

# Isolation of Melanin Pigment Producing Bacteria from Marine Water and Study of Photoprotective Role of the Pigment

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**ABSTRACT** : The aim of this study was to obtain melanin pigment producing isolate from marine water and study of photoprotective role of the pigment. Two isolates producing melanin pigment was isolated from marine water obtained from the sea shore of Marve beach, Malad (Mumbai) by isolation on L-tyrosine agar medium and its morphological and biochemical characteristics was studied. One of the two organisms was identified as *Burkholderia cepacia* by molecular technique of VITEC 2. Optimization of parameters like temperature and pH on melanin production was studied. Melanin pigment production was observed in the range of pH 7- pH 10, maximum found to be at pH 10. Temperature range of 27°C to 37°C was found to be suitable for melanin production, 37°C showing maximum pigmentation. The melanin pigment obtained was water soluble and was confirmed to be photoprotective using Ultraviolet-Visible spectrum analysis which showed maximum absorption in the UV range- (250-350nm) and decreasing towards visible region. The pigment also showed antioxidant activity (DPPH inhibition % was found to be 60%). Fourier Transmission Infra-red spectrum (FTIR) analysis confirmed the crude melanin extract obtained as melanin.

**KEYWORDS** : Melanin, Photoprotective, Optimisation, Antioxidant

## I. INTRODUCTION

Melanin is the predominantly indolic polymer and the major pigment present in surface structure of vertebrates. The origin of the name is from melons (Greek dark) is usually attributed to the Swedish chemist Berzelius in 1840. The synthesis of Melanin is one of the most universal and is widely dispersed in the animal and plant kingdoms and also synthesized from micro organisms.[3]

Melanins are diverse group of dark coloured negatively charged high molecular weight pigments synthesized ubiquitously in living organisms by oxidative polymerization of various phenolic and/or indolic compounds.[5]

There are three types of Melanin i.e eumelanins, pheomelanins and allomelanins based on their colour and structural classes. Eumelanins are black to brown colour pigments produced by Manson- Rapper pathway which involves action of tyrosinases on tyrosinase, pheomelanins are brown, red or yellow colour pigments produced by oxidation of tyrosine and or dihydroxyphenylalanine (DOPA), Allomelanins are formed from catechol precursors.[3]

The most noteworthy function of melanin is to act as a photoprotector against radiation, especially UV region of the spectrum.[4] Also melanin is known to be associated with numerous survival mechanisms and offers virulence thus contributing to growing resistance of the pathogenic bacteria towards antibiotics.[5]. Melanin plays major role in innate immune system of insects to damage microbial intruders. The pigment also has versatile biological activities such as radical scavenging, antioxidant, antitumor, anti-inflammatory and as immune stimulating agent and metal chelating ability. There are reports of Anti- HIV properties of melanin and their role in photo voltage generation and fluorescence studies.[4]

Melanin obtained from microbes has great advantages over melanin from animals and plants. Microorganisms don't cause the problems of seasonal variations and are selected as they modify them according to the medium and

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conditions provided to them. Since melanin is known for absorption of harmful UV- radiation, the pigment can be used in various areas that require UV protection. One such area is the packaging industries.

Considering the potential uses and increasing demand for the melanin pigment there is a need to conduct studies on the production of melanin from microbes. The photo protective role of the microbial melanin pigment should be ascertained by its extraction from the pigment producing micro organism and studying its property of UV absorption.

## II. RELATED WORK

Bacterial melanins have been observed in a great number of species as their cultures have black or dark brown colors. However, it is important to note that not all dark pigments in bacterial cultures are indeed melanin pigments. For instance, molasses from high polluting industries are a suspension of dark color bacterial subproducts in wastewater, but they are not really melanin and should be treated in a different way for detoxification and discoloration. Bacterial melanins were well first characterized in *Streptomyces*, as these species have been actively pursued in the research of antibiotics [6].

Certain bacterial species, such as *Azotobacter*, can also form melanin from catechols to create high respiration rates necessary for protection of the nitrogenase system in nitrogen fixation. Some bacteria are also able to carry out the pentaketide biosynthetic pathway leading to DHN-melanin, although the bacterial polyketide synthases have different features from those of the fungal and plant enzymes and possibly are able to incorporate other small aromatic metabolites to the melanin pigment. [6] L-Dopachrome increases tyrosinase activity, whereas L-tyrosine induces melanosome synthesis, and increases tyrosinase activity. L-Tyrosine serves as the starting material for biosynthesis of melanin. The immediate product, dopa, upregulates melanin synthesis.[11]

In agreement with the bacterial diversity, other species such as *S. marcescens* form a brown pigment by oxidation and polymerization of intermediates of catabolic pathways. In that species, the melanin precursor is 3,4-dihydroxyphenylacetate. When the enzymes of this catabolic pathway are induced and the 3,4-dihydroxyphenylacetate-2,3-dioxygenase remained at low activity; the accumulation of the diphenolic intermediate leads to pigment production as a form to eliminate its excess. Nowadays, the polymer is considered allomelanin although the original paper doubted about the melanin-like nature of that product as it was formed without the involvement of a polyphenol oxidase. Similar data were reported with some *Pseudomonas* due to a reduced 4-hydroxyphenylpyruvate dioxygenase activity, and the pigment is considered pyomelanin. Regarding this concept, other bacterial species have no polyphenol oxidase but are able to form a yellowish pigment that is also named pyomelanin. Its color resembles pheomelanin, but, in fact, these pyomelanins are different from those formed by *Pseudomonas* as they are derived from the homogentisic acid, HGA-melanin, an intermediate of the tyrosine catabolic pathway. *Vibrio cholerae* [7] synthesizes pyomelanin in response to specific physiological conditions that are stressful to the bacteria. Under those conditions, there is accumulation of homogentisic acid because of the low activity of homogentisate dioxygenase. Thus, homogentisic acid is subsequently oxidized and an ochre-colored polymer is formed.

More recently, this type of melanin has also been characterized in *Burkholderia cenocepacia*, a gram-negative opportunistic pathogen that can survive within phagocytic cells, and the synthesis of this melanin is a defense mechanism against the host cell [6], similarly to some pathogenic fungus, such as *Aspergillus fumigates*.

The spectroscopic properties of the melanin pigment from *Klebsiella* culture correlated with those of melanin produced by various microorganisms as reported previously as well as with the properties of the synthetic melanin. Different concentrations of purified melanin were prepared using the initial concentrations of 100 mg/l in 0.1 N NaOH, and diluted to 1:1, 1:2, and 1:3. Each alkaline solution was scanned from 180 to 900 nm wavelengths. A 0.1 N NaOH was used as the blank. The spectroscopic property of the melanin pigment obtained from *Klebsiella sp. GSK* was compared with synthetic melanin. The UV-visible wavelength scan (180 to 900 nm) increased in the UV region at 200-300 nm, but decreased towards the visible region, which is the characteristic property of melanin. This phenomenon is due to the presence of the very complex conjugated structure in melanin. This property of melanin is confirmed by comparing with the previous descriptions of melanin pigment and measurements of synthetic melanin.. [ 8]. Melanin pigment produced by the garden soil *Streptomyces* Speciesthrough solid state fermentation will be a potential source of antioxidant like trolox and tocopherol [9]. The melanin pigment produced by *N. alba* MSA10 can be used for environmentally benign synthesis of silver nanostructures and can be useful for food packaging materials..[10]

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Melanins are well known for its antioxidant property, which can help reduce ROS generation. Minimizing ROS minimizes inflammation as both these properties are interrelated to each other. The antioxidant and anti-inflammatory property of bacterial melanin produced by a marine bacteria Bacillus spp. BTCZ31 was explored. [12]

## III. MATERIALS AND METHODS

### 1. Isolation and Enrichment of melanin producing bacteria

Marine water sample was collected from the sea shore of Marve beach in Malad (Mumbai). The sample was streaked on the selective medium – Nutrient agar containing L-tyrosine ie Tyrosine agar medium since the melanin pigment is produced by the oxidation of L-tyrosine..

Enrichment was done in tyrosine broth medium . 2 ml of water sample was added to 2 flasks each containing 50 ml of broth and one incubated on rotary shaker and the other at static condition at RT for 5-7 days.

Pigment producing colonies obtained on Tyr-agar medium was isolated on Luria Bertani agar medium for separation of mixed cultures & identification of different isolates producing melanin. MacConkey agar medium was also used for isolation. Similarly isolation from Tyr broth medium was also carried out on LB agar medium

### 2. Identification of the bacteria producing the pigment

The bacterium was identified by morphological and biochemical methods . Gram staining was carried out for study of its morphological characteristics.

Biochemical tests included:

Sugar Fermentation tests of following sugars : D-Glucose, Sucrose, D-Maltose, D-Fructose, D- Lactose, Mannitol, IMViC tests ( Indole test, Methyl Red test, Voges-Proskauer test, Citrate utilisation), TSI ( Triple Sugar Iron Test), Lysine Decarboxylase test, Nitrate Reduction test  
Catalase test, Oxidase test, Gelatin hydrolysis, Starch Hydrolysis

### Identification of organism by Molecular technique

VITEC-2 compact automated instrument No- 0000172D6A1F(15667) was used to identify the marine bacteria found to be producing melanin pigment.

### 3. Extraction and purification of the pigment

#### 3 (a) From Tyrosine broth

The inoculated broth medium showing pigmentation after incubation was centrifuged at 2000rpm for 20mins. 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. Then this mixture was again centrifuged to obtain the melanin crude extract. Then equal volume of chloroform, ethyl acetate and acetone (1:1:1) was added and mixed to remove proteins. This step was repeated twice. An aqueous phase was collected by two phase separation using a separating funnel . Finally the aqueous phase was collected then kept in oven to dry at 60°C for 24 hrs.

#### 3 (b) From Peptone yeast extract broth and Tyrosine casein broth

The melanin pigment was extracted by the following method from Filtrate of tyrosine-casein and peptone-iron broth culture :

(50ml each) was adjusted to pH-7. then, 0.5g of potassium persulphate was added to each of the filtrate. Allowed to stand for two hours at room temperature with intermittent shaking. After 2 hours, 50ml methanol was added to each of the filtrate, allowed to stand for 3 days at room temperature. After 3 days, both the filtrates was centrifuged at 10,000rpm for 10 minutes. Supernatant discarded and pellet was collected, dried and weighed [1]

### 4. Analysis of the Pigment

**4 (a) Confirmation of the pigment :** The isolates was inoculated into 50 mL of 0.1% tyrosine substrate solution with few drops of Chloroform in 100mL Erlenmeyer flask and incubated at 37°C for 48hrs. The deep red color shows the positive result. [2]

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**4 (b) Chemical Analysis :** The solubility of extracted Melanin pigment in distilled water and organic solvent like ethanol, methanol, acetone, chloroform, 1M NaOH ; action of strong oxidizing agents such as H<sub>2</sub>O<sub>2</sub>, KMnO<sub>4</sub>, K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub> also its precipitation with 3N HCl and reaction with NaOCl was determined.[2]

**4 (c) Optimization studies for Melanin production :**The effect of two physical parameters (pH and Temperature) on the production of melanin pigment was studied. The isolate was cultured at temperatures (0°C , 37°C , 55°C ) and pH (6- 12) and the range for maximum pigmentation was optimised. Tyrosine broth medium was used for the optimization studies.

**4 (d) Physical analysis and study of photoprotective role :**UV-visible spectrum of the melanin extract obtained from the bacterial isolate was analyzed from 200nm to 900 nm using UV-Visible spectrophotometer . (For photoprotective role ). The undiluted and 1:2 dilution of Initial concentration 0.05g/ml in 0.1 N NaOH of the melanin extract were prepared. A 0.1 N NaOH solution was used as a blank. Each alkaline solutions was scanned from (200 to 900 nm ) The FTIR analysis of pigment was carried out after mixing with IR grade KBr (1:10) using FTIR spectrophotometer . ( For physical analysis of the pigment).

### 5. Study of antibacterial property of the pigment

The antimicrobial activities of crude pigment extracted was studied against Gram positive organisms such as *Staphylococcus aureus* and *Bacillus species* and Gram negative organisms such as *Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris* bacteria. The well diffusion method using Muller- Hinton medium was used for testing antibacterial activity. These cultures were swabbed on the Muller Hinton agar medium and 10 uL of the melanin extract was placed in the wells and incubated at 37°C for 24hrs.

### 6. Study of antioxidant property of the pigment

#### DPPH assay :

The radical scavenging activity by melanin ie its antioxidant property was investigated by using DPPH assay. 10mg/ml of the melanin extract was added to 2 ml of DPPH solution and absorbance was read photometrically using colorimeter at 500 nm before and after incubation at 37°C for 30 mins. DPPH inhibition % was calculated using the formula :**DPPH inhibition (%) = [ control absorbance – test absorbance ] x 100**

## IV. RESULTS

1. Isolation and Enrichment of melanin producing bacteria
- 2.

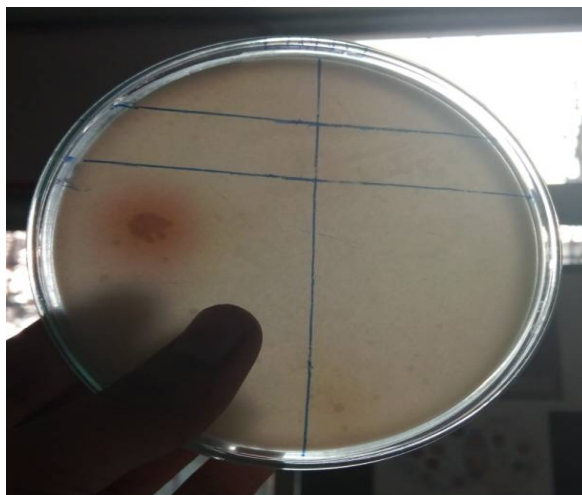


Figure 1 –Isolation of sample on Tyrosine agar medium

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The micro organisms producing melanin pigment showed brown colouration on the tyrosine agar medium. The pigment producing colony was then inoculated in tyrosine broth medium..

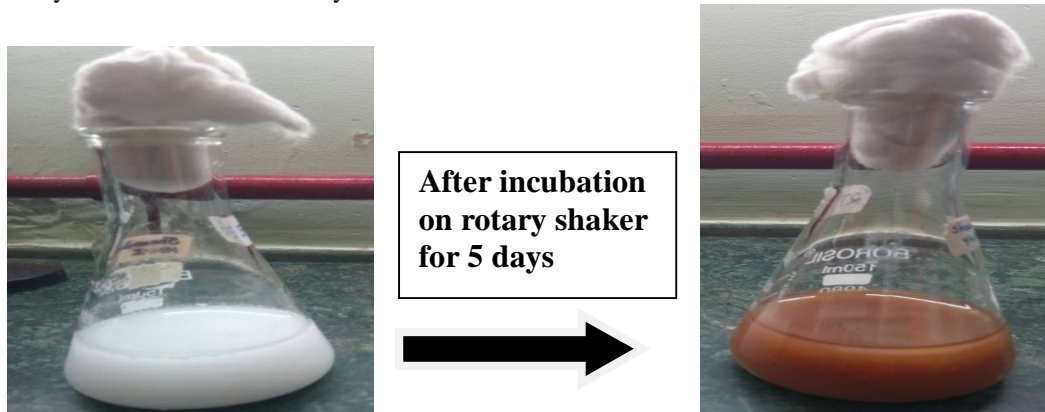


Figure 2 – Enrichment of marine sample in tyrosine broth medium

The pigment producing organism was enriched in the tyrosine broth medium by incubating the inoculated medium on rotary shaker for five days.

## 2. Identification of the bacteria producing the pigment

Table 1 :Biochemical characteristics of the melanin producing isolate

Characteristics	Results
<b>Sugar fermentation</b>	
<i>Aerobic:-</i>	Negative
Glucose	Negative
Maltose	Negative
Lactose	Negative
Sucrose	Negative
Mannitol	Negative
Fructose	Negative
<i>Anaerobic:-</i>	
Glucose	Negative
Maltose	Negative
Lactose	Negative
Sucrose	Negative
Mannitol	Negative
Fructose	Negative
<b>Indole test</b>	Negative
<b>Methyl red test</b>	Negative
<b>Vogesproskauer test</b>	Negative
<b>Citrate utilization</b>	Positive
<b>Nitrate reduction</b>	Positive
<b>Triple sugar iron test</b>	Acid/Alkaline/No gas/No H <sub>2</sub> S
<b>Lysine decarboxylase test</b>	



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Aerobic	Negative
Anaerobic	Negative
Catalase test	Positive
Oxidase test	Positive
Starch hydrolysis	Positive

The molecular identification of the bacterial isolate was done using VITEK 2 and the bacteria was identified as *Burkholderia cepacia*.

### 3.Extraction and purification of pigment

#### 3 (a) From Tyrosine broth

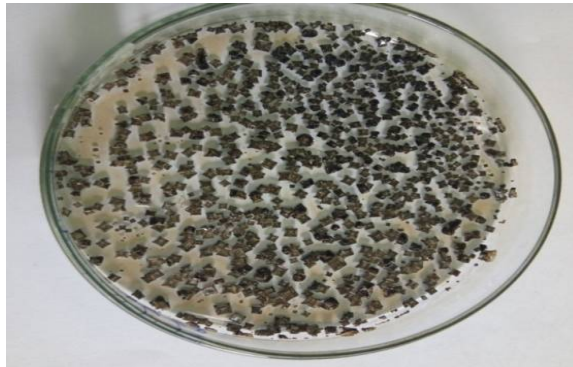


Figure 3- (a) Melanin crystals obtained after the extraction procedure

#### 3 (b) From Tyrosine casein broth and Peptone yeast extract Broth

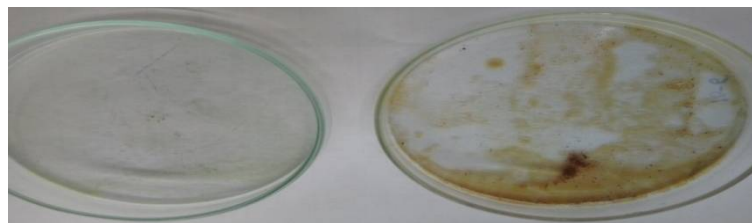


Figure 3 (b)- Extracts obtained from TCB and PIB

Crude melanin extract obtained : **0.005 grams/100ml** of Tyrosine casein broth ,**0.047 grams/100 ml** of Peptone iron broth, **2.265 grams/100 ml** Tyrosine broth was obtained.

Higher yield of the melanin extract was obtained from Tyrosine broth in the form of crystals.

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### 4. Analysis of the Pigment

#### 4 (a)Confirmation of the pigment

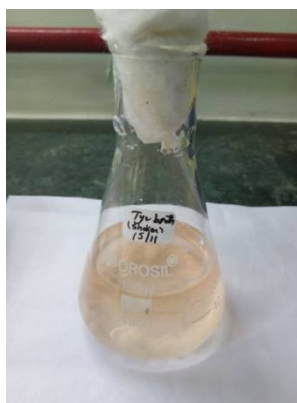


Figure 4(a) – Red colouration of the 1% tyrosine substrate

#### 4 (b)Chemical Analysis

Table 2 : Chemical Analysis

Reagents	Solubility/Reaction
Water	Soluble
Ethanol	Soluble
Methanol	Insoluble
Acetone	Insoluble
Chloroform	Insoluble
1 M NaOH	Soluble
H <sub>2</sub> O <sub>2</sub>	Soluble
KMnO <sub>4</sub>	Insoluble
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	Insoluble
3 N HCl	Soluble
NaOCl	Soluble

**Keys :-** 1M NaOH - Sodium hydroxide, H<sub>2</sub>O<sub>2</sub> - Hydrogen Peroxide, 3N HCl - Hydrochloric acid, NaOCl - Sodium hypochlorite, M – Molarity, N – Normality

#### 4 (c) Optimization studies for Melanin production

- ❖ **pH Optimization** : Pigment production was observed at pH-7 and pH-10. OD was checked at both the pH at 530 nm. OD for pH-7 was 0.5 and OD for pH-10 was 0.8. This showed that Tyr broth at pH 10 gave the maximum melanin pigmentation.
- ❖ **Temperature Optimization** : Pigment production was observed at Temp - 27°C and 37°C. OD was checked at both the temp at 530 nm. OD for 27°C was 0.43 and OD for 37°C was 0.52. This showed that Tyr broth at temp 37°C gave the maximum melanin pigmentation.

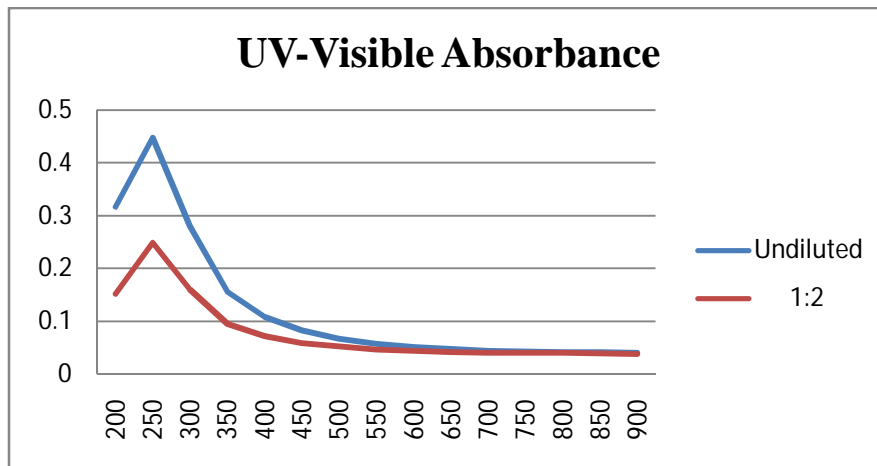
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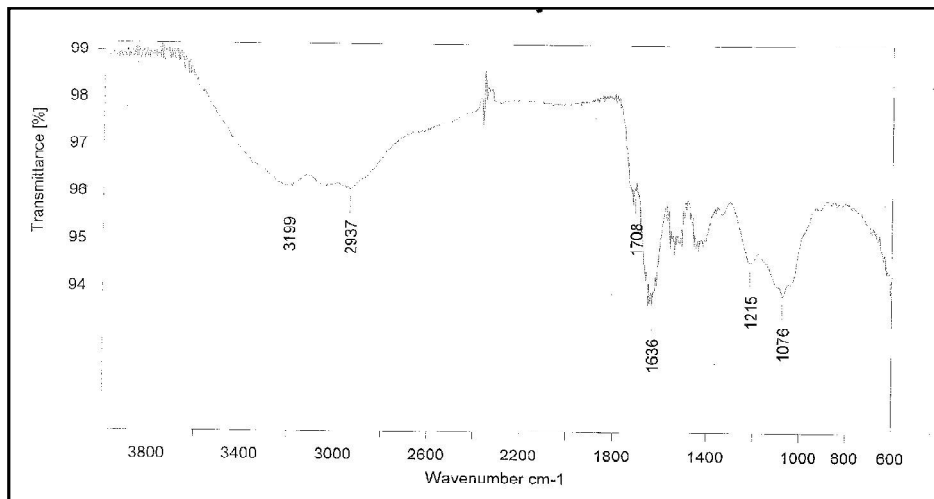
## 4 (d) Physical analysis and study of photoprotective role



Graph 1 - UV- Visible absorbance by Melanin extract

Maximum absorbance was seen in the UV range (250nm-350nm) and decreases towards visible range.

## FTIR Spectroscopy :



Graph 2- FTIR spectrum analysis of Melanin extract

Table 3: Functional groups detected through FTIR analysis

Wavenumber (cm <sup>-1</sup> )	Range	Functional Group	Mode of Vibration
1076	1440 cm <sup>-1</sup> -1220 cm <sup>-1</sup>	C-OH	Bending
1215	1600 cm <sup>-1</sup> -1475 cm <sup>-1</sup>	C=C ( aromatic ring)	Ring Stretch
1636	1700 cm <sup>-1</sup> -1640 cm <sup>-1</sup>	C=O (amide)	Stretch
3199	>3000 cm <sup>-1</sup>	C-H (aromatic)	Stretch



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These observations of FTIR indicate that the pigment extracted is Melanin. These characteristics were found to be much similar to that of bacterial melanin-pheomelanin.

## 5. Study of antibacterial property of the pigment

The obtained pigment did not show antibacterial activity against gram positive organisms *Staphylococcus aureus* and *Bacillus species* and Gram negative organisms such as *Escherichia coli*, *Salmonella typhi* and *Proteus vulgaris* bacteria in a well diffusion method carried out.

## 6. Study of antioxidant property of the pigment

### DPPH assay:

DPPH inhibition % was calculated using the formula :**DPPH inhibition (%) = [ control absorbance – test absorbance ] x 100**

**Before incubation :** DPPH inhibition (%) =  $[0.85 - 0.52] \times 100 = 33\%$

### After incubation:(At 37°C for 30 mins )

DPPH inhibition (%) =  $[1.02 - 0.42] \times 100 = 60\%$

Increase in DPPH inhibition % was seen after incubation at 37°C for 30 mins .

## V. CONCLUSION

The Melanin pigment producing organism, *Burkholderia cepacia* was isolated from marine water using tyrosine agar medium. It produced a brownish colour pigment. Peptone yeast extract broth and tyrosine casein broth also showed melanin pigment production, but large amount of the pigment could be extracted from Tyrosine broth - 2.265 grams/100 ml tyrosine broth. By performing melanin formation test using L-tyrosine as a substrate (1% tyrosine substrate solution), red colouration obtained confirmed that the pigment was melanin. The pigment obtained was found to be soluble in Water, Ethanol, 1M NaOH(Sodium hydroxide),H<sub>2</sub>O<sub>2</sub>(Hydrogen Peroxide),3N HCl(Hydrochloric acid), NaOCl(Sodium hypochlorite) and insoluble in methanol, acetone, chloroform,KMnO<sub>4</sub>(Potassium Permanganate), K<sub>2</sub>Cr<sub>2</sub>O<sub>7</sub>(Potassium dichromate). *Burkholderia cepacia* could grow well in the pH range 7-10 and showed pigmentation in this range giving maximum pigmentation at pH 10 and could tolerate temperature range of 27°C - 37°C with maximum pigmentation at 37°C.Maximum absorbance was seen in the UV range (250nm-350nm) decreasing towards visible range. This showed the photoprotective role of the extracted melanin pigment. The FTIR spectrum analysis technique confirmed that the pigment obtained was melanin and indicated the presence of the functional groups – [C=O, C-OH, C-H, C=C], much similar to the functional groups present in pheomelanin. The pigment did not show antibacterial activity against neither the gram positive nor the gram negative organisms. Melanin showed antioxidant activity. The antioxidant property was analysed by DPPH assay .The DPPH inhibition (%) for the pigment was found to be 60% after incubation at 37°C that is more as compared to what was obtained before incubation.

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