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Isolation and characterization of pigment producing marine actinobacteria from mangrove soil and applications of bio-pigments

Ishita Chakraborty, Priyanka Redkar, Minki Munjal, S. R. Sathish Kumar and K. V. Bhaskara Rao*

Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University, Vellore, Tamil Nadu, India

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ABSTRACT

The present study is focused on the extraction of intracellular pigment from actinobacteria isolated from the wetland soil sample of Sundarbans, West Bengal. Out of a total 20 isolates, only one actinobacterial isolates showed pigmented growth. Further, the potential isolate was identified by using 16s rRNA sequencing and confirmed as Streptomyces fradiae VITIPMSB. The intracellular pigment was extracted by cell disruption technique using ethyl acetate as the solvent. Partial characterizations of the pigment were carried out using UV-Visible spectrophotometry and FT-IR spectroscopy. The extracted pigment was applied for the preparation of lip balm and assessing its textile dyeing property. In addition, extracted pigment was analyzed for antioxidant and hemolytic activity. The obtained results showed potent antioxidant activity and confers the bio-pigmentas non-toxic to human erythrocytes.

Keywords: Actinobacteria, pigment, wetlands, lip balm, textile dyeing.

INTRODUCTION

Colors are one of the significant visual properties of food, textiles and cosmetics. It has been an age old practice to impart various colors to the entities in our daily commodities. This practice has amplified many folds with the invention of synthetic colorants principally due to their physical properties of good stability and better coloring ability [1]. Extracted pigments from living entity are termed as pigments of biological origin or bio-pigments [2]. Natural pigments are preferred over synthetic colorants due to the potential health hazard effects [3]. Natural pigments obtained from plants are usually limited, unstable, highly priced, and require more complex and tedious process for production [4]. In comparison, pigment obtained from microbes can be easily produced in sufficient amount, are cost effective and have a simpler extraction and purification process [5]. The range of shades that can be possible obtained is more varied. Moreover, these are safer and serve better as an economical substitute for the commercially available colorants [6].

Currently, the whole world is looking towards the usage of traditional products and adopting a natural approach towards life through increased usage of biological products. People are more interested in natural food, herbal medicines and traditional practices for healthy life. Higher demands were seen for the natural products from biological substances such as, plants and microorganisms. Recently, bio-cosmetics are pushing through the cosmetics industry in the world and the demand for the bio-cosmetics is rising and quite significant [7].

Actinobacteria is considered to be the vital genera in production of secondary metabolites and often might possess unique compounds that can exhibit virtually important traits that can be exploited in industries. Sometimes they also produce pigments, which play an important role in characterization of these organisms. They are widely used as coloring agents in industries such as, dyeing industry, printing industry, food industry, textile industry,

pharmaceutical industry and cosmetic production [8, 9]. These colorants also show properties such as antioxidant activity, antimicrobial activity, anti-coagulating property and food preserving capability [10]. In additionally, bio-pigments can be implemented in other related medical purpose [11] like anti-tumor and anti-cancer treatments or as indicators like stains and markers in laboratory research works [12, 13]. Often they might also possess rich nutritional value so as to be used as dietary supplements [14] or as food additives [15].

However, pigment extraction from actinobacteria on an industrial level is not much practiced due to the unavailability of protocols for large scale fermentation and downstream processing. In certain cases, the ability of the microorganism to produce pigment is extremely enhanced or completely degraded due to improper nutrition, culturing techniques or some other unknown reasons[16]. Hence, it may be assumed that incidence of such atypical strains which produces pigment with consistency is of research interest, further more if it is obtained from an unusual or comparatively less exploited habitat. The present study was focused on the isolation, characterization of the pigmented actinobacteria and the commercial applications of the extracted pigment.

MATERIALS AND METHODS

Sample Collection, Processing and Isolation

Soil samples were collected from mangrove forest in Sundarban Wetlands, West Bengal, India. Soil samples were collected at a depth of 10- 15 cm from the top layer of the soil surface. The pH of the collected soils was analyzed simultaneously. The collected soil samples were transferred into sterile polyethylene bags and transported to the Molecular and Microbiology Research Laboratory, Environmental Biotechnology Division, School of Bio Sciences and Technology, VIT University. The collected soil samples were stored at 4 °C for further studies.

The soil samples were serially diluted using sterile distilled water up to 10^{-7} dilution and one milliliter of the serially diluted samples were inoculated into different plates. Actinomycetes isolation agar and starch casein agar was used for the isolation of actinobacteria and the media was supplemented with nalidixic acid and cyclohexamide (100µg/mL) to avoid bacterial and fungal contamination respectively. The plates were incubated at 28°C and monitored periodically up to30 days of incubation period foractinobacterial growth [17]. Morphologically distinct colonies were separated, purified and maintained on actinomycetes isolation agar plates [18].

Fermentation Process and Extraction of Bioactive Compounds

The pigmented actinobacterial isolate (VITIPMSB) was inoculated into 50 mL of production media (SS broth-containing soluble starch-25g, glucose-10g, yeast extract-2g, $CaCO_3$- 3g, trace elements- 1mL, Distilled water -1000 mL) in 100 mL Erlenmeyer flasks separately and incubated at 28° C for 7 days on a rotary shaker (REMI) at 100 rpm. After incubation, the production media was harvested and centrifuged at 10,000 rpm for 10 min in a cooling centrifuge (REMI). The cell pellet was collected to extract intracellular pigments. Further, cell pellet was subjected to ultra sonification and the intracellular pigments were extracted by using different polarity solvents such as, ethyl acetate, chloroform, n-butanol, methanol, and ethanol in the ratio of 1:1 (w/v) [19-21].

16S rRNA sequencing of the potent isolates

The potent isolate was screened for 16S rRNA molecular sequencing in Amnion Biotech Ltd. Bangalore. The genomic DNA of VITIPMSB was isolated from the pure culture plate, and then ~1.4kb rDNA fragment was amplified using high fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the 16S forward primer 5'- CWG RCC TAN CAC ATG SAA GTC -3' and16S reverse primer 5'- GRC GGW GTG TAC NAG GC -3'. The result was analyzed by BLAST search tool in National Centre for Biotechnology Information (NCBI) database. The phylogenetic tree for the obtained sequence and its homology was prepared and compiled using the Clustal W and Tree View software.

Partial Characterization of Pigment

UV-Visible Spectrophotometry

The λmax of the isolated compound was observed by UV-Visible spectrum analysis. The λmax is useful to detect the absorbance range of the extracted pigment. The extracted pigment was dissolved in ethyl acetate solvent, subjected to UV analysis in the range level of 200-800 nm with the help of UV-visible spectrometer(shimadzu).

FT-IR Analysis

The FT-IR spectra of sample were recorded in order to characterize the presence of functional groups in isolated bioactive. Two mg of the isolated pure compound was added in 200 mg of potassium bromide (KBr- FT-IR grade) and prepared as dry pellet. All measurements were carried out in the range of 400-4000 cm-1 at resolution of 4.0 cm-1(Thermo Nicolet – Avatar-330, USA).

Applications of Bio-Pigment

Preparation of Bio-lip balm

The bio-lipstick was prepared using shredded bee wax along with coconut oil and lanolin were mixed in a ratio 2:4:1 (w/v/w) in a china bowl. The container was kept in water bath and heated until the wax melts completely and all the ingredients are homogeneously mixed. The pigment was added to it to impart color. The mixture was then poured into a container and allowed to cool. The lip balm formulation was developed and evaluated in the Preliminary Stability Tests which includes color, odor, appearance and spread ability over a minimum of three days at room temperature (28 ºC) and oven temperature (50 ºC).

Textile Dyeing

The extracted colors from actinobacterial cells were used for dyeing wool, absorbent cotton, and thread. The samples were pre-mordant with 5% of ferrous sulphate and copper sulphate separately. Finally, thewool, absorbent cotton, and thread each weighing 1 g were dyed in 50 mL of colored filtrate using MLR 1:50, dyeing time 45 min and incubated at 70-80°C. After dyeing, washing of the samples were carried outto boil for 5 min in lissapol followed by rinsing with cold water.

Antioxidant activity

2, 2-diphenyl-1-picrylhydrazylradical scavenging activity (DPPH) was carried out by following the method of Brand-Williams et al.,[22]. Actinobacterial solvent crude extracts was prepared at different concentrations (20, 40, 60, 80 and 100 µg/mL) using Milli Q water for DPPH radical scavenging activity. In this experiment, 2 mL of actinobacterial pigmentwas taken from different concentration and then added with 1 mL of 0.2 mM/mL DPPH solution. The reaction mixture was incubated at 20 °C for 30 min in dark. After incubation absorbance was noted at 517 nm using UV-Vis spectrophotometer. Methanol was used as a blank solution and ascorbic acid was used as a positive control. The percentage of DPPH activity of the test samples was detected using the following formula::percentage scavenging = [(Ac-At)/Ac]*100

Hemolytic Assay

The hemolytic activity of bio-pigment extracted from actinobacteria was measured using the following protocol [23]. In this assay, 5 mL of blood containing heparin was centrifuged at 15000 rpm for 30 minutes. Supernatant containing plasma was discarded and pellet containing red blood cells was washed with 0.75% saline by centrifugation at 1500 rpm for 5 minutes. The cells were suspended in normal saline. 0.5mL of cell suspension was then mixed with sample. The mixture was incubated for 30 minutes at 37°C and then centrifuged at 1500 rpm for 10 minutes. The free hemoglobin in supernatant was measured using UV-Vis spectrophotometer at 540nm. Distilled water and phosphate buffer saline was used as minimal and maximal hemolytic controls. The activity was calculated as follows:

percentageHemolytic activity =[(At- An)/(Ac- An)]*100

Where At is absorbance of sample,An is absorbance of negative control and Ac absorbance of control.

RESULTS AND DISCUSSION

Isolation and identification of pigment producing Actinomycetes

The wetland soil samples were collected from the mangrove forest of Sundarbans, (West Bengal, India) and 20 different actinobacterial isolates were isolated. Among them, only one isolate showed a dark pink colored growth and an aerial whitish mass, which turned grey on ageing (Fig. 1). Actinomycetes isolation agar and starch casein agar were used for the isolation of actinobacteria, among these two media starch casein agar enhanced more number of actinobacterial isolates. Similar study was carried out by Quadri et al., [24] where a total of 63isolates were isolated from limestone quarries and screened for melanin pigment producing actinomycetes. Among them only eight isolates showed positive results for the production of melanin pigment.The intracellular pigment was extracted by cell disruption technique using ethyl acetate as the solvent.The BLAST search of 16S rRNA sequence showed highest similarity of 98% with *Streptomyces fradiae*. The 16S rRNA sequence of the strain *S. fradiae* VITIPMSB was deposited in the Gene Bank of NCBI under the accession number kc991094. A phylogenetic tree was constructed with bootstrap values. Based on the molecular taxonomy and phylogeny, the potent isolate wasidentified and designated as *S.fradiae* VITIPMSB (Fig. 2).

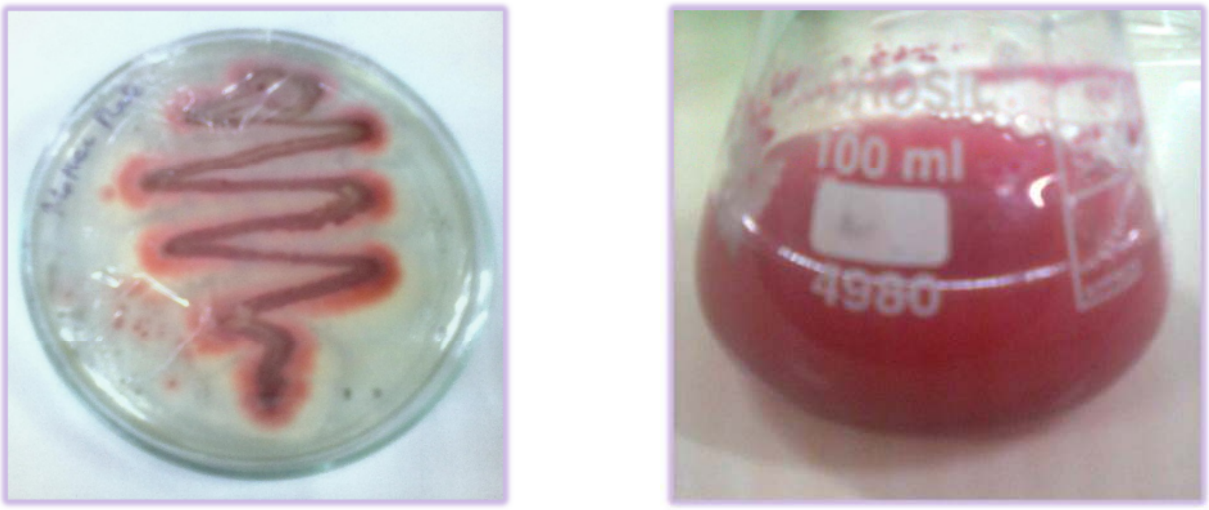

Fig. 1. A) Petri plate showing growth of *S. fradiae* VITIPMSB

B) Flask showing pink colored *S.fradiae*VITIPMSB culture

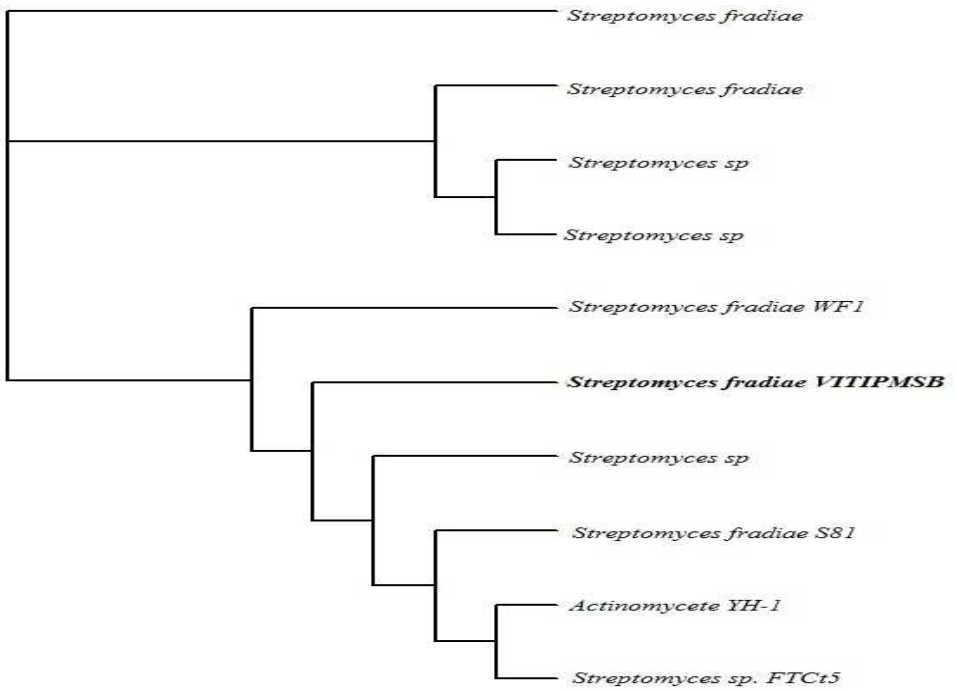

Fig.2. Phylogenetic tree of *S. fradiae* VITIPMSB

UV-Visible Spectrophotometry and FT-IR Analysis

The absorption spectrumwas observed at 263nm and 294nm that showed the maximum optical density of 0.206055 and 0.180173 respectively (Fig.3).The FT-IR spectrum report was analyzed and interpreted corresponding to the standard peak values. 3550, 3471 and 3412 Symmetrical stretching of Hydroxyl groups (-OH), 3232 Symmetrical stretching Amine group (-NH), 2958, 2926 and 2872 Stretching vibrations of methyl groups (-CH), 1637 Asymmetrical stretching of hydroxyl groups (-OH), 1616 stretching of aromatic ring carbons, 1400 asymmetrical stretching of -NH groups, 1161,1091 and 1051 symmetrical and asymmetrical stretching vibrations of carboxylic groups (-COOH)(Fig.4).

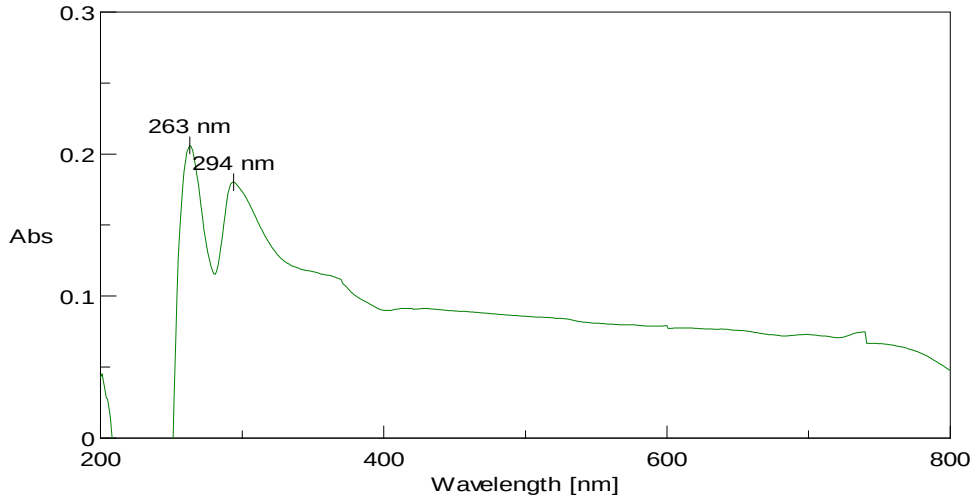

Fig. 3: UV-Visible spectra for λ_{max}

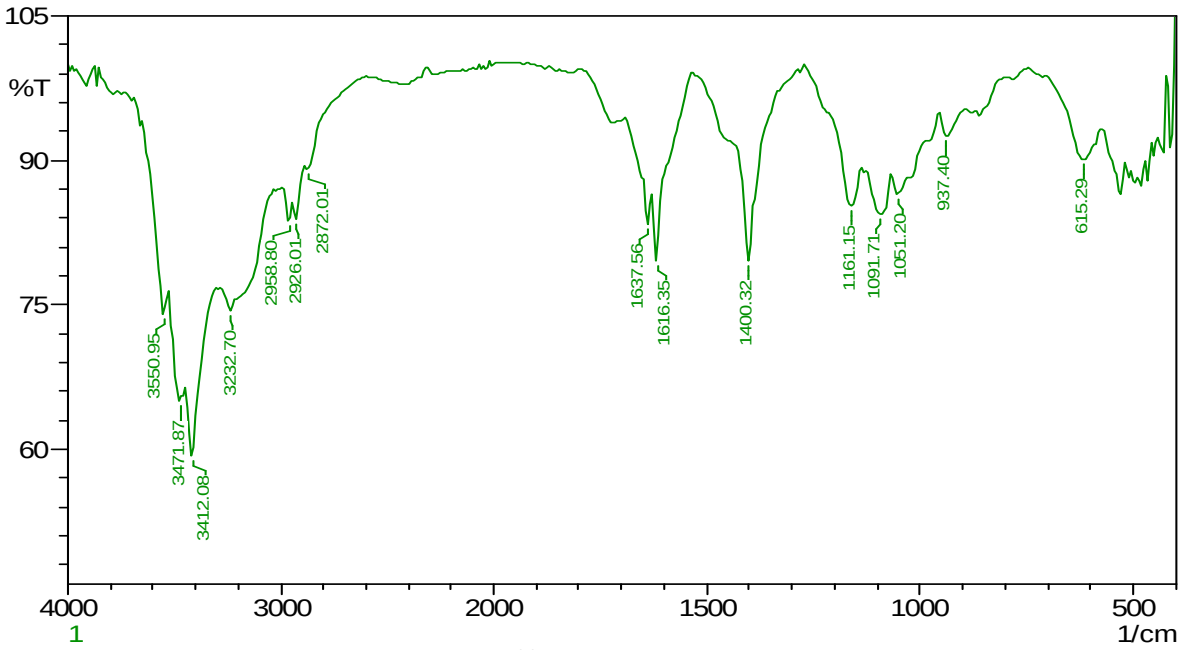

Fig. 4. The FT-IR spectral analysis of functional groups present in ethyl acetate extract

Applications of Pigment

Cosmetic Production

The formulated lip balm exposed suitable characteristics such as color, odor, and uniformity and solidified at the temperature of 45°C. It was noticed that there were no water formation, bleeding, streaking and blooming after three days of observation when the lip balm was maintained in room temperature at (28 °C) and oven temperature (50°C). The color formation of the lip balm was observed under room temperature, but there were slight changes under the oven condition, from pin to pale pink after one month of incubation.Therefore, it was evident that this formulation of lip balm was not stable after being subjected to a temperature of 50 ºC. The color formation of the product was stable under room temperature and odor characteristic of the lip balm remained stable throughout the 60 days of testing under all conditions evaluated. The visual aspect was considered uniform under the room temperature (Fig.5).

Textile Dyeing

Different types of textile materials were used to check the dyeing property of the extracted pigment. The textile materials were subjected to three consecutive normal water wash treatments and the pink color imparted was retentive for all the textile materials.In textile industries, the pigment extracted from biological source was used as an alternative to the synthetic colorants and also which are safe and cost effective. A piece of cotton, synthetic material, cotton thread and wool strands were used as textile materials to observe the coloring capacity of the ethyl extracted pigment. Usually any sort of dye requires a fixative or a mordant, which helps in the attachment of the dye

to the material. In our study, we observed that the extracted pigment did not require any fixative to incorporate the colored texture to the textile materials. The pink color was retentive enough to withstand a consecutive three normal water wash treatment (Fig. 6). Amal et al., [25] has studied similar fastness properties in textile materials using different shades of brown pigment isolated from *Streptomyces* sp.

Fig. 5. Natural lip balm formulation

Fig.6. Different types of textiles used to check dyeing property of the pigment

Antioxidant and Hemolytic Activity

Oxidative stress occurs in every cell due to metabolic processes. Environmental factors such as UV-radiation, ozone or smoke can induce oxidative stress. Antioxidant potential is the capability of a substance to scavenge free radicals available in its surroundings. DPPH (2,2-diphenyl-1-picryl-hydrazyl) is a light sensitive crystalline colored compound which contains stable free radicals [26].The ability of a particular substance to liberate these free radicals in the solution in the form of hydrogen ions, determines the scavenging potential of that substance. In this

experiment, the actinobacterial pigment of *S. fradiae* VITIPMSB was used in the DPPH radical scavenging activity. The actinobacterial pigment of *S. fradiae* VITIPMSBshowed maximum activity when compare to standard (Fig. 7). *In vitro*hemolytic assay using spectroscopic methods provides an effective and easy method for the quantitative measurement of hemolytic. This method helps to evaluate the effect of different concentration on biomolecules of the human erythrocytes.Hemolytic activity of the extracted pigment was screened against normal human erythrocytes and there was no hemolytic activity against human erythrocytes, indicating that the extracted actinobacterial pigment is non-toxic to the human red blood cells.

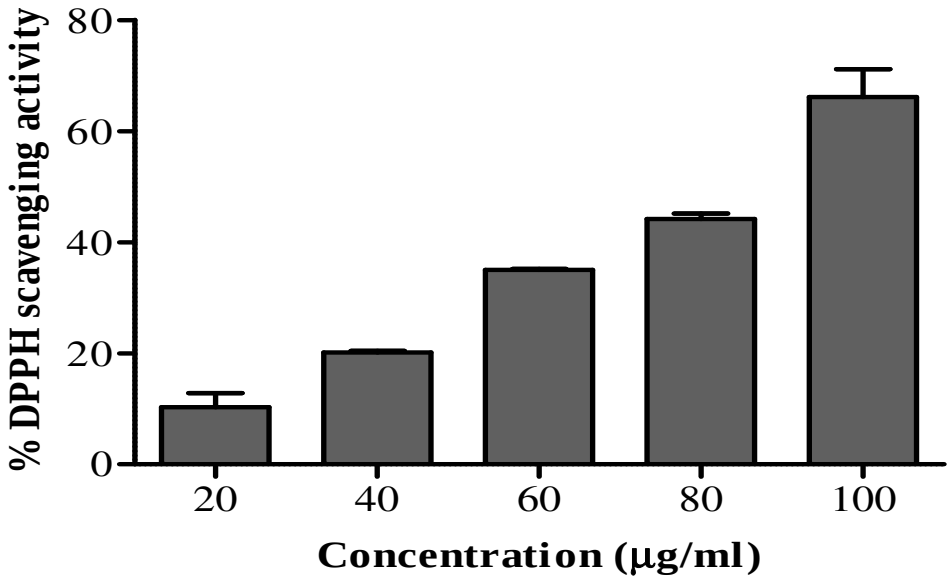

Fig. 7. Graph showing antioxidant potential of the pigment

CONCLUSION

The pigment extracted from actinobacteria showed significant results in the preparation of lip balm. Furthermore, pigment was tested for textile dyeing applications and showed prominent results for the retention of dye. Therefore, the current finding depicts the potential of pigments that could be manipulated as a prominent source to replace the synthetic chemicals for the preparation of cosmetics and textile dyeing.

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