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Research Article

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Production and Partial Characterization of Yellow Pigment Produced by *Kocuria flava* Isolate and Testing its Antioxidant and Antimicrobial activity.

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Abstract: Recently, Natural pigments produced great interest within the market and made need to find eco-friendlier and less hazardous pigments than synthetic colorants, particularly microbial pigments. This work was undertaken with an aim of isolating pigment producing bacteria from air, then production and extraction pigment from isolated bacteria with antioxidant and antimicrobial properties against pathogenic microorganisms. Bacterium was identified by morphological characteristics and 16S rRNA sequencing as *Kocuria flava* SIF3, submitted to GenBank (accession number MN162713). Pigment production was carried out using nutrient broth medium (NB). Yellow pigment extracted by using methanol as solvent, showed maximum absorption spectrum (λ_{max}) at 437 nm by UV-VIS spectroscopy and the degree of cell pigmentation was 0.472. Partial characterization of the extracted yellow pigment using UV- VIS spectroscopy and Fourier-Transform Infrared Spectroscopy analysis (FTIR) revealed it to be a carotenoid which has numerous applications. This novel extracted yellow pigment was screened for antimicrobial activity against pathogenic and antioxidant activity. This particular pigment showed inhibitory effect against all tested microbes so it could be chosen as a broad spectrum antimicrobial agent. The maximum zone of inhibition was (20 mm) against *Penicillium sp.* then *Pseudomonas aeruginosa* (12mm). The yellow pigment was discovered to have DPPH radical-scavenging activity with an IC50 value of 1.25mg / ml in vitro antioxidant assay. Present findings highlight the significance of yellow pigment extracted from *Kocuria flava* SIF3. Further purification of pigment will be useful in discovering a promising drug.

Keywords: *Kocuria flava*, yellow pigment, characterization, antimicrobial, antioxidant, carotenoids.

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1. INTRODUCTION

Color is an integral part of both human culture and in human life. Colors have been used to enhance the aesthetic value of everyday human life. It is the most important characteristic of food, clothes in everyday life. Objective of color in food is to make things appealing and recognizable[1].Natural pigments are preferred over synthetic colorants due to the potential health hazard effects[2]. This is due to the harmful effects of synthetic pigments on humans and the environment[3].The new found perception in human protection and environmental reservation has instigated novel enthusiasm for colors from natural sources. Dyes or natural colorants resulting from living organisms are thought to be harmless due to non-carcinogenic, non-toxic and naturally biodegradable[4]. Natural pigments refer to pigment products of plants, animals, insects, microorganisms and microalgae. Natural pigments extracted from microorganisms have been used for the pharmaceutical, textile and food industry for decades. Many microorganisms have been exploited for the production of color[1]. Microbial pigments are a promising alternative to other color additives which are extracted from animals or vegetables as they are considered natural, show a higher productivity and give no seasonal production problems. The importance of biocolors from microorganisms is gaining importance due to their ability of inhibiting mutagenesis, tumor, photo oxidation and ability to enhance immune system[5].Microorganisms have become a substantial source for pigment production due to high growth rate, cheap culture medium, and easy extraction process; all these offer more advantages for microorganisms than for other biological resources[3]. The various types of pigments produced by microorganisms are carotenoids, melanins, flavins, monascins, violacein and indigo[6]. These colorants also show properties such as antioxidant activity, antimicrobial activity, anti-coagulating property and food preservation capability[7]. In addition, biopigments can be implemented in other related medical purpose[8], like anti-tumor and anti-cancer treatments or as indicators like stains and markers in laboratory research works[9,10]. Often they might also possess rich nutritional value so as to be used as dietary supplements[11] or as food additives[12]. Among these, carotenoids are the largest and most complex class of natural colors with over 700 structures identified in plants, algae, fungi and bacteria[13]. Today, microbial carotenoids are exploited as the major source for pigment production owing to its availability. It also confers several biological properties which are advantageous to humans. They act as provitamin A as well as a chemoprotective agent in biological systems[14]. In addition, they also show antioxidant and antimicrobial properties[15,16].Considering the scope of microbial-derived pigments, the present investigation was carried out with an aim to isolate and identify pigment-producing bacteria from natural habitat. Production and extraction of pigment from isolated bacteria, then partially characterized to evaluate the antioxidant activity and antimicrobial activity against human pathogenic microbes.

2. MATERIALS AND METHODS

2.1 Chemicals and media

Nutrient broth (NB) and nutrient agar (NA) used for screening, isolation and production studies were procured from Al-Rowad Modern Est. Glycerol, DPPH (2,2-diphenyl-1-picrylhydrazyl) and Chemicals were obtained from Al-Rowad Modern Est, Saudi Arabia. All other chemicals used throughout the analysis were of analytical reagent grade.

2.2 Screening, isolation and purification of pigment-producing bacteria

Pigment-producing bacteria are isolated from various samples collected from different locations in Saudi Arabia. The isolated strain of our study was isolated from air on NA medium with 5% glycerol and incubated at 37^{0}C for 24-48 hrs. Pigment producing bacteria were recognized by the presence of colored colonies (diffusible) in agar plates. Pigmented bacterial isolates were purified by streaking onto NA plate with 5% glycerol and it was incubated for 24 hrs at 30-37 ^{0}C. It was sustained on NA slants at 5°C until further use.

2.3 Identification of potential pigment-producing bacteria

The isolate producing higher intensity of color pigmentation selected and identified according to their morphological, cultural, biochemical tests[17] and the strain was validated by 16S rRNA gene sequencing analysis which was carried out by Special Infectious Agents Unit - Biosafety Level 3, King Fahd Medical Research Center, Saudi Arabia

2.3.1 16SrRNA sequencing of the potent isolate

The potent isolate was screened for 16S rRNA molecular sequencing in Special Infectious Agents Unit - Biosafety Level 3. Sequencing of the generated PCR products was performed by direct sequencing using the ABI Prism BigDye Terminator Cycle Sequencing Kit v3.1. The cycle sequencing reaction was performed on Veriti® 96-Well Thermal Cycler (Applied Biosystems USA). The 16S forward primer was5′-GGTTACCTTGTTACGACTT -3' and16S reverse primer 5′-AGAGTTTGATYMTGGCTCAG -3' where Y is C or T. The sequencing reaction consisted of 1.75 µl Sequencing Buffer (5X), 0.32µl of primer (3.2µM),2µl sequencing RR and a varying volume of water based on the volume of the purified PCR product. The concentration of the PCR product for sequencing was measured by absorbance at 260 nm on a BioSpec-nano (Shimadzu, USA). The amount of the PCR product added to the sequencing reaction is based on its size (5-20 ng for fragments 500-1000bp in size and 10-40 ng for fragments 1000- 2000bpin size). The cycle sequencing reaction was performed in both the forward and reverse directions. Internal primers were used sometimes in sequencing of RSV type A because of it had long fragment length. The cycle sequencing condition in Veriti® 96-Well was as the following: the initial denaturation at 96°C for 1 min, repeat the following for 25 cycles: 96°C for 10 sec, 50°C for 5 sec and 60°C for 4 min then hold at 4°C until used to purify using Sephadex with MultiScreen-HV Filter Plate (0-45um).Nucleotide sequences were compared with GenBank database(http://www.ncbi.nlm.nih.gov/) by using the program of BLASTN followed by sequence alignment. A phylogenetic tree was developed by the neighbor-joining algorithm[18,19].

2.4 Pigment production

NB has been used for preparation of inoculum and production of pigment. Loop full of culture was added to 50 ml NB in 250 ml flasks, then incubated at 30 ^{0}C on a rotary

shaker at 200 rpm for 24hrs. One ml of inoculum size was transferred to 250 ml Erlenmeyer flask containing 50 ml of NB then incubated at 30 °C on a rotary shaker at 150 rpm for 48 hrs[20]. The medium was centrifuged at 5000 rpm for 15 min after incubation period to separate the cells pelt, then suspended in distilled water and again centrifuged to collect the supernatant. One milliliter culture diluted to 10 ml by 25% sodium chloride solution to measure growth (OD) at 660 nm using a spectrophotometer[21].

2.5 Extraction of pigment and determination of its absorption spectra

The culture medium was harvested and centrifuged to remove cells at 5000 rpm for 10 min then supernatant was discarded. Cell pellets were resuspended by using deionized water, followed by centrifugation 5000 rpm for 10 min to recover the cells by discharging the supernatant again. The 10 ml of methanol (with a purity of 99.7%) was added to the cell for extracting bacteria pigment and the mixture was vortexed, extracted twice with the same solvent. The mixture of the cells and methanol was treated by ultra sonication (59 KHz, 35-40 °C, 90 min) and kept overnight in light protection. The pigment extract was separated by centrifugation at 5000 rpm for 10 min and filtered by whatman filter paper. Pigment extract was analyzed by using absorbance scan in wavelength region of 200-700 nm using Genesys 10s UV-VIS spectrophotometer. The solvent was evaporated to dryness in evaporator at 40 °C and crude pigment was collected in screw cap bottle and kept light protected at -20 °C[20,21]. The pigmentation degree of cell was calculated according to the ratio of OD of carotenoid to OD of the growth ($OD\lambda_{max}/OD660$)[20].

2.6 Partial Characterization of Pigment

2.6.1 UV-Vis Spectrometric Analysis of Pigment

Pigment solution has been analyzed by using UV-VisSpectrometry with 200-700 nm ranges of wavelengths of light, to Find the highest pigment absorption peaks. The specification of the instrument has been Genesys 10s UV-VIS spectrophotometer.

2.6.2 Fourier-transform infrared spectroscopy (FTIR)

The structural analysis of the extracted pigment was done using FTIR analysis, where an infra-red spectrum helped in identifying the presence and position of various functional groups of the pigments. The analysis was carried out by using the infrared spectra (KBr disks) in the range of 4000 to 400 cm^{-1} that was recorded on Perkin- Frontier spectrometer (USA)

2.7 Determination of antioxidant activity of extracted pigment

DPPH (1, 1diphenyl-2picrylhydrazyl) accepts an electron to form a stable diamagnetic molecule. The violet color DPPH methanolic solution has a high UV absorbance at 517 nm. The odd electrons of DPPH radical pairs with a reducing agent presence in this methanolic solution, then the solution stoichiometrically fade color and the solution absorbance decreases at 517 nm. The crude methanolic pigment extract was aliquoted into different concentrations (0.312, 0.625, 1.25, 2.5 and 5 mg/ml) for determining its ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals using Brand-Williams *et al.,*[22] method with slight alterations. One milliliter of 0.1mM DPPH solution in methanol was mixed with one milliliter of pigment extract solution by using various concentrations (0.312, 0.625, 1.25, 2.5 and 5 mg/ml). Corresponding blank samples were prepared and L-Ascorbic acid (20, 40, 60, 80 and 100 µg/ml) was used as reference standard. One ml methanol and one ml DPPH solution was mixed and used as control. The reaction was conducted in triplicate and UV-Vis spectrophotometer was used to measure the decrease in absorbance at 517 nm after 30,60, 90 and 120 min in dark. Inhibition % was calculated by using the following formula.

$$\text{Scavenging activity (\%)} = Ac\text{-}As/Ac\times100$$

Where Ac is the absorbance of the control and As is the absorbance of the sample.

2.8 Antimicrobial activity of yellow pigment extract

The antimicrobial activity of the yellow pigment was tested by Agar-well Diffusion Method. Briefly 20 ml of NA for bacteria and Potato Dextrose Agar (PDA) media for fungi were poured into the Petri-dish and 6 mm well bored in the agar. 100 µL of extract with different concentrations (7.5, 15 and 30 mg/ml) were dissolved in methanol and poured into the wells , methanol has been used as control . The plates were incubated for 24 hrs for bacteria and 48-72 hrs for fungi at 37°C, the zone of inhibition was measured in mm[23]. The experiment was done in triplicates and the average was calculated .Five pathogens belonging to both gram positive and gram negative groups such as *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli* and *Pseudomonas aeruginosa,* in addition to four fungi ,*Candida albicans, Aspergillus niger, Aspergillus flavus and Penicillium sp* were used in this study as test pathogens.

3. RESULTS AND DISCUSSION

3.1 Pigment producing bacteria isolation and identification

Isolation, screening, and identification of the bacteria-producing pigment five strains from a total of fifty single colonies were chosen from specimens isolated from different locations from Saudi Arabia. Based on their morphological variations (color) in agar plates. Finally, SIF3 strain was selected as one of the yellow pigment producers according to the highest intensity of extracted pigment,[24]in the wavelength region of 400-600nm as analyzed by the spectrophotometer, was selected for further studies. The spectral scan of the pigment produced by the most promising organism is represented in Fig. (1). It produced light yellow pigment and showed a λmax at 437nm indicating that the pigment produced may be a carotenoid Bacteria producing pigment were screened on the morphology of the colony (color). SIF3 colonies were light

yellow smooth circular slightly convex with entire edges, it was gram positive, aerobic, non-motile and coccoid cells and catalase positive (Table (1) and Fig. 2 (a and b)). According to 16S rDNA sequence, the nucleotide sequence was placed under the MN162713 accession number in the GeneBank. The phylogenetic tree was constructed using the neighbor-joining approach in view of the 16S rDNA sequence Fig.(3). The almost complete 16S rRNA gene sequence of SIF3strain was compared by BLAST to the other bacterial strains similar sequences of database in the GenBank. Results of BLAST clarified that SIF3 strain was most similar to *Kocuria flava* (100% similarity). The phylogenetic tree has been established by the neighbor-joining algorithm Fig. (3). The topology of the tree, backed by high bootstrap esteems, clearly showed that *Kocuria flava* strain is inside the Kocuria genus. In view of the16S rDNA sequence data analysis and morphological characteristics, SIF3 was described as *Kocuria flava* MN162713.

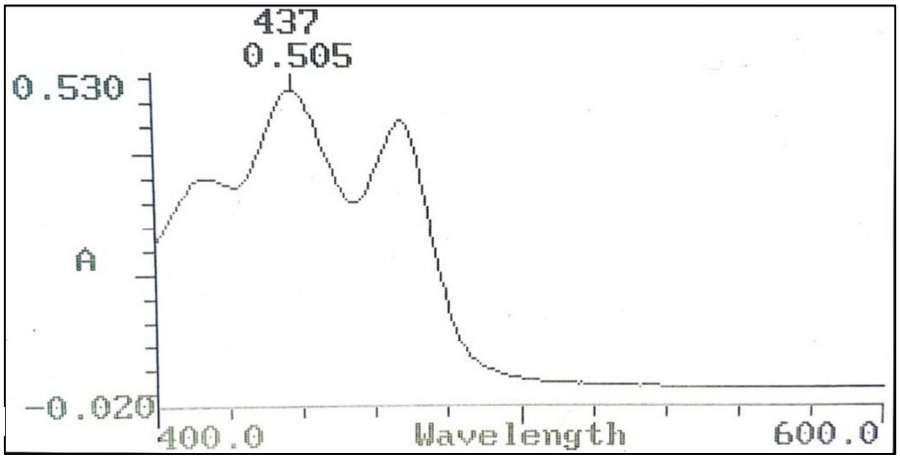

Fig.1. Light absorption spectra of yellow pigment produced by SIF3 isolate in methanol

Table 1. Morphological, cultural and physiological characteristics of SIF3 isolate

Character	Result
Gram's stain	Positive
Cell shape	Cocci
Motility	Non-motile
Colony shape	Light yellow smooth circular slightly convex colonies with entire edges .
Oxygen requirement	Aerobic
Catalase production	Positive

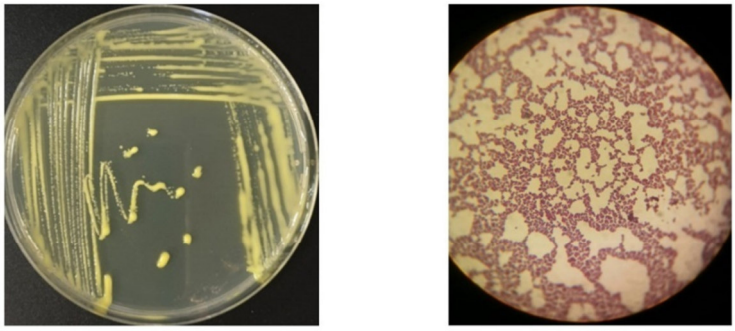

Fig 2. Pigment producing bacteria isolated from air

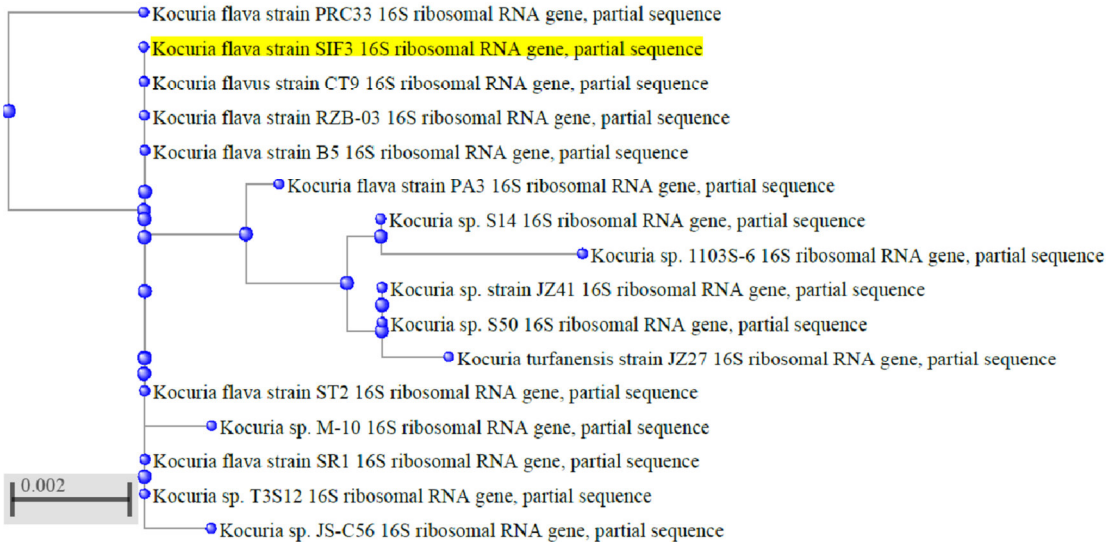

Fig 3. Phylogenetic tree of *Kocuria flava* SIF3obtained by neighbor-joining analysis of ITS region of 16 rDNA

3.2 Bacteria pigments production

After extraction of yellow pigment from isolated strain SIF3 with methanol (99.7%) the crude pigment extracted showed light yellow colour in Fig.(4). The productivity of crude pigment extract was 16.23 mg/g cells and 80 mg/l, while the cell pigmentation degree was determined according to the ratio ($OD\lambda_{max}/OD660$). The extracted pigment of the isolated bacteria strain had $OD\lambda_{max}$ at 437 nm (0.505) and OD of cell growth at 660 nm was (1.070), while the degree of cell pigmentation was (0.472).

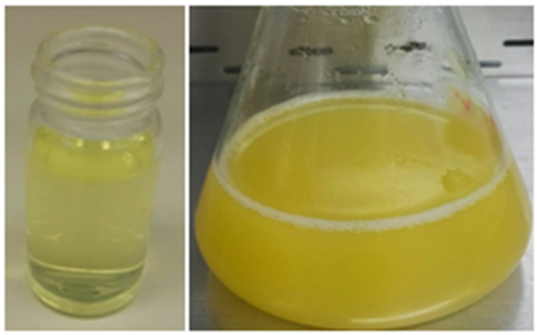

Fig 4. Extraction of yellow pigment from SIF3 isolate

3.3 Characterization of pigment

3.3.1 UV-Vis spectrometric analysis of pigment

Methanolic extract of yellow pigment was scanned in a visible region where it was seen to have three-peak spectra shown in Fig. (5). The three-peak spectrum is a characteristic feature of a carotenoid. The extracted pigment λ_{max} (maximum absorbance)appeared at 437 nm. The main reason for greater pigment absorptions at longer wavelengths was the effects of electron conjugation[25].Yellow Carotenoid pigments show three distinctive peaks within the range of absorption from 400 to 500 nm[26].

3.3.2 Fourier-transform infrared spectroscopy (FTIR)

FTIR spectra of crude pigment gave the prominent peaks at 2923.67, 2853.25, 1638.26 and 1460.36 cm^{-1} as depicted in Fig. (5) Other peaks were observed at 1150.04, 1074.92, 991.50 and 3435.84 cm^{-1}. Comparatively very less literature is available on bacterial carotenoid pigment using FTIR spectroscopy. Broad peak ranging from 3200-3500 cm^{-1}, indicative of OH group, which may also help explain why pigment in ethanol and methanol is extremely soluble (3435.84 cm^{-1}). Alkane stretching peaks were exhibited in the range of 2800-2950 cm^{-1}. Bands at 2923.67 cm^{-1} are due to asymmetrical stretching vibration of aliphatic CH group while at 2853.25 cm^{-1} are due to asymmetrical stretching vibration of the same group as is interpreted by Latha and Jeevaratnam[27]. The peak at 1460.36 cm^{-1} may be due to asymmetrical deformation vibration of CH3 groups. The C = C bonding was also observed as a characteristic of the pigment with a peak at 1600-1680cm^{-1}. Bands at 1638.26cm^{-1}may be due to the presence of the olefinic functional group. However the complete structure of compounds cannot be determined based on IR data. Vibrational peaks are most likely due to oxidation and/or deformation in polyene chain[28].

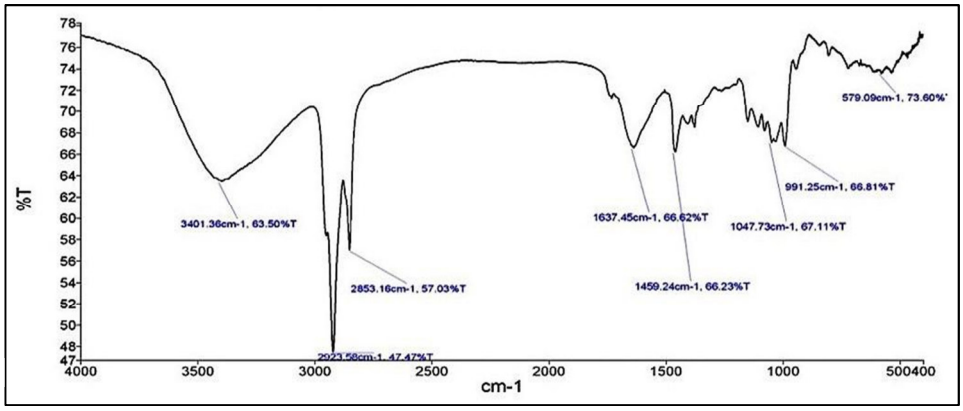

Fig 5. FTIR spectral scan of pigment produced by *Kocuria flava* SIF3

3.4 Scavenging activity of pigment using DPPH

Antioxidant activity test was based on the capability to quench DPPH, a stable free radical, and thus decolorize in the presence of antioxidants resulting in reduced absorbance values. Yellow pigment antioxidant activity at distinct levels (0.312, 0.625, 1.25, 2.5 and 5 mg/ml) was assessed as free radical DPPH scavenging and its outcomes are shown in Figure (6). It is evident that the largest scavenging activity for yellow pigment was found at 1.25 mg / ml for 90 min.(85.8%) followed by 2.5mg / ml for 120 min. (85.3%), 5.0mg / ml for 90 min. (83.9%) ,0.625mg / ml for 120 min. (63.4%) and 0.312mg/ml for 120min (52.5%). The yellow pigment was discovered to have DPPH radical-scavenging activity with an IC50 value of 1.25mg / ml *in vitro* antioxidant assay. Ascorbic acid (20, 40, 60, 80 and 100 µg/ml) was used as reference standard. Figure(7). The highest producing carotenoid *Exiguobacterium sp* isolated from soil and air was selected by Sasidharan *et al.,*[29]for scavenging activity which was about 70%. Carotenoid is usually potential for antioxidants, several times in vivo, because of the pro-oxidant effect they lack these properties[30]. Nevertheless, in antioxidant and anticarcinogenic properties, carotenoid compounds play a significant role[31].Mercy and Aruna[24] illustrated that the antioxidant assay of carotenoids produced by *K. flava* majod showed the IC50 value to be 3.2 (mg/mL). In another study, the carotenoid pigments isolated from *M. roseus* and *M. luteus* showed significant UV protective nature and antioxidant IC50 value of 3.5-4.5 mg/ml[32]. The difference in IC values was related to the presence of acyclic carotenoids with both large numbers of conjugated double bonds and of hydroxyl groups in the major carotenoid[33].The results of this investigation suggested that the pigment extracted from SIF3 isolate can be taken for industrial purposes after purification and chemical characterization processes.

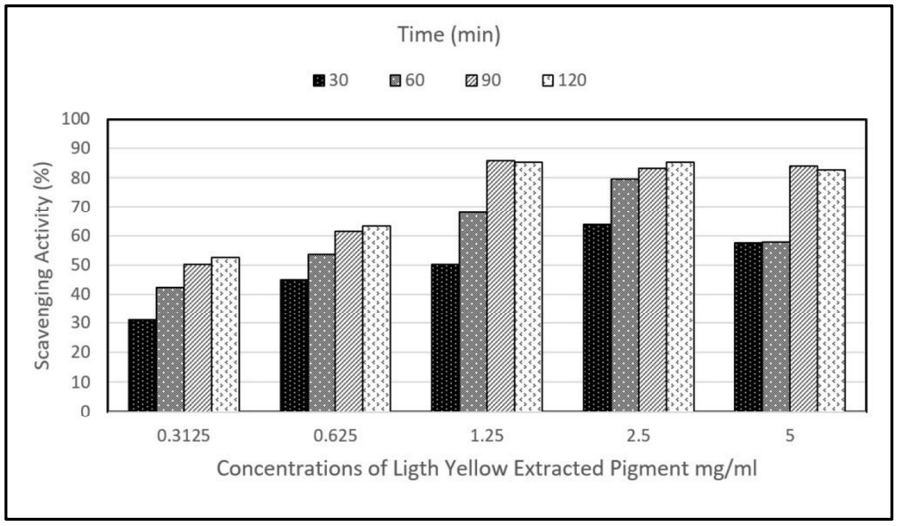

Fig 6. DPPH scavenging activity of light yellow extracted pigment

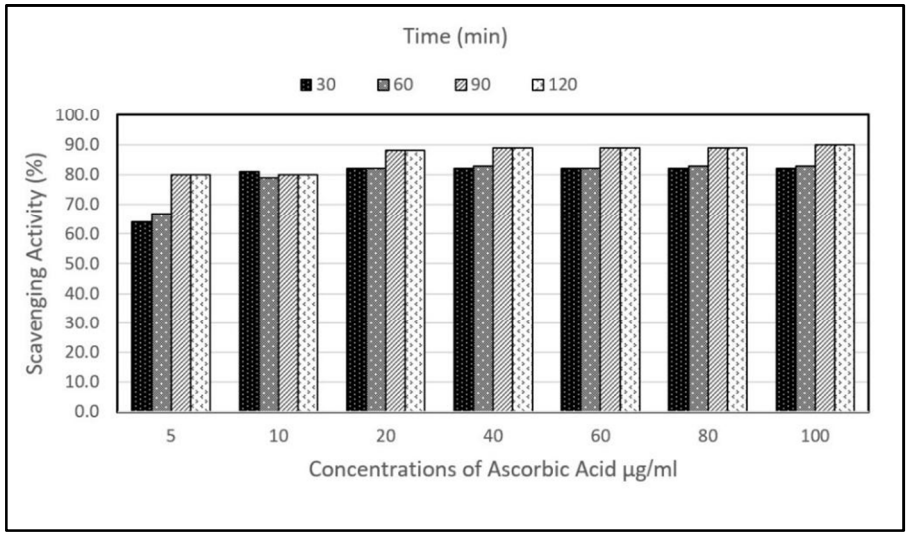

Fig 7. DPPH scavenging activity of ascorbic acid

3.4.1 Antimicrobial activity against bacterial and fungal pathogens

Antimicrobial activity of the extracted light yellow pigment from the isolated SIF3 pigment forming bacteria was determined by agar-well diffusion method using different concentrations of pigment.The isolated pigment showed antimicrobial activity against the test pathogens at different concentrations. Maximum zone of inhibition (20 mm) was shown by extracted pigment against *Penicillium sp*. by using 30mg/ml followed by *Pseudomonas aeruginosa* (12mm) inhibition zone at concentration 30 mg/ml Table (2). Antimicrobial activity of pigment extract against the test pathogens are shown in Table (2). The light yellow pigment from the isolate SIF3 was found to inhibit all tested microbes, thus might be selected as antimicrobial agents with a broad spectrum. Zone of inhibition formed by light yellow pigment varies in size even in case of isolates of the same genus (*Penicillium*) by using different concentrations of pigment. It was therefore concluded that the isolated bacteria were able to synthesize pigments that had antimicrobial activity against certain human pathogens. Pigments extracted from bacteria are reported to have antibacterial activity against Gram-positive and negative bacteria. Antibacterial activity of pigments separated from fifteen various bacteria was determined by Rashid *et al*.,[34]. Throughout their study, they reported that the pigments extracted from different isolates had antibacterial effects against different gram-positive and negative human pathogens, where it showed antibacterial activity more effective against gram-negative pathogens than gram-positive[34]. Monascus pigments which are colored in yellow, orange and red, possess weak antimicrobial activity[35].A pigment extracted from *Streptomyces sp.* D25 confirmed to have antibacterial activity, where the zone of inhibition ranged from 15 to 20mm against *Lactobacillus sp*., *Staphylococcus sp*., *Alcaligenes sp*., and *Bacillus sp*., at 25 mg/mL by using disc diffusion method[36]. Carotenoid pigment (yellow)from *Micrococcus luteus* exhibited inhibitory action against *Streptococcus faecalis* and *Staphylococcus aureus* with a wide range of inhibition zones at different concentrations from 2 to 10 mg/ml[32]. These studies have shown that bacterial pigments have potential bactericidal activity against various human pathogens.

Table 2. Antimicrobial activity of Light yellow pigment extracted from SIF3 isolate against various pathogens microbes using disc diffusion method.

Strain	Inhibition zone (mm)		
	Concentration of light yellow pigment (mg/ml)		
	7.5	15	30
Staphylococcus aureus	9	10	8
Bacillus subtilis	-	-	8
Bacillus cereus	-	8	-
Escherichia coli	8	10	9
Pseudomonas aeruginosa	-	9	12
Candida albicans	-	9	-
Aspergillus niger	10	7	-
Aspergillus flavus	-	10	9
Penicillium sp.	8	15	20

4. CONCLUSION

The production, extraction and characterization of yellow pigment produced by *Kocuria flava* SIF3 was successfully carried out in our study and it showed effective production of pigment. The isolated pigment was known to be of carotenoids as they showed λ_{max} absorbance at 437nm which is the indication for carotenoids. The extracted pigment showed antibacterial activity against all tested microbes. In addition it showed an antioxidant activity. In this regard, this study is an

initiative approach towards the use of bio-colors which find its applications in numerous sectors as an alternative for synthetic chemicals. To conclude, microbial pigment production is one of the emerging fields of research to demonstrate its potential for various industrial applications.

5. ACKNOWLEDGEMENT

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6. AUTHORS CONTRIBUTION STATEMENT

Sahar A. Mal, Ghada S. Ibrahim and Maha I. Al Khalaf conceivedand planned the experiments. Sahar A. Mal and Ghada S. Ibrahim carried out the experiments. Ahmed M. Al-Hejin, Noor Mohammed Bataweel and Mohamed Abu-Zaid helped to achieve the experiment and provided chemicals and lap equipment. Ghada S. Ibrahim and Sahar A. Mal wrote the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

7. CONFLICTS OF INTEREST

Conflict of interest declared none

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