Production and analysis of melanin pigment from bacteria isolated from farm soil

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Abstract: The aim of this research was production and analysis of melanin pigment producing isolate from farm soil sample and study of its photo-protective role. A pigment producing isolate was screened on Tyrosine casein media from the soil samples collected from various farms. The bacteria were identified as *Pseudomonas aeruginosa* by molecular characterization. Production of pigment was carried out by Submerged fermentation (SF) technique. Optimization parameters like Carbon and Nitrogen sources, along with effect of incubation time on pigment production was also studied. Maximum production was observed at 192nd hour to the incubation and pigment production was observed in Starch and L-tyrosine containing medium. The pigment extracted was found to be water soluble and was confirmed to be photo-protective using UV-Vis spectroscopic studies. The pigment showed reducing power capacity (Oyaizu 1986) which serves as potent antioxidants. Fourier Transform Infra-red spectrum (FTIR) analysis confirmed the crude melanin extract obtained as melanin.

Index Terms **– Melanin, L-tyrosine, Photo-protective, Antioxidants.**

I. INTRODUCTION

"Color" is a visual way of communication and rather very important one in foods, drugs, and cosmetic for creating and maintaining their acceptability and appeal in market. Natural colors are generally extracted from fruits, vegetables, roots and microorganisms as well. Microbial pigments are suitable for mass production when compared to others. Microbial pigments are of industrial interest because they are often more stable and soluble than those from plants or animal source (Gunasekaran *et al*., 2008). Melanin is to the animal kingdom like chlorophyll to the vegetal kingdom (Dadachova *et al*., 2007). Melanin is a broad term for a group of natural pigments that is found in most of the organisms be it Human beings, animals, as well as in microorganisms. In humans, melanin pigment is produced in a specialised group of cells known as melanocytes, which are found in basal layer of epidermis and melanin are "Primary Determinants" of skin colour (Prajapati *et al*., 2017). Melanin are enigmatic pigments that are produced by wide variety of microorganisms including several species of pathogenic bacteria, fungi and helminths. The ability of certain microbes to produce melanin has been linked with virulence and pathogenicity for their respective hosts (Nosanchuk *et al*., 2003).

Melanin term originates from Greek word "melanos" which means dark. Melanin form a diverse group of pigments synthesised in living organisms in the course of hydroxylation and polymerization of organic compounds (Shrishailnath *et al*., 2010). Melanin are nearly ubiquitous pigments, which are negatively charged, black amorphous pigments of high-molecular weight compounds (Nosanchuk *et al*., 2003). These pigments are irregular, dark brown polymers that are product of various microorganisms by fermentative oxidation of phenolic and indolic compounds (Dharmik *et al*., 2013). Melanin synthesis in mammals is catalysed by tyrosinase (Sanchez-Ferrer *et al*., 1995). The pathway can utilize L-tyrosine or dihydroxyphenylalanine (L-DOPA) for synthesis of melanin. Microbes can synthesize melanin via phenoloxidases (such as tyrosinases, laccases or catacholases).

Melanin confers resistance to UV light by absorbing a broad range of electromagnetic spectrum and preventing photo-induced damage (Hill,1992). Melanin is used for mimicry, and against high temperatures and chemical stresses. Bacterial melanin genes have been used as reporter gees to screen recombinant bacterial strains. It has anti-HIV and is useful in photo-voltage generation and fluorescence studies. Melanin is also used to generate monoclonal antibodies for the treatment of human metastatic melanoma (Plonka *et al*.,2006; Surwase *et al*., 2013).

There are various types of melanin: Eumelanin, Pheomelanin, Allomelanin and Neuromelanin. From all the type, Eumelanin is extensively studied. This pigment is extensively used in cosmetics, photo protective creams, eyeglasses, and immobilization of radioactive waste such as Uranium. Melanin is used to generate monoclonal antibodies for the treatment of human metastatic melanoma (Plonka *et al*., 2006; Surwase *et al*., 2013).

II. RESEARCH METHODOLOGY

1 MEDIA AND REAGENTS

Inoculum medium and pigment production medium were used. L-tyrosine (ThermoFisher Scientific India Pvt. Ltd.), Casein hydrolysate, Sodium nitrate, Agar-agar, MH agar powder (Hi-media), Peptone, Potassium persulphate, Potassium permanganate, Sodium thiosulphate, di Potassiumorthophospate, Ferric ammonium citrate were used. All these media were of analytical grade from Merck Ltd., ThermoFischer Scientific India Pvt. Ltd., and Finar Ltd. The reagents and media were prepared in distilled water.

2 SAMPLE COLLECTION

The farm soil samples were collected from three different areas *i.e*., Nanakwada, Segvi and Tithal of Valsad city, Gujarat. Soil samples were collected from a depth of 10-15 inches from the top of the soil. The samples were collected in clean jars using a spatula.

3 SCREENING AND ISOLATION

The soil samples collected earlier were serially diluted till 10^{-6} dilutions and were plated on sterile Tyrosine Casein Agar (TCA) plate. Tyrosine casein agar medium was used for screening and isolation of melanin producing species, L-tyrosine is used as precursor through the action of tyrosinase to produce melanin. Plates were incubated at 30°C for 7-8 days. After incubation, diffusible dark brownish-black pigment around the colony was observed. The pigment producing colonies were selected from mixed plate culture and maintained on the fresh medium to get pure culture and were preserved. Gram's staining was performed for the morphological characterization and colony characteristics were also recorded.

4 CHARACTERIZATION AND IDENTIFICATION OF MELANIN PRODUCING ISOLATE:

The most attractive potential uses of 16S r-RNA gene sequence informatics is to provide genus and species identification of isolates.

DNA extraction was carried out using Bacterial Genomic DNA purification kit. The quality of DNA was evaluated on 1% agarose gel. The DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using thermal cycler and electrophoresis was performed to analyze the size of amplified PCR product. The PCR product was purified using PCR clean up kit. DNA sequence reaction to PCR amplicon was carried out using 357F and 1391R primers using BDT v3.1 Cycle Sequencing Kit on ABI 300x1 Genetic Analyzer. The DNA sequences were analyzed using online BLAST-n facility of National Centre for Biotechnology Information (NCBI).

5 INOCULUM PREPARATION:

The prime objective of inoculum preparation is to achieve a high level of viable biomass in a suitable physiological state to boost up the main production media. In this research from the preserved culture slant, an isolated colony was transferred to three Erlenmeyer flasks containing 50ml of Peptone iron broth (PIB), Tyrosine Casein broth (TCB) and Tyrosine broth (TB) each, respectively. Each flask was incubated at 30°C for 7 days while maintaining static condition, as agitation or shake flask method drops the production of melanin pigment. To inoculate in production medium containing PIB, TCB and TB respectively, freshly grown 7 days old culture with 1.0 O. D at 600nm was used as inoculum.

6 PRODUCTION MEDIUM:

Melanin production was carried out in three 250ml flak containing 100ml of PIB, TCB and TB each through submerged fermentation. Each production medium was inoculated with 5% inoculum with 1.0 O. D of selected strain and then incubated at 30°C for 5-6 days.

7 EXTRACTION AND PURIFICATION OF THE PIGMENT:

7.1 From tyrosine casein broth

The production medium showing pigmentation after incubation was centrifuges at 2000 rpm for 20 minutes. The pH of the supernatant was adjusted to 10 with 5 M NaOH to ensure complete polymerization of melanin, then to pH 2 with 5 M HCl. This mixture was again centrifuged to obtain the melanin crude extract. Then equal volume of organic solvent- Chloroform: Ethyl acetate: Acetone was added in 1:1:1 ration and mixed to remove proteins. An aqueous phase was collected by two phase separation using separating funnel. Then, aqueous phase was collected and kept for drying overnight, weighed and stored.

7.2 From Peptone iron broth and tyrosine casein broth

Melanin pigment was extracted by following method from filtrate of Tyrosine casein and Peptone iron broth medium:

50 ml of each broth was adjusted to pH 7, then 0.5gms of Potassium persulphate ($K_2S_2O_8$) was added to each of the filtrate and allowed to stand for two hours with intermittent shaking. After incubation was over 50 ml of methanol was added to each and allowed to stand for 3 days. Then, both the filtrates were centrifuged at 10,000 rpm for 10 minutes. Supernatant was discarded and colored pallet was collected, dried and weighed (Deshmukh, 2012).

8 OPTIMISATION STUDIES OF MELANIN PIGMENT

8.1 Carbon sources

To study the effect of various C –sources on pigment production, isolated organisms were inoculated in tubes containing tyrosine medium with 1% Starch, Glucose and Sucrose respectively (Deshmukh, 2012). Tubes were incubated at 30°C for 5-6 days.

8.2 Nitrogen sources

To study of effect of N-source, isolated organism was inoculated in tubes containing basal medium with 0.4% L-proline, L-tyrosine and L-lycine (Santhanalakshmi *et al*., 2017). The tubes were incubated at 30°C for 5-6 days.

8.3 Effect of incubation time on melanin production

Time course of melanin pigment production was studied in melanin pigment production media using static flask condition. Optimum incubation time for maximum melanin production was determined by incubating media for total of 192 hours and analyzing sample at regular interval of 24 hours for melanin production at 30°C, static condition by noting down the absorbance at 480nm (Prajapati *et al*., 2017).

9 ANALYSIS OF PIGMENT

9.1 Confirmation of pigment

The isolates were inoculated into 50ml of 0.1% tyrosine substrate solution with few drops of Chloroform in 100ml flask and incubated for 48 hours at 30°C. The red coloration after incubation shows positive results.

9.2 Chemical analysis

The chemical analysis of melanin pigment was carried out by the modified method of Fava *et al*. The solubility of the brownish-black pigment in Distilled water, Ethanol, Chloroform, Acetone, Methanol, 1N NaOH and 3N HCl were determined and reaction with strong oxidizing agent *i.e.*, Hydrogen peroxide (H_2O_2) was also recorded.

9.3 Bleach test

For the analysis of bleaching reaction, slide test was carried out, in which crude melanin solution (dissolved in water) was kept on a clean slide and diluted solution of Potassium permanganate was added to it. Potassium permanganate serves as bleaching agent which bleaches the melanin and turns the brown color of pigment to colorless (Hongwu *et al*). Use of melanin bleach, is to remove excess melanin present in histological diagnosis of Ocular carcinomas.

9.4 Thin Layer Chromatography (TLC)

Thin layer chromatography is a technique to isolate non-volatile mixtures. The confirmation of the pigment was performed by Thin Layer Chromatography (TLC). TLC is one of the purification method and solvent system used was Hexane: Chloroform (15:15) and the chromatogram generated was later developed in UV chamber. Band was observed under UV chamber where the spot of pigment was loaded. Retention factor (R_f)value was noted down (Diraviyam *et al*., 2011).

10 SPECTROSCOPIC ANALYSIS OF THE PIGMENT

10.1 UV-Vis Spectroscopy

UV-VIS spectroscopy is used for quantitative determination of different analyte. It is one of the important criteria for melanin for analysis of photo-protective property. The crude melanin extract obtained from bacterial isolate was determined by UV-Visible light absorption spectrometer. 0.1 mg of pigment was dissolved in distilled water at pH 7.0, then it was analysed using the UV-Visible light at wavelength ranging from 200-500nm.

10.2 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR of the pigment was carried out for the determination of chemical/ functional groups present. The FTIR analysis was carried out in PerkinElmer Thermo Niocolet, AVATAR 330 compact, high performance, spectrometer at Centre of Excellence, Vapi. The control and melanin extract samples were mixed with spectroscopically pure Potassium bromide (KBr) and then finely pulverized and kept into a pellet forming die, then fixed in the sample holder and the analysis was carried out.

11 APPLICATIONS OF MELANIN

11.1 Reducing power assay

Substances thought to have reduction potential have a tendency to react with "potassium ferricyanide" (Fe^{+3}) to form "potassium ferrocyanide" (Fe^{+2}), which then reacts with ferric chloride to form Pearl's Prussian blue coloured complex that has an absorption maximum at 700nm.

$$K_3[Fe(CN)_6] + FeCl_3 \longrightarrow K_4[Fe(CN)_6] + FeCl_2$$

The reducing power of crude melanin extract was determined by the method described by Oyaizu (1986) with modification. Different amounts of crude melanin (10-40mg/mL) in water was mixed with 1 ml of 0.2 M phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide. The reaction mixture was incubated at 50°C for 20 minutes followed by addition of 1 ml of 10% trichloro acetic acid and centrifuged at 2000 rpm for 10 minutes. An aliquot of supernatant (1ml) was mixed with 1 ml of deionised water and 250 µl of 0.1% (v/v) ferric chloride. Absorbance was measured at 700nm wavelength against the diluent as blank. Higher absorbance indicates greater reducing power. The assay was carried out in triplicate and the results were expressed as mean ± standard deviation. Butylated hydroxyl toluene (BHT) was used a standard antioxidant (Manivasagan *et al*., 2013).

11.2 Anti-microbial property of pigment

Test organisms, including *Staphylococcus aureus*, *Escherichia coli*, *Proteus vulgaris* and *Bacillus cereus* were provided by Dolat-Usha Institute of Applied Sciences and Dhiru-Sarla Institute of Management & Commerce, Valsad microbiology department's laboratory.

All these organisms were separately cultivated on Muller-Hinton agar plates; swab inoculation of the microbes were made on the surface to produce lawn growth and wells of diameter 8 mm were made using cup borer. Antimicrobial activity of 10 µl of extracted pigment was tested by well diffusion method (Vasanthabharathi *et al*., 2011) alongside, using ethanol as negative control. The plates were incubated at 37°C for 24-48 hours. Presence of halo zone around the well indicates a positive antimicrobial activity results.

III RESULTS AND DISCUSSION

1 SCREENING AND ISOLATION

After incubation, diffusible dark brownish-black pigment producing mucoid, opaque colonies were screened and isolated on Tyrosine casein agar medium (TCA). Melanin is produced using tyrosine as precursor (Prajapati *et al*., 2017), so Tyrosine casein agar medium was used. Pigment producing colonies were selected from mixed plate and purified on same medium and later stored at 4°C for further analysis. Morphological characteristics of pigment producing colonies were recorded and Gram's staining was performed.

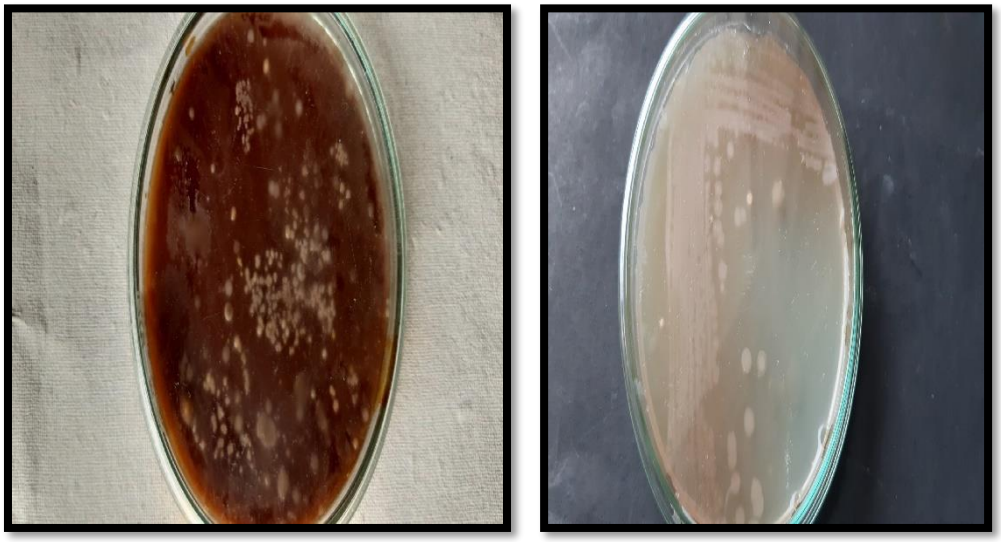
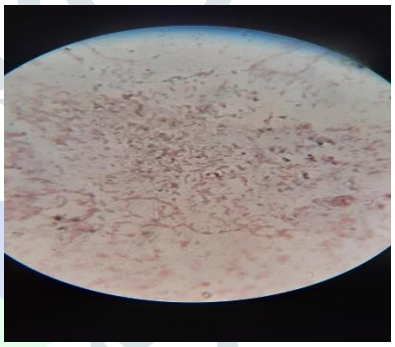


Figure 1: melanin pigment producing colonies on tyrosine casein agar (TCA) medium

Table 1: Morphological characterization and Gram's reaction of pigment producing colonies

Colony characteristics	**Gram's reaction and morphology**	**Figure**
Medium, mucoid, irregular, opaque, low convex colonies	Gram negative rods occurring singly and in chain	

2 CHARACTERIZATION AND IDENTIFICATION OF MELANIN PIGMENT PRODUCING BACTERIA

On the basis of 16S rRNA sequencing, the comparison with the available sequences at NCBI GeneBank, the sequence of pigment producing isolate showed highest homology (100%) with 100% query cover to *Pseudomonas aeruginosa* strain DSM 50071.

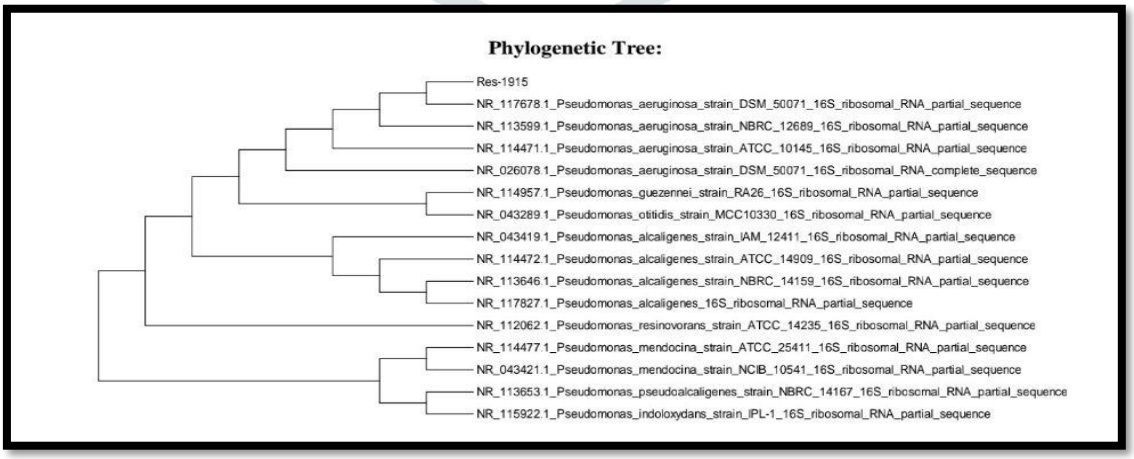


Figure 2: phylogenetic tree for *P. aeruginosa* on basis of 16S rRNA sequencing analysis

3 PRODUCTION MEDIUM

Pigment production was carried out in Tyrosine casein broth (TCB), Peptone iron broth (PIB) and Tyrosine broth (TB) respectively, through Submerged fermentation (SF) method. After incubation period of 7-8 days at 30°C for production medium, pigment production was visible in each media as the colour of the medium have changed and became opaque indicating melanin pigment production.

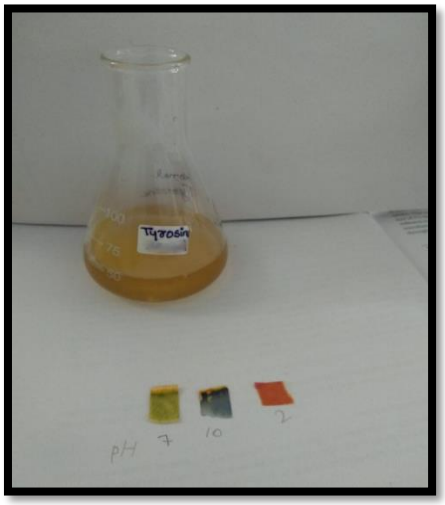
Figure 3: pigment production media before incubation

Figure 4: pigment production media after incubation

4 EXTRACTION OF PIGMENT

4.1 From tyrosine broth

For extraction of pigment from Tyrosine broth, complete polymerization of broth was done by adjusting the pH of the medium to 10 and then to 2**,** for removal of proteins and lipid content from the mixture equal ratio of organic solvents i.e., Chloroform: ethyl acetate: methanol was added to the mixture. After addition of organic solvents, aqueous phase was obtained which was collected using separating funnel. The content was dried overnight on evaporator, weighed (0.10gms/50ml of production medium) and stored in cool and dark place for further usage. Amongst all the three media that were used, highest amount of crude was extracted from Tyrosine Broth.



(A) **(B)**

Figure 5: A) complete polymerization of pigment B) Dried crude extract obtained from Tyrosine broth (0.10gm/50ml)

4.2 From Peptone iron broth and Tyrosine casein broth

After addition of potassium persulphate ($K_2S_2O_8$), a strong oxidising agent and later on addition of methanol which precipitates melanin from the broth, the content was centrifuged for 10 minutes at 10,000 rpm. Supernatant was discarded and brown coloured pallets obtained were collected and dried on evaporator, weighed (0.03gm/50ml and 0.07gm/50ml from PIB and TCB, respectively), and stored in glass vial in a cool, dark place.

(A) **(B)**

Figure 6: A) adjusting the pH of the filtrate B) brown coloured pallets

A) **B)**

Figure 7: crude extracted from A) PIB (0.03gm/50mL) and B) TCB (0.07gm/50mL)

5 OPTIMISATION STUDIES OF MELANIN PIGMENT

5.1 Carbon source

Amongst various carbon sources used for pigment production, medium containing 1% starch showed melanin pigment production, studies suggested that melanin pigment producing bacteria are considered to contribute to the breakdown of complex carbohydrates and recycling of organic compounds (Sunanda *et al*., 2009). Effect of various carbon sources in pigment production is shown in table 2.

Table 2: effect of various carbon source on pigment production

Parameter		**Melanin pigment production**
Carbon source	Starch	Observed
	Glucose	Not observed
	Sucrose	Not observed

5.2 Nitrogen source

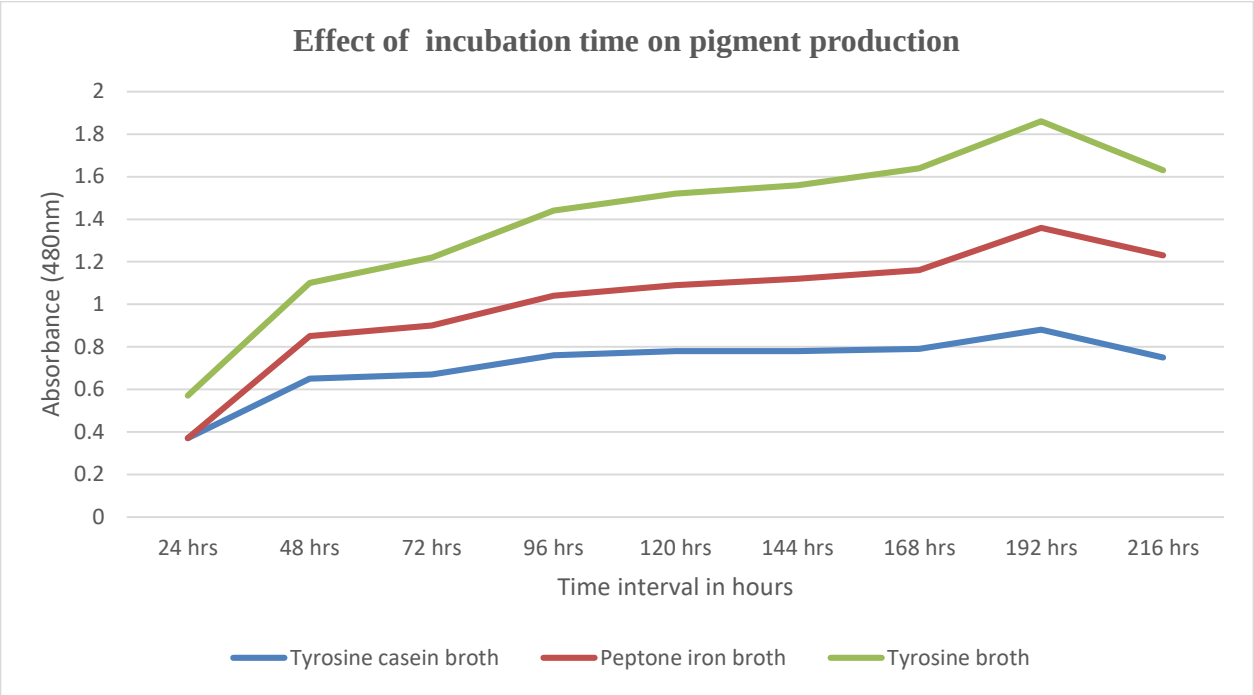
Amongst various other nitrogen sources, melanin pigment production was observed in basal medium containing L-tyrosine, as melanin is synthesized by oxidation of amino acid tyrosine followed by polymerization. The effect of other nitrogen sources is as shown in table 3.

Table 3: effect of various nitrogen source on pigment production

Parameter		**Melanin pigment production**
Nitrogen source	L-tyrosine	Observed
	L-lycine	Not observed
	L-proline	Not observed

5.3 Effect of incubation time on pigment production

The pigment production was affected by incubation time and gradual increase in pigment production was achieved with increasing the time period. Highest absorbance was noted at 192nd hours. But, further incubation showed decrease in pigment production. Same results were obtained, when effect of incubation time on pigment production was compared to results obtained by Prajapati *et al*., 2017.



Graph 1: effect of incubation time on melanin pigment production

6 ANALYSIS OF PIGMENT

6.1 Confirmation of pigment

In-vitro confirmation of melanin pigment was done by using substrate *i.e.*, Tyrosine solution. Reddish colour tinge was observed in the flask after 3 days of incubation at room temperature. The appearance of red color indicates positive results for melanin pigment confirmation.

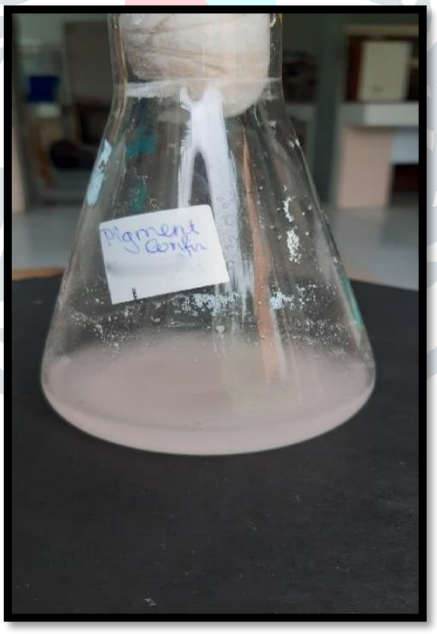


Figure 8: reddish tinge observed in tyrosine substrate solution after incubation

6.2 Chemical analysis

The chemical analysis of melanin pigment was conducted by determining its solubility in wide range of solvents. Melanin that was extracted was soluble in Distilled water and Ethanol. For further usages of the pigment in other tests, the crude melanin was stored in its soluble form using distilled water as it was easily available, cost effective and not-harmful compared to ethanol. Solubility reaction of crude with other solvents are noted down in table 4.

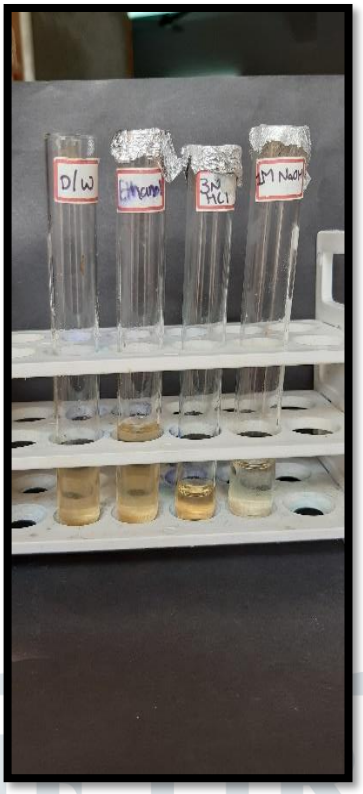


Figure 9: solubility reaction of melanin in various solvents

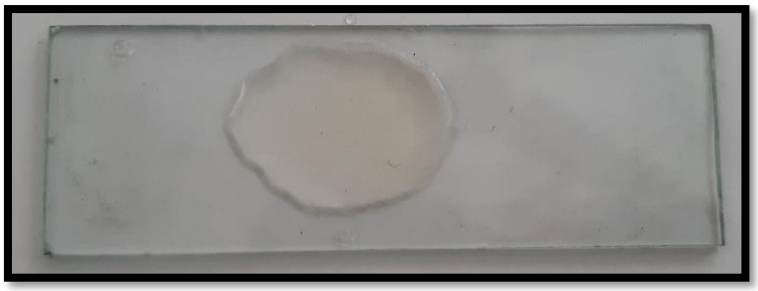
Table 4: solubility reaction of extracted melanin

Reagent	**Solubility reaction**
Distilled water	Soluble
Ethanol	Soluble
Acetone	Insoluble
Chloroform	Insoluble
1M NaOH	Soluble
3N HCL	Soluble
Reaction with H_2O_2	Decolorized

6.3 Bleach test

Potassium permanganate ($KMnO_4$) has been used as a bleaching agent, which is economical as well as non-polluting. When melanin is present in large amounts, cell details may be obscured. Also, ability to be bleached serves as an identifying factor for melanin. The bleaching of excessive melanin helps in diagnosis of ocular tumours based on histological reports (Pillaiyar *et al.*, 2017). In the experiment conducted, when crude melanin was subjected to dilute $KMnO_4$ solution, bleaching of melanin was observed as brown colour of melanin turned colourless due to the action of strong oxidising agent. Though the use of $KMnO_4$ as a bleaching compound is harmful and restricted in certain countries, it is still being used in combination with Oxalic/ Sulphuric acid.

(A)



(B)

Figure 10: Bleach test of melanin (Brown to colourless)

6.4 Thin layer chromatography

TLC is a type of purification and separation technique. Separation was done using n-Hexane: Chloroform (15:15) ratio. After the chromatogram was generated, separated spots of pigment was observed in UV lamp. Retention factor (R_f) value of the spot of melanin pigment was calculated to be 0.6 which is near to the analysis of melanin done by Diraviyam *et al*., 2010.

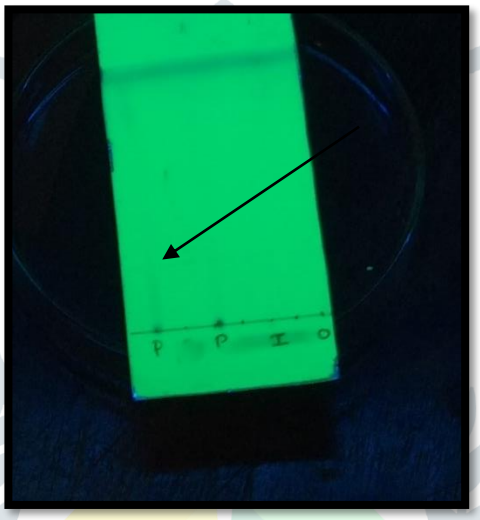


Figure 11: chromatogram developed in UV chamber showing spot of melanin pigment

7 SPECTROSCOPIC ANALYSIS OF THE PIGMENT

7.1 UV-Vis Spectroscopy

The UV-Visible spectrum of the pigment, showed strong absorbance in the UV region. The absorption spectrum of melanin showed a typical peak at 290 nm and then progressively decreased in the visible wavelength as for the typical absorption profile of synthetic melanin.

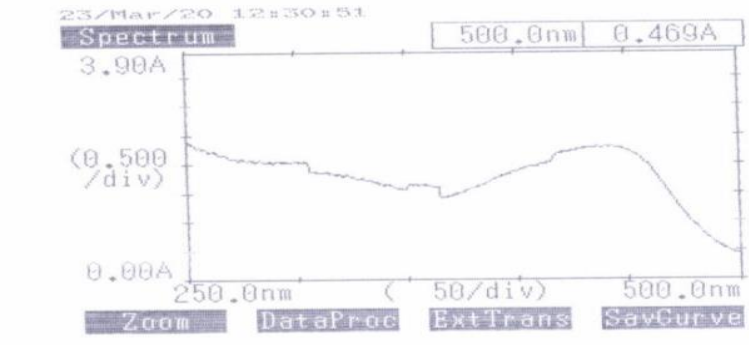


Figure 12: UV-Vis spectroscopic analysis of crude extract

7.2 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectral study was conducted to get information about melanin functional groups and structure. The FTIR spectra of synthetic melanin and the crude extract obtained in this research work showed common signals. Resemblance in the main absorption band centered near 3432 cm^{-1} reveals presence of OH group bonding with –NH group. The band centred at 1635 cm^{-1} is attributed to vibrations of aromatic ring C=C bounds and/or of aromatic conjugated C=O groups. The one centred at 1471 cm^{-1} must correspond to aliphatic C-H bending or C-N stretch, and/or carboxylate ion groups. The other little peaks may be ascribed to alkene C-H substitution in the melanin pigment. These absorptions are common and characteristics for melanin from several biological materials.

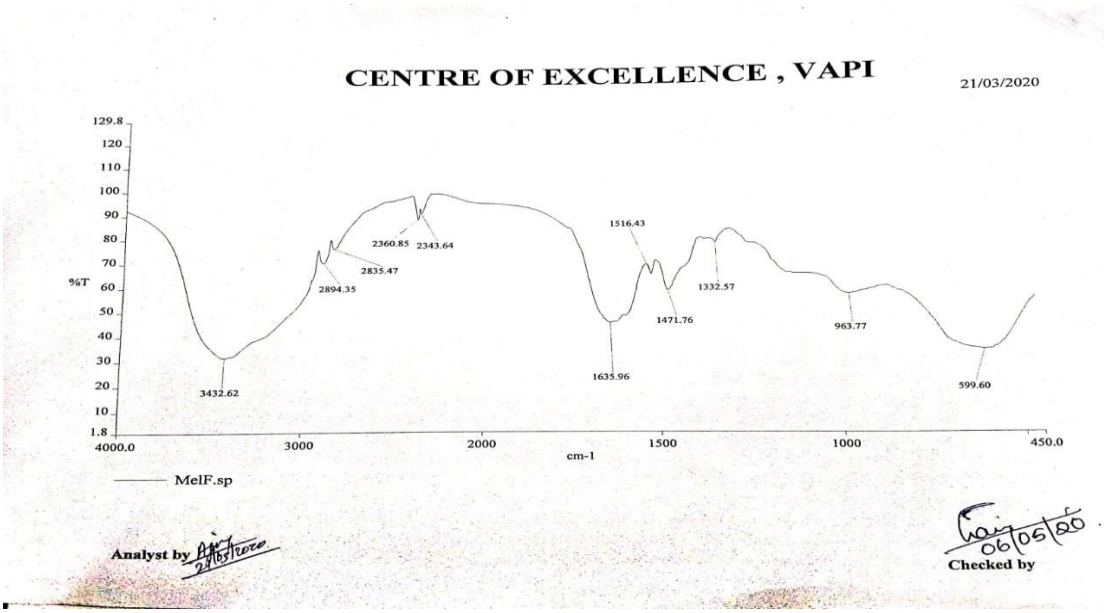


Figure 13: FTIR spectra of extracted melanin

8 APPLICATION OF PIGMENT

8.1 Reducing power assay

Reducing power assay modified method of Oyaizu (1986). Butylated hydroxyl toluene (BHT) was used as a standard, as BHT serves as highly active purified antioxidant. Some scientists (Vimala *et al*., 2013) have observed a direct correlation between antioxidant activity and reducing power of melanin. The reducing properties are generally associated with the presence of reductones, which have been shown to exert antioxidant action. Melanin interacts with free radicals and other reactive species readily due to the presence of unpaired electrons in its molecules, thus preventing peroxide formation and acts as an antioxidant, suggesting its use as a raw cosmetic material to minimize toxin induced tissue destruction (Naggar *et al*., 2017).

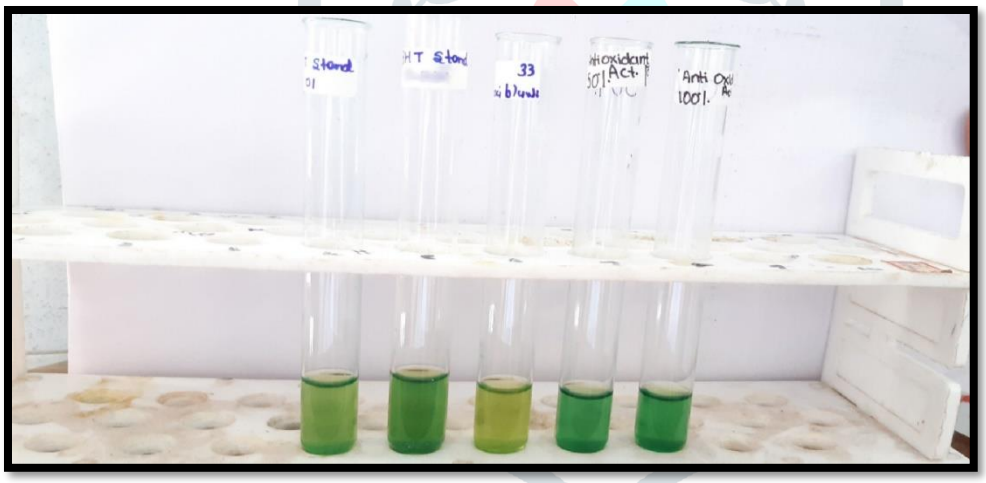
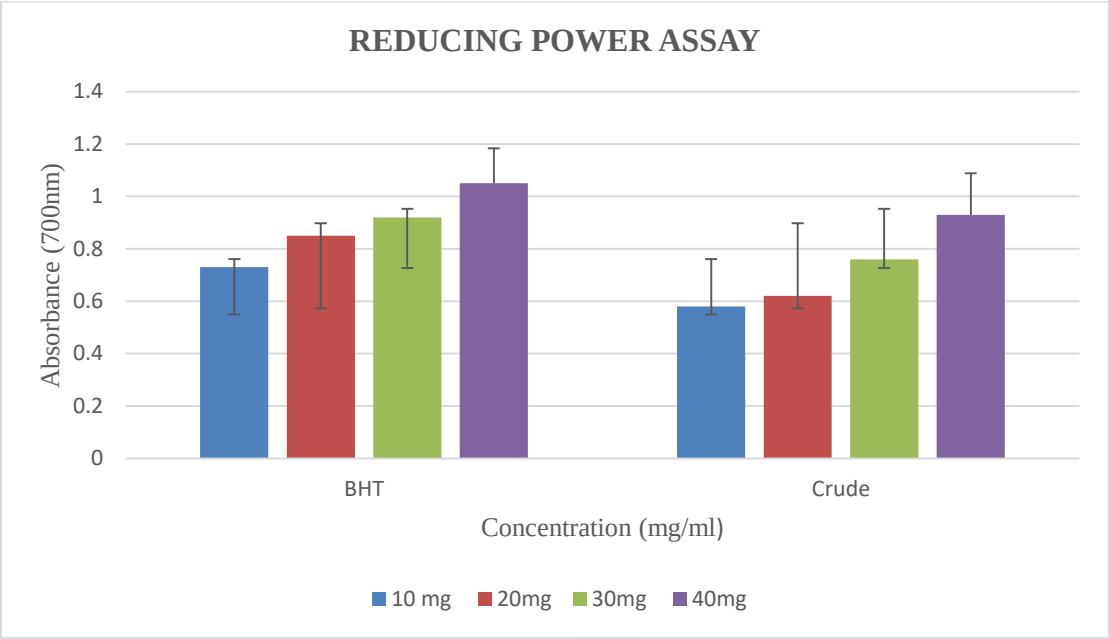


Figure 14: Reducing power assay by modified method of Oyaizu (1986)
[From Left to right: BHT standard, blank, and crude extract]



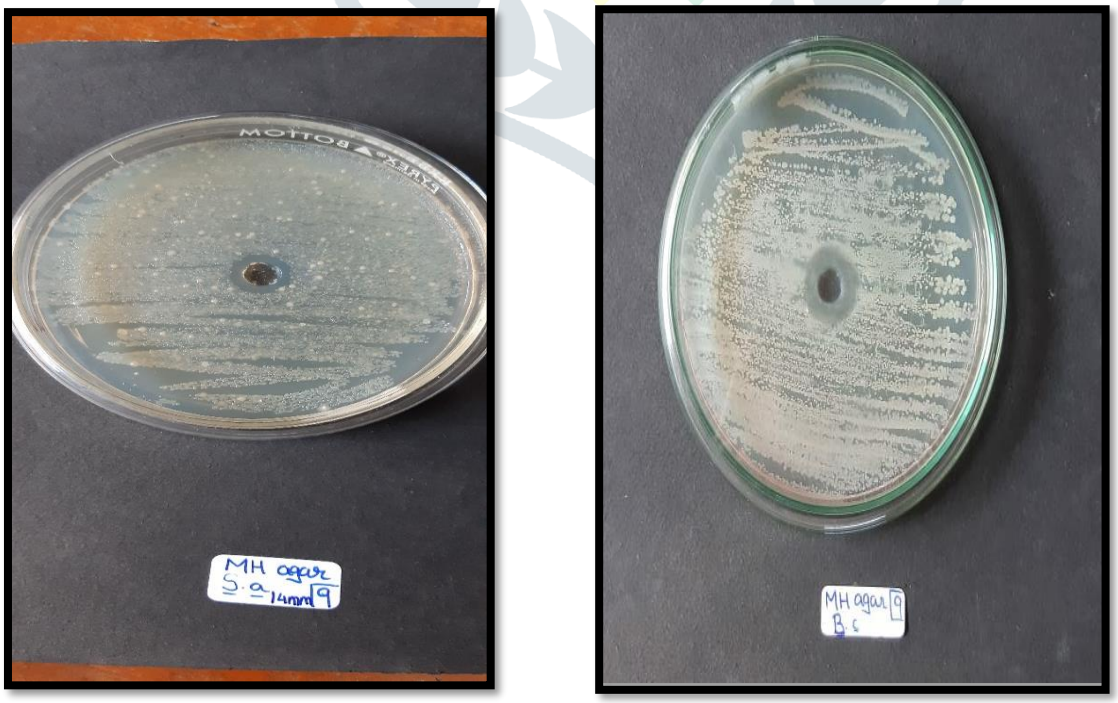
Graph 2: Reducing power of Melanin with respect to BHT depicted at 700nm. All the values are expressed ad mean ± standard deviations

8.2 Antimicrobial activity

The extracted pigment showed zone of clearance against Gram Positive Bacteria *Staphylocccus aureus* (14mm) and *Bacillus cereus* (15mm) and no zone was observed in Negative control. From the zone of inhibition observed on MH agar medium against organisms, it can be concluded that melanin possesses antimicrobial activity and could possibly act as a suitable source of new antimicrobial natural products and after certain confirmatory test, it may be used in pharmaceutical preparations also.

Table 5: Antimicrobial property of melanin

Organisms	**Diameter of inhibition (mm)**
Staphylococcus aureus	14mm
Bacillus cereus	15mm
Proteus vulgaris	No zone
Escherichia coli	No zone



(A) **(B)**

Figure 15: clear zone of inhibition observed in A) *S. aureus* and B) *B. cereus*

IV CONCLUSION

The melanin pigment producing organism, *Pseudomonas aeruginosa* strain DSM 50071 was isolated from farm soil sample using tyrosine casein agar medium. It produced diffusible brownish-black pigment. Amongst all the three production media used *i.e*., Peptone iron broth (PIB), Tyrosine casein broth (TCB) and Tyrosine broth (TB), highest amount of pigment was extracted from Tyrosine broth – 0.10grams / 50ml. By performing melanin formation test using tyrosine substrate solution, red coloration obtained confirmed that the pigment was melanin. The pigment obtained was found to be soluble in Water, Ethanol, 1M NaOH and 3N HCl and insoluble in acetone, methanol, chloroform. Maximum absorbance was seen in UV region (290nm) decreasing towards visible range. This showed the photo-protective role of extracted melanin. The FTIR spectrum analysis confirmed that the pigment obtained was melanin and indicated the presence of functional groups- C=C, C-H, C=O, much similar to the functional group present in different types of melanin. Reducing capacity of the pigment was also studied using modified method of Oyaizu (1986) against BHT as a standard. Antimicrobial activity of melanin was also recorded using well diffusion assay.

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