first 5 nm from 290 nm to 295 nm and it decreased continuously until 320 nm. Instead, the reference sunscreen exhibited a continuous increase in the absorption from 290 nm to 320 nm (Fig. 5B). The results of our study demonstrated that the methanolic extract of the yellow pigment (2 mg/ml) exhibited mild sun protection, with a measured SPF value of 7.69 ± 0.01. In comparison, the reference sunscreen displayed a significantly higher SPF value of 28.54 ± 0.02. To our knowledge, this is the first report on the sunscreen potential of *K. flava*. However, similar observations were made with pigments obtained from two *K. palustris* isolates, FT-7.22 and FT-5.12, which had low SPF values of 9.36 ± 0.52 and 8.66 ± 0.23, respectively[43]. Although these pigments exhibit low sun protection (SPF < 14.9) based on their SPF values, they can be used as a natural additive to enhance the sun protection potential of sunscreen formulations. Furthermore, their antioxidant ability could potentially help in mitigating the damaging effects of reactive oxygen species (ROS) generated by UV radiation[89].

Application as a fabric colorant
The exploration of microbial pigments as fabric colorants holds significant promise within the textile industry due to their potential to offer sustainable and environmentally friendly alternatives to traditional synthetic dyes.

A comprehensive study was conducted on the use of the yellow pigment extract of *K. flava* PUTS1_3 as a fabric colorant with ten different commercially available fabrics: representing both natural and synthetic fibers. Each fiber type exhibited a distinct color performance, as illustrated in Fig. 6A. Despite these variations, the resulting color tones were uniformly of high visual quality across all fiber types. The attachment of pigments to fibers may depend on the fiber type, fiber properties, the nature of carotenoids, and the role of mordants in enhancing binding[90]. Carotenoids, being hydrophobic molecules, may interact with fibers through van der Waals forces and hydrophobic interactions, with natural fibers like cotton or wool potentially exhibiting better retention when pretreated with mordants[91]. Similarly, Lawsone, a naphthoquinone pigment extracted from henna plant binds with polyester fabrics through hydrogen bonding, van der Waals forces, and dipole-dipole interactions[92]. These mechanisms enhance the affinity and stability of the interaction between the pigment and fabric molecules.

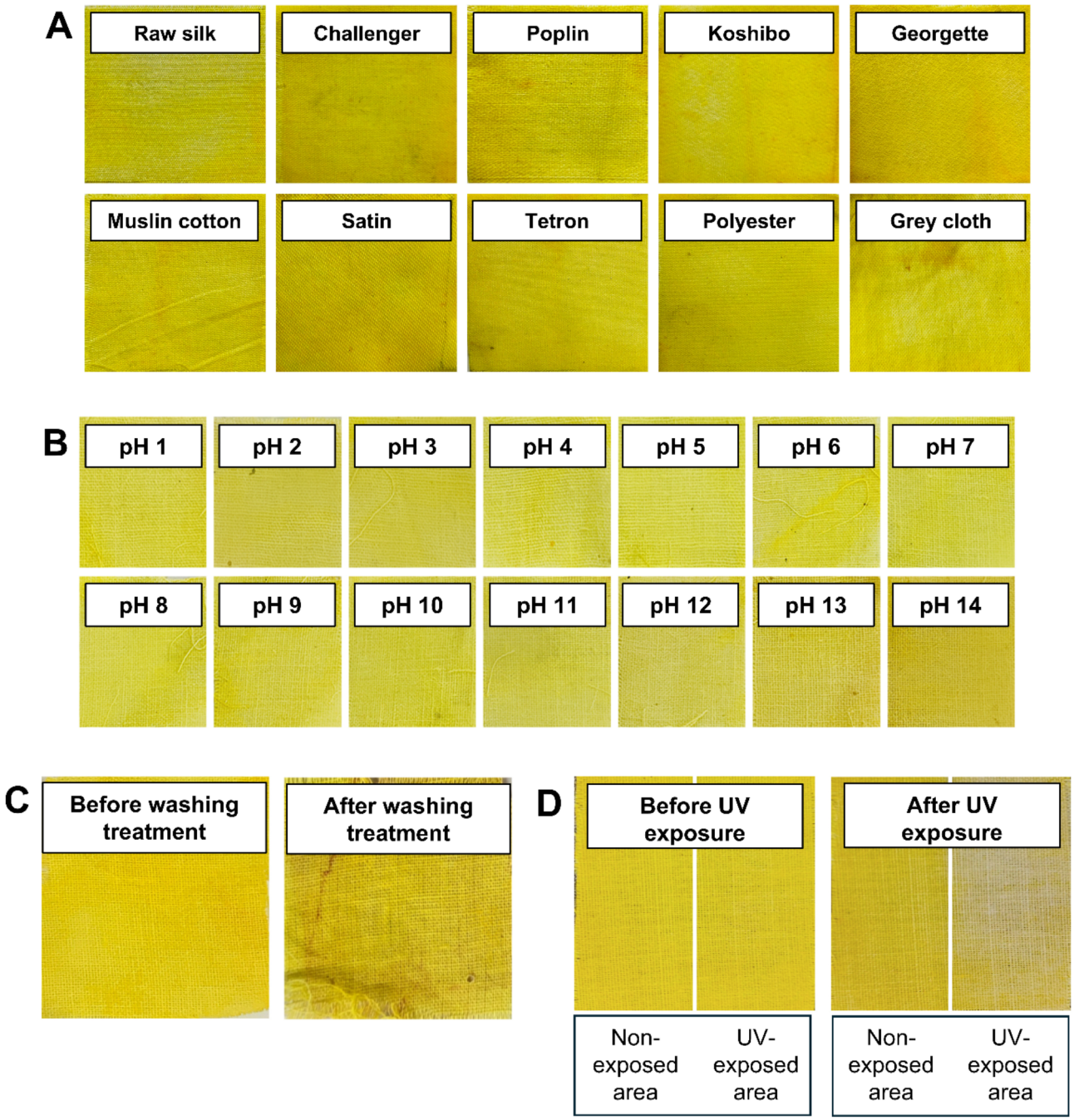

Fig. 6. Application of yellow pigment extract of PUTS1_3 as a fabric colorant and its color fastness properties. Ten different fabric types were dyed with the pigment extract (**A**). The color stability of the dyed fabric was observed after different pH treatments (**B**) and after the washing treatment with cold water (**C**). A color fading effect was observed after the exposure to UV radiation for 7 days (**D**).

	S. aureus	E. coli
Muslin cotton	0 mm zone diameter with no growth under the cloth **(Good effect)**	0 mm zone diameter with slight growth under the cloth **(Limited effect)**
Raw silk	0 mm zone diameter with no growth under the cloth **(Good effect)**	0 mm zone diameter with slight growth under the cloth **(Limited effect)**
Control	0 mm zone diameter with a considerable growth **(Insufficient effect)**	0 mm zone diameter with a considerable growth **(Insufficient effect)**

Table 3. Antibacterial activity of fabrics dyed with yellow pigment extract based on the ISO 20645:2004 method.

Subsequent tests conducted to determine the pH stability (Fig. 6B) and washing fastness (Fig. 6C) of dyed fabrics did not lead to any considerable color loss in the dyed fabrics. The degradation of the dyed fabrics' color became apparent with prolonged exposure to UV light (Fig. 6D), likely due to the inherent UV sensitivity of carotenoids[93]. This limitation could potentially be mitigated through the incorporation of suitable stabilizers. A similar study carried out with the pigment from *K. flava* HO-9041 found that the silk and wool fabrics dyed with the yellow pigment exhibited a good dye uptake with excellent resistance to rubbing and moderate stability when washed and exposed to light[36]. Further research is needed to explore and identify effective stabilizers for enhancing the lightfastness of the pigment extract.

Antibacterial activity of dyed fabrics

Functional fabrics with antibacterial activity hold significant value in healthcare settings and hygiene-critical applications, offering protection against the spread of harmful microorganisms and promoting improved hygiene[94,95]. Hence, the yellow pigment extract's ability to impart antimicrobial properties to dyed fabrics was assessed according to the method described in the ISO 20645:2004 standard[56,57]. Both synthetic (raw silk) and natural fabrics (muslin cotton) dyed with the yellow pigment extract were used to evaluate the antibacterial properties after the dyeing process. Undyed fabrics were used as negative controls. Results were interpreted based on the ISO protocol. No bacterial growth was observed underneath the dyed muslin cotton and raw silk fabrics against *S. aureus* and no inhibition zone surrounding the fabrics was observed. For *E. coli*, a slight growth was observed underneath both fabric types with no clear zones around the fabric indicating its limited efficacy against Gram negative bacteria like *E. coli*. Undyed control fabrics showed considerably more bacterial growth compared to dyed fabrics (Table 3). Although no previous studies are available on the antibacterial activity of fabrics dyed with *K. flava* pigment, some research has highlighted the antibacterial effects of fabrics dyed with microbial pigments, such as prodigiosin. Consistent with our findings, silk fabrics dyed with the red pigment derived from *Serratia marcescens* ATCC 8100 showed maximum antibacterial activity, with rates of 25.12% against *E. coli* and 97.17% against *S. aureus* when the pH of the dye liquor was adjusted to 2.1[96]. These findings, together with our results, highlight the potential use of microbial pigments in the future functional fabric industry.

Conclusion

Here we report that a Gram positive soil bacterium, *K. flava* PUTS1_3, isolated from the sediments of the mangrove ecosystem associated with the Puttalam lagoon, Sri Lanka, produced yellow-colored pigments, consisting of three carotenoid compounds, including β-carotene. The pigment extract exhibited antibacterial activity against Gram positive bacteria tested. Further, the yellow pigment extract demonstrated free radical scavenging ability, suggesting its potential as an antioxidant. Despite exhibiting a lower SPF value, the pigment's antioxidant properties hold a promise for sunscreen formulations. These properties could potentially neutralize free radicals (ROS) generated during UV damage, enhancing the overall photoprotective effect. Although, as a textile dye, the pigment displayed a good color uptake and stability against washing and pH treatments, UV-induced fading remains a challenge. Further research is needed to explore possible strategies for the improvement of lightfastness properties of dyed fabrics. Notably, the pigment showed selective antibacterial activity against Gram positive bacteria on dyed fabrics, highlighting the need for further exploration of its mechanism and potential applications in the functional fabric industry. The findings of this study greatly contribute to the growing interest in utilizing natural pigments from microbial sources for various biotechnological applications.

Data availability

The 16S rRNA gene sequence of the bacterial isolate PUTS1_3 can be accessed under the GenBank accession number OQ442354.1.

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Author contributions

Authors confirm the contribution to the final manuscript as follows: K.E.W.: Methodology, Formal Analysis, Investigation, Data curation and interpretation, Visualization, Writing: Original draft; A.T.K.: Formal Analysis, Investigation; R.N.A.: Data curation and interpretation, Validation, Resources, Supervision, Writing: Review and editing; C.S.K.R.: Conceptualization, Methodology, Data curation and interpretation, Validation, Resources, Supervision, Writing: Review and editing; A.P.H.: Conceptualization, Methodology, Data curation and interpretation, Validation, Resources, Supervision, Project administration, Funding Acquisition, Writing: Review and editing. All authors have read and agreed to the submitted version of the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

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