STUDIES ON ISOLATION, BIOPRODUCTION AND CHARACTERIZATION OF MELANIN PRODUCED FROM NEWLY SCREENED BACTERIAL CULTURES FROM DTU LAKE

A MAJOR PROJECT-II

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

FOR THE AWARD OF DEGREE OF

MASTER OF TECHNOLOGY

IN

INDUSTRIAL BIOTECHNOLOGY

Submitted by

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Under the supervision of

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CANDIDATE'S DECLARATION

I, Aditi Singh, 2K17/IBT/01, student of M.Tech (Industrial Biotechnology), hereby declare that the report entitled "**Studies on Isolation, Bio Production and Characterization of Melanin Pigment Produced from Newly Screened Bacterial Cultures from DTU Lake**", which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi, in the partial fulfilment of the requirements for the award of the degree of Master of Technology, is original and has not been copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associateship, Fellowship or other similar title or recognition.

Place: Delhi
Date: July 29, 2019 (**Aditi Singh)**

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CERTIFICATE

I hereby certify that the dissertation entitled "**Studies on Isolation, Bio Production and Characterization of Melanin Pigment Produced from Newly Screened Bacterial Cultures from DTU Lake**", which is submitted by Aditi Singh (2K17/IBT/01), Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Technology, is a record of the project work carried out by the student under my supervision. To the best of my knowledge that work has not been submitted in part or full for any Degree or Diploma to this university or elsewhere.

PROF. JAIGOPAL SHARMA
Supervisor & Head
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ABSTRACT

Melanins are composed of polymerized phenolic and/or indolic compounds, commonly found in organisms across all biological kingdoms. In this study, a total of 11 melanin producing bacterial strains were screened from lake water sample collected from DTU Lake, Delhi Technological University, India, using nutrient media supplemented with L-Tyrosine. Further these pigment producing strains were subjected for various biochemical tests to indicate the organism. Two strains RT3 and Ep1 were sent for microbial identification based on 16S rDNA molecular techniques, consensus sequences were obtained and were utilized for 16S rRNA sequence comparison and phylogenetic tree analysis. After phylogenetic identification, these two bacterial strains RT3 and Ep1 were identified as *Stenotrophomonas* spp. and *Bacillus oleronius* respectively. All the screened bacterial strains produced nearly black colored pigment which was tentatively inferred to as melanin only due to its ability to utilize L-tyrosine as precursor. One of the strains, designated **RT 3**, produced the maximum amount of pigment ~ 149µg/mL of bacterial culture utilized. The pigment was further characterized by UV and FT-IR spectroscopy, and its qualitative analysis was done by performing Thin Layer Chromatography and High Performance Liquid Chromatography. UV-vis wavelength scan showed the strong absorbance peaks in the region 200 to 240 nm, and further gradual decline when the wavelength is increased towards the infrared region. FT-IR and Thin Layer Chromatography results also confirmed the pigment to be melanin. However, after performing High Performance Liquid Chromatography for the synthetic and bacterial melanin pigment, no trend could be obtained for significant peak analysis in order to quantify the pigment. It still needed further improvisation by trying more solvent systems, so that the results obtained could be relatable.

Keywords: Melanin pigment, L-Tyrosine, Bacterial identification, UV-vis spectroscopy, FT-IR Spectroscopy, Thin Layer Chromatography, High Performance Liquid Chromatography.

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Abbreviations and Symbols Used

AHP	Amino hydroxyphenylalanine
bp	Base pairs
CTL	cytotoxic T-lymphocytes
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole- 2-carboxylic acid
DHN	1,8-dihydroxynaphthalene
DNA	Deoxyribonucleic acid
DOPA	3,4-dihydroxyphenylalanine
DTU	Delhi Technological University
FT-IR	Fourier Transformation Infrared spectroscopy
FDA	Food and Drug Administration
GGT	Gamma glutamyl transpeptidase
GST	Glutathione-S-transferase
h	Hours
HIV	Human Immunodeficiency Virus
HPLC	High performance liquid chromatography
mg	Milligram
min.	Minutes
mL	Millilitre
nBLAST	Nucleotide Basic Local Alignment Search Tool
NCBI	National Centre for Biotechnology Information
ºC	Degree Celsius
PDCA	Pyrrole-2,3,5-tricarboxylic acid
psi	Pounds per square inch
rpm	Revolution per minute
TLC	Thin Layer Chromatography
TRP	Tyrosine related proteins
UV-Vis Spectrum	Ultraviolet-Visible Spectrum

1. INTRODUCTION

1.1 Prologue

The use of pigments as colorants has been practiced since prehistoric times in different parts of the world. According to certain evidences discovered by some world archaeologists revealed the usage of paint by early humans for aesthetic purposes. It was further evidenced by the discovery of some 350,000-400,000 year old grinding equipments, which were found in a cave at Twin Rivers, near Lusaka, Zambia. It was even practiced during the Bronze Age in ancient Europe and during the Indus Valley period in India (2500 BC). Usage of Henna prior 2500 BC, mentioning of saffron in the Bible, and utilizing plants, barks, and insects for dyeing purposes 5,000 years are some reported evidences in the history of pigment. (Rao *et al., 2017*).

An upward trend towards replacing the synthetic colorants with natural pigments has been observed since last few decades due to the strong consumer demand towards biologically-derived products. Dyes such as tartrazine, sunset yellow, etc. provoke allergies individually or in combination with certain colorants. Even some synthetic colorants that had been approved by the Food and Drug Administration (FDA) for usage in foods, pharmaceuticals, and cosmetic industry were later found to promote cancer. For example, carbon black (widely used as printing ink pigment) is thought to be a potential carcinogen. From the environmental point of view, unethical discharges of untreated industrial dye effluents produce toxins and persist for long time due to long stability period.

These drawbacks of synthetic color have elevated the global demand for biologically synthesized pigments. Such pigments have wide applications in the fields of food and beverages, paper production, textile industries, drug and cosmetic industries, agricultural practices, water science and technology, etc. on everyday basis (Tuli *et al.*, 2015).

1.2 *Why Microbial Pigments?*

Major sources of natural pigments include either plants, microorganisms, aquatic and terrestrial animals; plant pigments derived from agro-industrial wastes such as grape (anthocyanins), tomato (lycopene) and palm (carotenoids) processing residues. However, variability in source and presence of pigment in low concentration in those target fruits

require processing of large amounts of agro-industrial waste, which isn't feasible enough. (Babitha, 2009)

On the other hand, microorganisms selected or modified, in search of suitable color additives have enormous advantages over plant pigments:

- Simple and rapid growth in low cost medium,
- Smooth processing,
- High concentration & quality
- Selection of pigment-producing microorganisms is straightforward: by observing colored colonies in agar plates.

The isolation of new microbes may be directed towards an acid stable pigment by regulating pH of the medium. (De Carvalho *et al.*, 2014)

However, microbial pigment has some limitations of high-priced, low stability and variation in pigment color due to changes in pH. Despite that, such pigments in food processing field are an area of promise with a large economic potential. These pigments not only have the capacity to increase the pr0duct marketability, but they also display antioxidants, antimalarial, antimicrobial, antineoplastic and anticancer properties (Rajasekaran *et al.*, 2008; Venil *et al.*, 2013).

Using bacteria for pigment bioproduction has several benefits over fungi, such as short life cycle and ease for genetic modification. However, in comparison to fungal pigments, most of bacterial pigments are still at the research and development stage (*Rao et al., 2017*) (refer **Table 1.1**);

Fungi/ Bacteria	**Color**	**Pigment**	**Status**
Fungi			
Monascus sp.	Yellow	Ankaflavin	Industrial production
Monascus sp.	Orange	Rubropunctatin	Industrial production
Ashbya gossip	Yellow	Riboflavin	Industrial production
Cordyceps unilateralis	Deep blood red	Napthoquinone	Industrial production
Monascus sp.	Red	Monascorubrin	Industrial production
Penicillium oxalicum	Red	Anthraquinone	Industrial production
Blakeslea trispora	Red	Lycopene	Development stage
Blakeslea trispora	Yellow-orange	Beta-carotene	Industrial production
Mucor circinelloides	Yellow-orange	Beta-carotene	Development stage
Bacteria			
Bradyrhizobium sp.	Orange	Canthaxanthin	Research project

Streptomyces sp.	Yellow	Carotenoids	Development stage
Streptomyces echinoruber	Red	Rubrolone	Development stage
Paracoccus zeaxanthinifaciens	Yellow	Zeaxanthin	Research project
Paracoccus carotinifaciens	Pink-red	Astaxanthin	Research project
Bradyrhizobium sp.	Dark-red	Cantaxanthin	Research project
Pseudomonas sp.	Blue, green	Pyocyanin	Industrial production
Flavobacterium sp.	Yellow	Zeaxanthin	Development stage
Agrobacterium aurantiacum	Pink-red	Astaxanthin	Research project

Table 1.1: Several reported Fungal and bacterial pigments for research or commercial application.

Hence, work on bacterial pigments production should be intensified to make them available in the market. Pigment producing bacteria are ubiquitously present in various ecological niches, such as rhizospheric soil, desert sand, fresh water, and marine samples. They were reported in low and high temperature regions, can even persist in salt regions and in endophytes. Various genera such as *Streptomyces, Micromospora, Nocardia, Rhodococcus, Thermomonospora, Actinoplanes, Microbispora, Actinomadura, Streptosporangium,* and *Kitasatospora* have been reported for producing a wide variety of pigments (Usman *et al*., 2017). The genus *Streptomyces* has been reported for highest pigment production so far. Like fungi, several bacteria have been reported to produce a wide range of pigments such as carotenoids, prodigiosin, zeaxanthin, pyocyanin, melanin, violacein, and actinorhodin (Rao *et al.,* 2017). Pigmentation may contribute to virulence by allowing a given microbe to evade host immune killing or by provoking inflammatory damage to cells and tissues.

Melanin is one such pigment ubiquitous in all biological kingdoms (Hill, 1992) due to its protective nature. These are predominant indolic polymers, negatively charged, amorphous, and hydrophobic with high molecular weight compounds (Butler and Day, 1998).

1.3 Aim and Objective

The aim and objective of this study was designed as follows:

1. Isolation and screening of melanin-producing bacterial strains from water sample collected from DTU Lake, Delhi Technological University, New Delhi.
2. Biochemical characterization of melanin producing bacterial strains.

3. Bacterial identification of strains RT3 and Ep1 using 16S rDNA sequence and their DNA sequence analysis and phylogenetic relationship analysis
4. Extraction and Purification of melanin pigment from bacterial strains and further characterization of pigment using UV-vis spectroscopy, FT-IR spectroscopy, Thin Layer Chromatography and High Performance Liquid Chromatography.

2. REVIEW OF LITERATURE

Melanins are indolic macromolecules, synthesized by oxidative polymerization of phenolic / indolic compounds (El-Naggar & El-Ewasy, 2017). It is a polymer of various groups with the ability to donate or accept an electron. Therefore, it can act as a final acceptor or a shuttle in the electron exchange with insoluble compounds of iron (Menter and Willis, 1997). On having accepted numerous electrons, such "reduced' melanin serves the bacteria as a reducer of insoluble ferric (III) oxides to ferrous (II) state. (Figure.2.1.)

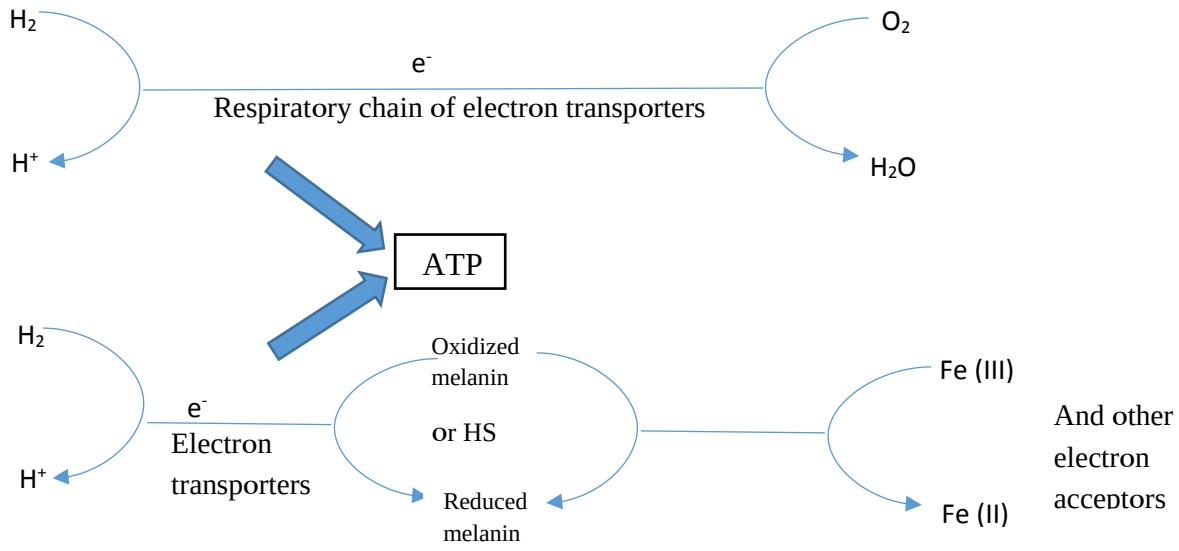


Figure.2.1. Function of melanin as an electron acceptor in the respiratory chain of a marine bacteria, in comparison to the aerobic variant of the process. (Plonka and Grabacka, 2006)

The pigments are often brown or black in color but many other colors have also been observed. There are several categories of melanin based on color and structural classes, which includes:

- **Eumelanin**, are often black to brown coloured pigments, produced by melanisation by classic Mason-Rapper pathway (Figure.2.2.), which produce some tyrosine intermediates / metabolites by the action of tyrosinases (Figure.2.3.).

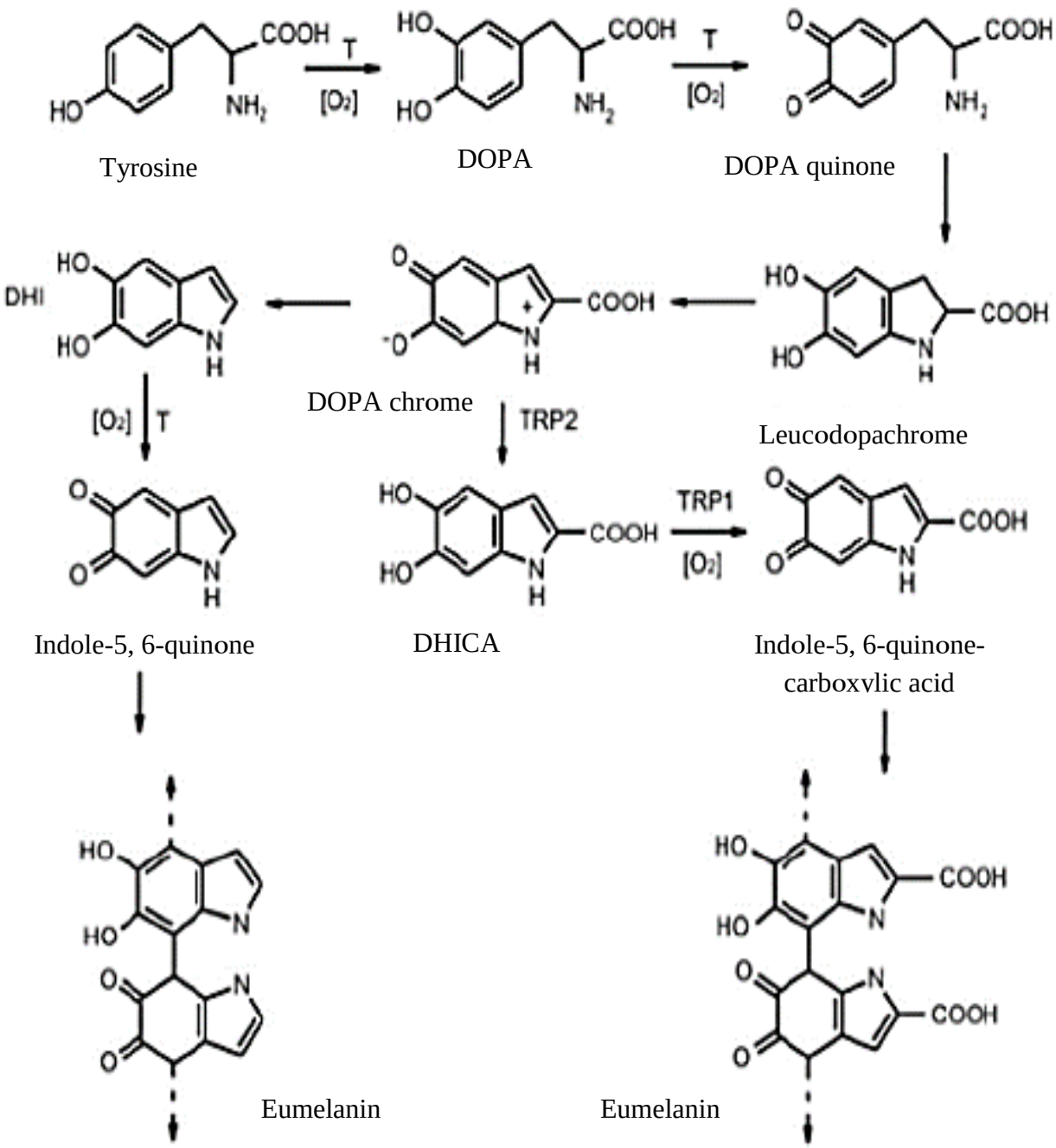


Figure.2.2. Mason Rapper pathway (Adapted from Plonka and Grabaka, 2006)

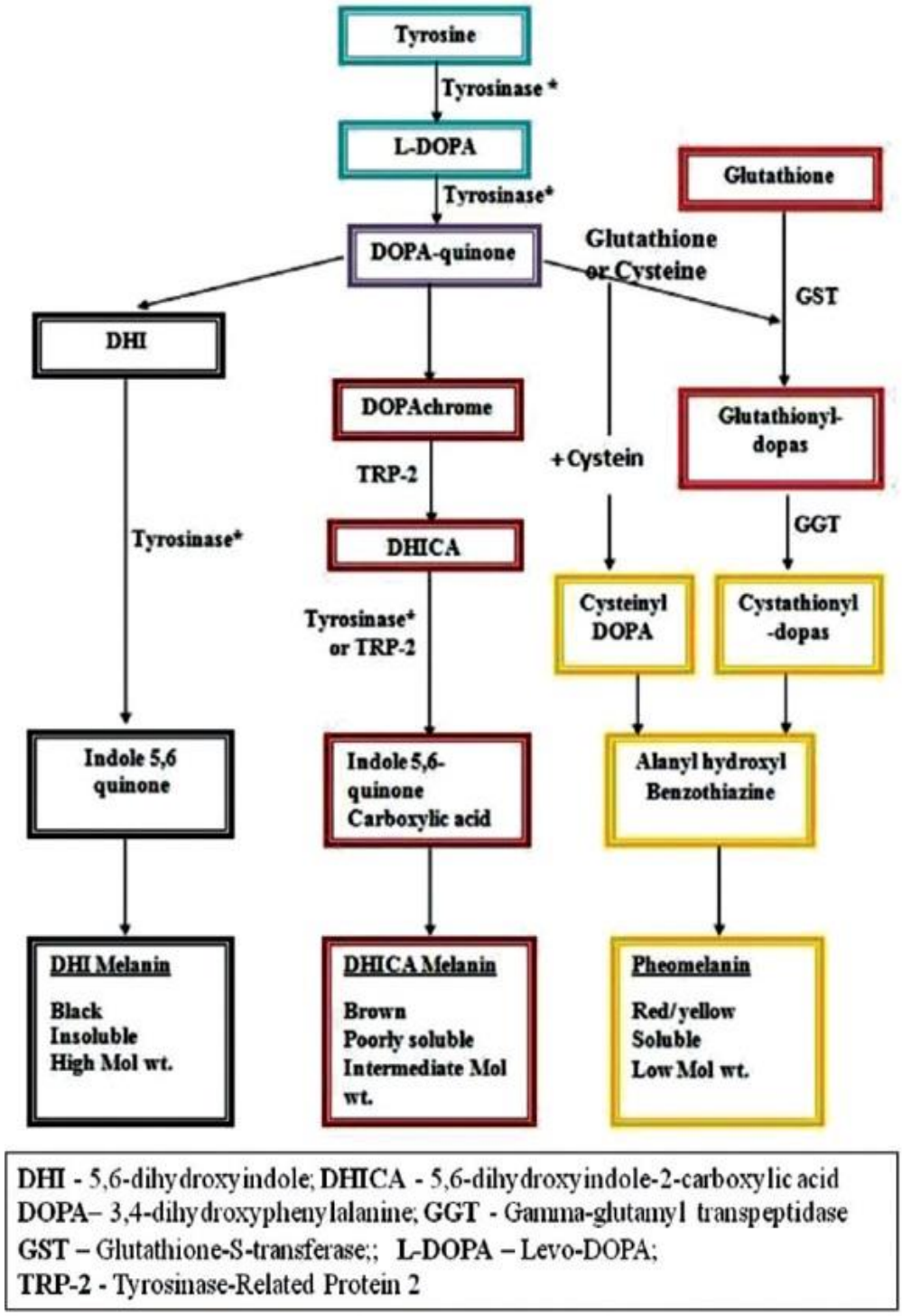


Figure.2.3. Eumelanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

- **Pheomelanin**, generally brown, red or yellow in colour, are produced by oxidation of tyrosine and/or phenylalanine to dihydroxyphenylalanine (DOPA) and dopaquinone (Tarangini, K., and Mishra, S., 2013). Pheomelanin results from cysteinylation of DOPA and these are sulphur containing compounds. (Figure.2.4.)

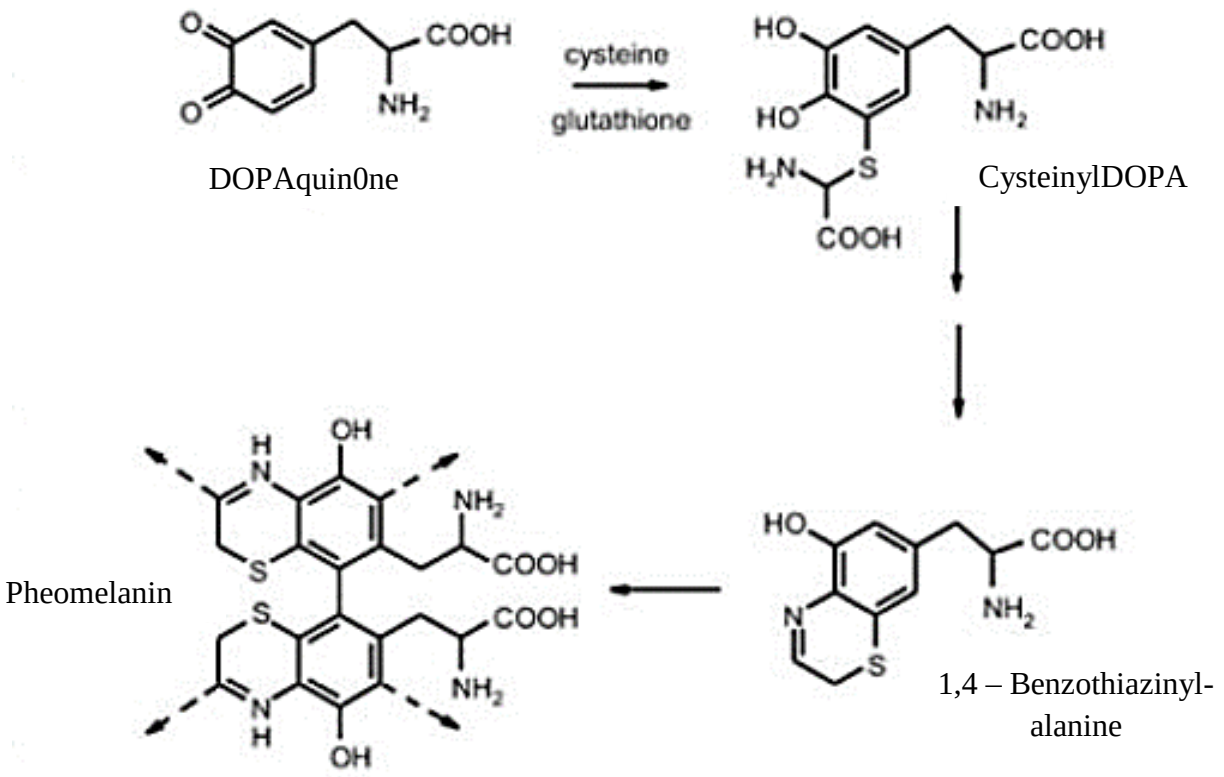


Figure.2.4. Pheomelanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

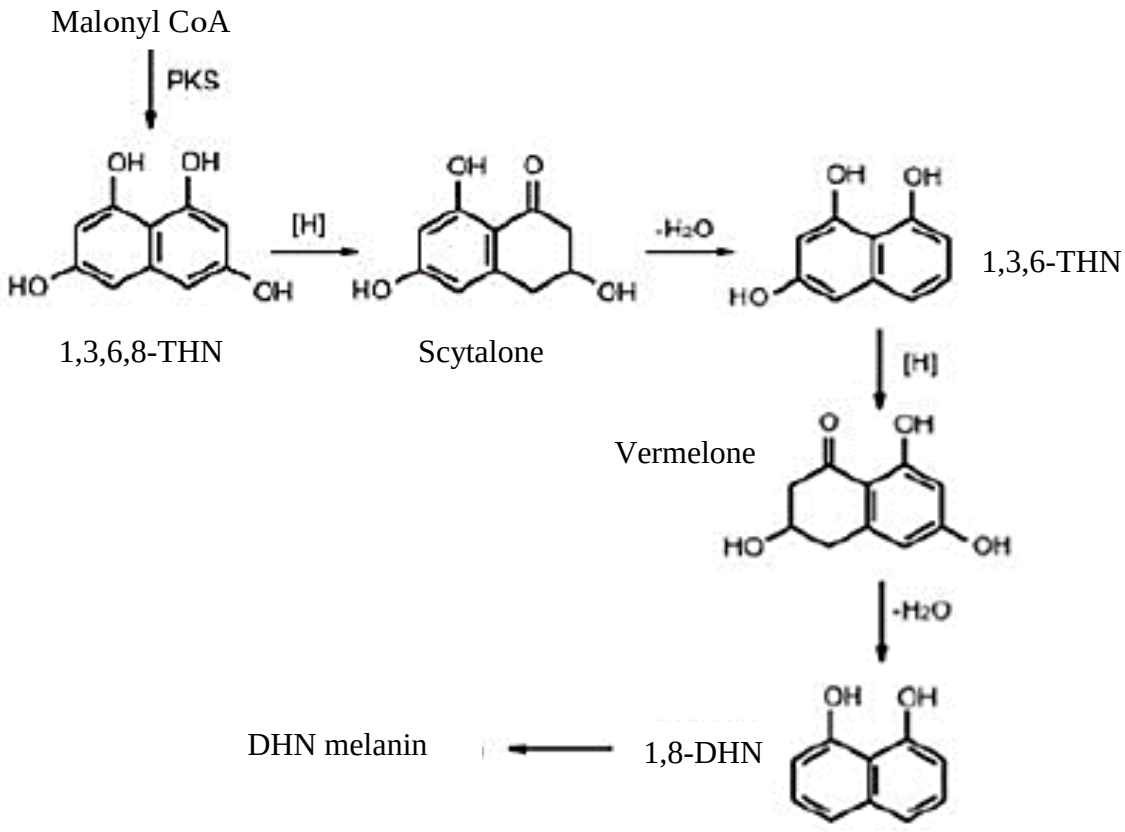


Figure.2.5. DHN-melanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

- **Allomelanin**, include nitrogen free heterogeneous group of polymers synthesized from catechol precursors. These melanins are least studies and most heterogeneous group of melanins which are synthesized by the polymerization of di (DHN) or tetrahydrofolate via pentaketide pathway leading to formation of various colored polymers including **DHN-melanin** (Figure.2.5.), homogentisic acid (**pyomelanin**) (Figure.2.6.), γ- glutaminyl-4-hydroxybenzene, catechols, as well as of 4-hydroxyphenylacetic acid.

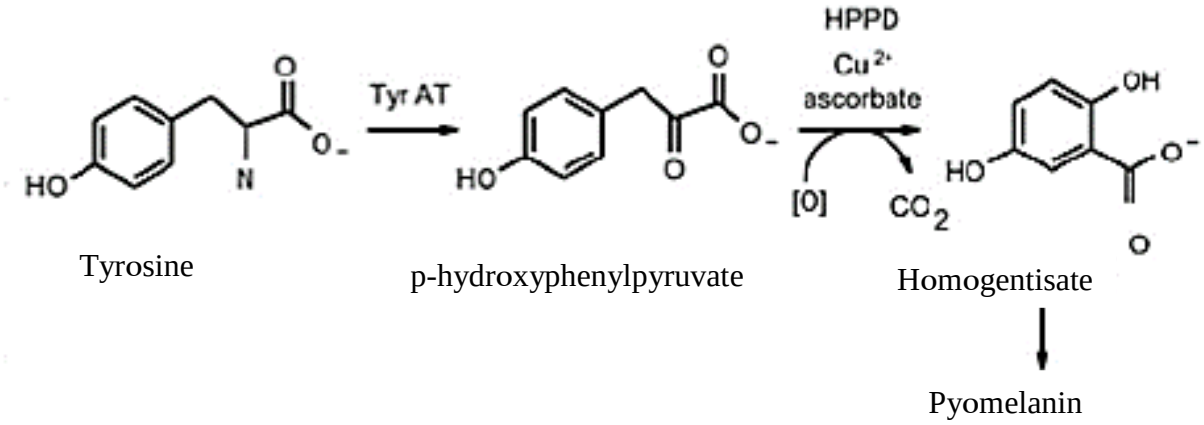


Figure.2.6. Pyomelanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

Generally, enzymes such as Tyrosinase, laccamases, polyketide synthases are responsible for producing the pigment in numerous microorganisms, depending upon the precursor availability.

Melanin production has already been reported in fungus, such as *Cryptococcus neoformans, Sporothrix schenckii, Sepia officinalis, Aspergillus niger, Penicillium marneffei, Paracoccidioides brasiliensis, Histoplasma capsulatum, C. neoforman*. Also in bacteria, for example, *Rhizobium sp*. (Cubo *et al.*, 1988), *Pseudomonas aeruginosa* (Rodríguez *et al.*, 2009), *Bacillus thuringiensis* (Aghajanyan *et al.*, 2005), *Klebsiella sp*. (Sajjan *et al.*, 2010) and *Modestobacter versicolor* (Reddy *et al.*,2007), etc.

Hence, isolating a high melanin producing microorganisms and identifying a suitable promotional medium is essential for enhancing biological melanin production. Conventionally produced melanin is often priced at $350 per gram. These conventional optimization methods are laborious, time-consuming and yield unreliable results because they ignore interaction effects. (Guo *et al*., 2014)

Often being insoluble in both aqueous and organic solvents, melanin is negatively charged and hydrophobic in nature (Butler and Day, 1998); however, synthesis of water soluble melanin has also been reported (Kimura *et al*., 2015).

Technical Applications of Melanin

Melanin confers resistance to UV light by absorbing a broad range of the electromagnetic spectrum and preventing photo induced damage. They have several biological functions including photo protection, thermoregulation, and action as free radical sinks, and cation chelators (Rao *et al.*, 2017; Zerrad *et al.*, 2014).

Even some melanin producing bacteria were also reported to be resistant to antibiotics.
Other than contributing to microbial virulence by allowing the organism to cause disease in a host, it can also elicit intense inflammatory responses that may result in host damage. (Nosanchuk *et al.*, 2003)

The genes responsible for the melanin synthesis from bacteria were used as a reporter gene to screen the recombination in host bacteria. For instance, melanin producing genes can be a best alternative to generally used blue white screening method in *E. coli* (Tseng *et al.*, 1990; Adham *et al.*, 2003).
Melanins are mostly used in cosmetics as a component of creams and sunscreen lotions basically for UV- protection and free radicals scavenging properties (Riley, 1997; Babitha, 2009). Melanins can be used as UV-protective agent in the bio-insecticide preparation such as in the *Bacillus thuringenesis* (Bt) insecticidal crystals (Wan *et al*., 2007; Zhou *et al.*, 2008). The melanin producing organism can also be used in bioremediation of radioactive waste such as Uranium (Plonka and Grabacka, 2006; Surwase *et al*., 2013)

The melanin can be used in vaccine preparation against human melanocyte cancer (melanoma); the lymphocytes of melanoma patients can be restimulated in vitro with autologous tumour cells to generate antitumor cytolytic T-lymphocytes (CTL). Such antitumor CTL clones which appear to recognize melanin as an antigen. The melanin antigen may therefore constitute a useful target for specific immunotherapy of melanoma (Brichard *et al*., 1993, Nosanchuk *et al*., 1998).

The anti-HIV (Human Immunodeficiency Virus) property of melanin was also reported, as soluble melanin found to be inhibiting replication of HIV *in vitro.* Melanin is also used to generate monoclonal antibodies for the treatment of human metastatic melanoma (Montefiori and Zhou, 1991).

ANALYTICAL INSTRUMENTATION

Weighing Balance

Weighing balance is a digital balance used to measure small mass in the grams, milligrams and other small units of mass. The pan is confined from all the sides with the glass walls so that there is no inhibition from the dust or air, while measuring the sample. This is usually known as draft shield. The instrument should be calibrated before measuring the mass on pan.

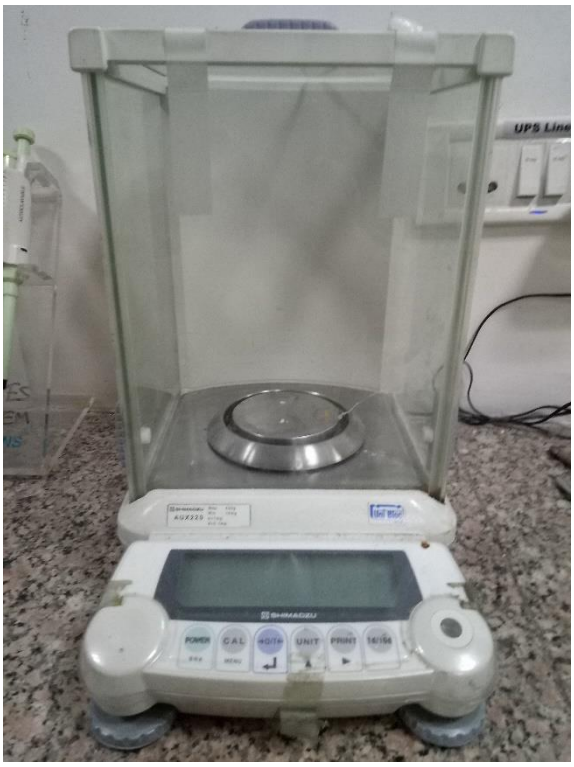


Figure.2.7. Shimadzu Weighing Balance

High Speed Centrifuge: The Centrifuge 580 R is a high speed centrifuge for sedimentation of denser particles at the bottom of the tube. Swing-bucket and fixed-angle rotors can be used to operate the centripetal acceleration on the particles inside the sample.

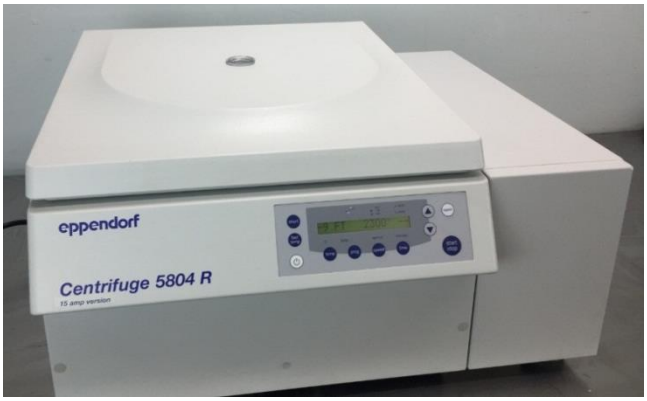


Figure.2.8. Eppendorf Centrifuge 580 R

LAMINAR HOOD

It provides the sterile working environment free from dust and other air-borne impurities by maintaining a unidirectional and constant flow of **HEPA-filtered air** over the working slab. The flow can be vertical, air drifting from top of the cabinet to the working slab else it can be horizontal**,** where filter is placed at the back of the working slab.

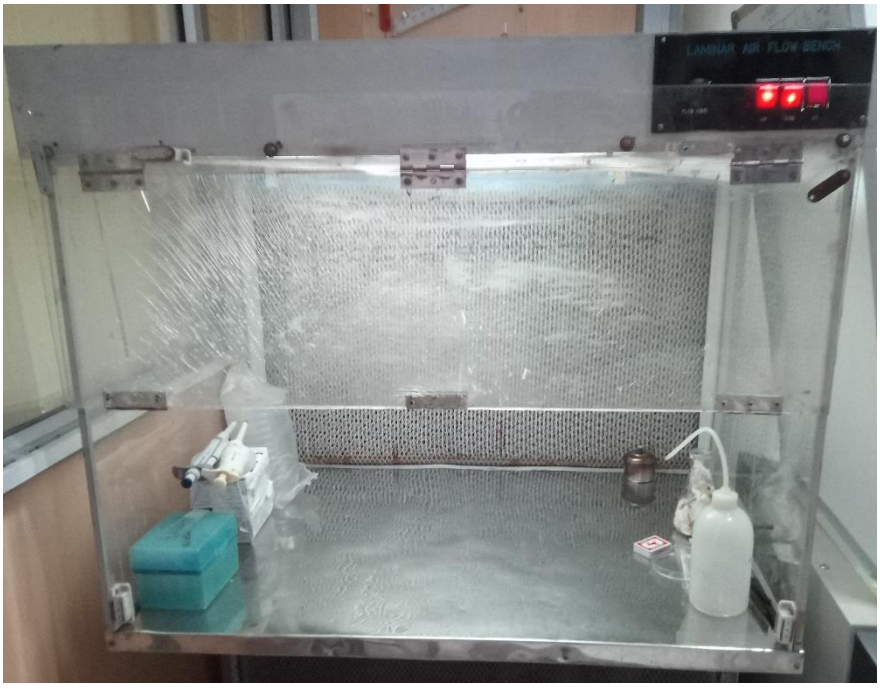


Figure.2.9. Laminar Flow Hood

ULTRASONICATOR

Sonication works on the principle of using sound waves to agitate the particles in a solution. Ultra-sonication typically uses ultrasound waves with frequencies of 20 kHz or higher. These frequencies are above what you can hear, but ear protection is still recommended during

sonication because the process that creates a loud screeching noise. The greater the frequency, the stronger the agitation of particles.

Sonication can either be done by using ultrasonic bath or probe-sonicators in a laboratory. These are required for many processing applications, such as nano-crystallization, nano-emulsification, deagglomeration, extraction, cell disruption, etc.

Figure.2.10. Water bath ultrasonicator

UV-VIS SPECTROPH0T0METER

Ultraviolet & **visible** (**UV-Vis**) absorption **spectroscopy** is used for the measurement of the attenuation of a beam of light after it passes through a sample or after reflection from a sample surface. Absorption measurements can either be at a single / multiple wavelength or over an extended spectral range.

Figure.2.11. Eppendrof BioSpectrophotometer

FOURIER TRANSFORM INFRARED (FT-IR) SPECTROSCOPY

Fourier Transform Infrared Spectroscopy is one of the spectroscopy method which uses Infrared of the Electromagnetic spectrum. The principle is as follows: Infrared radiation passes through the sample. Some radiation is absorbed by the sample while the remaining radiation is passed through or transmitted.

Figure.2.12. Spectrum Two FT-IR Spectrophotometer - Perkin Elmer

The spectrum obtained depicts the molecular absorption and transmission; which forms the molecular fingerprints of the sample. These molecular fingerprints are different for different molecular structures. Thus this technique is useful for various types of analysis as follows:

1) It is used to ascertain the unknown materials.
2) The quality or consistency of sample is discerned.
3) Can discern the amount components in a mixture

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a type of chromatography which employs a liquid mobile phase and a very finely divided stationary phase. In order to obtain satisfactory flow rates, the liquid must be pressurized to several hundred pounds per square inch or more. This chromatography technique improved the performance if compared to classical column chromatography that's why known as high-performance chromatography. Most of the drugs in multicomponent dosage forms can be analysed by HPLC method because of its various advantages like specificity, accuracy, fast, precision, and ease of automation in this method (Bhardwaj *et al.*, 2015). HPLC method reduces tedious extraction and isolation procedures.

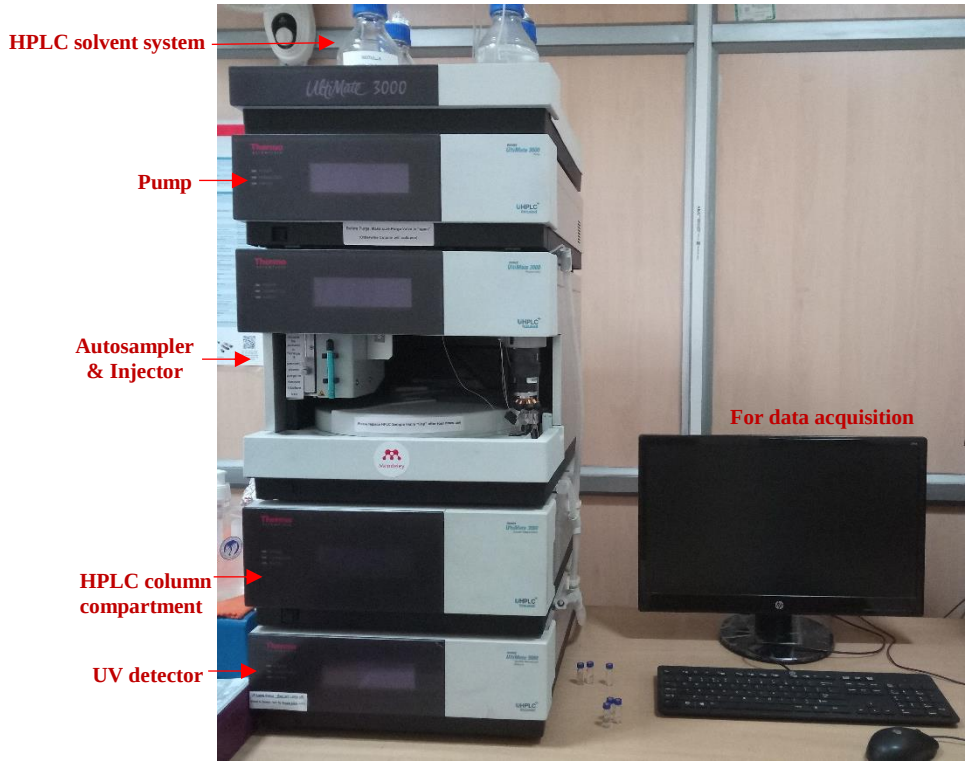


Figure.2.13. Thermofisher Scientific Ultimate 3000 UHPLC System

Some of the advantages of HPLC are:

- Speed (analysis can be accomplished in 20 minutes or less),
- Greater sensitivity (various detectors can be employed),
- Improved resolution (wide variety of stationary phases)

- Columns are reusable (expensive columns but can be used for many analysis),
- Ideal for substances 0f low volatility,
- Easy sample recovery, handling and maintenance,
- Instrumentation lends itself to automation and quantitation (less time and labour),
- Precise and reproducible, and
- Calculations are done by integrator itself

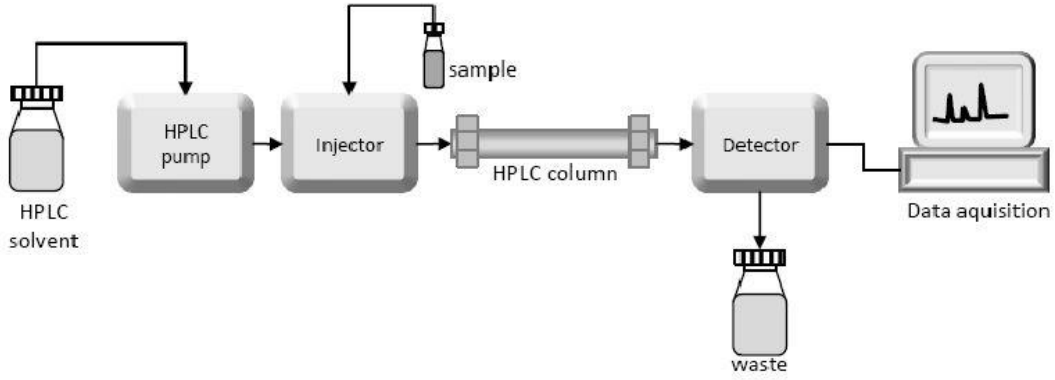


Figure.2.14. HPLC block diagram

HPLC instruments consist of a reservoir of the mobile phase, a pump, an injector, a separation column, and a detector. Components are separated by injecting the sample mixture into the column. The different compounds in the mixture move through the column and get separated because of the differences in their partition behaviour between the mobile phase and the stationary phase. The mobile phase must be degassed to remove the formation of air bubbles. The pump provides a steady high pressure without pulsation and can be programmed to vary the composition of the mobile phase during the course of separation. The detector relies on the change in refractive index, UV-VIS absorption, and fluorescence after excitation with a suitable wavelength in order to detect the separated compounds.

C18 Column

C18 has 18 carbons in the column packing that are bonded to the silica (Si). In general, C18 column retains more than C8 column, for instance, if a similar compound eluted on these two columns, it will elute later on the C18 column. The reversed-phase HPLC column is the most adaptable and commonly used type of column and it can be used for a broad range of different types of analytes. Normal-phase HPLC columns have polar packing. C18 column is dense and because of denser packing of column surface area get increased which leads mobile phase to travel per unit of length of the column.

THIN LAYER CHROMATOGRAPHY (TLC)

In this mode of Chromatography, stationary phase is attached to a suitable matrix which is coated thinly on to a glass, plastic or metal foil plate. The mobile liquid phase passes across the thin layer plate, held either horizontally or vertically by capillary action. This form of chromatography is one of the older forms. It has a practical advantage that a large number of samples can be studied simultaneously.

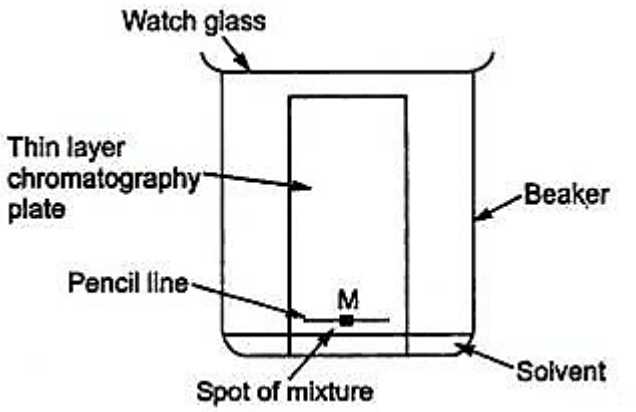


Figure.2.15. Thin Layer chromatography

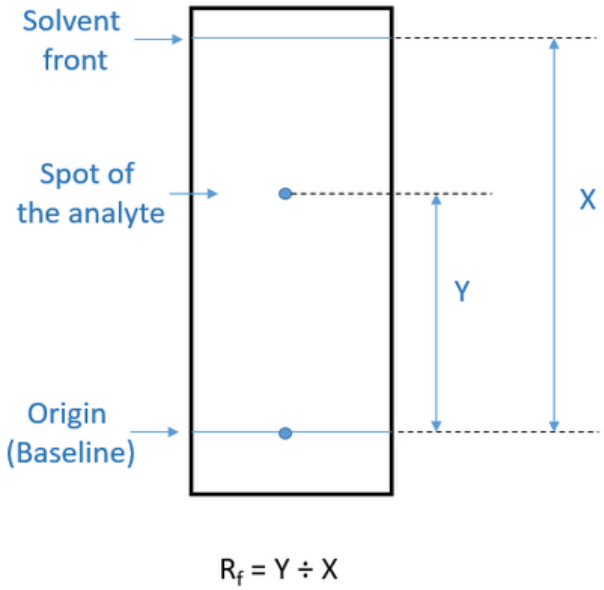


Figure.2.16. Measurement of Retention factor after TLC plate development

The relationship between the distance travelled by the solvent front and the compound is usually expressed in terms of Retention factor, **R_f** value.

R_f value = distance travelled by compound ÷ distance travelled by solvent front.

3. MATERIALS & METHODS

3.1 MATERIALS

The composition of all the media and reagents used in this study are given in Appendix I and II.

3.2 COLLECTION OF SAMPLES

Water sample was collected from DTU Lake. The samples were collected in plastic bottles and were carried to laboratory for further investigation.

3.3 ISOLATION OF MELANIN PRODUCING BACTERIA

Various bacterial strains were primarily isolated from collected water samples by pour-plating technique at different dilutions of the sample prepared in sterile 0.66% saline and Nutrient Agar media.

The media, saline and the glassware should be autoclaved at 15 psi (121 ºC) for 15 min prior to the experiment; these agar plates with media have to be incubated at 37ºC for 4 days. Selective colonies then were separated out for sub culturing and characterization.

Melanin producing bacteria were screened by using quadrant streaking of above obtained pure bacterial cultures on media composed of by Nutrient agar supplemented with L-tyrosine.

3.4 PHENOTYPIC CHARACTERIZATION OF MELANIN PRODUCING BACTERIA

3.4.1 Gram Staining

The morphological characteristics *i.e.* shape, and arrangement of individual organisms were determined by standard Gram staining procedure of heat fixed smears of activated screened cultures as depicted in Fig.3. (a) and (b).

Fig.3.1. (a)

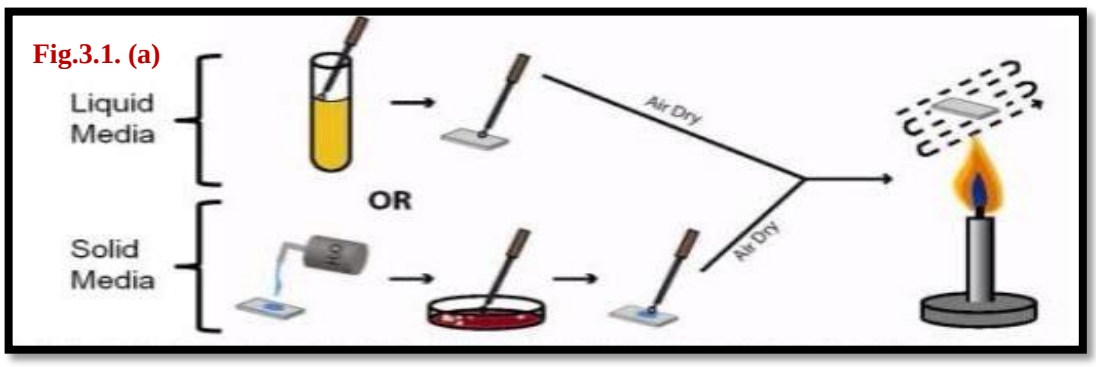


Fig.3.1. (b)

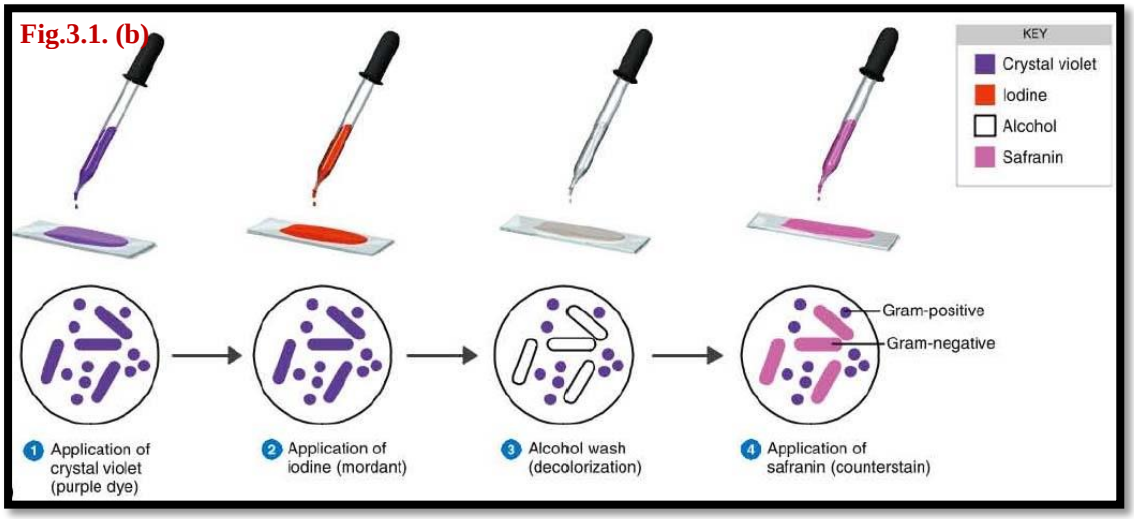


Figure.3.1. (a) Heat fixing and smear preparation of bacterial strains on a clean glass slide (b) Stepwise gram staining of bacterial slide

3.4.2 Catalase Production

The catalase test was performed by the slide method. Using a micropipette, 12-18 h old culture was poured on a clean glass slide. A drop of 3% H_2O_2 solution was added on to this culture and closely observed for the effervescence.

Few drops of 3% H_2O_2 solution was added on to test cultures freshly grown on nutrient agar plates, which was observed for bubble formation due to liberation of nascent oxygen. This indicated the test for production of catalase.

3.4.3 Test for motility and oxidative/ fermentative reactions

Stab inoculation of the test cultures were performed in the sterile tubes of Hugh Leifson medium. Each culture was inoculated in duplicate. To one set of the inoculated tubes, few drops of sterile mineral oil was added to overlay the agar medium (for anaerobic conditions). The other set of tubes was kept without any overlay.

Both set of tubes were incubated at 37ºC for 24-48 h. Incubated tubes were observed for the spreading of the culture from the line of inoculation, which indicated the motile nature of the culture.

Acid production from glucose was indicated by color change of medium from light green to yellow. Positive tubes for acid production under aerobic condition indicate the oxidative nature, while positive tubes under anaerobic condition indicated fermentative nature.

3.4.4 Urease Production

Individual test cultures were inoculated into the prepared slants of Kohn two-tube medium No.1, by making a stab in the butt and streaking on the slant. Inoculated tubes were incubated for 24-48 h at 37ºC. Positive urease reaction (i.e., alkaline) was indicated by a deep cerise (cherry red/ bright red) color of the whole medium.

3.4.5 Indole Production

Individual test cultures were inoculated into prepared tryptone broth tubes and incubated at 37ºC for 24 h. To each of the incubated tubes, 0.2-0.3 mL of Kovac's reagent was added. Formation of dark red color in the surface layer of the culture broth indicated a positive test for indole.

3.4.6 Nitrate Production

Individual test cultures were inoculated into the prepared nitrate broth and incubated at 37ºC for 24 h. The culture broth was tested for nitrate reduction using the following reagents:

Solution (1): Sulphanilic acid 8 g, 5N Acetic Acid 1000 mL

Solution (2): α-napthol 5 g, 5 N Acetic Acid 1000mL

To 5 mL of the 24 h old- culture broth, was added 2 drops of each solution (1) and (2). Development of orange/brick red color indicated the reduction of nitrate to nitrite.

3.4.7 Citrate Utilization

Freshly prepared slants of Simmons's Citrate agar were inoculated with individual test organisms and incubated at 37ºC for 24-48 h. Formation of deep blue color in the incubated slants indicated positive reaction for citrate utilization.

3.4.8 Gelatin Hydrolysis

Nutrient Agar was prepared with 8% gelatin and plates were pre-poured. A loop full of 20 h-old culture was spotted onto portions of the prepared plates and incubated for 24-48 h at 37ºC. Incubated plates were examined for gelatin hydrolysis by pouring a mixture of 1.5% $HgCl_2$ and 10% hydrochloric acid solution over the individual cultures. The formation of clear transparent zone around the growth area indicated positive reaction for gelatin hydrolysis.

3.4.9 Ammonia from Arginine

The prepared arginine dihydrolase broth tubes were inoculated with a loop full of 20 h old culture, mixed well and incubated at 37ºC for 24-48 h. Incubated tubes were

observed for colour change in the medium due to ammonia production from orange to rosy red. (as reflected by indicator phenol red)

3.4.10 Ornithine

The cultures were inoculated into the ornithine decarboxylase broth and incubated at 30ºC for 24-48 h. These tubes were then observed for the change in color, initially to yellow and then to purple.

3.4.11 Lysine

The cultures were inoculated into the lysine decarboxylase broth and incubated at 3oºC for 24-48 h. These tubes were then observed for the change in color, initially to yellow and then to purple.

3.4.12 Production of H_2S

The slants of triple sugar iron agar were prepared with a butt of about 1 inch long. The organisms were inoculated and incubated at 30ºC for 24-48 h and observed for the formation of black precipitate in the butt region.

3.4.13 TDA

The slants of triple sugar iron agar were prepared with a butt of about 1 inch long. The organisms were inoculated and incubated at 30ºC for 24-48 h. The change in color in both butt and slant was observed. Gas production was observed with the formation of cracks in the butt region.

3.4.14 Oxidase Test

Freshly grown culture (12-15 h old) was smeared on a piece of filter paper. A drop of 1% aqueous solution of N,N-dimethyl-p-phenylene diamine applied onto it, and that filter paper was observed for the color change within 10-15 seconds. Violet color formation indicated a positive reaction, however development of whitish to pinkish color within those 10-15 seconds indicated a negative reaction for oxidase.

3.4.15 Methyl Red Test

About 5-6 drops of Methyl Red reagent (refer Appendix II) was added to the fresh grown culture broth. Pinkish color development indicated the positive reaction.

3.4.16 Voges-Proskauer Test

To a 5mL freshly grown culture broth, 0.6 mL of solution A and 0.2 mL of solution B were added (refer Appendix II), mixed well and tubes were kept unplugged to facilitate them with aerobic environment. Eosin pink color formation indicated the positive reaction.

3.4.17 ONPG Test

o-Nitrophenyl-β-D-galactopyranoside (ONPG) is structurally analog to lactose. It is used as a substrate to detect β-galactosidase enzyme, which hydrolyses galactoside bond to yield galactose and o-Nitrophenol (yellow color compound) from ONPG; and glucose and galactose from lactose.

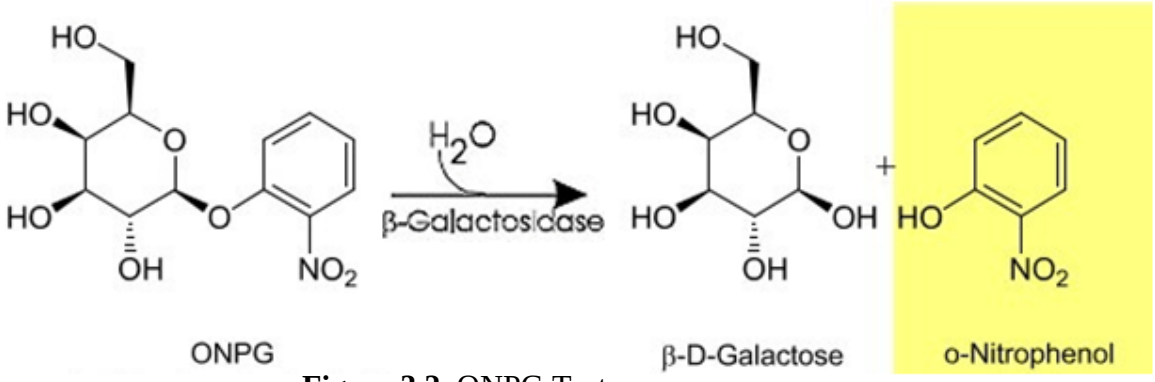


Figure.3.2. ONPG Test

ONPG discs (from HiMedia Laboratories) were used to detect β-galactosidase enzyme activity. The culture was inoculated into fresh sterile saline tubes with ONPG discs, and were incubated for 24-48 hours. The tubes were observed for yellowish color development.

3.4.18 Acid Fermentation from sugars

Sugar fermentation test is used to determine whether or not bacteria can ferment a specific sugar. Sugar fermentation patterns are useful in differentiating among bacterial groups or species.

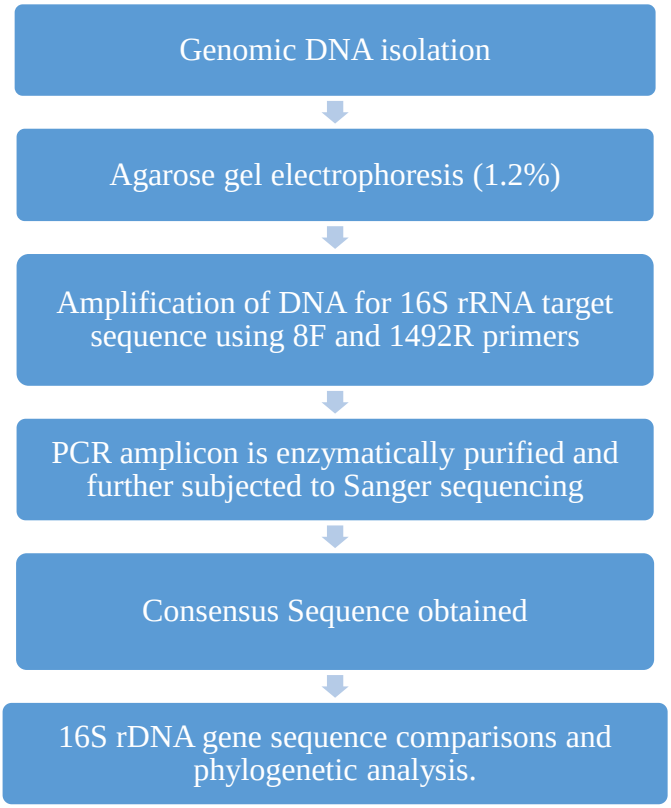
To each individual tubes of 3mL of sugar fermentation basal medium, 0.3mL each of the individual 10% membrane filtered sugar solutions were added and mixed well. These sugars were namely glucose, lactose, arabinose, raffinose, rhamnose, xylose, sucrose, mannitol, inositol, sorbitol, adonitol and salicin, separately, which was kept at -20º C for storage until usage.

These sterile media tubes containing sugar solution were then inoculated with the individual test cultures, mixed well and incubated at 37º C for 5 days. These incubated tubes were checked for the color change in the interval of every 24 h, from purple to yellow in the media, due to acid production from sugars.

3.5 BACTERIAL IDENTIFICATION OF STRAINS RT3 AND Ep1

Two of the melanin producing bacterial strains Ep1 and RT 3 were sent to Xcleris Lab Ltd. (Gujarat, India) for identification using 16S rDNA based molecular techniques.

3.5.1. Experimental Method



DNA sequences obtained in FASTA format were analysed using online BLASTn tool (Nucleotide Basic Local Alignment Search Tool) facility of National Centre for Biotechnology Information (NCBI).

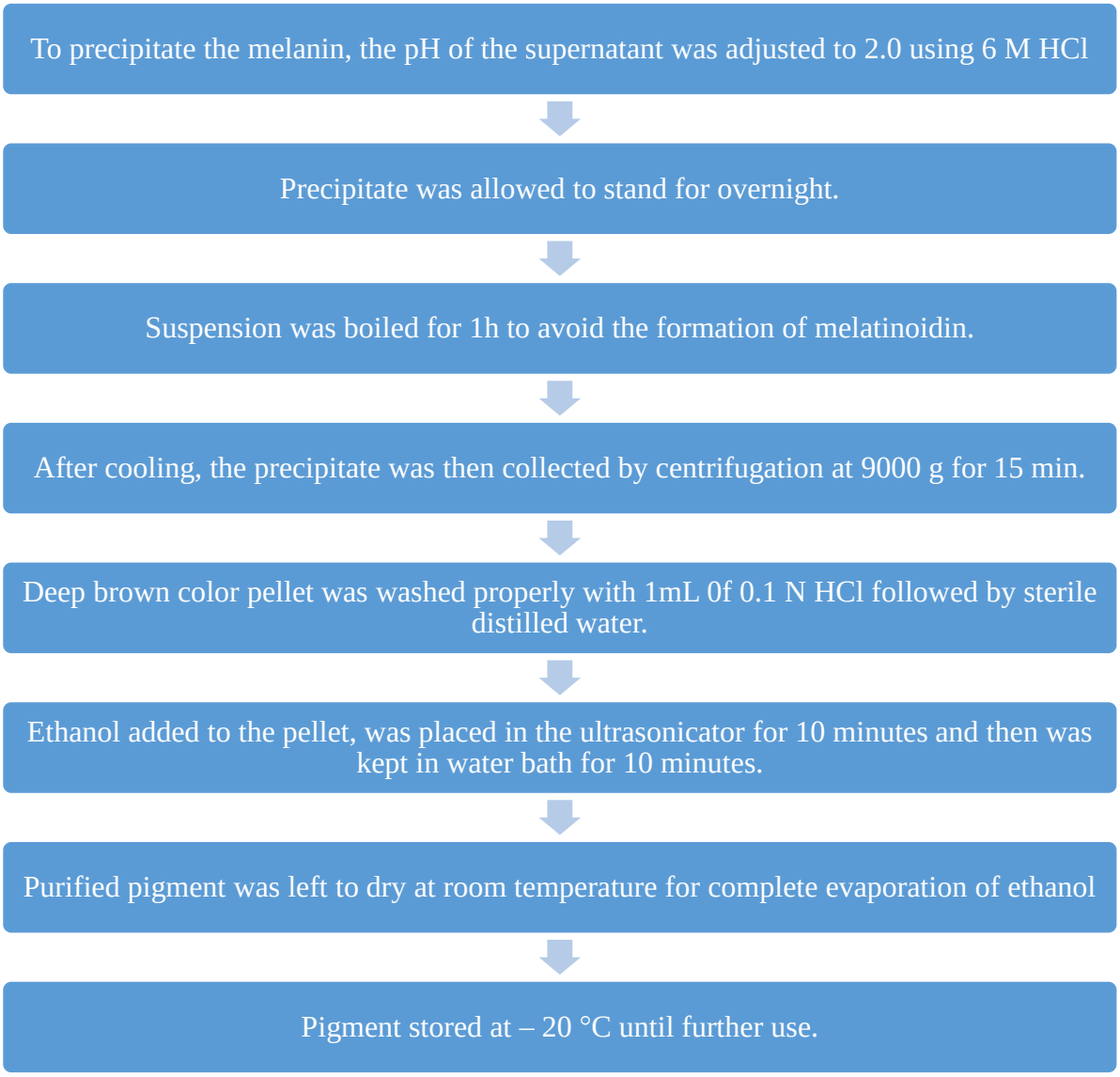
The BLAST results were used to find out evolutionary relationship with bacterial strains RT3 and Ep1. Altogether sixteen sequences, including the bacterial sample were used to generate the phylogenetic tree. The tree was constructed by Neighbour-Joining method using MEGA 7 software (Saitou N. and Nei M., 1987).

3.6 PIGMENT PRODUCTION

- Nutrient broth supplemented with L-tyrosine is used for inoculums preparation and pigment production. Bacterial cultures are added to 100 mL nutrient broth in 250 mL flasks, which is incubated at 37˚C at 100rpm. All the media used for the study has to be sterilized by autoclaving unless elsewhere stated.

- After 5-7 days incubation until the liquid medium become darkly pigmented and nearly opaque. After the incubation time, the medium is centrifuged at 9000 g for 15 min to collect the supernatant, and remove cells and debris.

3.7 PIGMENT EXTRACTION AND PURIFICATION



3.8 PHYSIOCHEMICAL ANALYSIS OF PIGMENT

To determine the solubility of bacterial melanin, 1 mg of purified pigment powder was separately mixed with H_2O, 0.1 N NaOH, 0.1 N HCl, 0.1N KOH, Dimethyl sulfoxide, Methanol, Ethanol, Chloroform, Acetone, Isoamyl alcohol, Isopropanol, Butanol, Ethyl acetate, etc.

3.9 UV-VIS ANALYSIS OF MELANIN

UV-visible spectrum scanning of the standard as well as bacterial melanin was performed in a solution of 0.5M NaOH to a concentration of 0.01 g/l, and was scanned from 200 to 1100 nm wavelengths. Standard synthetic melanin was used as standard and 0.5 M NaOH as blank.

3.10 FTIR ANALYSIS OF MELANIN

Fourier transform infrared spectroscopy (FT-IR) is most useful for identifying the types of chemical bonds (functional groups) and therefore, can be used to elucidate the compound structures. The FT-IR analysis of pigment should be carried out after mixing with KBr using FT-IR spectrophotometer.

The melanin powder and KBr (infrared quality) powder are mixed in an agate mortar in the ratio of 1:10 and pressed into disks under high pressure using a pellet maker. The mixed disc was scanned at 4000–400 cm^{-1} in a Fourier Transformation infrared spectrophotometer.

3.11 THIN LAYER CHROMATOGRAPHY OF MELANIN

In order to standardize the best solvent for melanin, TLC was performed using various combinations and proportions of different organic solvent system such as Acetonitrile, Ethanol, Methanol, Acetic acid, Butanol, etc. Acetonitrile: Methanol: H_2O (9:9:2) was observed to be most suitable for TLC analysis in comparison to others solvent systems.

TLC of the extracted melanin pigment was performed on a 6 x 10 cm silica gel 60 F254 TLC plate(stationary phase), using the mixture of Acetonitrile: Methanol: H_2O as the mobile phase system, in the ratio 9:9:2. Synthetic melanin was taken as standard. Samples and standard pigment were prepared by mixing the pigment powder in 10 mM NaOH. Detection was done by placing the TLC plate under the UV chamber. The R_f values for the separated spots were calculated and compared with R_f value of standard melanin.

3.12 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MELANIN

For the quantification and qualitative analysis of biologically synthesized melanin HPLC is performed. Also, to know its retention time. Till now there is no official HPLC methods are available for melanin so we have tried to establish a protocol for biologically synthesized melanin.

Selection of solvent

Based on the sample's solubility, stability and suitability different mobile phase compositions were tried to achieve for good separation and resolution with sharp peaks.

From Thin Layer Chromatography (TLC) we were able to find that Sodium hydroxide is a suitable solvent for both synthetic and biologically synthesized melanin. According to the required working pH of HPLC setup molarity of NaOH was altered. For final HPLC protocol 0.001M NaOH, 7.5 pH was used to dissolve melanin. 0.1% Formic acid was also tried as a solvent but this idea was dropped due to its high corrosiveness and also it breaks melanin.

Selection of detection wavelength

The sensitivity of an HPLC method that used UV detection depends upon the proper selection of the wavelength. An ideal wavelength is one which gives good response for all the components to be detected. The UV spectrums 0f 10 µg/ml of standard melanin in selected solvents were recorded individually. The spectrums were superimposed to get overlay spectrums. From this overlaying spectrum detection wavelength 200-220 nm was fixed because at this wavelength it shows good absorbance.

Sample Preparation

Sample should be prepared of four different concentration i.e. 50, 100, 150 and 200ppm by dissolving in appropriate solvent. For good results, the sample should be prepared freshly before every cycle and the freshly prepared sample should be placed in ice because it is highly unstable.

Optimized chromatographic conditions

Based on the studies, the following chromatographic conditions were selected for the estimation of melanin in selected formulations.

Stationary phase: C18 column

Mobile phase: Acetonitrile: Methanol: milliQ water

Mobile phase ratio: 45: 45:10 % v/v/v

Detection wavelength: 220 nm

Flow rate: 1 ml / min

Injection Volume: 10 µl

Temperature: 35˚C

Time Window: 10-12 min

To run the HPLC Purging and equilibration are two important steps. In purging flow rate is generally high than equilibration i.e. 6ml/min, where as in equilibration it is 0.5 t0 1 ml/min.

4. RESULTS & DISCUSSION

Melanin is one such pigment ubiquitous in all biological kingdoms. (Hill, 1992) Because of its ubiquitous nature and various properties such as antimicrobial, anti-oxidative, anti-HIV, thermoregulatory properties, etc., several researches has been done on effective high-yield of this pigment.

Melanin pigment-producing bacterial colonies were isolated from DTU Lake.

4.1 ISOLATION OF MELANIN PRODUCING BACTERIAL STRAINS

Streaked agar plates were observed each day for dark brown pigment-producing colonies. At ambient conditions, during the end of fourth day (~96 h) visible dark color colonies with diffused blackish-brown color was evident on Nutrient agar plates supplemented with L-tyrosine, inoculated with sample or streaked with a purified culture.

Figure 4.1. Streaking on Agar Plate (a) No melanin pigment obtained from bacterial strain DTU J (b) Development of brownish pigment from strain RT-7

Total 11 dark brown to black bacterial strains were screened by pour plating followed by quadrant streaking technique to purify the isolate. The pure cultures were maintained by subsequent sub-culturing and plating. After 5-7 days of incubation, these strains produced diffused black pigment.

4.2 SCREENING OF BACTERIAL STRAINS

Streaked agar plates were observed each day for dark brown pigment-producing colonies. At ambient conditions, during the end of fourth day (~96 h) visible dark color colonies with diffused blackish-brown color was evident on Nutrient agar plates supplemented with L-tyrosine, inoculated with sample or streaked with a purified culture.

Figure.4.2. Variation in pigment intensity for different strains cultured in test tubes and Erlenmeyer flasks

4.3 BIOCHEMICAL CHARACTERIZATION OF BACTERIAL STRAINS

The detailed taxonomic studies involving various physiological and biochemical characteristics to identify the isolates are documented in the Table 4.1 below.

Table 4.1. Results of various biochemical tests for identification of melanin producing bacterial strains

Biochemical Tests	**RT1**	**RT3**	**RT4**	**RT6**	**RT7**	**T2 4**	**T2 8**	**T31.1.2**	**T3.1.2.1**	**Ep1**	**G**	***S. aureus* (control)**
Gram Character	Negative	Negative	Negative	Negative	Negative	Negative	Negative	Positive	Positive	Positive	Negative	Positive
oxidase	+	+	+	+	-	+	-	-	+	+	+	-
Motility	+	+	+	+	+	+	+	+	+	-	+	-
Catalase Prod.	+	+	-	-	+	+	+	-	+	+	-	+
Citrate	+	+	+	+	+	+	+	+	+	-	-	+
TDA (Acid production from Glucose & Lactose) & Gas bubble formn.	No fermentation	Glucose only No gas formation	Glucose only No gas formation	Glucose only No gas formation	Glucose only No gas formation	Glucose only No gas formation	Glucose only No gas formation	Glucose only No gas formation	No fermentation No gas formation	Both lactose & Glucose	Both lactose & Glucose Gas bubble formation	Both lactose & Glucose No gas formation
H_2S Prod.	-	-	-	-	-	-	-	-	-	-	-	-
Urease Prod.	+	+	+	-	+	+	+	+	+	-	-	+
Methyl red	-	-	+	+	-	+	+	-	-	-	+	+
Voges-Proskauer	-	-	+	-	-	+	+	-	-	-	+	+
Arginine Hydrolysis	+	+	+	+	+	+	+	+	+	-	+	+
Nitrate Reduction	+	+	+	+	-	+	+	+	+	+	+	+
Indole**	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin Hydrolysis	+	+	+	+	-	-	-	-	-	-	-	+

ornithine Decarboxylase	-	+	+	-	-	-	-	-	-	+	+	+
Lysine Decarboxylase	-	+	+	+	-	-	-	-	-	+	+	+
Oxidative / Fermentative	Oxidative	Oxidative	Oxidative	Oxidative	Oxidative	Oxidative & Fermentative	Oxidative & Fermentative	Oxidative	Oxidative	none	Oxidative & Fermentative	none
ONPG	-	-	+	+	-	+	+	+	-	-	+	+
Acid Fermentation from Sugars:												
Glucose	-	+	-	+	+	+	+	+	+	+	+	+
Lactose	-	-	+	-	-	+	-	-	-	-	-	-
Arabinose	-	-	-	+	-	-	-	-	-	-	-	-
Raffinose	-	-	-	+	-	+	+	-	-	-	-	-
Rhamnose	-	-	-	-	-	-	-	-	-	-	-	-
Xylose	-	-	+	-	-	-	-	-	-	-	-	-
Sucrose	-	-	+	+	-	+	+	-	-	-	+	+
Mannitol	-	-	-	-	-	+	+	-	-	+	+	+
Inositol	-	-	+	+	-	+ (weak)	+ (weak)	-	-	-	+	- (weak)
Sorbitol	+	+	+	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-

4.3.1 Gram Staining

All the isolated colonies on the nutrient agar media were studied for its gram behaviour and its morphology (see Figure.4.3. (a) & (b))

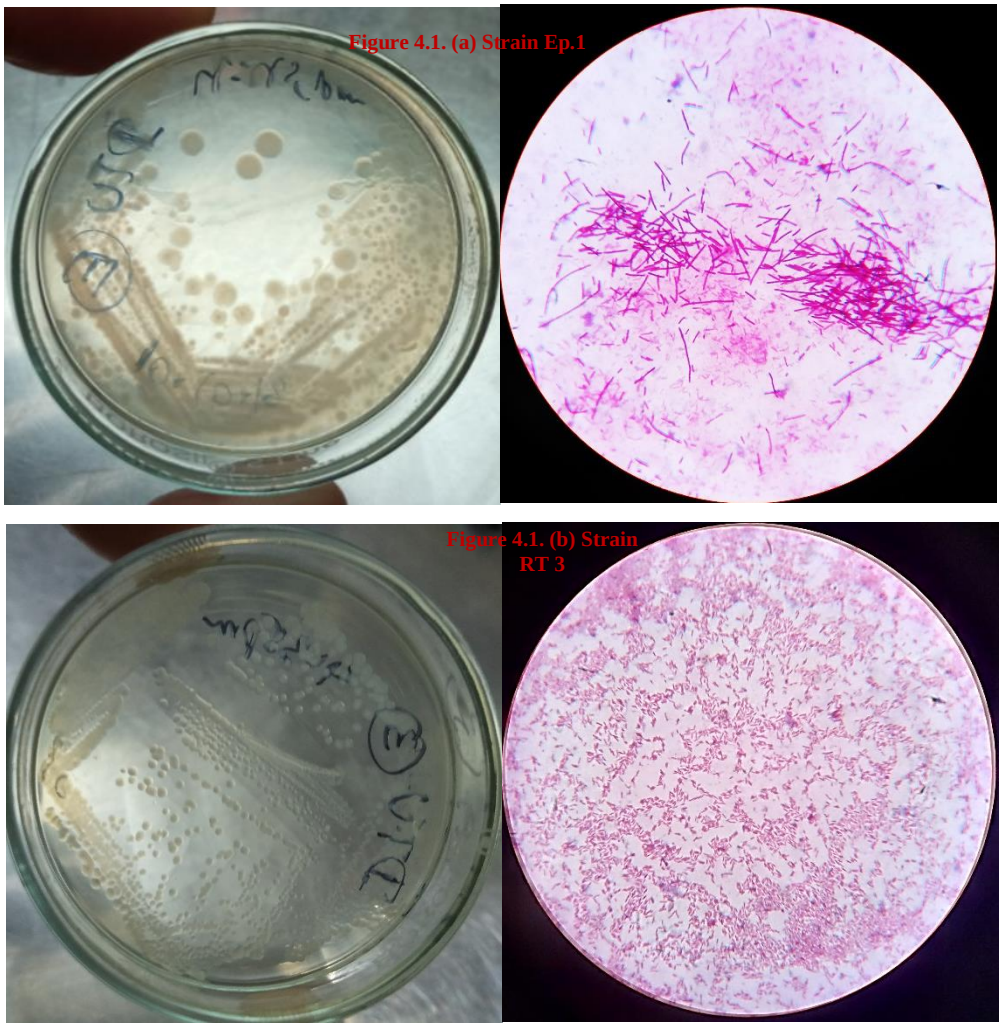


Figure.4.3. Gram Staining & bacterial colony morphology of (a) Strain Ep.1 (Gram positive, medium-long sized rods); (b) Strain RT 3 (Gram negative, small bacilli or rods)

4.3.2 Catalase Production

Bubble formation due to liberation of nascent oxygen on addition of 3% hydrogen peroxide onto the fresh test culture indicated the test for production of catalase.

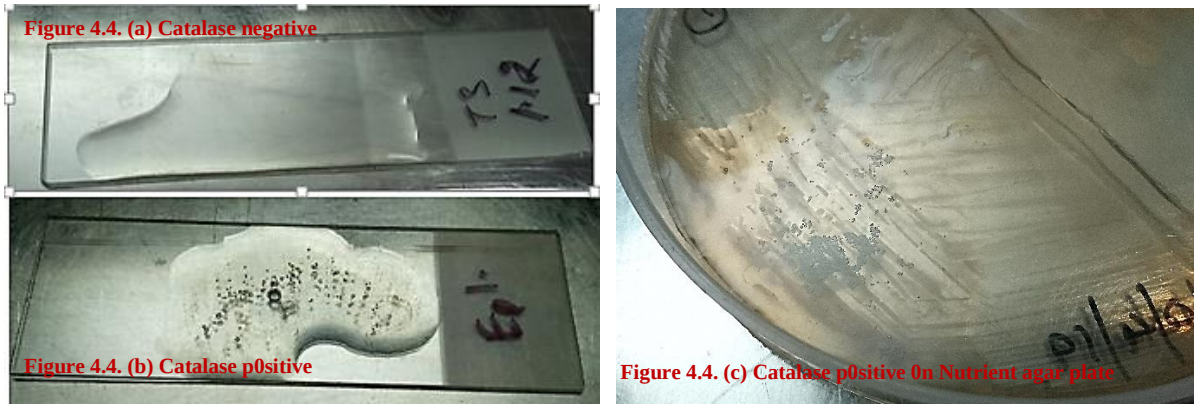


Figure 4.4. (a) Catalase negative (b) Catalase positive, for test cultures taken on glass slide, (c) Catalase positive culture grown on nutrient agar

4.3.3 Oxidase Test

Filter paper onto which freshly grown bacterial culture was applied, was observed for a color change upon addition of drop of 1% aqueous solution of N,N-dimethyl-p-phenylene diamine onto it within 10-15 seconds.

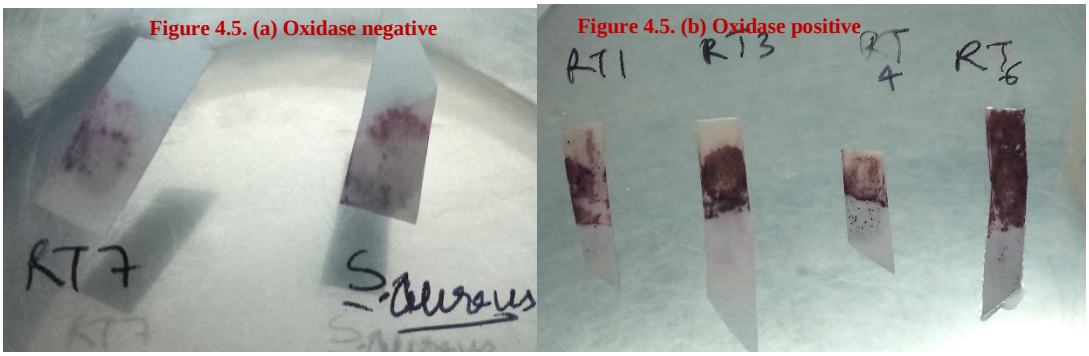


Figure 4.5. (a) Oxidase negative (b) Oxidase positive

Oxidase Test was indicated by violet color formation within 10-15 seconds, however development of whitish to pinkish color within those 10-15 seconds indicated a negative reaction for Oxidase.

4.3.4 Test for motility and oxidative/ fermentative reactions

Incubated tubes were observed for the spreading of the culture from the line of inoculation, which indicated the motile nature of the culture.

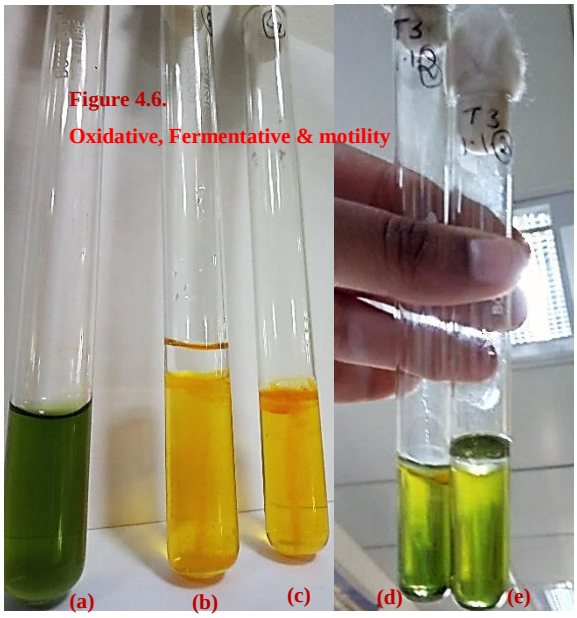


Figure 4.6. Test for oxidative and fermentative reactions, and for its motile nature
(a) Blank
(b) Fermentative (overlaid with sterile mineral oil for anaerobic condition) & motile
(c) Oxidative & motile
(d) Oxidative & motile
(e) Fermentative & motile

Acid production from glucose was indicated by color change of medium from light green to yellow. Positive tubes for acid production under aerobic condition indicate the oxidative nature, while positive tubes under anaerobic condition indicated fermentative nature.

4.3.5 Urease Production

Positive urease reaction (i.e., alkaline) was indicated by a deep cerise (cherry red/ bright red) color of the medium. (Figure.4.7.)

4.3.6 Indole Production

Formation of dark red color due to addition of Kovac's reagent in the surface layer of the culture broth indicated a positive test for indole. But indole test was negative for all the test cultures, since no red color ring formation was observed. (Figure.4.8.)

Figure 4.7.

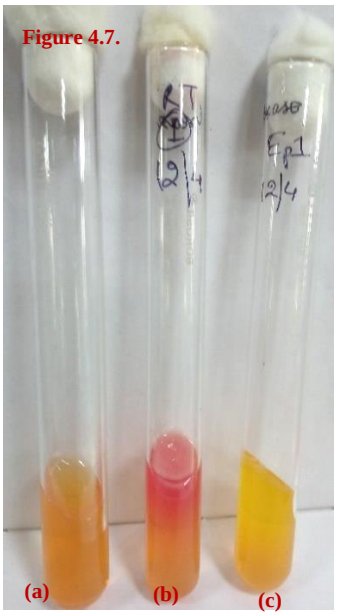


Figure 4.7. Urease production (a) blank (b) positive (c) negative

Figure 4.8.

Figure 4.8. Indole Test (a) negative (b) negative - control

4.3.7 Nitrate Production

Development of orange/brick red color indicated the reduction of nitrate to nitrite.

4.3.8 Citrate Utilization

Formation of deep blue color in the incubated slants indicated positive reaction for citrate utilization.

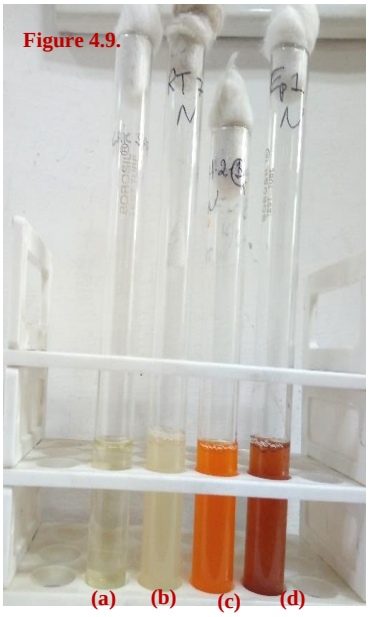


Figure 4.9. Nitrate production (a) blank (b) negative (c) positive

Figure 4.10. Citrate production (a) positive (b) blank (c) negative

4.3.9 Methyl Red Test

Development of pink color indicated the positive reaction.

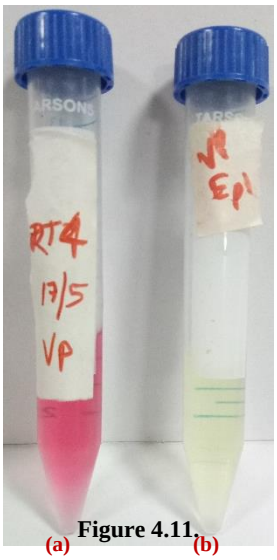


Figure 4.11.

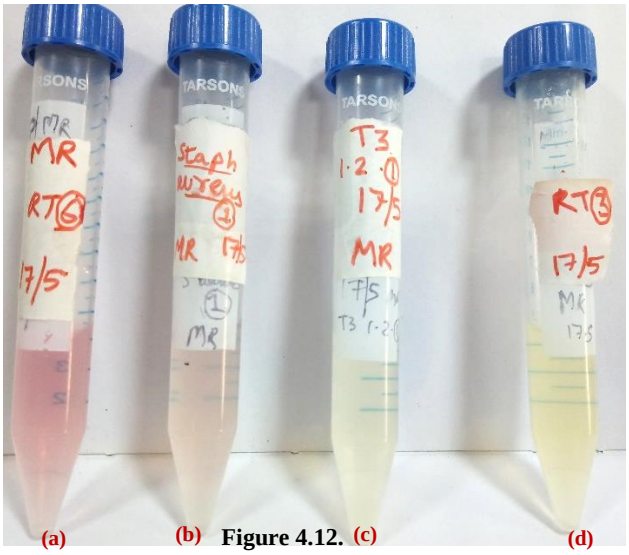


Figure 4.12.

Figure 4.11. Methyl Red Test (a) positive (b) negative

Figure 4.12. Voges-Proskauer Test (a) positive (b) positive (c) negative (d) negative

4.3.10 Voges-Proskauer Test

Formation of eosin pink coloration indicated the positive reaction.

4.3.11 ONPG

Formation of yellowish color indicated the formation of o-nitrophenol due to hydrolysis of oNPG (which is an analog of Lactose), through the action of the enzyme beta-galactosidase present in the bacteria.

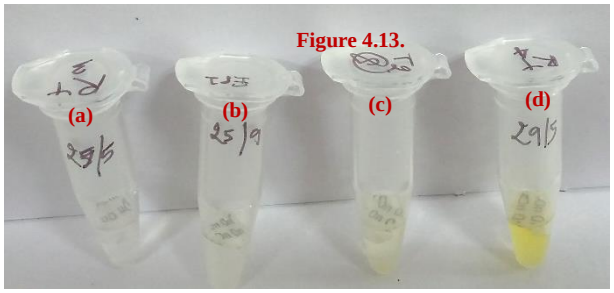


Figure 4.13. (a) & (b) no color change - ONPG Test- negative; (c) Lightish yellow/ creamish color (d) Yellow color formation - ONPG- positive

4.3.12 Gelatin Hydrolysis

Incubated plates were examined for gelatin hydrolysis. The formation of clear transparent zone by pouring a mixture of 1.5% $HgCl_2$ and 10% hydrochloric acid solution around the growth area indicated positive reaction for gelatin hydrolysis.

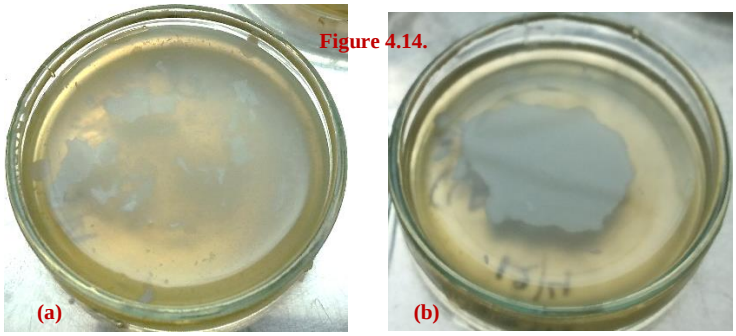


Figure 4.14. (a) Formation of transparent zone - gelatin hydrolysis - positive (b) Gelatin hydrolysis - negative

4.3.13 Ammonia from Arginine

Incubated tubes were observed for color change in the medium due to ammonia production from orange to rosy red. (as reflected by indicator phenol red) (Figure.4.15.)

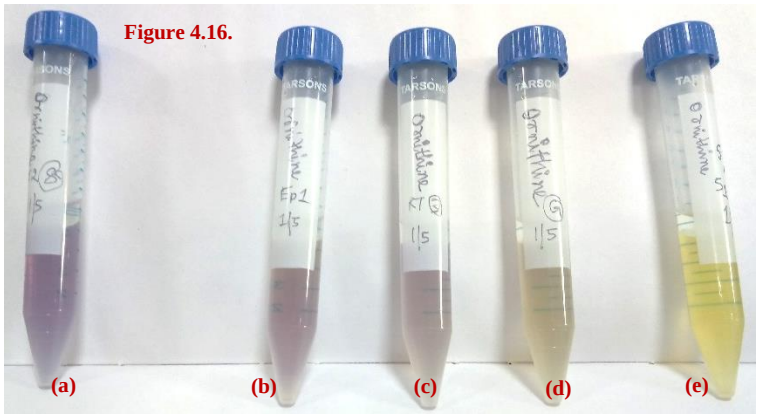


Figure 4.15. Arginine hydrolysis (a) negative (b) positive (c) positive

Figure 4.16. Ornithine decarboxylase test (a) negative (b) positive (c) positive (d) positive (e) positive (e-b, reverse order- increase in intensity of color change)

4.3.14 Ornithine

Incubated cultures in ornithine decarboxylase broth tubes were then observed for the change in color, initially to yellow and then to purple. (Figure.4.16.)

4.3.15 Lysine

Incubated cultures in lysine decarboxylase broth were then observed for the change in color, initially to yellow and then to purple.

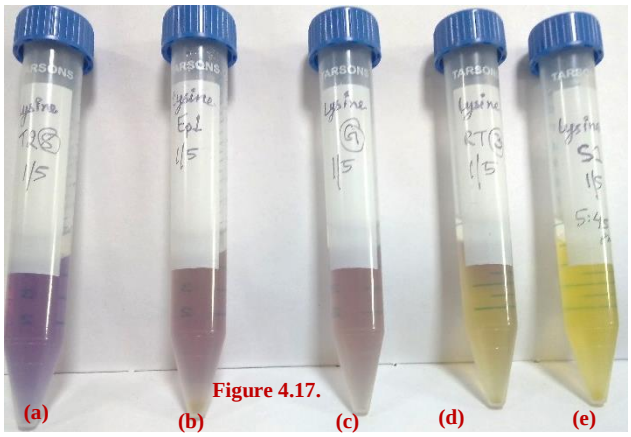


Figure 4.17. Lysine decarboxylase test (a) negative (b) positive (c) positive (d) positive (e) positive (e-b, reverse order- increase in intensity of color change)

4.3.16 Production of H_2S

The slants of triple sugar iron agar inoculated with test cultures were observed for the formation of black precipitate in the butt region. No color formation implies that no iron sulphide is formed, indicating the negative result.

Figure 4.18. TDA and Production of H_2S (a) blank (b) no acid production from both glucose and lactose (c) acid production from both glucose and lactose positive, and gas production - positive; (d) acid production from both glucose and lactose positive, gas production – positive (e) acid production from both glucose and lactose positive, but no gas production (control)

4.3.17 TDA

The slants of triple sugar iron agar inoculated with test cultures were observed for the change in color in both butt and slant (Figure 4.18.). Gas production was observed with the formation of cracks in the butt region. Refer Table 4.2.

Table 4.2. The expected results of TSI Agar test

Results (slant/butt)	**Symbol**	**Interpretation**
Red/yellow	K/A	Glucose fermentation only; Peptone catabolized
Yellow/yellow	A/A	Glucose and lactose and/or sucrose fermentation
Red/red	K/K	No fermentation; Peptone catabolized
Red/no color change	K/NC	No fermentation; Peptone used aerobically
Yellow/yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation; Gas produced
Red/yellow with bubbles	K/A,G	Glucose fermentation only; Gas produced
Red/yellow with bubbles and black precipitate	K/A,G, H_2S	Glucose fermentation only; Gas produced; H_2S produced
Red/yellow with black precipitate	K/A, H_2S	Glucose fermentation only; H_2S produced
Yellow/yellow with black precipitate	A/A, H_2S	Glucose and lactose and/or sucrose fermentation; H_2S produced
No change/no change	NC/NC	No fermentation
A=acid production; K=alkaline reaction; G=gas production; H_2S=sulfur reduction; NC = no change in color		

4.3.18 Acid Fermentation from Sugars

Different sugars, namely glucose, lactose, arabinose, raffinose, rhamnose, xylose, sucrose, mannitol, inositol, sorbitol, adonitol and salicin, were individually utilized to determine the sugar fermentation by different test cultures.

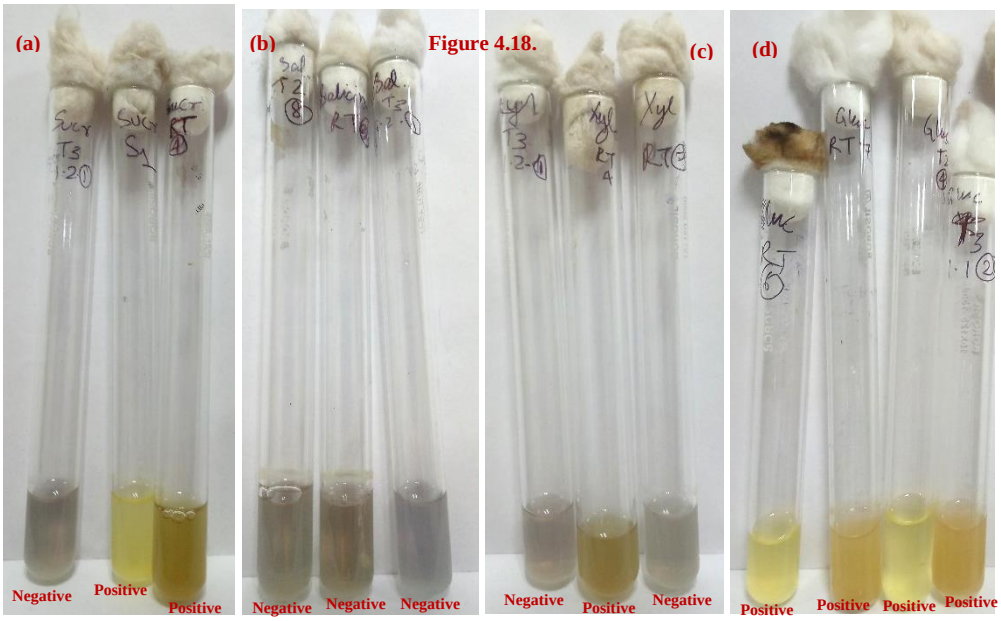


Figure 4.18.

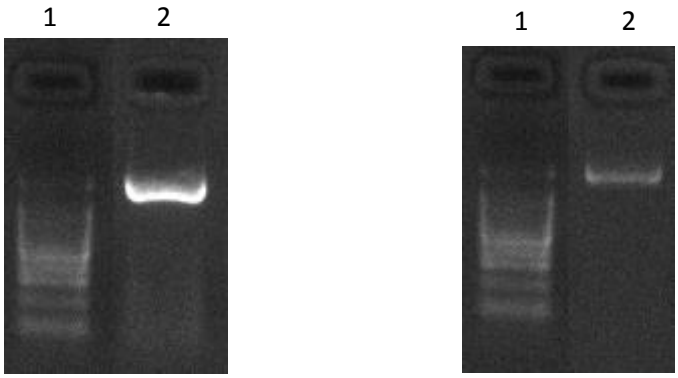
Figure.4.19. Acid Fermentation tests of the bacterial test cultures for sugars (a) Sucrose (b) Salicin (c) Xylose (d) Glucose

Incubated tubes were checked for the color change from purple to yellow in the media, due to acid production from sugars. No color change indicated no acid production.

4.4 BACTERIAL IDENTIFICATION OF STRAINS Ep1 AND RT3

i. DNA was isolated from the cultures -**Strain-Ep1** and **RT3**. Quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.

ii. Isolated DNA was amplified with *16S rRNA* Specific Primer (**8F**and **1492R**) using Veriti® 96 well Thermal Cycler (Model No. 9902). A single discrete PCR amplicon band of **1500 bp** was observed (**Figure 4.20**).



(a) Figure 4.20. (b)

Figure 4.20. 1.2% Agarose gel showing single 1500 bp of *16S rDNA* amplicon. (a) Ep1 (b) RT3 Lane 1: 100bp DNA ladder; Lane 2: *16S rDNA* amplicon (Report, Xcleris lab, 2019).

iii. The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing.

iv. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with **8F** and **1492R** primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

v. Consensus sequence of **1481 bp** (for Ep1) and **1455bp** (for RT3) **16S rDNA** was generated from forward and reverse sequence data using aligner software.

Consensus Sequence of Strain-EP1 (1481 bp)

CGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATCTGATGGGAGCTTGCTCCTGATGATAGCGGCGGACGG
GTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACTTTTTTC
TTCGCATGAAGAGAGAATTGAAAAATGGGCTTCGGCTATCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACTTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAG
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTATCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAA
AGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAATCTTGCGGCTCAACCGCAAGCGGCCATTGGAAACTGGGAGACT
TGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGG
CGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGG
AGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGC
AACGCGAAGAACCTTACCAGGTCTTGACATCCTCTTGACCTCCCTAGAGATAGGGATTTCCCTTCGGGGACAGGAGTGA
CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGACCTTA
GTTGCCAGCATTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATC
ATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCGAGGTTTAGCCAATC
CCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGT
GAGGTAACCTTTTGGAGCCAGCCGCCGAAAGTGGGACAGATGATGGGGGTGAAGTCTCA

Consensus Sequence of Strain-Rt3 (1455 bp)

GCTAACACATGCACGTCGAACGGGCAGCACAGAGGAGCTTGCTCCTTCGGGTGTCGAGTGGCGGACGGGTGAGGAATAC
ATCGGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGG
GGATCTTCGGACCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACG
ATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAGCCCTTT
TGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGC
CAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAGCGTGCGTAGGTGGTCGTTTAAG
TCCGTTGTGAAAGCCCTGGGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGTAGAGGGTAGCGGAA
TTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACA
CTGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCGAACTGGATGTT
GGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTC
AAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCT
TGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCT
CGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTC
TAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACAC
ACGTGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCGG
GCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGTTGCTCCAGAAGTAGCTAGTCTAACCGCATGGAGGACGGTTA
CCACGGAGTGATTCATGACTGGGGTGAAGTCGT

vi. The ***16S rDNA*** sequence was used to carry out BLAST alignment search tool of NCBI Genbank database.Based on maximum identity score first Fifteen sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using MEGA 7 and the Phylogenetic tree was constructed using MEGA7.

16S rRNA gene sequence comparisons and phylogenetic analysis.

For strain Ep1:

A BLAST search of the GenBank database using 1481 bp 16 S rRNA gene sequence of strain Ep1 showed its similarity to different strains of *Bacillus oleronuis.* 16 S rRNA gene sequence similarities between the strain Ep1 and these strains were between 99 to 100%.

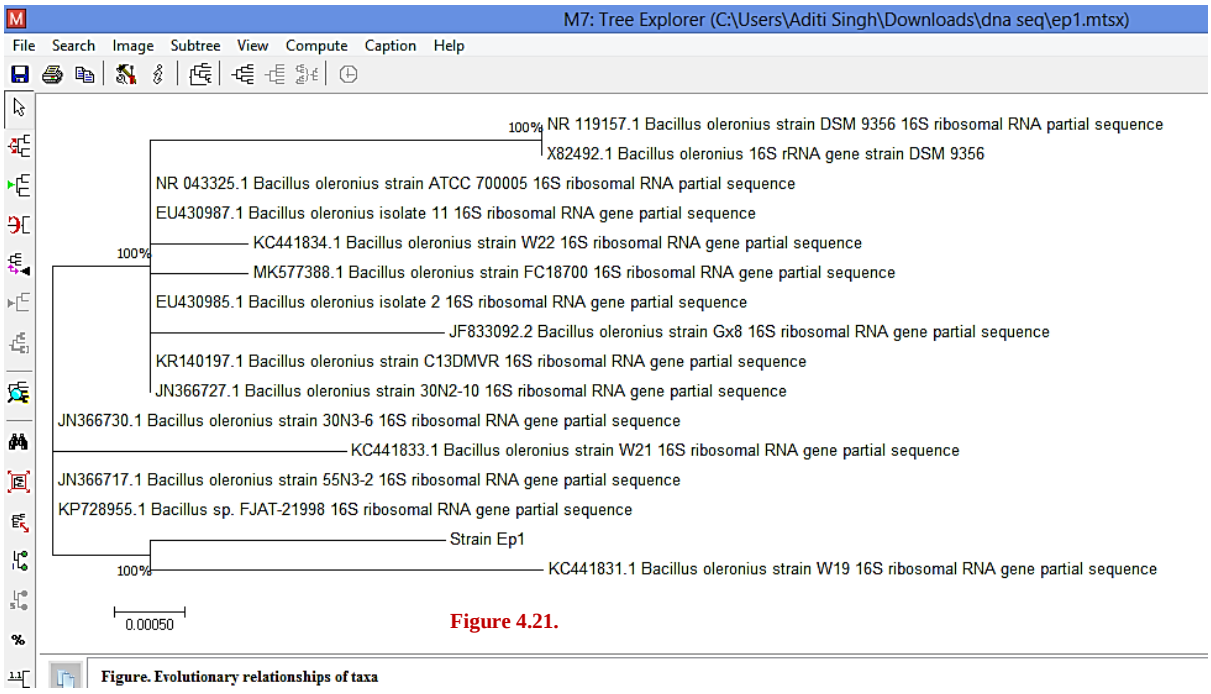


Figure 4.21. Neighbour-joining phylogenetic tree based on 16 S rRNA gene sequences, showing the relationships between strain Ep1 and related species of genus *Bacillus* sp. Only bootstrap values above 50%. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA7. Bar, 0.1 substitution per nucleotide position.

A phylogenetic tree (Figure.4.21.) based on 16 S rRNA gene sequences of members of bacteria *Bacillus oleronius* was constructed according to the bootstrap test of neighbor-joining algorithm method of Saitou and Nei with MEGA7. Phylogenetic analysis indicated that the strain Ep1 consistently falls into a clade together with *Bacillus oleronius* strain W19 (similarity 99.3%), *Bacillus* sp. FJAT-21998(similarity 99.2%),

Bacillus oleronius strain 55N3-2 (similarity 99.2%), *Bacillus oleronius* strain 30N3-6 (similarity 99.1%), etc. Pairwise global alignment using EMBOSS Stretcher, based on classic dynamic programming algorithm, was performed for determining the similarity of RT3 with different strains.

But it is observed to be most closely related to the type strain of *Bacillus oleronius* strain W19, the highest degree of similarity 99.3 %). Therefore, this strain was identified as *Bacillus oleronius* strain Ep1.

For strain RT3:

A BLAST search of the GenBank database using 1455 bp 16 S rRNA gene sequence of strain RT3 showed its similarity to different strains of the genus *Stenotrophomonas*.

16 S rRNA gene sequence similarities between the strain RT3 and these strains were between 90 % and 94.3%.

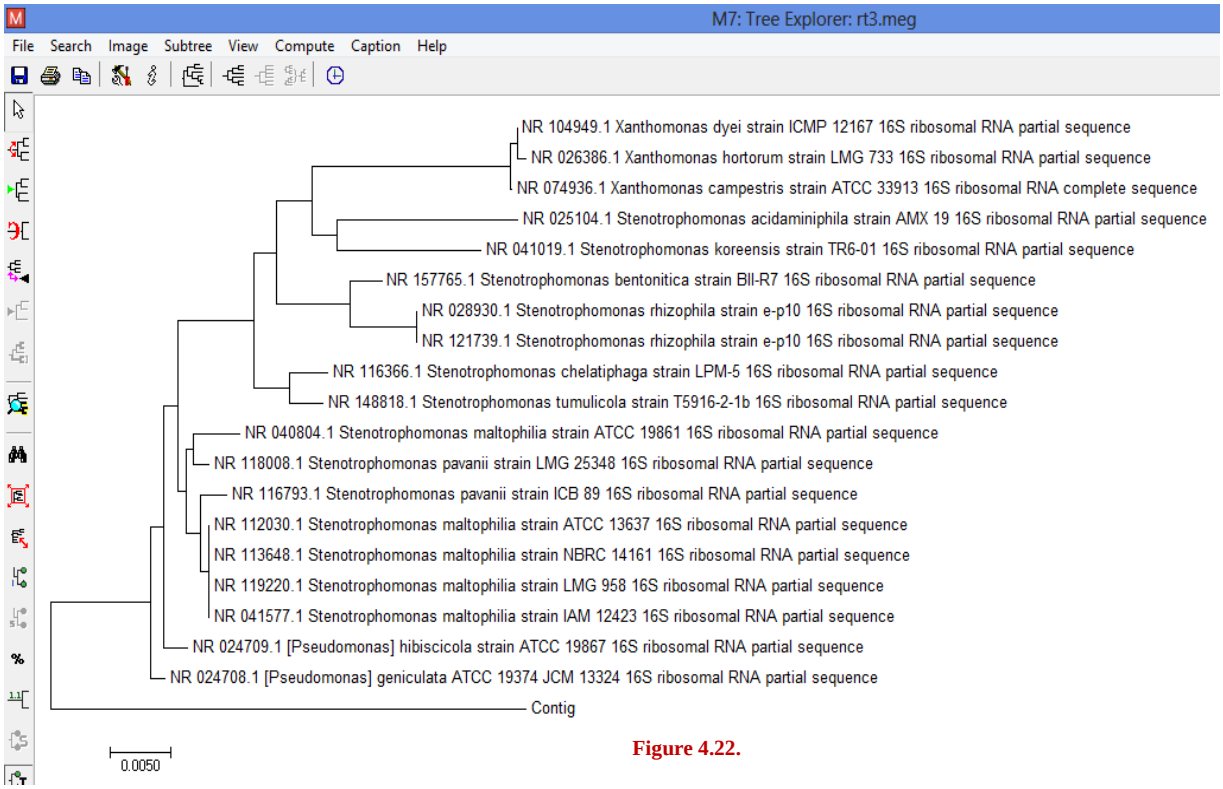


Figure 4.22.

Figure 4.22. Neighbour-joining phylogenetic tree based on 16 S rRNA gene sequences, showing the relationships between strain Ep1 and related species of genus *Stenotrophomonas.* Only bootstrap values above 50%. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA7. Bar, 0.1 substitution per nucleotide position.

A phylogenetic tree (Figure.4.22.) based on 16 S rRNA gene sequences of members of the genus *Stenotrophomonas* was constructed according to the bootstrap test of neighbor-joining algorithm method of Saitou and Nei with MEGA7. This tree shows the close phylogenetic association of strain RT3 with certain other *Stenotrophomonas* species. Phylogenetic analysis indicated that the strain RT3 consistently falls into a clade together with *[Pseudomonas] hibiscicola* strain ATCC19867 (similarity 94.0%), *[Pseudomonas] geniculata* strain ATCC 19374 JCM 13324(similarity 93.7%), *Stenotrophomonas maltophilica* strain IAM 12423 (similarity 94.2%), *Stenotrophomonas maltophilica* strain LMG 958 (similarity 94.1%), *Stenotrophomonas maltophilica* strain ATCC 13637 (similarity 94.1), etc. However both of these [*Pseudomonas*] species were later reclassified under the genus *Stenotrophomonas.* (Anzai *et al.,* 2000); Pairwise global alignment using EMBOSS Stretcher, based on classic dynamic programming algorithm, was performed for determining the similarity of RT3 with different strains.

Thus, pairwise 16S rRNA gene sequence similarities of RT3 to these strains were below 98.0%, but can be classified under the genus of *Stenotrophomonas*.

4.5 EXTRACTION AND PURIFICATION OF MELANIN

Extraction and purification of pigment from bacterial cultures was performed. Immediately after adding 6N HCl to the cell free supernatant, the clumping of particles started and was left disturbed for at least 4 h to settle at the bottom; however the same wasn't observed in case of non-pigment producing strains. After centrifugation, the pellet formed was subjected to further purification process.

Purified pigment was oven dried and stored at -20 ºC for prolong use.

One of the strains, designated **RT 3**, produced the maximum amount of pigment ~ 149µg/mL of bacterial culture utilized.

4.6 PHYSIOCHEMICAL CHARACTERISTICS OF MELANIN PIGMENT

Assuming that the extracted pigment may contain melanin, solubility in different polar and non-polar solvents, oxidation with H_2O_2, etc. were tested employing standard methods to check for various chemical characteristics of the pigment.

Both synthetic and melanin from bacteria were mostly insoluble in almost all the inorganic solvents (refer Table.4.3), however, some exceptions were also observed in case of melanin from bacterial origin, which were readily soluble in some solvents such as water, dimethyl sulfoxide, but partially soluble in methanol and 0.1N HCl.

Table 4.3 Chemical characterization of test samples of melanin

Properties	**For melanin extracted from bacterial sources**	**For synthetic melanin**
Color	Dark brown	Dark brown to blackish
Solubility in water	Soluble	Insoluble
Solubility in 0.1N NaOH	Soluble	Soluble
Solubility in 0.1N KOH	Soluble	Soluble
Solubility in 0.1 N HCl	Partially soluble	Insoluble
Solubility in Methanol	Partially soluble	Insoluble
Solubility in Dimethyl sulfoxide	Soluble	Insoluble
Solubility in Ethanol	Insoluble	Insoluble
Solubility in Acetone	Insoluble	Insoluble
Solubility in Chloroform	Insoluble	Insoluble
Solubility in other organic solvents (such as n-Hexane, Ethyl acetate, Isopropanol, Isoamyl alcohol, Butanol, Diethyl ether, Acetic acid)	Insoluble	Insoluble
Reaction with H_2O_2	Turns colorless	Turns colorless

4.7 UV-Vis ANALYSIS OF MELANIN

UV-Vis absorption spectrum in the region of 200 to 800 nm was observed (Gadd, 1982) for the characteristic property of both, the samples and the chemically synthesized melanin of known concentration.

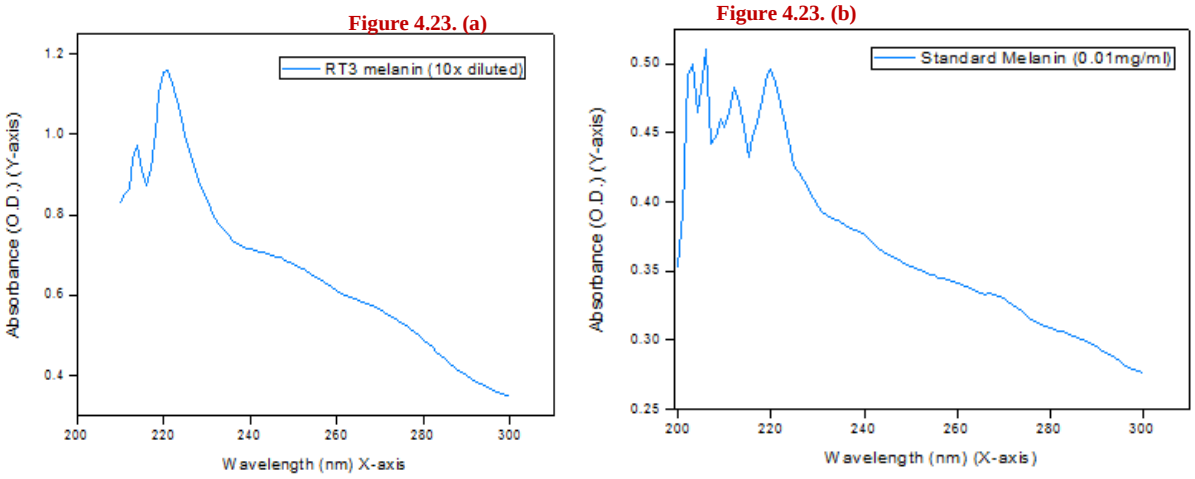


Figure.4.23. UV spectrum of melanin from 200 nm to 300 nm (a) For melanin pigment extracted from bacterial strain RT3 (b) For synthetic melanin (10 mg/L concentration)

Sample pigments initially extracted from 1.5 mL of bacterial culture were further diluted to ten-fold to check for the distinct pattern similarity with reference to the standard synthetic melanin.

However, few strong absorbance peaks between 200 to 225 nm were observed in both, the melanin for unknown and given concentrations (in mg/mL), but the absorbance peak at 220 to 222nm were precisely similar in for all samples and standard of the pigment. (**Figure.4.23.**)

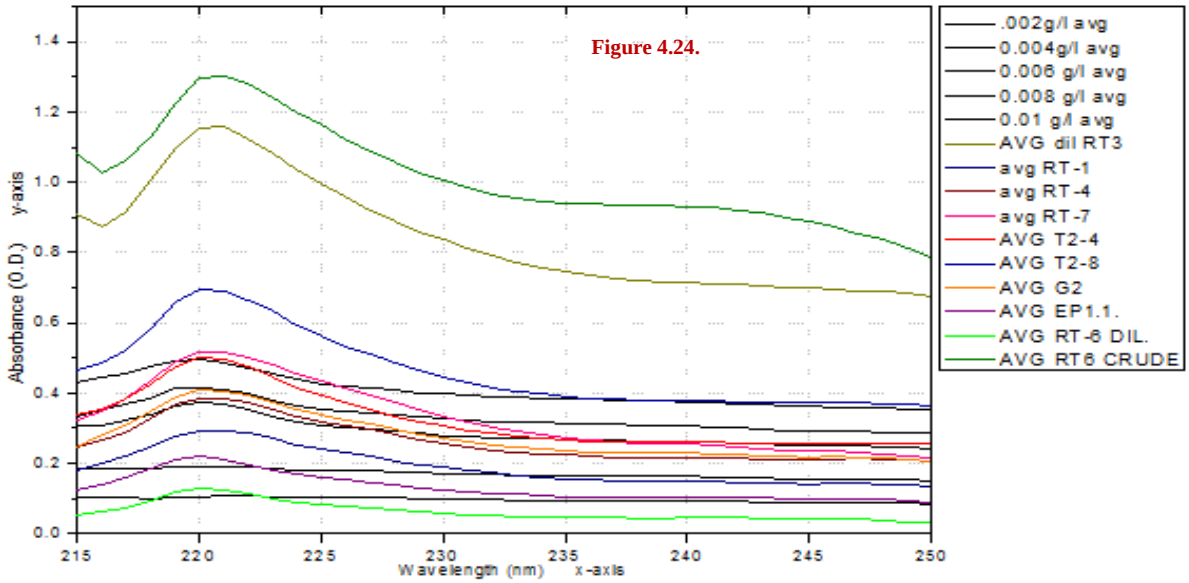


Figure.4.24. UV-Vis spectrum of the melanin sample from bacterial cultures vs standard melanin (in concentrations ranges of 0.002 – 0.01 g/l), with strong peaks at 220nm.

A sharp increase in the peak size with gradual increase in known concentrations (ranging from 2 ppm to 10 ppm) of standard melanin was also observed at the peak range of 220-222 nm in contrast to the bacterial melanin (**Figure.4.24.**) Further gradual decline when the wavelength is increased towards the infrared region is also observed as previously reported by Guo *et al.*, 2014.

4.8 FT-IR ANALYSIS OF MELANIN

FT-IR spectroscopy was performed for the melanin extracted from bacterial strains, namely RT3, RT4 and T2-8 (Figure.4.25. (b), (c), (d)) in contrast to synthetic melanin (Figure.4.25. (a)).

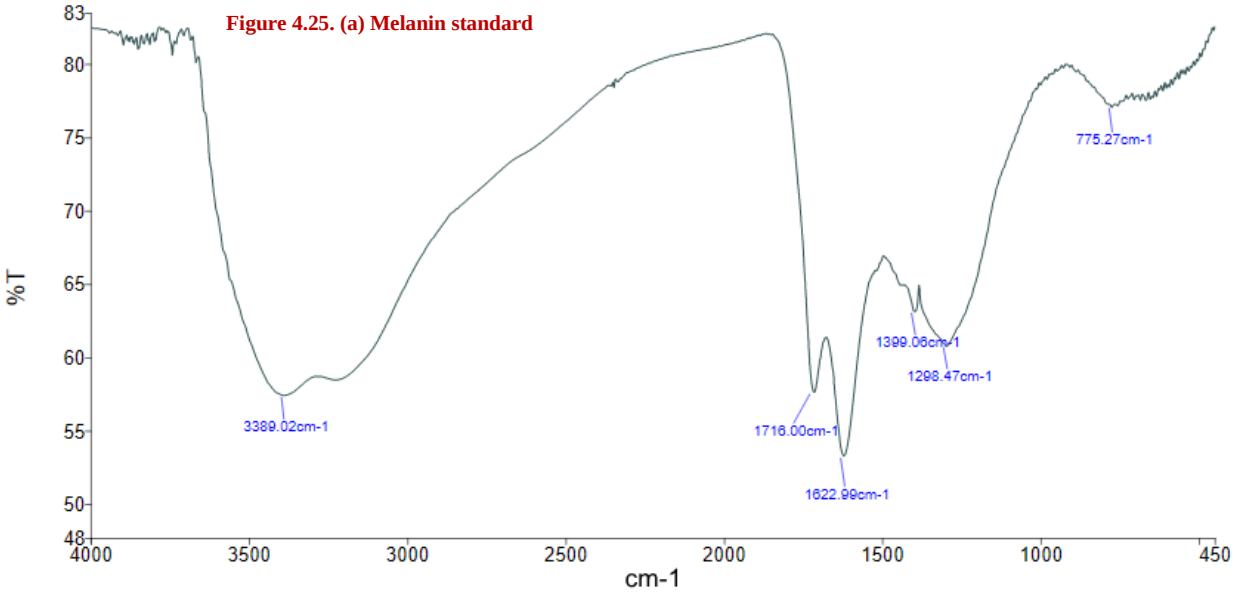


Figure 4.25. (a) Melanin standard

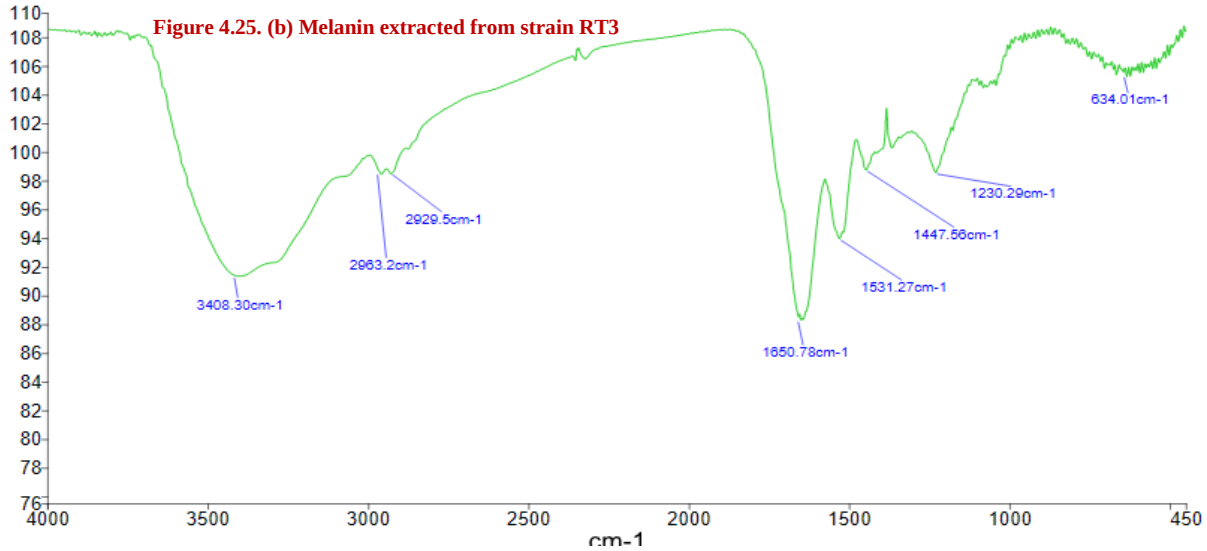


Figure 4.25. (b) Melanin extracted from strain RT3

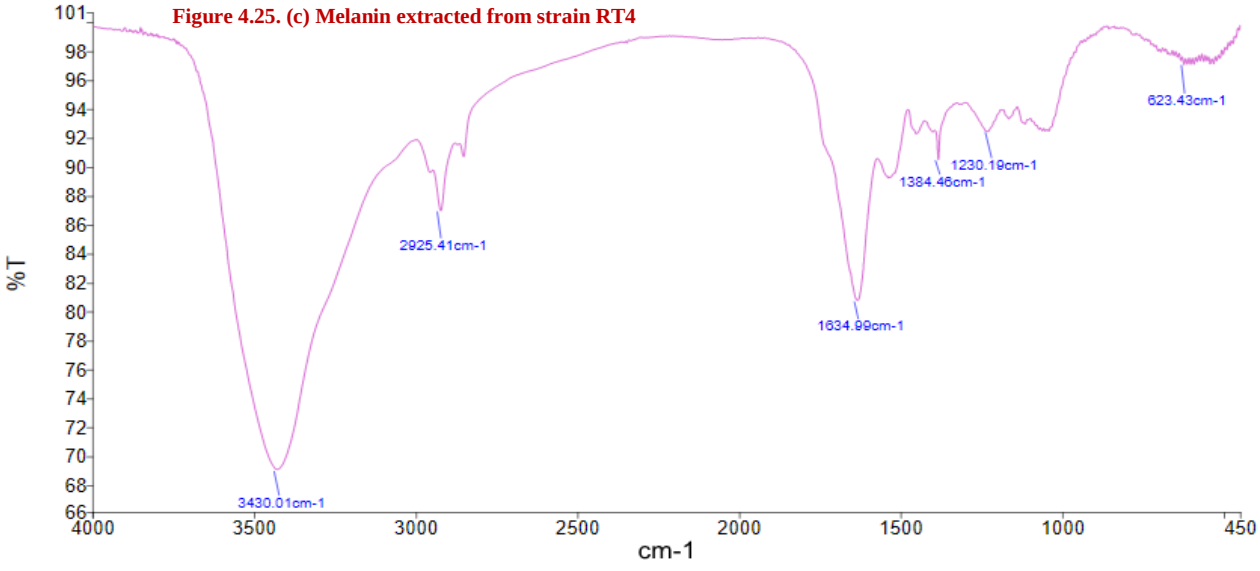


Figure 4.25. (c) Melanin extracted from strain RT4

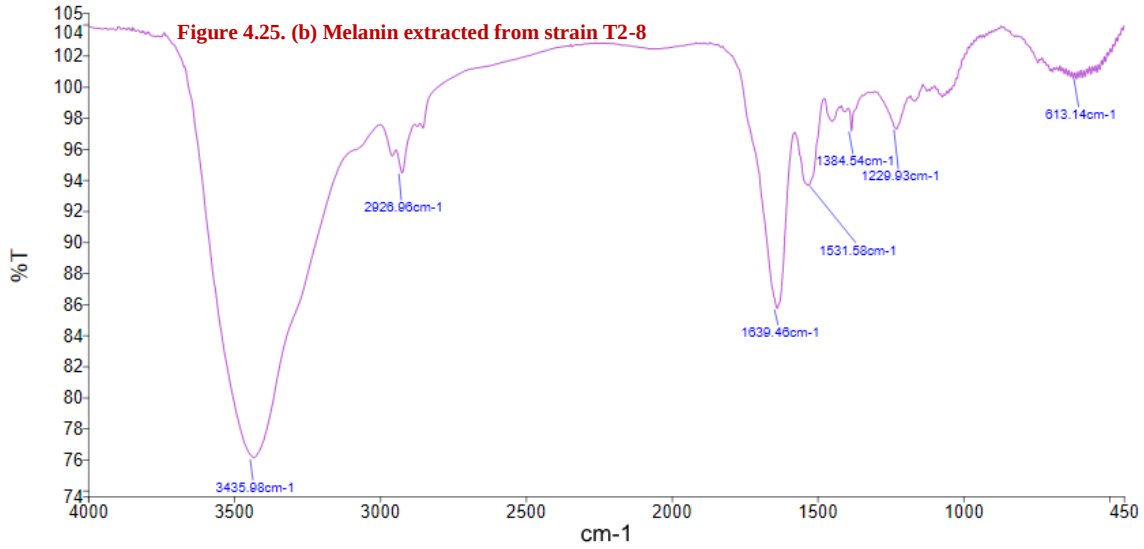


Figure.4.25. FT-IR Spectral analysis of (a) Synthetic melanin (standard), and melanin pigment extracted from bacterial strains (b) RT3 (c) RT4 (d) T2-8

The FT-IR spectrum of the synthetic melanin shows a peak around 3389.02 cm^{-1}, and melanin extracted from RT3, RT4 and T2-8 shows a peak at 3408.30 cm^{-1}, 3430.01 cm^{-1} and 3435.98 cm^{-1} respectively, which correspond to the OH group.

A small band at the range of 2900-2970 cm^{-1}observed in bacterial extracted melanin, but not in synthetic pigment, can be assigned to stretching vibration of aliphatic C-H group. The signals in the range of 3600–2800 cm^{-1} area are attributed to the stretching vibrations (O-H and N-H) of amide, amine, carboxylic acid, phenolic and aromatic amino functional groups present in the indolic and pyrrolic systems. The simple C-H stretching vibrations for saturated aliphatic species occur between 3000 and 2800 cm^{-1}, and the corresponding simple bending vibrations nominally occur between 1500 and 1300 cm^{-1} (Coates, 2000).

Peak observed in the range of 1600-1620 cm^{-1} can be attributed to the vibrations of aromatic ring C = C of amide / C = O and/or of COO- groups, indicates a typical indole structure of melanin. Phenolic COH stretching at relates to phenolic compounds. It was proposed that peaks at 1243 to 1305 cm^{-1} relates to the anhydride group (C-O) in synthetic melanin and all extracted microbial pigments (Hewedy & Ashour, 2009). Weak bands below 700 cm^{-1} ascribed to alkene C-H substitution in the bacterial melanin pigment (Coates, 2000). The spectroscopic properties of the pigment extracted from RT 3, RT4 and T2-8 correlated with those of melanin extracted from various different microorganisms previously reported. (El-Naggar & El-Ewasy, 2017; Hewedy & Ashour, 2009; Sajjan *et al.*, 2010; Sivaperumal *et al.*, 2014; Aghajanyan *et al.*, 2011).

4.9 THIN LAYER CHROMATOGRAPHY OF MELANIN

TLC of the extracted melanin pigment was performed on a 6 cm x 10 cm silica gel 60 F254 TLC plate(stationary phase), using the mixture of Acetonitrile: Methanol: H_2O as the mobile phase system, in the ratio 9:9:2. The plate was observed under the UV chamber, however, the spot of the pigment solution was visible normally, even without the utility of the UV chamber.

R_f values for both the sample and standard melanin was calculated after TLC plate development (refer Table 4.4). A single R_f value with an intense spot was obtained for the tests was matching with R_f value of the standard melanin.

Distance travelled by Solvent front = 7.5 cm

Distance travelled by

- Standard melanin = 6.35 cm
- pigment extracted from
    - Strain T.3.1.1.2. = 6.3 cm
    - Strain RT4 = 6.5 cm

Table 4.4: Results of TLC analysis of melanin pigment

Sample	**R_f value**
Standard melanin (Lane 3)	0.847
Melanin samples extracted from bacterial strains:	
T.3.1.1.2. (Lane 1)	0.84
RT4 (Lane 2)	0.867

The ImageJ software was further used to analyse the chromatogram of the pigment developed on TLC plate. The image was converted to greyscale for better resolution prior plotting the peaks. Different lanes of melanin samples runs with the intense spots were selected, and then was plotted to create the corresponding chromatogram peak signifying the actual number of intense peaks or bands formed on the TLC plate after development.

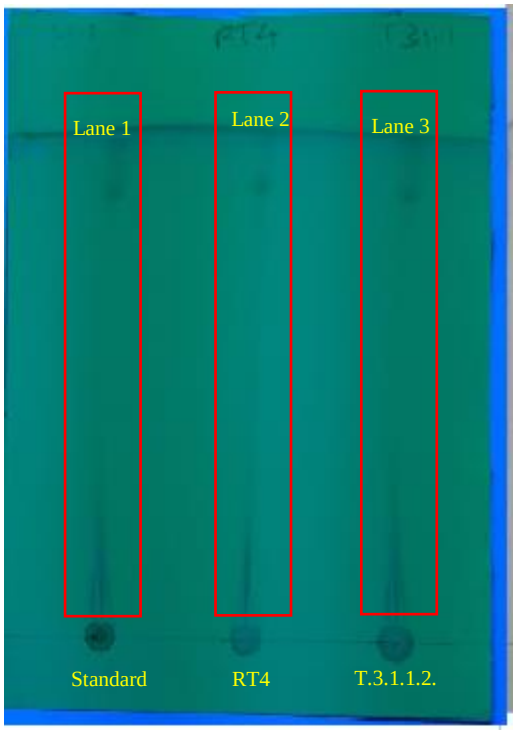


Figure.4.26.

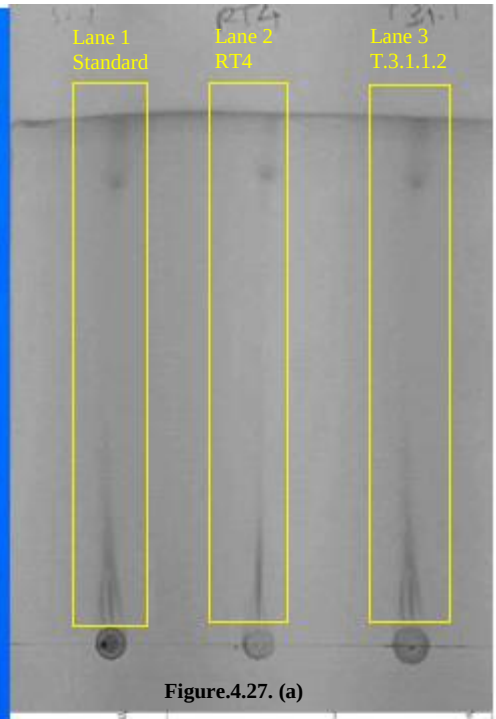


Figure.4.27. (a)

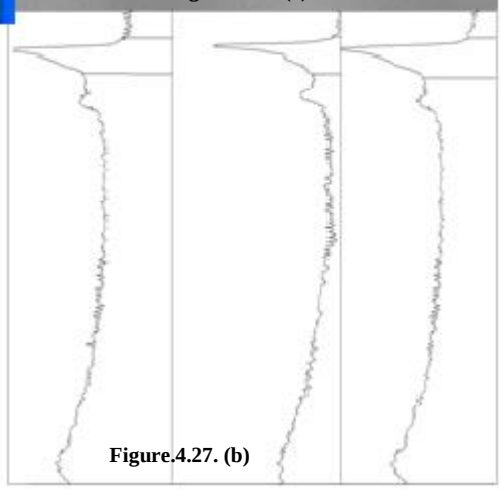


Figure.4.27. (b)

Figure.4.26. TLC analysis of melanin pigment after plate development

Figure.4.27. (a) Greyscale version of developed TLC plate, further utilized for plot analysis of pigment using ImageJ software. (http://imagej.nih.gov/ij/, ver. 1.52a, Rasband W. National Institutes of Health, USA).
(b) Respective chromatogram of melanin obtained by the converted greyscale image.

A significant characteristic chromatogram peak was observed against the run of each sample and the standard of the melanin pigment.

4.10 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF MELANIN

HPLC method was performed for the qualitative analysis and determination of retention time of biologically synthesized of melanin from bacterial sources as well as for synthetic melanin.

However, a high-performance liquid chromatography method for the quantitative analysis of the contents of eumelanin and pheomelanin has already been reported (Ito, 1993), to study the regulation of melanogenesis in tissue samples without any isolation procedures. The pigment was subject to acidic permanganate oxidation to yield derived monomer products such as Pyrrole-2,3,5-tricarboxylic acid (PTCA) and hydriotic acid hydrolysis to yield amino hydroxyl-phenylalanine (AHP) isomers, so that these specific products could later be analysed using HPLC. But a major drawback of this method is that it doesn't produce the appreciable amounts of PDCA from natural eumelanin and DHI-melanin (Wakamatsu & Ito., 2002).

Till now there is no official HPLC methods are available for melanin pigment derived from microbial sources. This is largely because of the chemical properties of melanins, such as their insolubility over a broad range of pH, the heterogeneity in their structural features, and due to lack of methods that can split melanin polymers into their monomer units (all other biopolymers can be hydrolysed to the corresponding monomer units). Yet we have tried to establish a protocol for bacterial synthesized melanin without derivatization or degradation of the pigment.

Upon looking at the chromatogram in Figure.4.28, and its analysis of peak heights at different retention times, we couldn't find any trend which could be used for the analysis of the peaks in order to quantify them (Refer Appendix III). Upon further investigation we infer that in order to analyse melanin we need to further improvise our approach and try more solvent systems. We suggest finding out such solvents which do not disturb the stability of the pigment and therefore the results could be obtained relatable.

Figure.4.28. HPLC chromatogram peaks for the standard melanin of concentrations (a) 50 ppm (b) 100 ppm (c) 150ppm; and test samples of melanin (d) RT3 (e) RT3 (g) RT4 (h) T2 8 (i) T2 8 ; and blank (f).

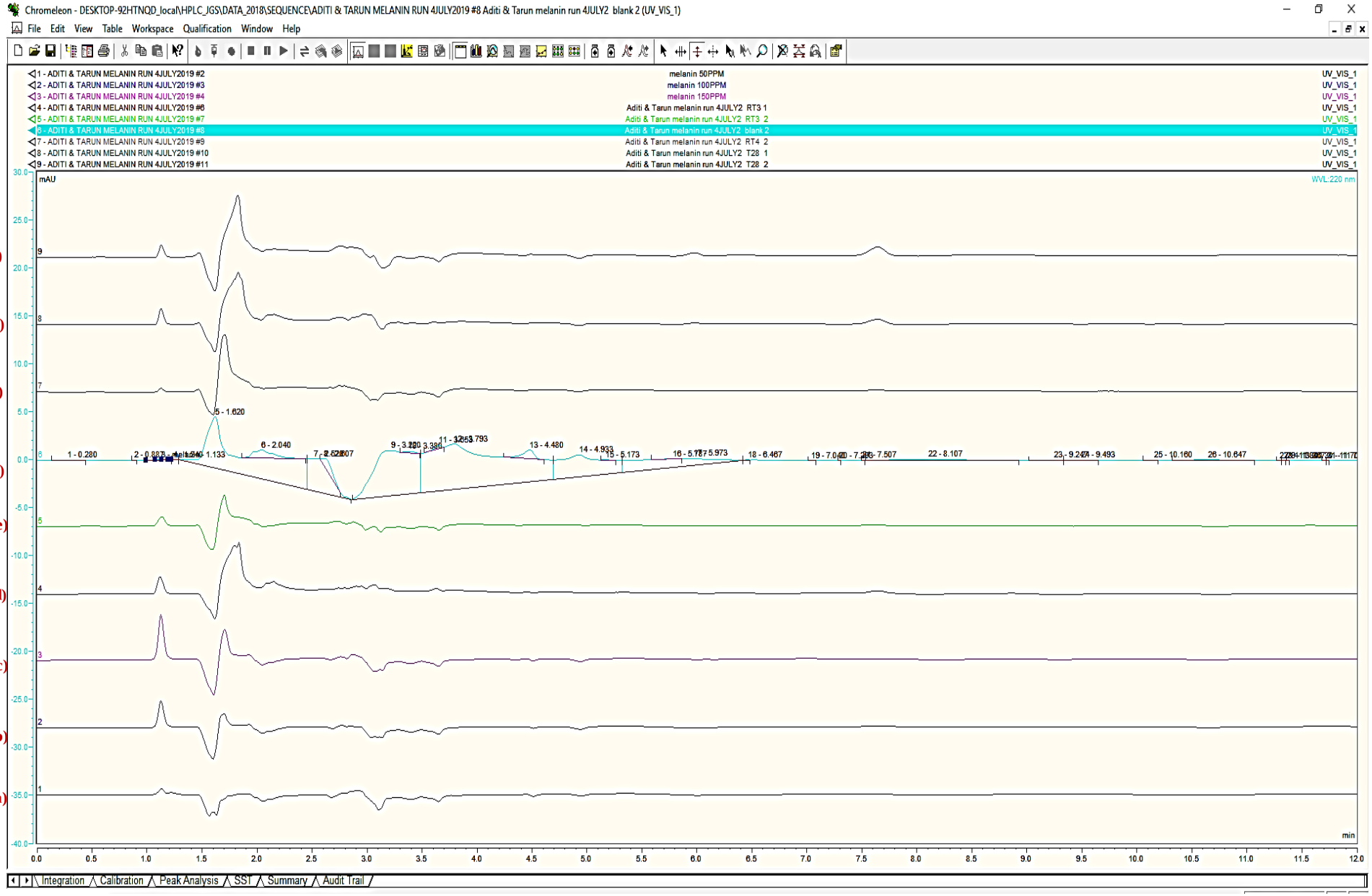

Chromeleon - DESKTOP-92HTNQD_local\HPLC_JGS\DATA_2018\SEQUENCE\ADITI & TARUN MELANIN RUN 4JULY2019 #8 Aditi & Tarun melanin run 4JULY2 blank 2 (UV_VIS_1)
File Edit View Table Workspace Qualification Window Help
1 - ADITI & TARUN MELANIN RUN 4JULY2019 #2 melanin 50PPM UV_VIS_1
2 - ADITI & TARUN MELANIN RUN 4JULY2019 #3 melanin 100PPM UV_VIS_1
3 - ADITI & TARUN MELANIN RUN 4JULY2019 #4 melanin 150PPM UV_VIS_1
4 - ADITI & TARUN MELANIN RUN 4JULY2019 #6 Aditi & Tarun melanin run 4JULY2 RT3 1 UV_VIS_1
5 - ADITI & TARUN MELANIN RUN 4JULY2019 #7 Aditi & Tarun melanin run 4JULY2 RT3 2 UV_VIS_1
6 - ADITI & TARUN MELANIN RUN 4JULY2019 #8 Aditi & Tarun melanin run 4JULY2 blank 2 UV_VIS_1
7 - ADITI & TARUN MELANIN RUN 4JULY2019 #9 Aditi & Tarun melanin run 4JULY2 RT4 2 UV_VIS_1
8 - ADITI & TARUN MELANIN RUN 4JULY2019 #10 Aditi & Tarun melanin run 4JULY2 T28 1 UV_VIS_1
9 - ADITI & TARUN MELANIN RUN 4JULY2019 #11 Aditi & Tarun melanin run 4JULY2 T28 2 UV_VIS_1
mAU
WVL:220 nm
(i)
(h)
(g)
(f)
(e)
(d)
(c)
(b)
(a)
5 - 1.620
1 - 0.280
6 - 2.040
13 - 4.480
14 - 4.933
22 - 8.107
min
Integration Calibration Peak Analysis SST Summary Audit Trail

Figure 4.28.

CONCLUSION

- In summary, total 11 melanin-producing strains from DTU Lake water sample has been successfully screened so far. For pigment production, nutrient broth supplemented with L-tyrosine had been used for inoculum preparation. After 5-7 days of incubation, these strains produced diffused black pigment.
- These strains were further subjected for gram staining behaviour; majority of them were observed to be rod or bacilli-shaped cell morphologies. And all the strains were also checked for their different biochemical behaviours such as catalase, oxidase, motility, acid fermentation from different sugars, gelatinase, urease, indole production, MR-VP, citrate, arginine, nitrate, ONPG, decarboxylases etc. tests. *Staphylococcus aureus* was taken for the reference in this study.
- Two strains among the screened isolates were subjected for the microbial identification RT3 and Ep1 using NCBI blast (local alignment), ClustalW (multiple sequence alignment) and Embl EMBOSS stretcher (pairwise global alignment). Phylogenetic tree were constructed using MEGA 7 tool.
- Ep 1 was observed to have highest similarity of 99.3% with many *Bacillus oleronius* strains, thus, can conformably be classified as *Bacillus oleronius*. Since RT3 had 94.3% highest similarity with *Stenotrophomonas maltophilica*, which is less than 97% to be classified with in the same species level. Thus, RT3 can be classified under *Stenotrophomonas* sp.
- Extraction and purification of pigment was also performed by acidification and subsequent washing using different solvents and palletisation. The obtained pigment was stored in dried state at – 20 º C in order to maintain stability. Hence, the isolated bacteria produced nearly black colored pigment which was tentatively inferred to as melanin due to its ability to utilize L-tyrosine as precursor.
- The obtained pigment was subjected to further characterization processes such as UV-vis and FT-IR Spectroscopy, and TLC and HPLC chromatographic analysis.
- Prior the extraction step, overall cell-free supernatant was being UV-vis spectrum scanned from 200 to 1100 nm wavelengths. All the pigment producing samples showed various multiple peaks in the region of 205-240 nm. However, after extraction, purification and drying process, melanin produced was characterized by its UV-vis absorption spectrum; and it was also found that the pigment produced by different bacterial strains yielded a similar spectrum to commercial synthetic melanin with a strong peak at 220nm.

- FT-IR analysis revealed that the functional groups were conserved in both, standard and sample melanin, and almost appeared to be same. The spectroscopic properties of melanin from bacterial sources correlated with those of melanin extracted from various different microorganisms previously reported.
- TLC analysis of melanin pigment revealed the R_f value with a single intense spot was obtained for the tests was matching with R_f value of the standard melanin, which was obtained in the range of 0.84-0.87. A single and strong chromatogram peak was also obtained signifying the actual number of intense peaks or bands formed on the TLC plate after development.
- HPLC analysis of the pigment gave us more insight about the pigment and we inferred a lot of observation such as the thermal stability of melanin in solution is very poor in thawed state. The C18 column could be a potential column for this analysis, the solvent system greatly affects the results of such an analysis, melanin can break down differently upon different types of temperature states and solvent system treatments, column temperatures etc.
- Stability can be greatly affected with temperature shocks upon addition of solvents to solubilize the pigment and HPLC analysis of the pigment needs further tweaking than what we could possibly perform in our time of experimentation.

FUTURE PERSPECTIVE

The melanin pigment extracted from the bacterial sources can further be studies for other characterization studies such as NMR spectroscopy, Mass spectroscopy and XRD analysis. Melanin extracted need to be checked for efficiency of free radical scavenging activity of a model DPPH radical. Antibacterial activity of melanin pigment against all tested pathogens and the zone of clearance, if present, are to be observed.

Further understanding of the properties of microbial pigments will not only enrich our instinctual curiosity about color, but also provide a scientific basis for therapeutic disarming of the pathogens responsible for microbial virulence in pigments.

A promising model obtained using RSM methodology on further optimization of factors like pH and temperature, incubation period, other nutrient supplements, etc. can be implemented for predictive estimation and modification of fermentation processes for maximizing melanin yields possibly up to industrial scale.

APPENDIX-I

Composition of different types of Media and Buffers, used in the study, are given below. Sterilization of media and buffers were performed at 121 ºC for 20 min under 15 p.s.i. pressure**.**

COMPOSITION OF DIFFERENT CULTURE MEDIA AND BUFFERS

1. **Arginine dihydrolase broth**

Peptic Digest of Animal Tissue	1.50g
Sodium Chloride	5.00g
Dipotassium hydrogenphosphate	0.30g
L- Arginine	10.00g
Phenol Red indicator	0.24g
Agar Powder	3.00g
Deionized Water	1000mL
Final pH	7.2±0.2

2. **Hugh Leifson medium**

Peptone	2.00g
Sodium Chloride	5.00g
Glucose	10.00g
Dipotassium hydrogenphosphate	0.30g
Bromothymol blue	0.05g
Agar	2.00g
Deionized Water	1000mL
Final pH	7.2±0.2

3. **Kohn two tube medium No.1**

Beef Extract	2.00g
Peptone	15.00g
Yeast Extract	2.00g
Dextrose	1.00g
Mannitol	10.00g
Phenol Red	0.05g
Agar	15.00g
Deionized Water	1000mL

Final pH	7.2±0.2

4. **Lysine Decarboxylase Broth**

Pancreatic digest of Animal Tissue	5.00g
Yeast Extract	3.00g
Dextrose	1.00g
L-lysine Hydrochloride	5.00g
Bromocresol purple	0.02g
Deionized Water	1000mL
Final pH	6.8±0.2

5. **Nitrate Broth**

Beef Extract	3.00g
Peptone	5.00g
Potassium Nitrate	1.00g
Deionized Water	1000mL
Final pH	7.2±0.2

6. **Nutrient Broth**

Peptone	10.00g
Beef extract	10.00g
Sodium chloride	5.00g
Deionized Water	1000mL
Final pH	7.0±0.1

7. **Nutrient Agar**

Peptone	10.00g
Beef extract	10.00g
Sodium chloride	5.00g
Agar Powder	15.00g
Deionized Water	1000mL
Final pH	7.0±0.1

8. **Ornithine Decarboxylase Broth**

L-ornithine monohydrochloride	5.00g

Yeast Extract	3.00g
Glucose	1.00g
Bromocresol purple	15.00mg
Deionized Water	1000mL
Final pH	6.8±0.2

9. **Simmons Citrate Medium**

$MgSO_4.7H_2O$	0.20g
Ammonium dihydrogenphosphate	1.00g
Sodium Citrate	2.00g
Sodium Chloride	5.00g
Dipotassium hydrogenphosphate	1.00g
Bromothymol blue	0.08g
Agar Powder	15.00g
Deionized Water	1000mL
Final pH	6.8±0.2

10. **Tryptone Broth**

Tryptone	10.00g
Sodium Chloride	5.00g
Deionized Water	1000mL
Final pH	7.2±0.2

11. **Triple Sugar Iron Agar**

Peptic digest of Animal Tissue	10.00g
Casein enzymatic hydrolysate	10.00g
Beef extract	3.00g
Yeast extract	3.00g
Lactose	10.00g
Sucrose	10.00g
Dextrose	10.00g
Sodium Chloride	5.00g
$FeSO_4$	0.20g
Sodium thiosulfate	0.30g

Phenol Red	24.00mg
Agar Powder	12.00g
Deionized Water	1000mL
Final pH	7.4±0.2

APPENDIX-II

1. L-Tyrosine, 2g/l supplemented in Nutrient Agar/Broth
2. Gram Staining reagents:
    a. Gram's Crystal Violet
    b. Gram's Iodine
    c. Decolorizer: Mixed equal volumes of 95 % ethanol and acetone
    d. Safranine solution: Dissolve 2.5 g of safranine O in 100 mL of 95 % ethanol to make a stock solution.
3. 3% H_2O_2 solution
4. Mineral Oil (sterile)
5. Kovac's reagent

p-Dimethyl aminobenzaldehyde	5.00g
Amyl alcohol	75.00mL
Conc. Hydrochloric acid	25.00mL

Dissolve p-Dimethyl aminobenzaldehyde in amyl alcohol and then slowly add conc. hydrochloric acid to it.

5. Solution (1) for Nitrate reduction

Sulphanilic acid	8.00g
5 N Acetic acid	1000mL

6. Solution (2) for Nitrate reduction

α-napthol	5.00g
5 N Acetic acid	1000mL

7. Methyl red reagent
Add 0.1g of Methyl red indicator in 300mL of 95% ethanol, and volume was later made upto 500mL using distilled water.

8. Voges-Proskauer Solution A

α-napthol	5.00g
Absolute ethanol	100.0mL

9. Voges-Proskauer Solution B

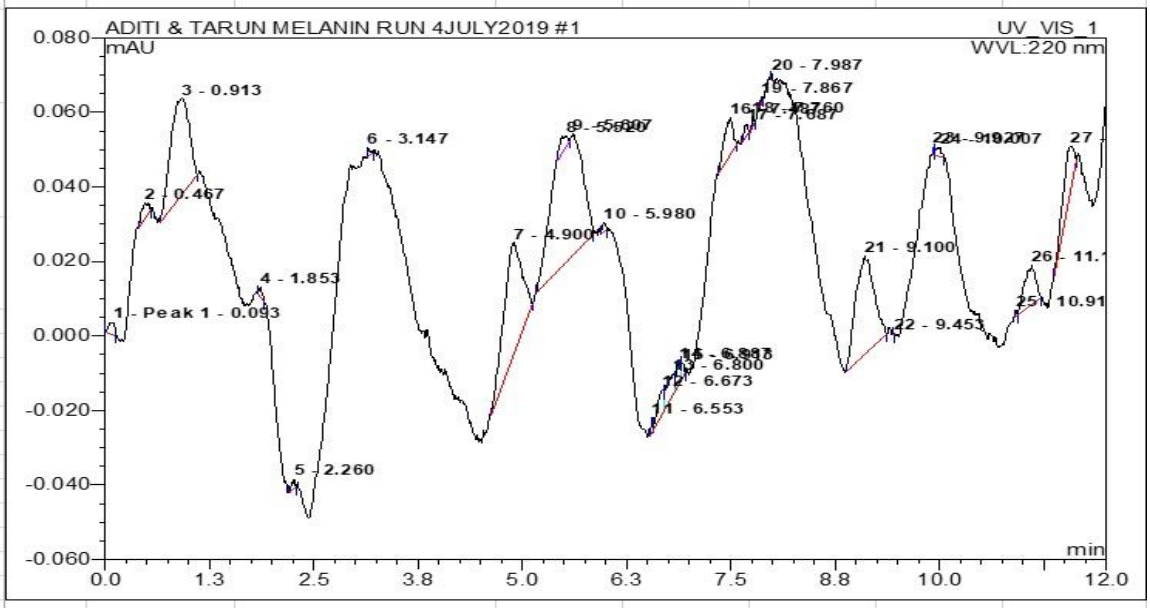
Potassium hydroxide	40.0g
Creatine	0.5g
Distilled water	100.0mL

10. Mixture solution of 1.5 % Mercuric chloride and 10% hydrochloric acid for gelatin hydrolysis test.

APPENDIX-III

1 BLANK

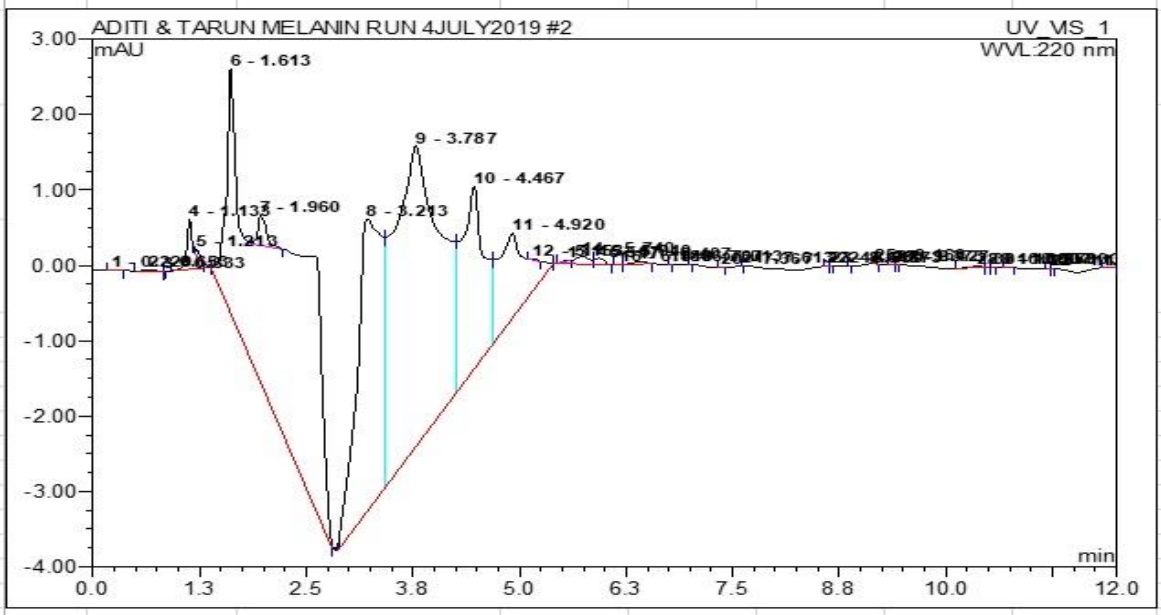
Sample Name:	**BLANK**	*Injection Volume:*	**10.0**
Vial Number:	**RA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**blank**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 14:09**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.09	Peak 1	0.003	0.000	0.59	n.a.	BMB
2	0.47	n.a.	0.004	0.000	0.92	n.a.	BMB
3	0.91	n.a.	0.026	0.007	15.38	n.a.	BMB
4	1.85	n.a.	0.003	0.000	0.28	n.a.	BMB
5	2.26	n.a.	0.003	0.000	0.26	n.a.	BMB
6	3.15	n.a.	0.003	0.000	0.19	n.a.	BMB
7	4.90	n.a.	0.030	0.008	17.33	n.a.	BMB
8	5.52	n.a.	0.003	0.000	0.95	n.a.	Ru
9	5.61	n.a.	0.032	0.014	31.64	n.a.	BMB
10	5.98	n.a.	0.003	0.000	0.26	n.a.	BMB
11	6.55	n.a.	0.004	0.000	0.26	n.a.	BM
12	6.67	n.a.	0.007	0.001	1.36	n.a.	M
13	6.80	n.a.	0.006	0.001	1.51	n.a.	M
14	6.89	n.a.	0.005	0.000	0.84	n.a.	M
15	6.91	n.a.	0.004	0.000	0.34	n.a.	MB
16	7.49	n.a.	0.010	0.001	2.84	n.a.	BMB
17	7.69	n.a.	0.003	0.000	0.39	n.a.	BMB
18	7.76	n.a.	0.003	0.000	0.16	n.a.	BMB
19	7.87	n.a.	0.002	0.000	0.09	n.a.	BMB
20	7.99	n.a.	0.002	0.000	0.08	n.a.	BMB
21	9.10	n.a.	0.026	0.007	15.33	n.a.	BMB
22	9.45	n.a.	0.001	0.000	0.01	n.a.	BMB
23	9.93	n.a.	0.002	0.000	0.09	n.a.	BM
24	10.01	n.a.	0.003	0.000	0.42	n.a.	MB
25	10.91	n.a.	0.002	0.000	0.07	n.a.	Ru
26	11.10	n.a.	0.011	0.002	3.83	n.a.	BMB
27	11.57	n.a.	0.012	0.002	4.60	n.a.	BMB
Total:			0.211	0.044	100.00	0.000	

2 melanin 50PPM

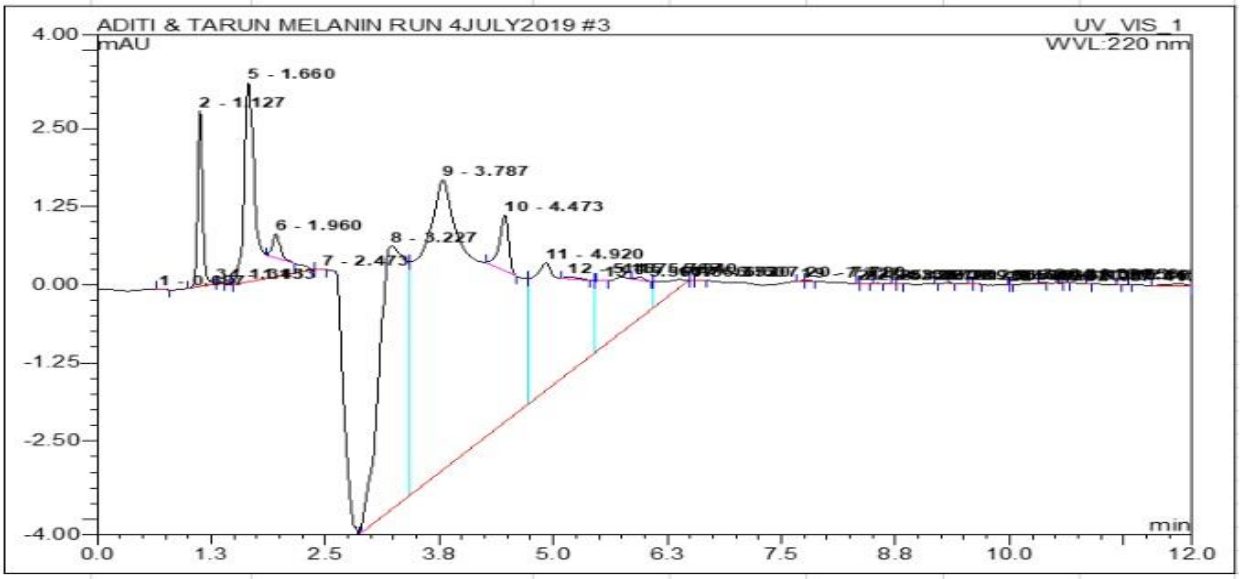
Sample Name:	**melanin 50PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA2**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 14:22**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	0.22	n.a.	0.011	0.001	0.02	n.a.	BMB
2	0.65	n.a.	0.023	0.004	0.05	n.a.	BMB
3	0.83	n.a.	0.001	0.000	0.00	n.a.	bMB
4	1.13	n.a.	0.665	0.064	0.79	n.a.	BMb
5	1.21	n.a.	0.083	0.005	0.06	n.a.	Rd
6	1.61	n.a.	3.243	2.794	34.78	n.a.	bMB
7	1.96	n.a.	0.397	0.045	0.57	n.a.	Rd
8	3.21	n.a.	3.875	1.320	16.43	n.a.	BM
9	3.79	n.a.	3.985	2.536	31.56	n.a.	M
10	4.47	n.a.	2.432	0.768	9.55	n.a.	M
11	4.92	n.a.	1.120	0.448	5.58	n.a.	MB
12	5.15	n.a.	0.021	0.002	0.02	n.a.	Rd
13	5.57	n.a.	0.010	0.001	0.01	n.a.	Ru
14	5.74	n.a.	0.102	0.023	0.29	n.a.	bM
15	5.94	n.a.	0.084	0.011	0.14	n.a.	MB
16	6.18	n.a.	0.004	0.000	0.00	n.a.	BMB
17	6.41	n.a.	0.029	0.005	0.07	n.a.	BMB
18	6.77	n.a.	0.003	0.000	0.00	n.a.	BMB
19	7.01	n.a.	0.002	0.000	0.00	n.a.	BMB
20	7.37	n.a.	0.005	0.000	0.00	n.a.	BMB
21	7.61	n.a.	0.001	0.000	0.00	n.a.	BMB
22	8.61	n.a.	0.003	0.000	0.00	n.a.	BM
23	8.66	n.a.	0.002	0.000	0.00	n.a.	MB
24	8.87	n.a.	0.002	0.000	0.00	n.a.	BMB
25	9.16	n.a.	0.003	0.000	0.00	n.a.	BMB
26	9.37	n.a.	0.006	0.000	0.00	n.a.	BM
27	9.43	n.a.	0.004	0.000	0.00	n.a.	MB
28	10.36	n.a.	0.015	0.003	0.04	n.a.	BMB
29	10.51	n.a.	0.002	0.000	0.00	n.a.	bMB
30	10.55	n.a.	0.002	0.000	0.00	n.a.	BMB
31	10.70	n.a.	0.006	0.001	0.01	n.a.	BMB
32	11.20	n.a.	0.003	0.000	0.00	n.a.	BM
33	11.25	n.a.	0.002	0.000	0.00	n.a.	MB
34	11.93	n.a.	0.012	0.001	0.02	n.a.	BMB
Total:			16.160	8.034	100.00	0.000	

3 melanin 100PPM

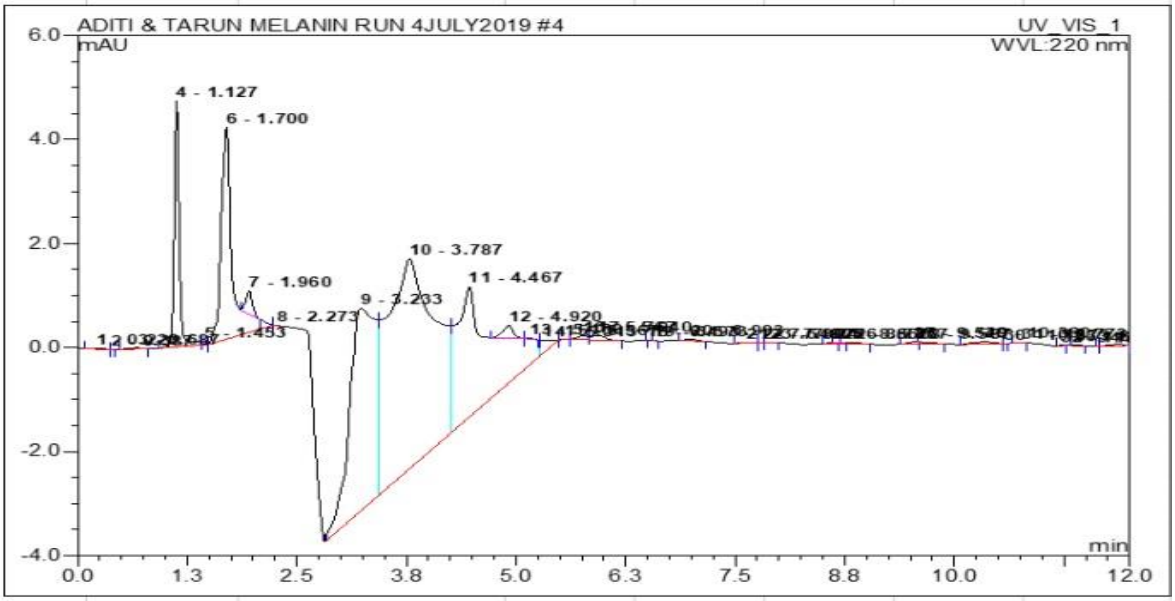
Sample Name:	**melanin 100PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA3**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 14:35**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.69	n.a.	0.007	0.001	0.01	n.a.	BMB
2	1.13	n.a.	2.808	0.181	2.18	n.a.	BM
3	1.31	n.a.	0.030	0.001	0.02	n.a.	Mb
4	1.45	n.a.	0.030	0.001	0.01	n.a.	bMB
5	1.66	n.a.	3.186	0.521	6.27	n.a.	BMB
6	1.96	n.a.	0.395	0.039	0.46	n.a.	Rd
7	2.47	n.a.	0.011	0.001	0.01	n.a.	bMB
8	3.23	n.a.	4.219	1.492	17.95	n.a.	BM
9	3.79	n.a.	4.644	4.154	49.98	n.a.	M
10	4.47	n.a.	0.893	0.104	1.25	n.a.	Rd
11	4.92	n.a.	2.043	1.197	14.40	n.a.	M
12	5.17	n.a.	0.037	0.007	0.08	n.a.	Rd
13	5.57	n.a.	0.006	0.000	0.01	n.a.	Ru
14	5.77	n.a.	0.867	0.511	6.15	n.a.	M
15	5.94	n.a.	0.044	0.005	0.06	n.a.	Rd
16	6.37	n.a.	0.136	0.084	1.01	n.a.	MB
17	6.52	n.a.	0.003	0.000	0.00	n.a.	bMB
18	6.61	n.a.	0.007	0.000	0.01	n.a.	BMB
19	7.73	n.a.	0.009	0.001	0.01	n.a.	BM
20	7.78	n.a.	0.009	0.001	0.01	n.a.	MB
21	8.33	n.a.	0.001	0.000	0.00	n.a.	BMB
22	8.42	n.a.	0.005	0.000	0.00	n.a.	BMB
23	8.56	n.a.	0.003	0.000	0.00	n.a.	BMB
24	8.73	n.a.	0.003	0.000	0.00	n.a.	BM
25	8.79	n.a.	0.003	0.000	0.00	n.a.	MB
26	9.20	n.a.	0.001	0.000	0.00	n.a.	BMB
27	9.36	n.a.	0.003	0.000	0.00	n.a.	BMB
28	9.58	n.a.	0.001	0.000	0.00	n.a.	BMB
29	9.62	n.a.	0.003	0.000	0.00	n.a.	BMB
30	9.99	n.a.	0.001	0.000	0.00	n.a.	BMB
31	10.02	n.a.	0.001	0.000	0.00	n.a.	BMB
32	10.36	n.a.	0.006	0.000	0.00	n.a.	BMB
33	10.57	n.a.	0.003	0.000	0.00	n.a.	BMB
34	10.65	n.a.	0.002	0.000	0.00	n.a.	BMB
35	10.87	n.a.	0.004	0.000	0.00	n.a.	BMB
36	11.21	n.a.	0.002	0.000	0.00	n.a.	BMB
37	11.32	n.a.	0.002	0.000	0.00	n.a.	BMB
38	11.86	n.a.	0.035	0.009	0.10	n.a.	BMB
Total:			19.464	8.311	100.00	0.000	

4 melanin 150PPM

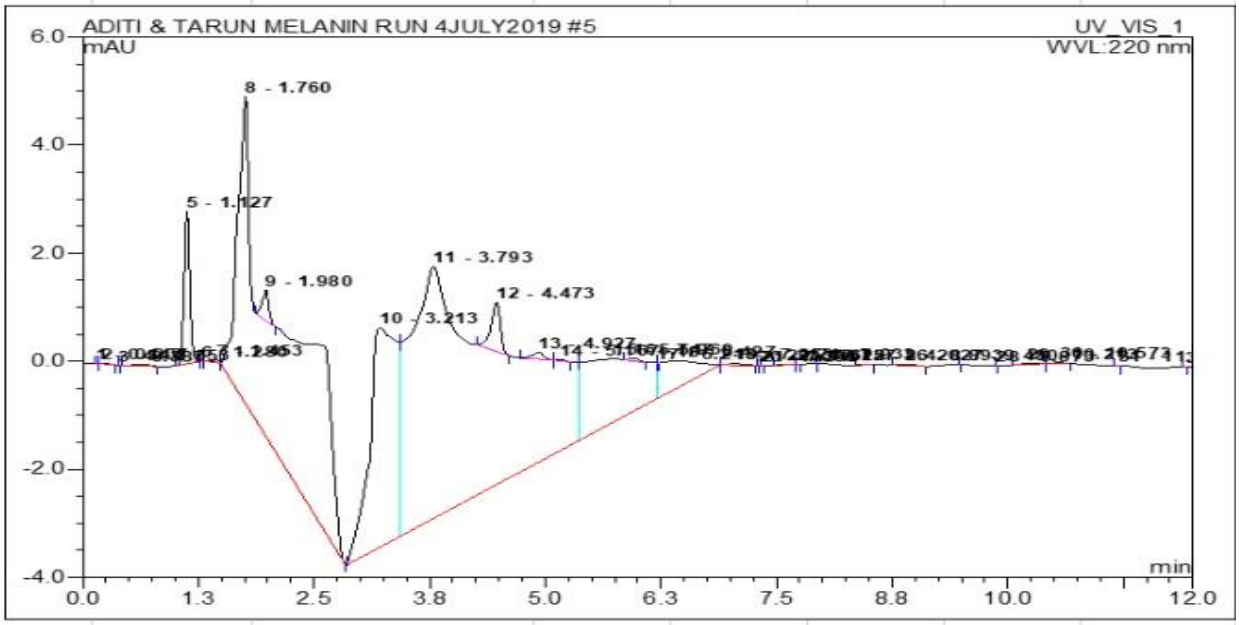
Sample Name:	**melanin 150PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA4**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 14:48**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.22	n.a.	0.022	0.003	0.05	n.a.	BMB
2	0.39	n.a.	0.003	0.000	0.00	n.a.	BMB
3	0.69	n.a.	0.022	0.004	0.06	n.a.	BMB
4	1.13	n.a.	4.723	0.314	5.04	n.a.	BM
5	1.45	n.a.	0.062	0.003	0.05	n.a.	MB
6	1.70	n.a.	4.079	0.610	9.77	n.a.	BMb
7	1.96	n.a.	0.464	0.039	0.62	n.a.	Rd
8	2.27	n.a.	0.015	0.001	0.01	n.a.	bMB
9	3.23	n.a.	3.884	1.314	21.06	n.a.	BM
10	3.79	n.a.	4.030	2.574	41.26	n.a.	M
11	4.47	n.a.	2.509	1.237	19.84	n.a.	M
12	4.92	n.a.	0.243	0.033	0.53	n.a.	Rd
13	5.16	n.a.	0.018	0.001	0.02	n.a.	Rd
14	5.31	n.a.	0.244	0.031	0.49	n.a.	MB
15	5.57	n.a.	0.007	0.001	0.01	n.a.	bMB
16	5.77	n.a.	0.073	0.010	0.17	n.a.	bM
17	5.94	n.a.	0.096	0.017	0.27	n.a.	MB
18	6.49	n.a.	0.001	0.000	0.00	n.a.	BMB
19	6.57	n.a.	0.002	0.000	0.00	n.a.	BMB
20	6.99	n.a.	0.028	0.005	0.08	n.a.	BMB
21	7.64	n.a.	0.014	0.002	0.03	n.a.	BMB
22	7.81	n.a.	0.004	0.000	0.00	n.a.	bM
23	7.91	n.a.	0.008	0.001	0.01	n.a.	MB
24	8.66	n.a.	0.004	0.000	0.00	n.a.	Ru
25	8.73	n.a.	0.002	0.000	0.00	n.a.	Ru
26	8.87	n.a.	0.028	0.007	0.12	n.a.	BMB
27	9.54	n.a.	0.043	0.012	0.20	n.a.	BMB
28	9.59	n.a.	0.001	0.000	0.00	n.a.	Rd
29	10.36	n.a.	0.042	0.012	0.19	n.a.	BM
30	10.57	n.a.	0.003	0.000	0.00	n.a.	MB
31	10.77	n.a.	0.007	0.001	0.01	n.a.	BMB
32	11.21	n.a.	0.003	0.000	0.00	n.a.	BMB
33	11.37	n.a.	0.004	0.001	0.01	n.a.	BMB
34	11.64	n.a.	0.002	0.000	0.00	n.a.	BM
35	11.88	n.a.	0.027	0.005	0.08	n.a.	MB
Total:			20.718	6.238	100.00	0.000	

5 melanin 200PPM

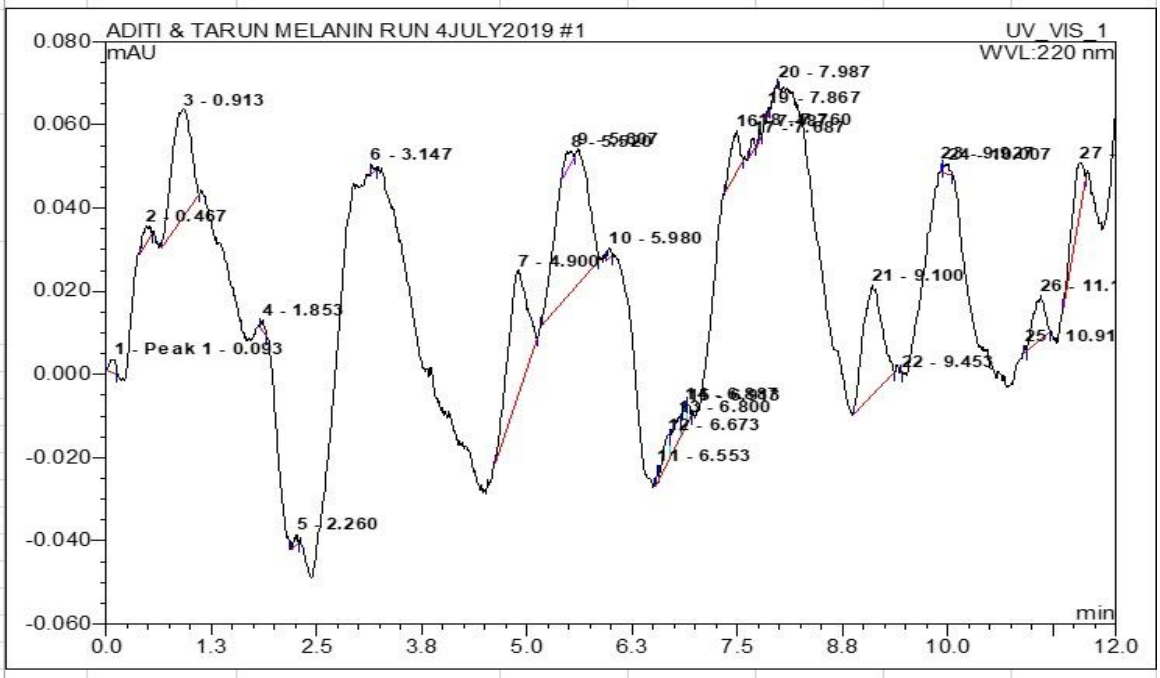
Sample Name:	**melanin 200PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA5**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 15:02**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.15	n.a.	0.002	0.000	0.00	n.a.	BMb
2	0.21	n.a.	0.011	0.001	0.01	n.a.	bMB
3	0.39	n.a.	0.001	0.000	0.00	n.a.	BMB
4	0.65	n.a.	0.031	0.006	0.05	n.a.	BMB
5	1.13	n.a.	2.826	0.202	1.76	n.a.	BM
6	1.28	n.a.	0.008	0.000	0.00	n.a.	Mb
7	1.45	n.a.	0.051	0.004	0.04	n.a.	bMB
8	1.76	n.a.	5.685	3.181	27.82	n.a.	bMB
9	1.98	n.a.	0.560	0.045	0.40	n.a.	Rd
10	3.21	n.a.	4.061	1.369	11.97	n.a.	BM
11	3.79	n.a.	4.662	5.305	46.39	n.a.	M
12	4.47	n.a.	0.912	0.106	0.93	n.a.	Rd
13	4.93	n.a.	0.132	0.018	0.16	n.a.	Rd
14	5.17	n.a.	0.031	0.003	0.03	n.a.	Rd
15	5.75	n.a.	1.170	0.918	8.02	n.a.	M
16	5.96	n.a.	0.050	0.006	0.05	n.a.	Rd
17	6.21	n.a.	0.001	0.000	0.00	n.a.	Ru
18	6.43	n.a.	0.500	0.248	2.17	n.a.	MB
19	7.05	n.a.	0.029	0.006	0.05	n.a.	bMB
20	7.30	n.a.	0.002	0.000	0.00	n.a.	BMB
21	7.34	n.a.	0.002	0.000	0.00	n.a.	BMB
22	7.61	n.a.	0.017	0.003	0.02	n.a.	BM
23	7.73	n.a.	0.005	0.000	0.00	n.a.	MB
24	7.93	n.a.	0.002	0.000	0.00	n.a.	BMB
25	8.42	n.a.	0.010	0.001	0.01	n.a.	BMB
26	8.89	n.a.	0.029	0.006	0.05	n.a.	BMB
27	9.48	n.a.	0.001	0.000	0.00	n.a.	BMB
28	9.87	n.a.	0.003	0.000	0.00	n.a.	BMB
29	10.21	n.a.	0.024	0.004	0.04	n.a.	BMB
30	10.57	n.a.	0.019	0.003	0.03	n.a.	bMB
31	11.19	n.a.	0.004	0.000	0.00	n.a.	BMB
32	11.93	n.a.	0.004	0.000	0.00	n.a.	BMB
Total:			20.844	11.436	100.00	0.000	

1 BLANK

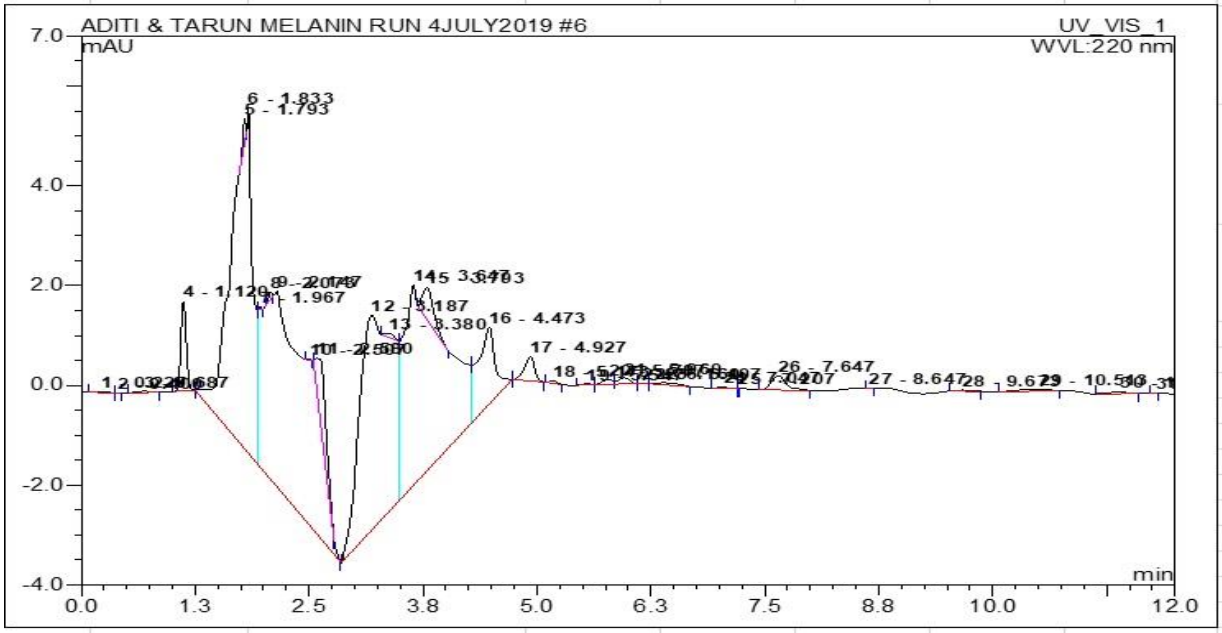
Sample Name:	**BLANK**	*Injection Volume:*	**10.0**
Vial Number:	**RA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**blank**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 14:09**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	0.09	Peak 1	0.003	0.000	0.59	n.a.	BMB
2	0.47	n.a.	0.004	0.000	0.92	n.a.	BMB
3	0.91	n.a.	0.026	0.007	15.38	n.a.	BMB
4	1.85	n.a.	0.003	0.000	0.28	n.a.	BMB
5	2.26	n.a.	0.003	0.000	0.26	n.a.	BMB
6	3.15	n.a.	0.003	0.000	0.19	n.a.	BMB
7	4.90	n.a.	0.030	0.008	17.33	n.a.	BMB
8	5.52	n.a.	0.003	0.000	0.95	n.a.	Ru
9	5.61	n.a.	0.032	0.014	31.64	n.a.	BMB
10	5.98	n.a.	0.003	0.000	0.26	n.a.	BMB
11	6.55	n.a.	0.004	0.000	0.26	n.a.	BM
12	6.67	n.a.	0.007	0.001	1.36	n.a.	M
13	6.80	n.a.	0.006	0.001	1.51	n.a.	M
14	6.89	n.a.	0.005	0.000	0.84	n.a.	M
15	6.91	n.a.	0.004	0.000	0.34	n.a.	MB
16	7.49	n.a.	0.010	0.001	2.84	n.a.	BMB
17	7.69	n.a.	0.003	0.000	0.39	n.a.	BMB
18	7.76	n.a.	0.003	0.000	0.16	n.a.	BMB
19	7.87	n.a.	0.002	0.000	0.09	n.a.	BMB
20	7.99	n.a.	0.002	0.000	0.08	n.a.	BMB
21	9.10	n.a.	0.026	0.007	15.33	n.a.	BMB
22	9.45	n.a.	0.001	0.000	0.01	n.a.	BMB
23	9.93	n.a.	0.002	0.000	0.09	n.a.	BM
24	10.01	n.a.	0.003	0.000	0.42	n.a.	MB
25	10.91	n.a.	0.002	0.000	0.07	n.a.	Ru
26	11.10	n.a.	0.011	0.002	3.83	n.a.	BMB
27	11.57	n.a.	0.012	0.002	4.60	n.a.	BMB
Total:			0.211	0.044	100.00	0.000	

6 Aditi & Tarun melanin run 4JULY2 RT3 1

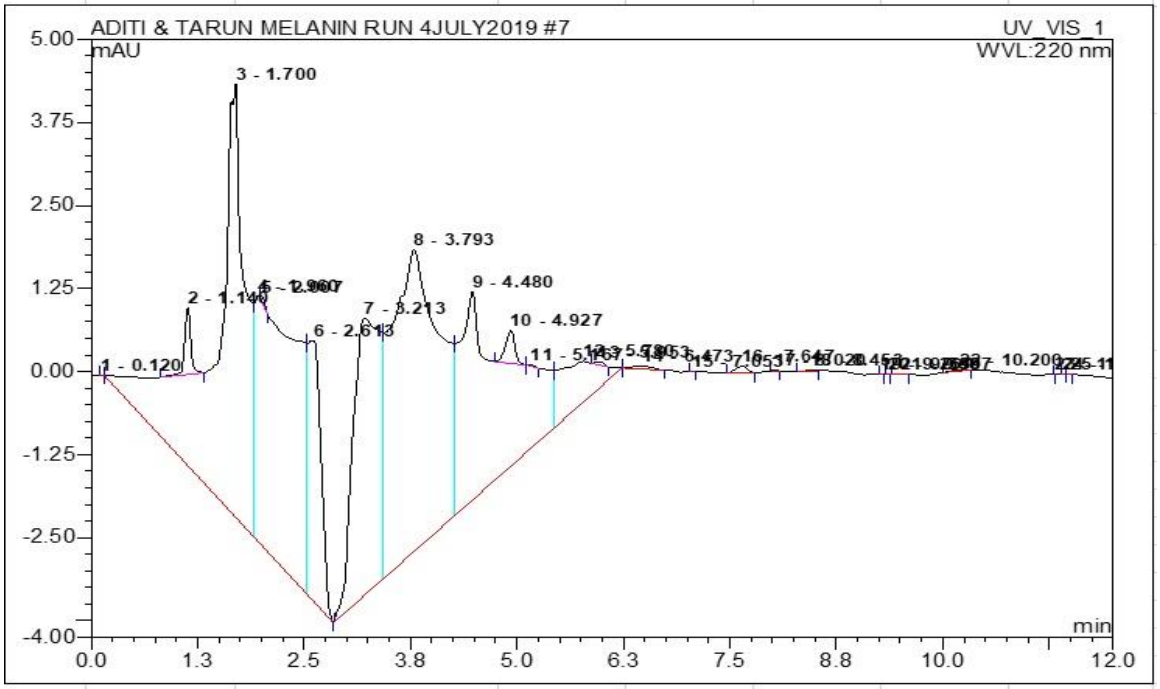
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT3 1**	*Injection Volume:*	**10.0**
Vial Number:	**RA6**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 15:15**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.23	n.a.	0.022	0.003	0.04	n.a.	BMB
2	0.40	n.a.	0.003	0.000	0.00	n.a.	bMB
3	0.69	n.a.	0.029	0.005	0.05	n.a.	BMB
4	1.12	n.a.	1.798	0.123	1.35	n.a.	BMb
5	1.79	n.a.	0.466	0.017	0.19	n.a.	Ru
6	1.83	n.a.	6.921	1.826	20.11	n.a.	bM
7	1.97	n.a.	0.048	0.001	0.02	n.a.	Ru
8	2.07	n.a.	0.159	0.006	0.07	n.a.	Ru
9	2.15	n.a.	3.921	2.482	27.33	n.a.	MB
10	2.51	n.a.	0.014	0.001	0.01	n.a.	Rd
11	2.58	n.a.	0.573	0.152	1.68	n.a.	Rd
12	3.19	n.a.	4.300	1.789	19.70	n.a.	BM
13	3.38	n.a.	0.081	0.009	0.10	n.a.	Rd
14	3.65	n.a.	4.004	2.010	22.14	n.a.	M
15	3.79	n.a.	0.646	0.092	1.01	n.a.	Rd
16	4.47	n.a.	1.547	0.366	4.03	n.a.	MB
17	4.93	n.a.	0.496	0.063	0.70	n.a.	bMB
18	5.17	n.a.	0.045	0.004	0.05	n.a.	BMB
19	5.55	n.a.	0.021	0.002	0.02	n.a.	BMB
20	5.79	n.a.	0.093	0.013	0.14	n.a.	bM
21	5.96	n.a.	0.121	0.017	0.19	n.a.	MB
22	6.16	n.a.	0.006	0.000	0.00	n.a.	bMB
23	6.41	n.a.	0.044	0.012	0.13	n.a.	BMB
24	7.05	n.a.	0.019	0.003	0.03	n.a.	BMB
25	7.21	n.a.	0.001	0.000	0.00	n.a.	BMB
26	7.65	n.a.	0.287	0.055	0.60	n.a.	BMB
27	8.65	n.a.	0.003	0.000	0.00	n.a.	BMB
28	9.67	n.a.	0.025	0.004	0.05	n.a.	BMB
29	10.51	n.a.	0.043	0.017	0.18	n.a.	BMB
30	11.41	n.a.	0.030	0.008	0.08	n.a.	BMB
31	11.79	n.a.	0.004	0.000	0.00	n.a.	BMB
Total:			25.772	9.081	100.00	0.000	

7 Aditi & Tarun melanin run 4JULY2 RT3 2

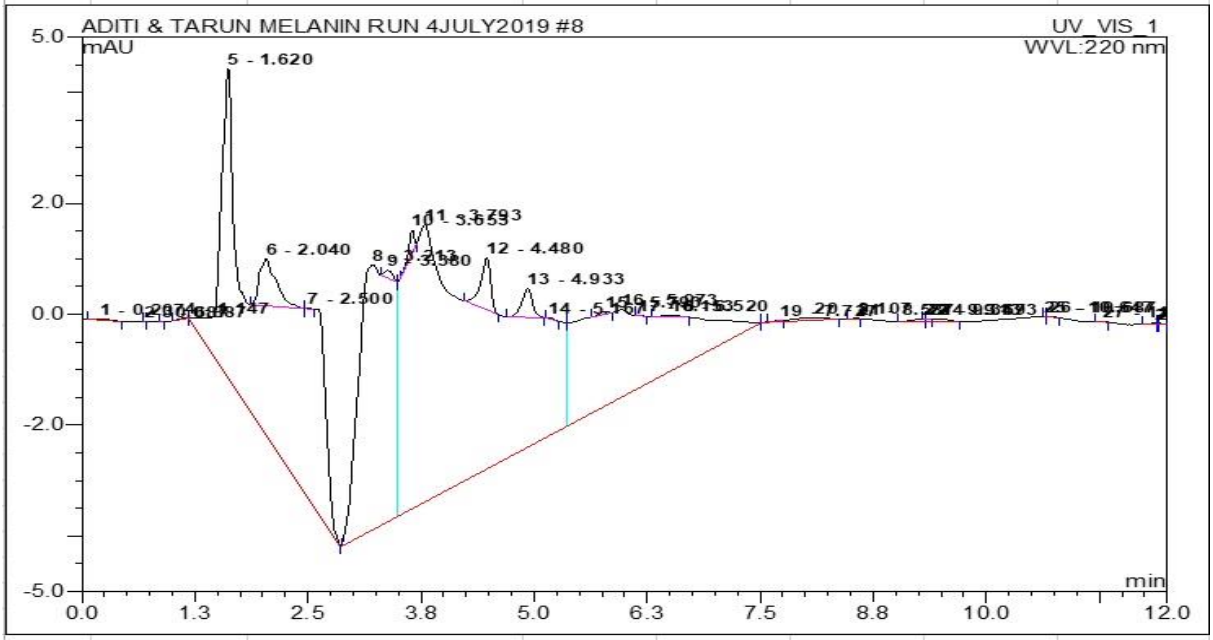
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT3 2**	*Injection Volume:*	**10.0**
Vial Number:	**RA7**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 15:28**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.12	n.a.	0.003	0.000	0.00	n.a.	BMB
2	1.14	n.a.	1.005	0.086	0.66	n.a.	Ru
3	1.70	n.a.	6.532	2.966	22.70	n.a.	bM
4	1.96	n.a.	3.707	2.264	17.32	n.a.	M
5	2.01	n.a.	0.068	0.005	0.04	n.a.	Rd
6	2.61	n.a.	3.929	0.683	5.22	n.a.	MB
7	3.21	n.a.	4.168	1.535	11.74	n.a.	BM
8	3.79	n.a.	4.543	2.999	22.94	n.a.	M
9	4.48	n.a.	3.128	2.048	15.67	n.a.	M
10	4.93	n.a.	0.495	0.063	0.48	n.a.	Rd
11	5.17	n.a.	0.024	0.002	0.01	n.a.	Rd
12	5.78	n.a.	0.614	0.380	2.91	n.a.	MB
13	5.95	n.a.	0.049	0.006	0.04	n.a.	Rd
14	6.47	n.a.	0.042	0.012	0.09	n.a.	bMB
15	7.05	n.a.	0.004	0.000	0.00	n.a.	BMB
16	7.65	n.a.	0.096	0.016	0.12	n.a.	BMB
17	8.02	n.a.	0.005	0.000	0.00	n.a.	BMB
18	8.45	n.a.	0.013	0.002	0.01	n.a.	BMB
19	9.27	n.a.	0.001	0.000	0.00	n.a.	BMB
20	9.36	n.a.	0.003	0.000	0.00	n.a.	BMB
21	9.51	n.a.	0.011	0.001	0.01	n.a.	BMB
22	10.20	n.a.	0.020	0.004	0.03	n.a.	BMB
23	11.32	n.a.	0.001	0.000	0.00	n.a.	BMB
24	11.41	n.a.	0.002	0.000	0.00	n.a.	BMB
25	11.49	n.a.	0.003	0.000	0.00	n.a.	BMB
Total:			28.467	13.070	100.00	0.000	

8 Aditi & Tarun melanin run 4JULY2 RT4 1

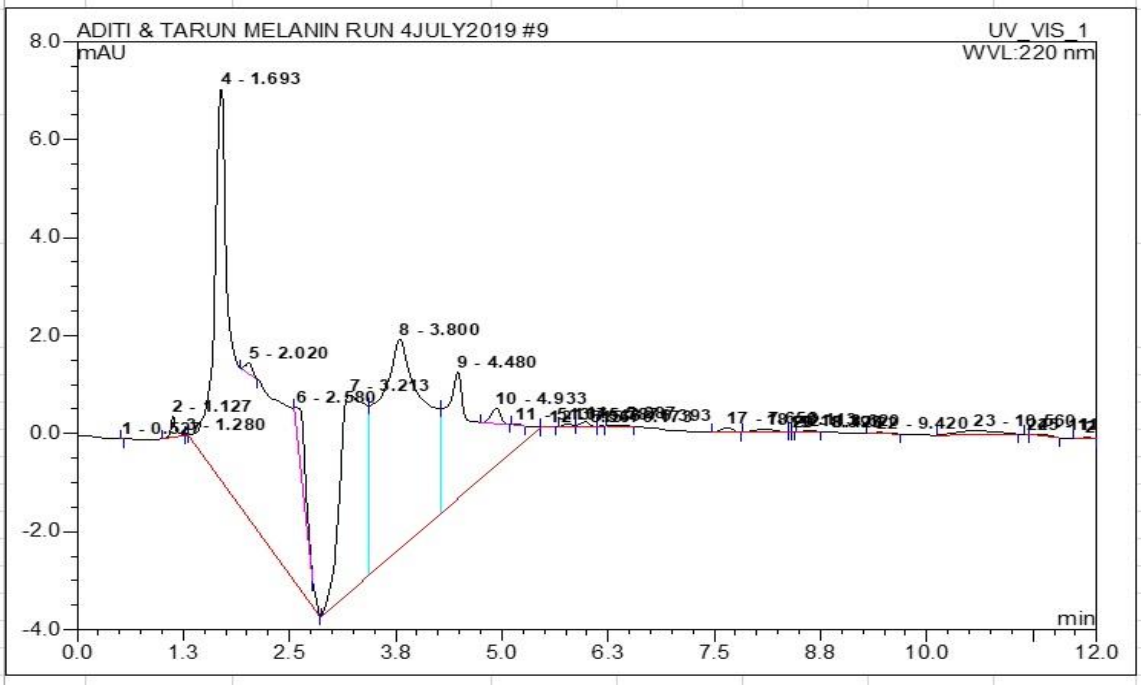
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT4 1**	*Injection Volume:*	**10.0**
Vial Number:	**RA8**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 15:41**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	0.21	n.a.	0.025	0.005	0.04	n.a.	BMB
2	0.69	n.a.	0.002	0.000	0.00	n.a.	BMB
3	0.89	n.a.	0.002	0.000	0.00	n.a.	BMB
4	1.15	n.a.	0.014	0.002	0.01	n.a.	BMb
5	1.62	n.a.	5.580	3.670	25.58	n.a.	bMB
6	2.04	n.a.	0.851	0.181	1.26	n.a.	Rd
7	2.50	n.a.	0.012	0.001	0.01	n.a.	Rd
8	3.21	n.a.	4.777	2.080	14.49	n.a.	BM
9	3.38	n.a.	0.138	0.015	0.10	n.a.	Rd
10	3.65	n.a.	0.411	0.027	0.19	n.a.	Ru
11	3.79	n.a.	4.997	5.928	41.31	n.a.	M
12	4.48	n.a.	0.938	0.120	0.84	n.a.	Rd
13	4.93	n.a.	0.522	0.070	0.49	n.a.	Rd
14	5.17	n.a.	0.026	0.002	0.02	n.a.	Rd
15	5.79	n.a.	0.043	0.005	0.04	n.a.	Ru
16	5.97	n.a.	1.571	2.183	15.21	n.a.	MB
17	6.15	n.a.	0.009	0.001	0.00	n.a.	Rd
18	6.52	n.a.	0.027	0.007	0.05	n.a.	Rd
19	7.73	n.a.	0.008	0.001	0.01	n.a.	Ru
20	8.11	n.a.	0.050	0.023	0.16	n.a.	bMB
21	8.59	n.a.	0.004	0.000	0.00	n.a.	BMB
22	9.31	n.a.	0.001	0.000	0.00	n.a.	Ru
23	9.37	n.a.	0.002	0.000	0.00	n.a.	Ru
24	9.49	n.a.	0.055	0.023	0.16	n.a.	BMB
25	10.65	n.a.	0.003	0.000	0.00	n.a.	BMb
26	10.69	n.a.	0.005	0.001	0.01	n.a.	bMB
27	11.29	n.a.	0.008	0.001	0.00	n.a.	BMB
28	11.87	n.a.	0.013	0.001	0.01	n.a.	BM
29	11.90	n.a.	0.012	0.000	0.00	n.a.	M
30	11.93	n.a.	0.011	0.000	0.00	n.a.	MB
Total:			20.118	14.348	100.00	0.000	

9 Aditi & Tarun melanin run 4JULY2 RT4 2

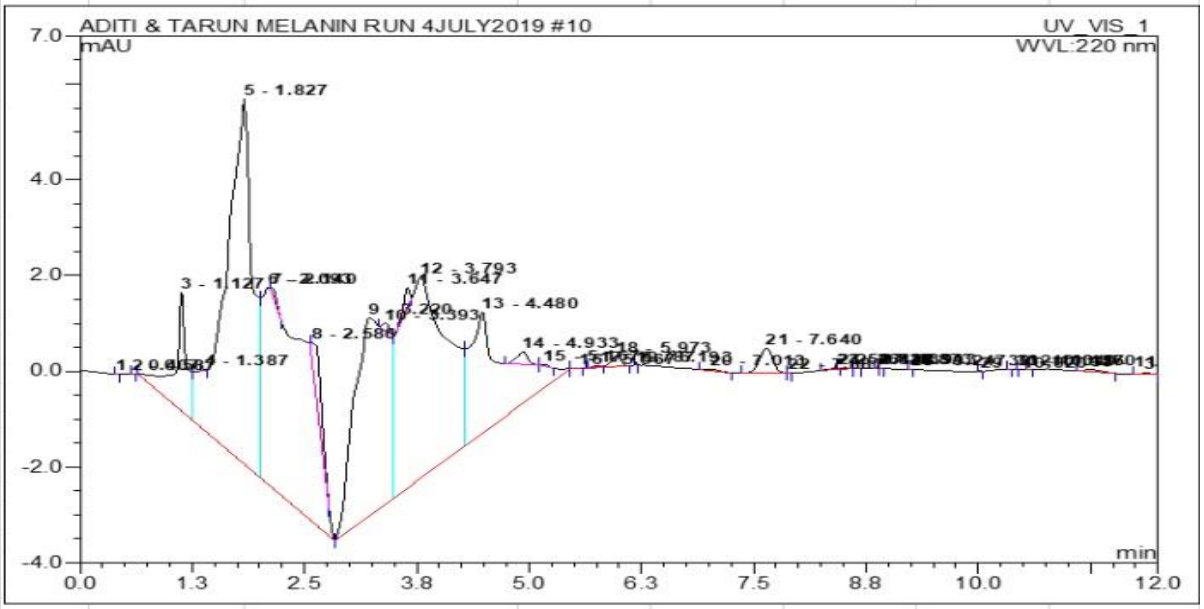
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT4 2**	*Injection Volume:*	**10.0**
Vial Number:	**RB1**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 15:54**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	0.53	n.a.	0.002	0.000	0.00	n.a.	BMB
2	1.13	n.a.	0.420	0.028	0.28	n.a.	BM
3	1.28	n.a.	0.009	0.000	0.00	n.a.	Mb
4	1.69	n.a.	7.987	4.141	41.87	n.a.	bMB
5	2.02	n.a.	0.218	0.021	0.22	n.a.	Rd
6	2.58	n.a.	0.445	0.135	1.36	n.a.	Rd
7	3.21	n.a.	4.012	1.402	14.17	n.a.	BM
8	3.80	n.a.	4.270	2.736	27.66	n.a.	M
9	4.48	n.a.	2.582	1.269	12.83	n.a.	MB
10	4.93	n.a.	0.324	0.040	0.41	n.a.	Rd
11	5.17	n.a.	0.023	0.002	0.02	n.a.	Rd
12	5.57	n.a.	0.014	0.001	0.01	n.a.	bMB
13	5.79	n.a.	0.056	0.007	0.07	n.a.	BM
14	5.99	n.a.	0.088	0.011	0.11	n.a.	MB
15	6.17	n.a.	0.002	0.000	0.00	n.a.	BMB
16	6.39	n.a.	0.020	0.004	0.04	n.a.	BMB
17	7.65	n.a.	0.097	0.015	0.15	n.a.	BMB
18	8.11	n.a.	0.052	0.015	0.15	n.a.	BM
19	8.39	n.a.	0.004	0.000	0.00	n.a.	M
20	8.42	n.a.	0.003	0.000	0.00	n.a.	MB
21	8.62	n.a.	0.017	0.003	0.03	n.a.	BMB
22	9.42	n.a.	0.021	0.005	0.05	n.a.	BMB
23	10.56	n.a.	0.089	0.045	0.46	n.a.	BMB
24	11.18	n.a.	0.002	0.000	0.00	n.a.	BMb
25	11.30	n.a.	0.025	0.007	0.07	n.a.	bMB
26	11.87	n.a.	0.025	0.004	0.04	n.a.	BMB
Total:			20.807	9.892	100.00	0.000	

10 Aditi & Tarun melanin run 4JULY2 T28 1

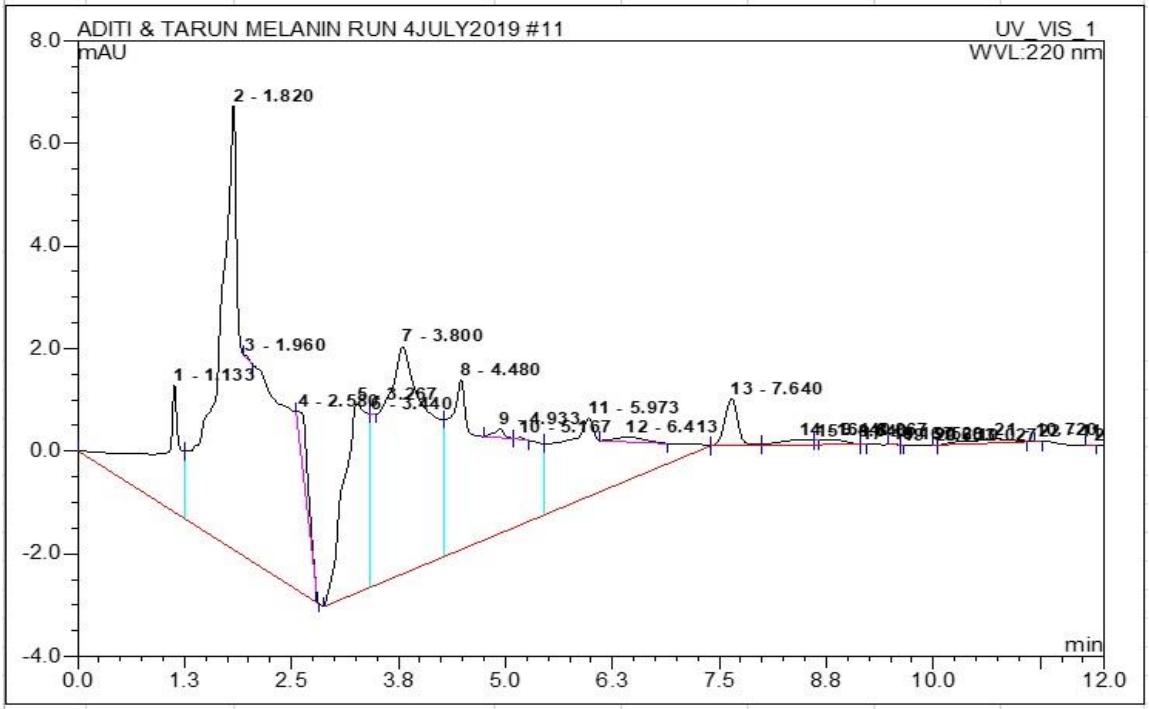
Sample Name:	**Aditi & Tarun melanin run 4JULY2 T28 1**	*Injection Volume:*	**10.0**
Vial Number:	**RB2**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 16:08**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
1	0.41	n.a.	0.002	0.000	0.00	n.a.	BMB
2	0.59	n.a.	0.003	0.000	0.00	n.a.	BMB
3	1.13	n.a.	2.474	0.377	3.28	n.a.	BM
4	1.39	n.a.	0.011	0.000	0.00	n.a.	Ru
5	1.83	n.a.	7.629	2.680	23.29	n.a.	M
6	2.09	n.a.	4.118	2.523	21.93	n.a.	MB
7	2.14	n.a.	0.125	0.011	0.10	n.a.	Rd
8	2.58	n.a.	0.331	0.124	1.08	n.a.	Rd
9	3.22	n.a.	4.135	1.839	15.98	n.a.	BM
10	3.39	n.a.	0.146	0.013	0.11	n.a.	Rd
11	3.65	n.a.	0.399	0.032	0.27	n.a.	Ru
12	3.79	n.a.	4.202	2.495	21.68	n.a.	M
13	4.48	n.a.	2.521	1.222	10.62	n.a.	MB
14	4.93	n.a.	0.266	0.036	0.31	n.a.	Rd
15	5.17	n.a.	0.031	0.003	0.03	n.a.	Rd
16	5.57	n.a.	0.008	0.001	0.01	n.a.	bMB
17	5.79	n.a.	0.022	0.002	0.02	n.a.	Ru
18	5.97	n.a.	0.208	0.030	0.26	n.a.	BMB
19	6.19	n.a.	0.001	0.000	0.00	n.a.	BMB
20	7.01	n.a.	0.027	0.005	0.05	n.a.	BMB
21	7.64	n.a.	0.521	0.091	0.79	n.a.	BMB
22	7.89	n.a.	0.002	0.000	0.00	n.a.	BMB
23	8.42	n.a.	0.029	0.006	0.05	n.a.	BMB
24	8.45	n.a.	0.001	0.000	0.00	n.a.	Rd
25	8.63	n.a.	0.004	0.000	0.00	n.a.	bMB
26	8.87	n.a.	0.003	0.000	0.00	n.a.	BMB
27	8.91	n.a.	0.003	0.000	0.00	n.a.	bMB
28	9.25	n.a.	0.003	0.000	0.00	n.a.	BMB
29	10.02	n.a.	0.002	0.000	0.00	n.a.	BMB
30	10.34	n.a.	0.003	0.000	0.00	n.a.	BMB
31	10.43	n.a.	0.002	0.000	0.00	n.a.	BMB
32	10.56	n.a.	0.003	0.000	0.00	n.a.	BMB
33	11.25	n.a.	0.038	0.010	0.08	n.a.	BMB
34	11.85	n.a.	0.027	0.004	0.04	n.a.	BMB
Total:			27.295	11.506	100.00	0.000	

11 Aditi & Tarun melanin run 4JULY2 T28 2

Sample Name:	**Aditi & Tarun melanin run 4JULY2 T28 2**	*Injection Volume:*	**10.0**
Vial Number:	**RB3**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
Recording Time:	**04-07-2019 16:21**	*Sample Weight:*	**1.0000**
Run Time (min):	**12.00**	*Sample Amount:*	**1.0000**



No.	Ret.Time	Peak Name	Height	Area	Rel.Area	Amount	Type
	min		mAU	mAU*min	%		
1	1.13	n.a.	2.472	0.843	5.81	n.a.	BM
2	1.82	n.a.	8.641	5.116	35.25	n.a.	MB
3	1.96	n.a.	0.043	0.003	0.02	n.a.	Rd
4	2.58	n.a.	0.405	0.127	0.88	n.a.	Rd
5	3.27	n.a.	3.673	1.174	8.09	n.a.	BM
6	3.44	n.a.	0.005	0.000	0.00	n.a.	Ru
7	3.80	n.a.	4.433	3.004	20.70	n.a.	M
8	4.48	n.a.	3.312	2.409	16.60	n.a.	M
9	4.93	n.a.	0.177	0.024	0.16	n.a.	Rd
10	5.17	n.a.	0.033	0.003	0.02	n.a.	Rd
11	5.97	n.a.	1.521	1.488	10.25	n.a.	MB
12	6.41	n.a.	0.091	0.038	0.26	n.a.	Rd
13	7.64	n.a.	0.912	0.157	1.08	n.a.	bM
14	8.44	n.a.	0.108	0.042	0.29	n.a.	M
15	8.64	n.a.	0.002	0.000	0.00	n.a.	Ru
16	8.87	n.a.	0.092	0.034	0.23	n.a.	M
17	9.17	n.a.	0.003	0.000	0.00	n.a.	MB
18	9.52	n.a.	0.004	0.000	0.00	n.a.	BM
19	9.65	n.a.	0.001	0.000	0.00	n.a.	MB
20	10.03	n.a.	0.003	0.000	0.00	n.a.	BM
21	10.72	n.a.	0.079	0.050	0.34	n.a.	MB
22	11.24	n.a.	0.004	0.000	0.00	n.a.	BMB
23	11.88	n.a.	0.004	0.000	0.00	n.a.	BMB
Total:			26.019	14.513	100.00	0.000	

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