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Original Article

PRODUCTION AND PARTIAL CHARACTERIZATION OF PIGMENTS PRODUCED BY *KOCURIA* SP BRI 36: INFLUENCE OF HEAVY METALS

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ABSTRACT

Objective: To study the production of pigments by *Kocuria* sp. BRI 36, their characteristics and influence of heavy metals on pigments.

Methods: The effects of various physical and chemical parameters on pigments production by *Kocuria* sp. BRI 36 were examined. Pigments were extracted and partially characterised by Thin Layer Chromatography (TLC) and Fourier Transform Infrared Spectroscopy (FTIR). The effects of heavy metals such as Pb^{2+}, Cd^{2+}, Ni^{2+} and Cr^{3+} were studied on pigment production. Antimicrobial activity and stability studies of crude pigment were also conducted.

Results: *Kocuria* sp. BRI 36 isolated from cold oceanic region maximally produced red-orange pigment in presence of glucose (5% w/v) and protease peptone (0.2% w/v) at pH 7.5, 10±1 °C. Thin layer chromatography (TLC) analysis revealed the occurrence of three different compounds in the crude pigment belonging to carotenoid and xanthophyll group. Metals like Ni^{2+} and Cr^{3+} adversely affected pigment production while $Pb^{2+}$and Cd^{2+} enhanced the yield. The significant features of *Kocuria* sp. BRI 36 pigment are i) antimicrobial activity against Gram-positive and Gram-negative bacteria, ii) maximum stability at pH 7.5 and 10±1 °C and iii) ~38% color loss at 50±1 °C in 5 h.

Conclusion: Our results suggest application potential of *Kocuria* sp. BRI 36 pigments in various biotechnological fields.

Keywords: Antimicrobial activity, Carotenoid, Halotolerant, Metals, Pigment

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INTRODUCTION

Pigments are colourful compounds that are produced naturally or synthetically. Natural pigments produced by bacteria, fungi, plants, insects etc. have better bio-degradability and environment acceptability over synthetic pigments. Bacterial pigments could play a key role as additives in colorful beverages, textile industries as natural colorant [1]. Biopigments are also known to posses antimicrobial and antitumor activity [2]. Among various types of pigments reported from bacteria, a carotenoid group of pigments are more widely studied with respect to their applications. Kulkarni *et al.* [3] reported the application of yellow pigment in the dyeing of fabric produced by *Kocuria flava* sp. HO-9041 similarly application of bright red pigment prodigiosin for dying of wool, nylon, acrylic and silk had been suggested by Alihosseini *et al.* [4] and Ahmed *et al.* [5]. While Alcantara *et al.* [6] have demonstrated the application of zeaxanthin from *Flavobacterium sp* in food as an additive in poultry feeds. *Bradyrhizobium* sp. strain was described as a canthaxanthin (4,4'-diketo-b-carotene) producer which has been used as aqua feed to impart the desired flesh colour in farmed salmonids [7].

Considering the demands of bio-pigments in various applications, better quality natural colorant with higher stability is the need of the hour. Among the genus *Kocuria,* seventeen species have been described so far [8] and are found to produce pigments like ethinenone, echinenone, beta-carotene, lycopene, canthaxanthin, alfa carotene etc [9]. However, *Kocuria* sp. from extreme habitats has not been studied in depth. Also, effects of heavy metals on pigment production and/or the role of *Kocuria* pigment in the detection of metals has not been examined yet. In view of this, the present paper deals with production and partial characterization of carotenoid pigment produced by *Kocuria* sp. BRI 36 [10], an isolate from the cold oceanic region. The present paper also discusses an effect of heavy metals on pigment production and its potential in heavy metal detection.

MATERIALS AND METHODS

Organism

The halotolerant (15% NaCl tolerance) *Kocuria* sp. *BRI 36* was used in this work. The organism was grown in Mineral Salt Medium (MSM) at 25±2 °C for 48 h with shaking at 120 rpm [11]. It was further used for inoculation in all the experiments at 10% concentration.

Chemicals and reagents

All chemicals used were of analytical grade. The media components were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). The stock solutions of cadmium, nickel, lead and chromium at a concentration of 1000 ppm each were purchased from Sigma-Aldrich.

Extraction and estimation

The culture of *Kocuria sp.* BRI 36 grown for 48 h was centrifuged at 8000 rpm for 15 min. The harvested cells were washed with sterile distilled H_2O and suspended in 1 ml chloroform. The pigment was extracted using the method described by Ahmad *et al.* 2012 [9]. The pigment was concentrated by rotary evaporator at 40±2 °C (IKA RV 10) and dried at 37±2 °C for 24 h. The powder was used as crude pigment for further experiments. Its λ_{max} was determined by using UV visible spectrophotometer (Thermo Fisher scientific 10 UV scanning) in the range of 200 nm to 700 nm.

Production

The effect of various physical and chemical parameters on pigment production was evaluated by varying one parameter at a time and keeping the other parameters constant. The one giving best result was used in further experiments. At the end of each experiment, a pigment was extracted and its absorbance was measured at its λ_{max}.

Effect of heavy metals

Kocuria sp. BRI 36 exhibits very high tolerance to heavy metals viz. lead, cadmium, chromium and nickel [12]. Immobilized cells of BRI 36 were used to determine the effect of metals on pigment colour. Immobilization was achieved using 2.5% sodium alginate and 50 × 10^{-3} mole calcium chloride [11]. The beads formed were exposed to 10-40 ppm concentration of each metal for 24 h.

To check the effect of heavy metals on pigment production, BRI 36 was cultivated in a previously standardized medium supplemented

with different concentrations (1 to 5 ppm) of $Pb^{2+}$and Cd^{2+}whereas it was 5 to15 ppm for $Ni^{2+}$and Cr^{3+}. At the end of incubation, cells were separated by centrifugation at 8000 rpm for 15 min, the pigment was extracted and its absorbance was measured at λ_{max}.

Characterization

The experiments for characterization of crude pigment were performed using the sample dissolved in phosphate buffered saline (PBS) at 5 mg/ml concentration.

Stability studies

The stability of crude pigment was determined in terms of its absorbance at λ_{max}. The effect of various conditions of pH (5.0, 7.0, 9.0) on stability was examined at room temperature. The pH showing maximum stability was selected to investigate effect of temperature (10 to 50 °C). These conditions were further used to analyse an effect of dark and light conditions for different time intervals (24, 48, 72 h) on stability. Percent color loss was determined by using the following equation.

$$\% \ color \ loss = \frac{ODi - ODt}{ODi} \times 100$$

Where ODi = Initial OD; and ODt= OD at time (t)

Antimicrobial activity

Antibacterial activity of the crude pigment (5-0.013 mg/ml) against several microbial strains was determined by the 96-well plate microdilution method [13]. Different clinical isolates used were *E. coli, Bacillus, Staphylococcus aureus, Pseudomonas aeruginosa, Shigella and Salmonella paratyphi.* 125 µl double strength growth medium was added to the first column of the 96-well microplate. After 48 h of incubation at 37 °C, the optical density was measured at 600 nm. The growth percentages at different pigment concentrations for each microorganism were calculated as:

$$\% \ growth = \frac{ODi - ODt}{ODi} \times 100$$

Where ODi = Initial OD; and ODt= OD at time (t)

Thin layer chromatography (TLC) and fourier transform infrared spectroscopy (FTIR)

Kocuria sp. BRI 36 was grown under optimized conditions and the pigment was extracted as described above. TLC analysis of the crude pigment was carried out as described by Vora *et al.* [14]. One mg of crude red-orange pigment was directly used for FTIR (Brucker, tensor 37) analysis. The conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400 and 4000 cm^{-1}.

Statistical analysis

The experiments were performed in triplicates and the standard deviation was calculated. One-way ANOVA was applied to determine the significant value ($p<0.05$).

RESULTS AND DISCUSSION

Estimation of pigment

The extracted pigment from *Kocuria* sp. BRI 36 was dissolved in PBS at a concentration of 5 mg/ml. Spectrophotometric analysis showed 475 nm as its λ_{max}. Previous reports on different species of *Kocuria* have also shown maximum absorbance of carotenoid pigment in the range of 471-477 nm [15].

Pigment production

Different parameters influencing the pigment production were studied individually by varying one parameter at a time. Taking one parameter at a time represented an efficient way to optimise production of microbial metabolites and/or biological processes [16, 17]. We observed maximum pigment production at 10 °C (0.29±0.01) (fig. 1a) and pH 7.5 (0.23±0.005) (fig. 1b). It decreased with increase in temperature. The response of microorganism to low temperature in terms of increasing proportion of unsaturated fatty acids is well documented [18]. It helps in increasing membrane fluidity. Medicharla *et al.* [19] have suggested a role of carotenoid in maintaining rigidity of membrane at low temperature. *Kocuria carniphila* MY and *Kocuria polaris* MO were also found to produce carotenoid optimally at 10 °C and at neutral pH [9]. As shown in fig. 1c, pigment absorption was highest at 5% glucose (0.22±0.005). *Kocuria* sp. K70 showed better pigment production at 1% lactose while for *Arthrobacter* sp.*, Serratia marcescens, Brevibacterium maris* glucose proved to be the better source [20]. We have used different organic and inorganic nitrogen compounds to examine their effect and among all, protease peptone (0.73±0.0005) was found to be the best (fig. 1d). Optimal effect of organic nitrogen on pigment production had been also observed by Kim and Park [21] in *Kocuria* sp. Smilarly El-Sharouny [22] and Subhasree *et al.* [23] have reported higher pigment production in presence of peptone and yeast extract from *Kocuria carniphila* MY and *Kocuria Polaris* MO, respectively. *Kocuria* sp. BRI 36 (this work) is a halotolerant isolate from cold region and can grow up to 15% w/v NaCl concentration [10]. However, increase in NaCl concentration above 5% decreased the pigment yield (fig. 1e). We have come across a few studies which focus on biotechnological production of pigments at NaCl concentrations of ≥ 15% w/v NaCl. For example, pigment production by photosynthetic halophiles at saturated NaCl (35% w/v NaCl), conferring diverse ecological advantages to the halophile which helps them to dominate their habitat [24, 25]. Another species of *Kocuria* sp. K70, have exhibited pigment production at 2% NaCl [21]. Similarly, *Pseudomonas* sp. isolated from marine environment had also displayed production at 2% w/v NaCl [26]. NaCl both reduces water activity and osmotic stress in microbial cells; the cellular stress imposed triggers increase in various types of metabolites [27, 28]. The reduction in water activity is pertinent to the current study as this is the parameter which stimulates an increase in secondary metabolites.

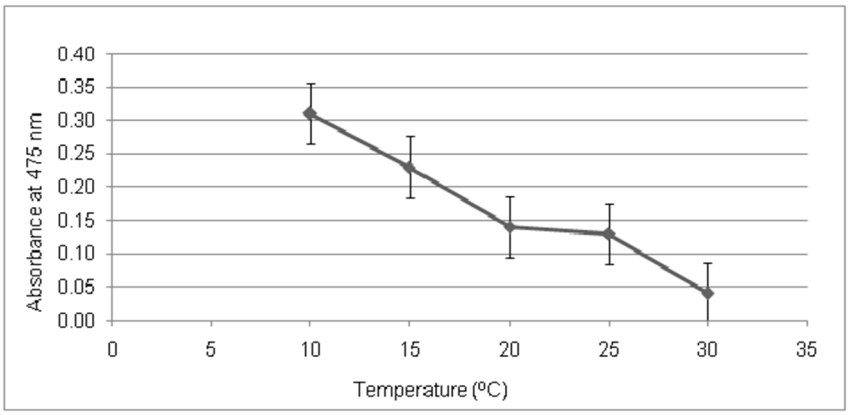
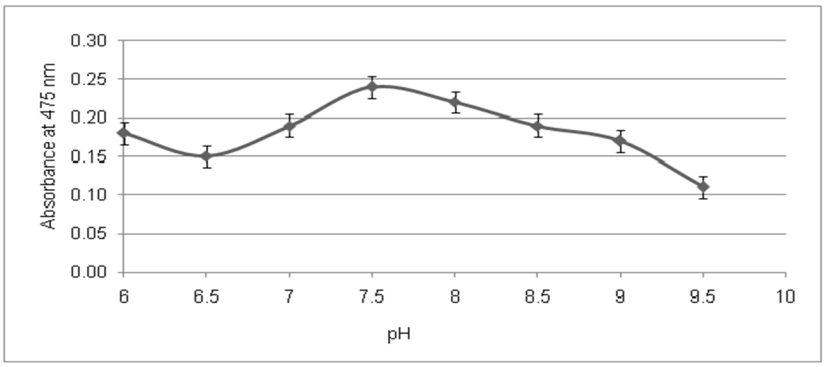


Fig. 1 (a)



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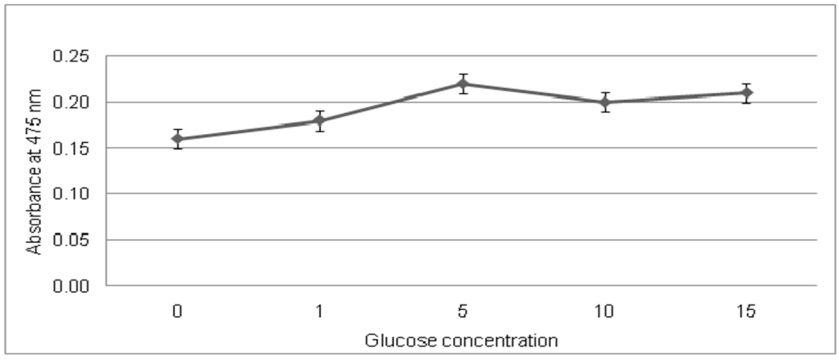

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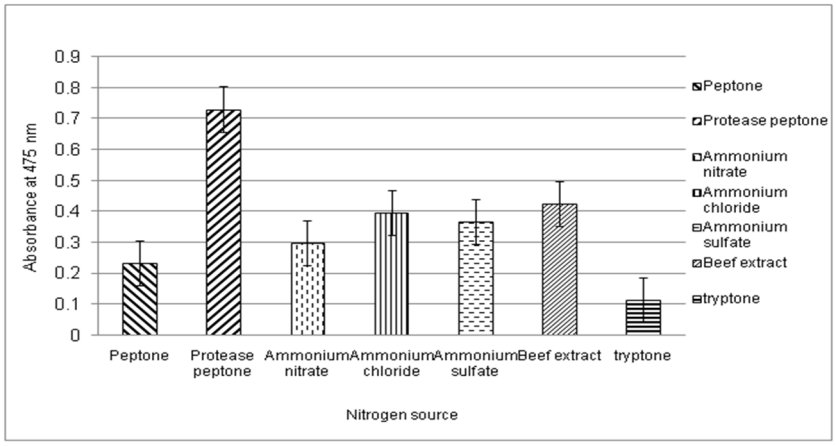

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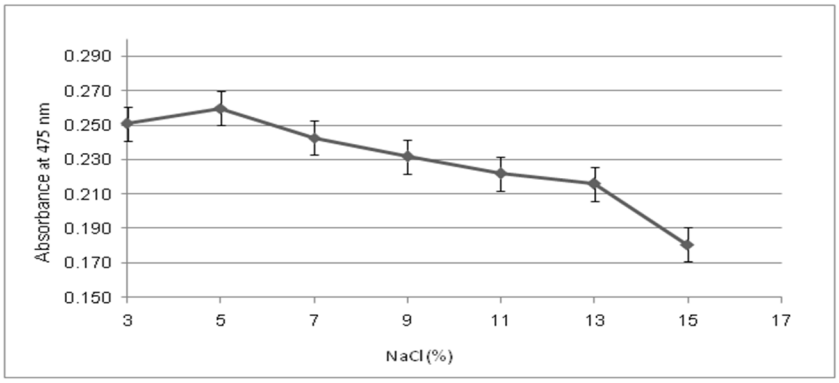


Fig. 1(e)

Fig. 1: Effect of various parameters on pigment production by *Kocuria sp.* BRI 36 by varying one factor at a time, a) temperature, n=6, [0.29±0.01] b) pH, n= 8, [0.23±0.005] c) glucose concentration, n=5, [0.23±0.005] d) nitrogen source, n=7, [0.73±0.0005] and e) NaCl concentration, n=7, [0.23±0.0005]. Data were analyzed by two–way ANOVA ($p<0.05$) and vertical bars represent standard error. Values in the square brackets indicate mean±SD

Effect of heavy metals

Experiments were carried out to evaluate the effect of Pb^{2+}, Cd^{2+}, $Ni^{2+}$and Cr^{3+}at various concentrations on pigment colour using immobilized pigmented biomass of *Kocuria* sp. BRI 36. Increase in metal concentration caused loss of pigment colour at the end of 24 h (fig. 2). These observations suggest a possible application of *Kocuria* pigment in the detection of metal contamination, although further experimentation is necessary. Application of immobilized cells of *Cynobacteria Anabaena cylindrical* for detection of Cu and Pb had been shown by Wong and Teo [29]. On similar lines, immobilized cells of *Kocuria* SP. BRI 36 may prove helpful in developing biosensor. With regards to the effect of metals on pigment production, a positive effect was observed when the medium was emended with $Pb^{2+}$and Cd^{2+} (fig. 3a,c). Pigment production increased with increase in metal concentration upto 5 ppm on the contrary, increase in concentration of $Ni^{2+}$and Cr^{3+}adversely affected pigment production (fig. 3b,d). Enhancing the effect of cadmium on pigment production by *Bacillus safensis* and *Pseudomonas aerogenosa* had been also reported by Priyalaxmi *et al.* [30] and Abdul-sada [31] respectively. Whereas, chromium was found to augment yellow pigmentation in *S. aureus* up to 100 µg/ml [32]. It may be attributed to increased pigment synthase (s) action as it was observed in case of red pigment producing *Monascus* sp. When cultivated in Fe, Zn and Mn [33]. To our knowledge, this is the first report analysing the effect of heavy metals on pigment production in *Kocuria* sp.

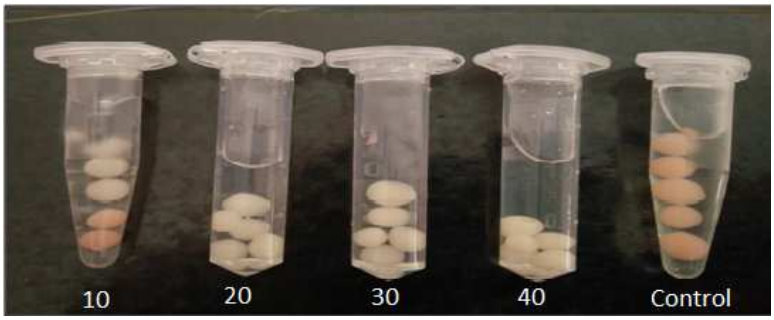
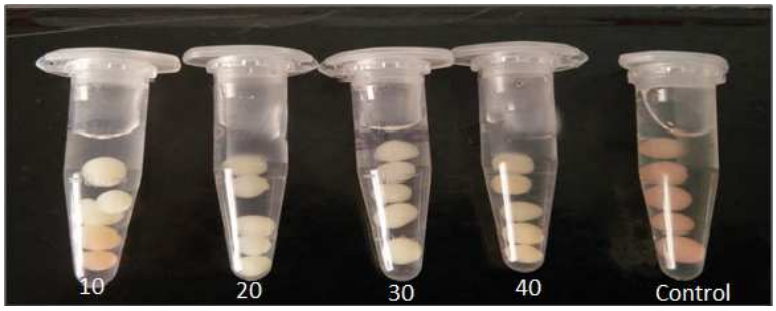


Fig. 2(a)

Fig. 2(b)



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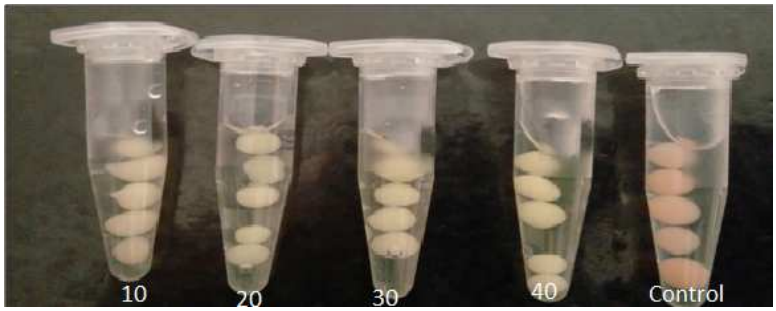


Fig. 2(d)

Fig. 2: Effect of different heavy metals on pigment colour produced by *Kocuria sp.* BRI 36 when exposed to different concentrations (10 ppm - 40 ppm) of a) Cd^{2+} b) Ni^{2+} c) Pb^{2+} and d) Cr^{3+}

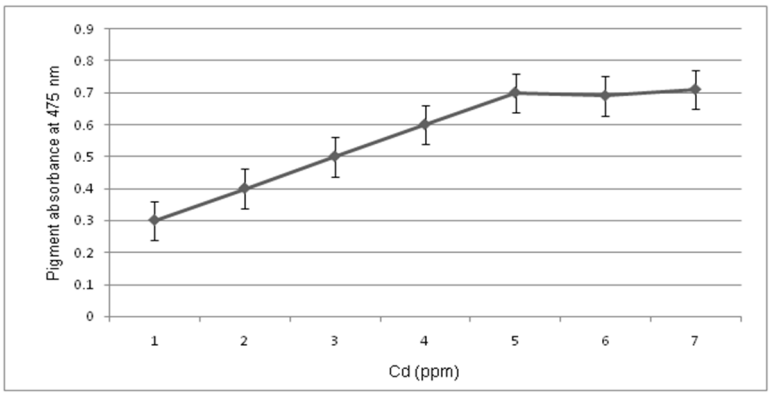


Fig. 3(a)

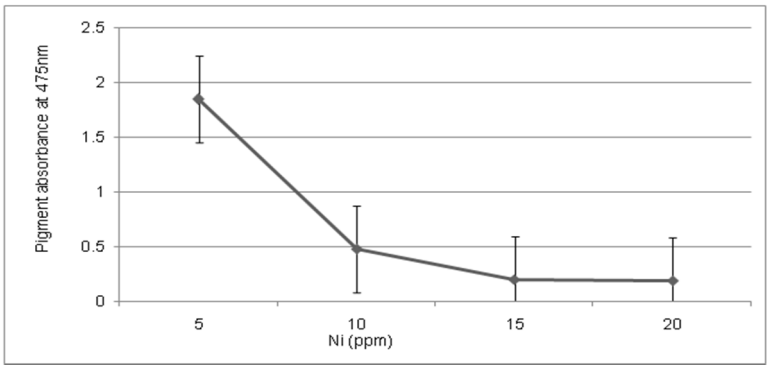
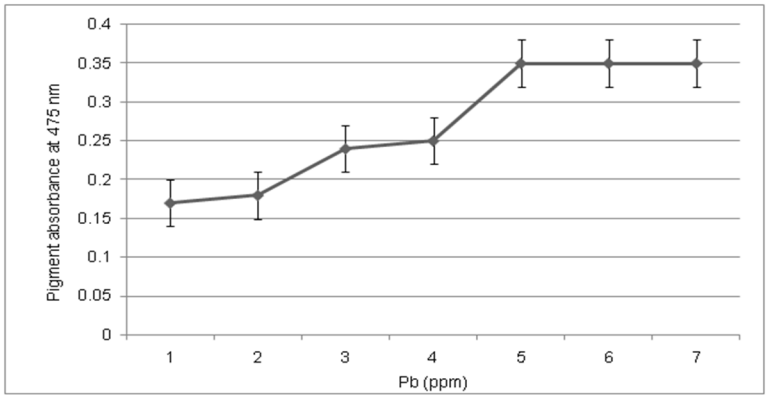


Fig. 3(b)



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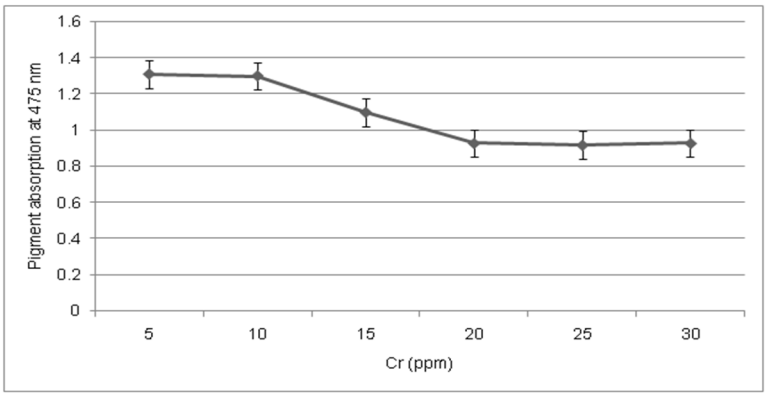

Fig. 3(d)

Fig. 3: Effect of different heavy metals on pigment production. *Kocuria* sp. BRI 36 was cultivated in previously standardized medium supplemented with different concentrations of a) Cd^{2+}, n=7, [0.6±0.05] b) Ni^{2+}, n=4, [1.85±0.005] c) Pb^{2+}n=7, [0.34±0.005] and d) Cr^{3+}, n=6, [1.2±0.05]. Data were analyzed by two–way ANOVA ($p<0.05$) and vertical bars represent standard error. Values in the square brackets indicate mean±SD

Pigment characterization

Stability

The absorbance of pigment at 475 nm at room temperature and pH 7.5 was considered 100% and colour loss was determined with respect to that. Crude pigment showed ~77% stability at 10 °C at the end of 5 h, while the increase in temperature caused a loss in stability with ~38% colour loss at 50 °C after 5 h (fig. 4a). Exposure of the pigment to various pH at room temperature indicated ~90% stability at pH 7.0 (fig. 4b). Studies on the effect of dark and light conditions on pigment stability showed ~ 80% and 50% stability respectively at the end of 72 h (fig. 4c). Similar studies were carried out by Shatila *et al.* [34] and they have shown 80% stability in orange colour pigment producing *Exiguobacterium aurantiacum FH* after exposure to light for 24 h. Thus our results indicated application potential of *Kocuria* sp. BRI36 pigment in textile, food, decorative articles as a colouring agent. Fig. 8 shows its use in preparation of colourful candle using paraffin wax.

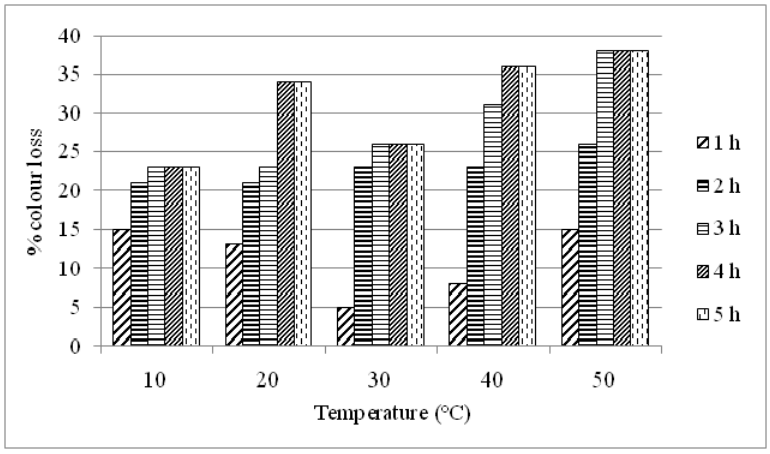

Fig. 4(a)

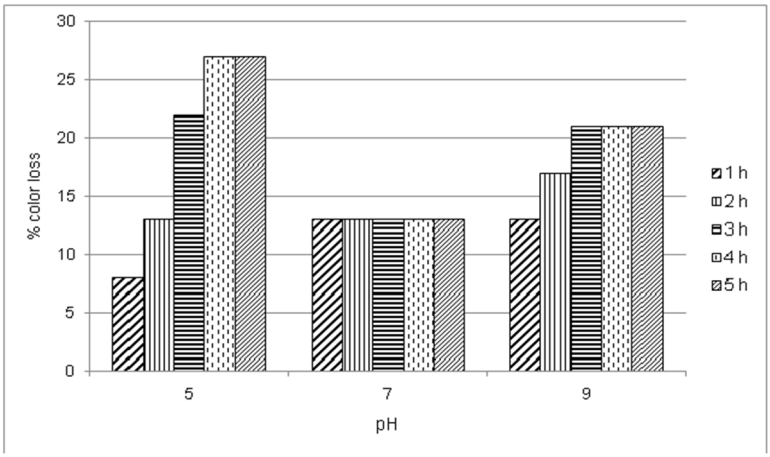

Fig. 4(b)

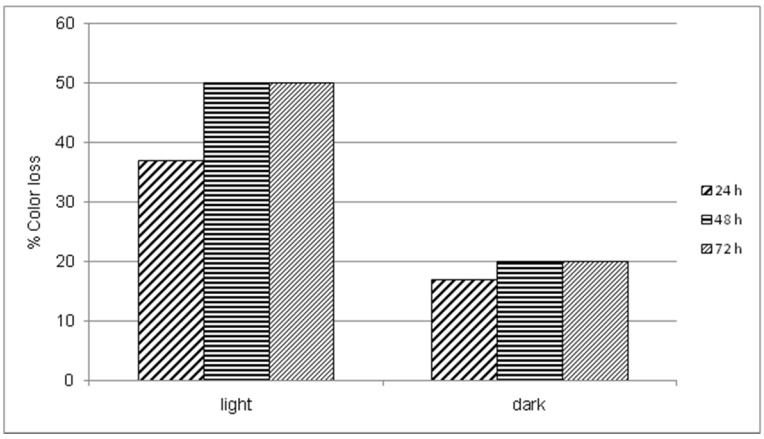

Fig. 4(c)

Fig. 4: Effect of a) temperature (10 to 50 °C), b) pH (5, 7 and 9), c) dark and light conditions on stability of crude pigment extracted from *Kocuria* sp. BRI 36

Antimicrobial activity

Antimicrobial activity of the crude pigment was studied against *E. Coli, Pseudomonas aerugenosa and Bacillus subtilis.* The activity increased with increasing concentration of pigment (0.015 to 5 mg/ml). We observed 83% growth inhibition in *Pseudomonas aeruginosa* and *Bacillus subtilis* while it was 75% in case of *E. coli* at 0.015 mg/ml concentration. The maximum effect was recorded in *Pseudomonas aeruginosa* with more than 95% inhibition at 5 mg/ml concentration (fig. 5).

Similarly, Kushwaha *et al.* [35] reported antimicrobial activity of the pigment produced by psychrotrophic *Kocuria* sp. against *Bacillus cereus*, *Staphylococcus aureus*, *Vibrio choler, Shigella dysenteriae* at 40 to 80 µg/ml pigment concentration. Carotenoid pigment isolated from *Micrococcus* sp. had also showed inhibitory effect on Gram-positive organism when tested in the concentration range of 0.25 to 2.0 mg/ml [36]. Thus, our results demonstrate the potential of BRI 36 pigment for application in pharmaceutical and cosmetic products.

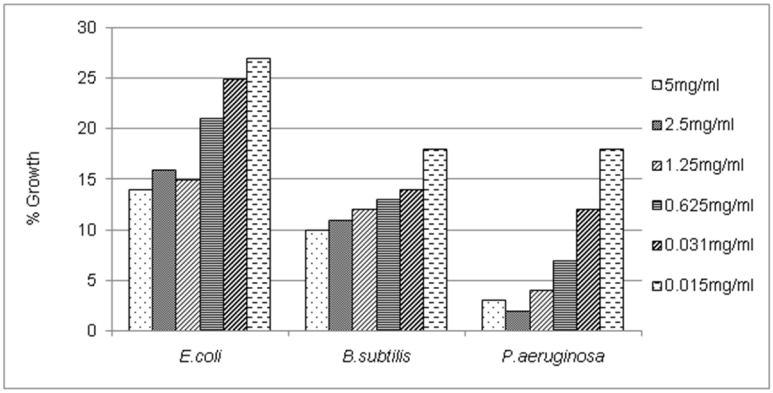

Fig. 5: Anti-microbial activity of crude pigment (5-0.013 mg/ml) against different clinical isolates viz. *E. coli, Bacillus subtilis', Staphylococcus aureus, Pseudomonas aeruginosa, Shigella dysenteriae' and Salmonella paratyphi*

TLC and FTIR

The crude pigment was found to be a mixture of three different compounds corresponding to the R_f values of 0.177, 0.387, 0.9182 (fig. 7) as observed in TLC experiments. The R_f values (0.387 and 0.9182) are in accordance to reported R_f values of carotenoid pigments [37]. R_f value of 0.177 indicates the presence of xanthophyll which is an oxygenated derivative of carotenoid [11]. FTIR spectra of crude pigment gave the prominent peaks at 2921.69, 2852.76 and 1066.72 cm^{-1} as depicted in fig. 6. Other peaks were observed at 1737.15, 1627.53, 1460.14, 1220.65, 518.85 cm^{-1}. Comparatively very less literature is available on psychrotrophic bacterial carotenoid pigment using FTIR spectroscopy. The bands at 2921.69 cm^{-1} are due to asymmetrical stretching vibration of aliphatic CH group while at 2852.76 cm^{-1} are due to asymmetrical stretching vibration of the same group as is interpreted by Latha and Jeevaratnam [38]. The peak at 1460 cm^{-1} may be due to asymmetrical deformation vibration of CH_3 groups. Bands at 1657 cm^{-1}may be due to the presence of the olefinic functional group. Peak at 1734.42 $cm^{-1}$is due to>C1=O group probably ester. 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 [39].

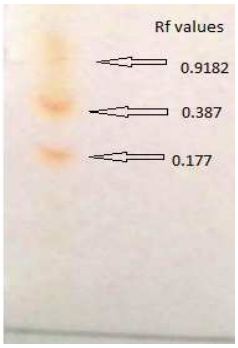

Fig. 6: Thin layer chromatogram of the crude pigment, the sample was resolved using butanol: ethanol: water (9:1:1) system

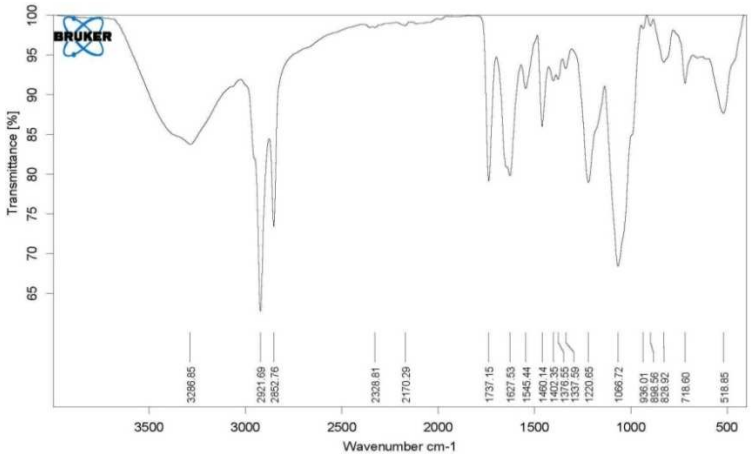

Fig. 7: FTIR analysis of the crude pigment. One mg of crude red-orange pigment was directly used for FTIR analysis. The conditions used were 16 scans at a resolution of 4 cm^{-1} measured between 400 and 4000 cm^{-1}

Fig. 8: Preparation of colour candle using crude pigment

CONCLUSION

Crude pigment produced by *Kocuria* sp. BRI 36 is a mixture of three different compounds with significant stability at high temperature and pH. Glucose and protease peptone found to affect pigment production positively while heavy metals like $Cr^{3+}$and Ni^{2+}had negative effect on pigment production. Other recent studies found that the ecophysiology of pigments produced by bacteria can be exploited for biotechnological purposes [40]. The findings of the current study suggest that phylogenetically diverse types of microbes may potentially yield biotechnologically valuable pigments, and further bio-prospecting efforts are needed to determine how much-untapped potential there is in hitherto uncharacterised microbial pigments.

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AUTHOR CONTRIBUTION

All the experiments were performed by Anuradha Mulik. Priyanka Kumbhar assisted her for optimization and characterization experiments. Planning of experiments, result analysis, manuscript writing and reviewing were carried out by Dr. Rama Bhadekar.

CONFLICT OF INTERESTS

No conflict of interest was reported by the authors

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