ESTIMATION OF LIPASE AND MELANIN PRODUCTION WITH MULTIPLE MICROBIOTA

A DISSERTATION

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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CANDIDATE'S DECLARATION

I Tarun Garg, Roll No. 2K17/IBT/04 of M. Tech Biotechnology, hereby declare that the Major project –II titled **" Estimation of Lipase and Melanin Production with Multiple Microbiota "** which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award of the degree of Master of Technology, is original and not copied from any source without proper citation. This work has not previously formed the basis for the award of any Degree, Diploma Associate ship, Fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the Major Project-II titled **"Estimation of Lipase and Melanin Production with Multiple Microbiota"** which is submitted by Tarun Garg, Roll No. 2K17/IBT/04, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfilment of the requirement for the award the Degree of Master of Technology, is a record of the project work carried out by the students under my supervision. To the best of my knowledge this work has not been submitted in part or full for any Degree or Diploma to University or elsewhere.

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ABSTRACT

Lipases are enzymes that catalyse the hydrolysis of triacylglycerols at the oil-water interface to release glycerol and free fatty acids. It holds considerable potential and thus it is prevalent in many industrial sectors. Massive research is employed in order to optimize its productivity and selecting specific variants for specific industrial application. This research project explores various dimensions of enzyme lipase eventually attempts to employ it for chiral resolution of dl-menthol into l-menthol. The primary aim of the project revolved around the cost factor involved for purchasing imported lipase to meet the needs of menthol industry. Some cultures were acquired from MTCC and some are isolated from DTU lake (unknown). The culture with the maximum lipase production efficiency was KT2 (unknown strain isolated from DTU lake water). The results proved that the enzyme is ubiquitously present in a number of strains.

Now to the next part,

Melanin is also an important pigment used in Industrial applications and its production is required for meeting demands of the industry. For this sole purpose different strains were analyzed for melanin production and screened for viability and reproducible Melanin production. This project aims at analyzing novel strains for melanin production and its quantification. For this we have melanin standard commercially available to the one synthesized in the lab, and is analyzed using techniques such as UV Vis spectroscopy, HPLC and TLC separations. The results obtained visually showed the production but upon analyzing with the different available techniques we came to know about various facts relating to melanin stability which are discussed in due course of this report.

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LIST OF ABBREVIATIONS

FFA	Free Fatty Acid
TAGs	Triacylglycerols
pNPP	Para-Nitrophenol palmitate
IUB	International Union of Biochemistry
DTU	Delhi Technological University
Eep	Enantiomeric excess of product
EC	Enzyme Commission
MTCC	Microbial Type Culture Collection
O.D.	Optical Density
SmF	Submerged Fermentation
SSF	Solid State Fermentation
ATPS	Aqueous two-phase system
conc.	Concentration
Min.	Minute
µL	Micro litre
ml	Milli litre
gm	Gram
M	Molar
mM	Milli molar
%	Percentage
^{o}C	Degree centigrade

CHAPTER - 1

INTRODUCTION

Enzymes are the biological molecules (usually proteins) which acts as a catalyst. They accelerate chemical reactions by providing an alternative reaction pathway of lower activation energy, but it does not get consumed in the process (Bugg, 2004). They can only modify the rate of reaction, but not the position of the equilibrium. The purpose of the active site is to create a favourable environment that makes the chemical reaction catalysed by the enzyme more likely to happen (Bartlett et al., 2002a). Change in pH and temperature of working environment could modify intermolecular and intramolecular bonds which holds protein in their secondary and tertiary structures. Thus, the catalytic activity of an enzyme is pH and temperature dependent (Peterson *et al.*, 2007).

Substrate(s) → Product(s)

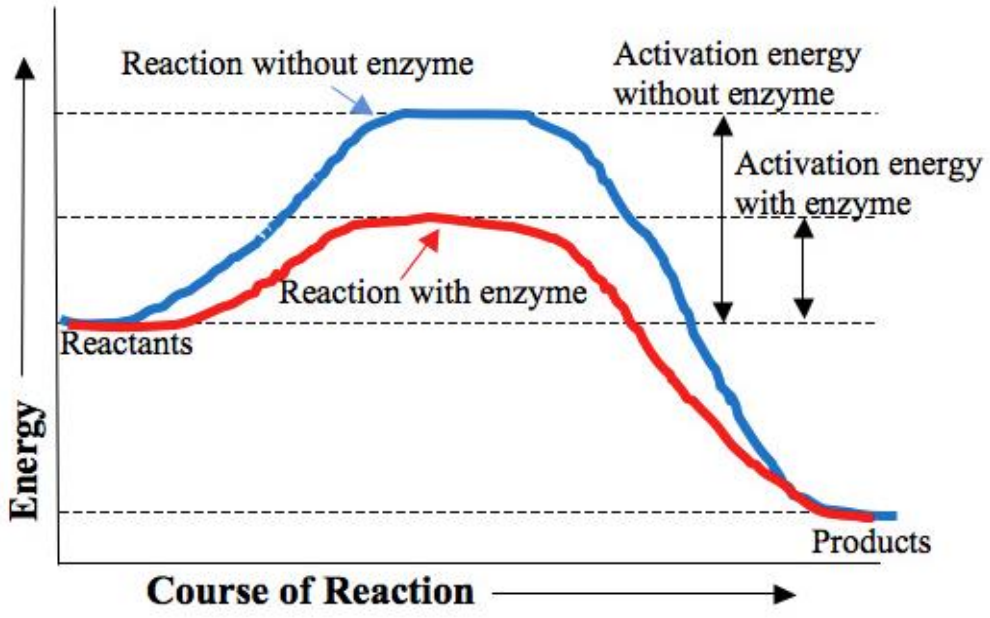


Fig. 1.1 : Effects of enzymes on chemical reaction

Catalytic process of an enzyme takes place at specific position known as active sites. Most of the active site is located in one of the largest clefts in enzyme's surface and often it is deeply enfolded. Some of the enzymes require only their amino acid residue instead of chemical

groups for activity. other enzymes require some supplementary chemical component which is called cofactor. Cofactors can be either organic group which permanently bound to the enzyme **(prosthetic groups),** or organic molecules which combine with the enzyme-substrate complex temporarily **(coenzymes)** usually made up from vitamins, and cofactor can also be a cation **(activator)** which binds temporarily to the active site (Union of Pure & Applied Chemistry, 2005–2009).

1.1 Nomenclature of Enzymes

Nomenclature of enzymes atre related to their functions, in other words on the basis of the type of reaction they catalyse. For numerical classification of enzyme, Enzyme Commission number is used. This means that the enzymes that catalyse the same reaction will have the same EC number. All known enzymes fall into six categories. Enzyme format is E.C. a.b.c.d; where each number represents progressively finer classification of the enzyme. IUB classification is given below:

Major Class (Type of reaction catalyzed)	Common exmaples	Kind of reaction	Specific Example
1. ***Oxidoreductases*** *(Transfer of electrons)*	Oxidases Reductases Dehydrogenase	$A^{+3} + B^{+2} \rightarrow A^{+2} + B^{+3}$	Alcohol + NAD ↓ *Alcohol dehydrogenase* Aldehyde + $NADH_2$
2. ***Transferases*** *(Transfer of functional groups)*	Transaminase Transketolase Transaldolase	$A - X + B \rightarrow A + B - X$	Glucose + ATP ↓ *Glukokinase or hexokinase* Glucose-6-Phosphate + ADP
3. ***Hydrolases*** *(Hydrolysis Reactions)*	Amylases Lipases Proteases Nucleases	$A–B+H_2O \rightarrow A–OH + B–H$	Sucrose ↓ *Sucrase* Glucose + Fructose
4. **Lyases or *Desmolases*** *(Group elimination to form double bonds without hydrolysis)*	Aldolase Decarboxylase Fumarase Citrate synthase	$A - B \rightarrow A = B + X - Y$ X Y	Histidine ↓ *Hlstidine decarboxylase* Histidine + CO_2
5. ***Isomerases*** *(Transfer of Groups within a molecule*	Isomerase Mutase Epimerase	$A - B \rightarrow A - B$ Y X X Y	Glucose – 6-Phosphate ↓ *Isomerase* Fructose-6-Phosphate
6. ***Ligases or Synthetases*** *(Bond formation couples with ATP hydrolysis)*	Synthetases Carboxylases	$A + B + ATP \rightarrow$ $A - B + ADP + Pi$	Pyruvate + CO_2 + ATP ↓ *Pyruvate carboxylase* Oxaloacetate + ADP + Pi

Table 1.1: Classification of enzymes

1.2 Chiral resolution of dl-menthol

Menthol is an important cyclic monoterpene alcohol which has eight optical isomers as a result of three chiral centres. Among these, levo-Menthol is an important flavouring chemical. This l-menthol can be obtained from enantioselective enzymatic hydrolysis or esterification reaction of dl-menthyl esters. Apart from being used as a flavouring agent, it is extensively used in oral products, pharmaceuticals, tobacco products, confectionaries and shaving products.

As a result, of such massive demand for l-menthol, its extraction from natural products like mint is no more feasible. Hence, considerable efforts have been made for the production of l-menthol by synthetic or semi-synthetic method.

Lipase is known to drive such enantioselective reactions of hydrolysis and esterification. The esterification reaction is represented below:

dl-Menthol + Acyl donor (acid) → l-menthyl ester (Extracted and converted to l-menthol)

Basically, three parameters are analysed during the reaction:

1) Extent of conversion (c)

2) Enantiomeric excess of product (Eep) = [d] – [l] / [d] + [l]

3) Enantiomeric ratio (E) = ln [1- c (1+ Eep)] / ln [1- c (1- Eep)]

The activity of lipase is influenced by acyl donors. Some of the commonly used acyl donors are:

- Acetic acid
- Propionic acid
- Valeric acid
- Caproic acid

Immobilized enzymes provide greater operational stability and thus attempts are made to improve the same. This can be done on MCNC (Magnetic Cellulose Nanocrystals), DEAE-Sephadex A-25, chitin etc.

1.3 Motivation for the project

According to data collected from a company namely “Virat Export” office situated in Rohini, the cost for purchasing lipase from AMANo Enzyme, Japan is approx. Rs. 7000 per kg. The company uses this lipase for chiral resolution of dl-menthol into l-menthol (similar to menthol extracted from natural sources). Enzymes from other companies in India and abroad were not able to give specific result as enzyme from AMANo. Hence,

the owners of the company were looking for a cost-effective alternative that will serve the purpose efficiently. This project was thus an attempt to fulfil the need of the company in a lucrative manner. As per the provided figures, the company operates 5 batches of 2 tonnes of substrate in a month. Also, each batch has a requirement of 4% of enzyme (immobilized). This means that the average cost spent on the enzyme per month can be calculated as following:

Amount of enzyme required monthly: 4% of (2*5*1000) kg = 400 kg Thus, Cost = 7000*400 = Rs 2,800,000 = Rs 28 Lakhs

Hence, the company has to shed large amount of money annually in this venture. Reducing the cost even by a fraction will result in huge profit to the company. Thus, the project endeavours to contribute to the mission as much as possible.

Now on the other focus point i.e. **MELANIN**,

The use of pigments as colorants has been practiced since prehistoric times in different parts of the world. Archaeologists have uncovered evidence that early humans used paint for aesthetic purposes. It was further proven when pigments and grinding equipment, which were about 350,000-400,000 years old, were found in a cave at Twin Rivers, near Lusaka, Zambia. In Europe, it was practiced during the Bronze Age. In China, dyeing with plants, barks, and insects has been traced back more than 5,000 years. In India, it occurred during the Indus Valley period (2500 BC). Henna was used before 2500 BC, while saffron has been mentioned in the Bible (*Rao et al., 2017*).

In last few decades, an upward trending is observed towards replacement of synthetic colorants with natural pigments because of the strong consumer demand towards biologically-derived products. Dyes such as tartrazine, sunset yellow, etc. provoke allergies either on their own or in combination with other 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 colour have increased the global demand for natural 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).

1.4 Why Microbial Pigments?

Main sources of natural pigments include either plants or microorganisms; plant pigments derived from agro-industrial wastes such as tomato (lycopene), grape (anthocyanins), and palm (carotenoids) processing residues. However, source variability and presence in low concentration of pigment 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, may be selected or modified, in search of suitable colour additives have enormous advantages over plant pigments:

- Easy and rapid growth in low cost medium,
- Easy processing,
- High concentration & quality
- Selection of colour-producing microorganisms is straightforward: the observation of coloured colonies in agar plates.

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

However, microbial pigment has some limitations of high cost, lower stability and pigment color variation 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 product marketability, but they also display antioxidants, antimalarial, antimicrobial, antineoplastic as well as anticancer properties (Rajasekaran *et al.*, 2008; Venil *et al.*, 2013).

Utility of bacteria for pigment production has several advantages over fungi, such as short life cycle and ease for genetic modification. However, compared with fungal pigments, most of bacterial pigments are still at the research and development stage (*Rao et al., 2017*) (refer **Table 1.2**);

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 1.2: Fungal and bacterial pigments studied or applied for commercial production.

Hence, work on bacterial pigments production should be intensified to make them available in the market. Pigment producing bacteria are ubiquitous and present in various ecological niches, such as soil, rhizospheric soil, desert sand, fresh water, and marine oregions, and even as endophytes. Various genera such as *Streptomyces, Nocardia, Micrmonospora, Thermomonspra, Actinoplanes, Microbispora, Streptosporangium, Actinomadura, Rhodococcus, and Kitasatospora* produce a wide variety of pigments (Usman *et al.*, 2017). The genus *Streptomyces* was reported for highest pigment production. Similar to fungi, bacteria also produce a wide range of pigments such as carotenoids, melanin, violacein, prodigiosin, pyocyanin, actinorhodin, and zeaxanthin (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 tissue-

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

CHAPTER - 2

LITERATURE REVIEW

2.1 LIPASES (E.C. 3. 1. 1. 3)

2.1.1 Definition and Mechanism of Action

Lipases are enzymes that catalyse the hydrolysis of triacylglycerols (TAGs) at the oil-water interface to release glycerol and free fatty acids (FFA). Lipases bear the international name of triacylglycerol acyl hydrolases (E.C. 3.1.1.3). The presence of water in lipase-catalysed reactions facilitates the breaking of covalent bonds in the substrate and subsequent assimilation of these molecules in product formation (Whitaker, 1996).

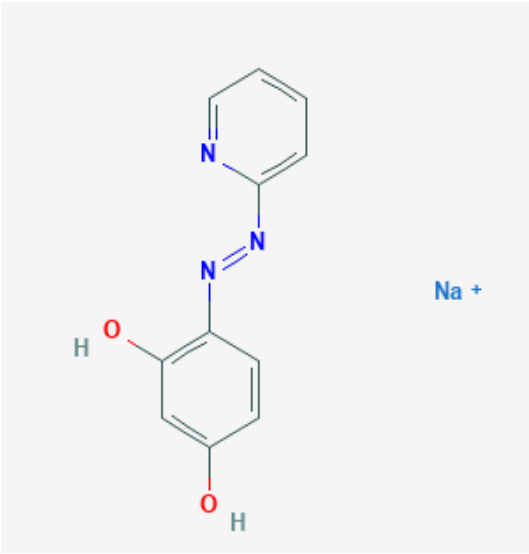


Fig 2.1: 2-D structure of Lipase

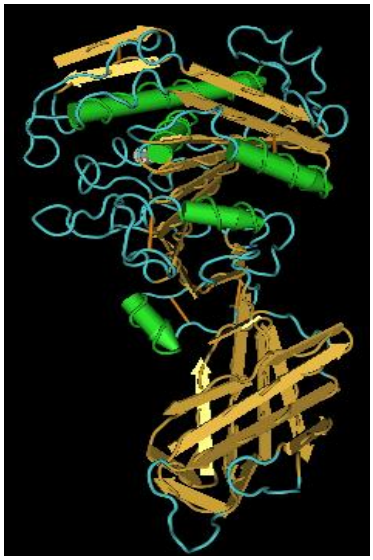


Fig 2.2: Computer-generated image of lipase

Besides, most lipases are able to catalyse the synthesis and translocation of ester linkages, mainly under low water content or non-aqueous conditions. In addition to their biological importance, lipases have important role in different biotechnological and industrial processes due to their diverse catalytic properties and substrate specificity. Their activities are utilized in the food, pharmaceutical, leather, and detergent industries, as well as in the production of fine chemicals and biodiesel. Most of the current commercial enzymes are derived from microbial sources produced by bacteria or filamentous fungi. The main advantage of enzyme production by microbes is that relatively large amounts of enzyme can be produced economically. In addition, lipases derived from diverse microorganisms

have different biochemical characteristics, namely substrate specificity, temperature and pH optimum and stability, etc. Among zygomycetes, many strains are known as good extracellular enzyme producers, however, a limited information available about their lipase production, and the biochemical characteristics of the produced hydrolytic and synthetic activities as well. Identification and characterization of fresh microbial lipase with encouraging hydrolytic or synthetic properties have importance for industrial and biotechnological development process.

Property Name	Property Value
Molecular Weight	238.202 g/mol
Hydrogen Bond Donor Count	2
Hydrogen Bond Acceptor Count	5
Rotatable Bond Count	2
Exact Mass	238.059 g/mol
Monoisotopic Mass	238.059 g/mol
Topological Polar Surface Area	78.1 A^2
Heavy Atom Count	17
Formal Charge	1
Complexity	247
Isotope Atom Count	0
Defined Atom Stereocenter Count	0
Undefined Atom Stereocenter Count	0
Defined Bond Stereocenter Count	0
Undefined Bond Stereocenter Count	0
Covalently-Bonded Unit Count	2
Compound Is Canonicalized	Yes

Table 2.1 Chemical and Physical properties of lipase (Pubchem)

Although lipases are designed by nature for the hydrolytic c1eavage of the ester bonds of TAGs (Quinlan and Moore, 1993), lipases can catalyse the reverse reaction; ester synthesis in a low-water environment (Bjorkling et al., 1991; Bomscheuer, 1995). Hydrolysis and esterification can occur simultaneously by continual removal of water molecules via distillation or evaporation under reduced pressure (Yan et al., 2002). It is also known that concurrent hydrolysis and esterification is not always practically possible, thus a lipase

that is efficient in hydrolysis may not necessarily have esterification capabilities (Wu et al., 1996).

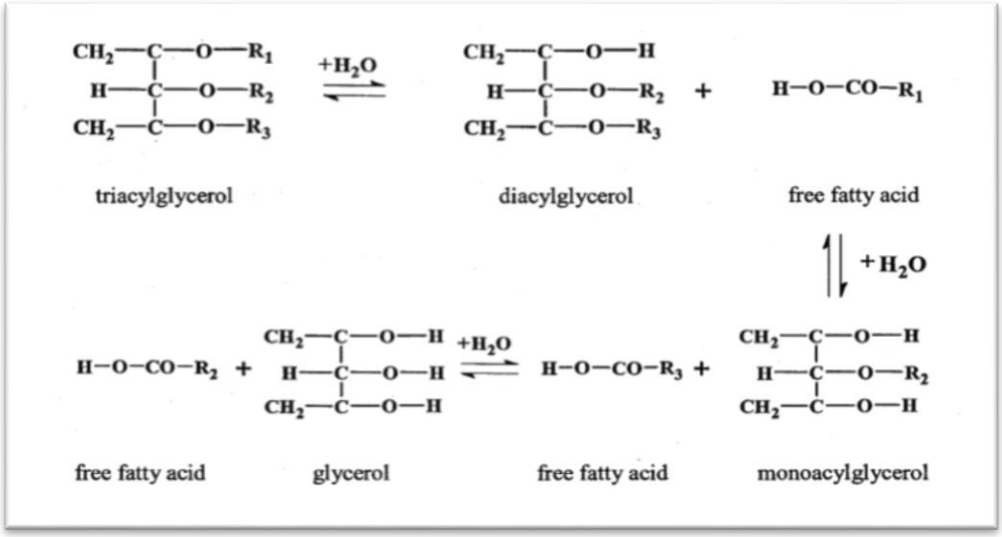


Fig 2.3: Lipase-catalysed hydrolysis and esterification reactions

Lipase-catalysed ester synthesis has been exploited. Depending on the substrates, lipases can catalyse the resolution of racemic mixture in non-aqueous media with water immiscible organic solvents, and transesterification reactions (Trincone et al., 1993; Wuet al., 1996; Gunstone, 1999; Johri et al., 2001; osório et al., 2001) including;

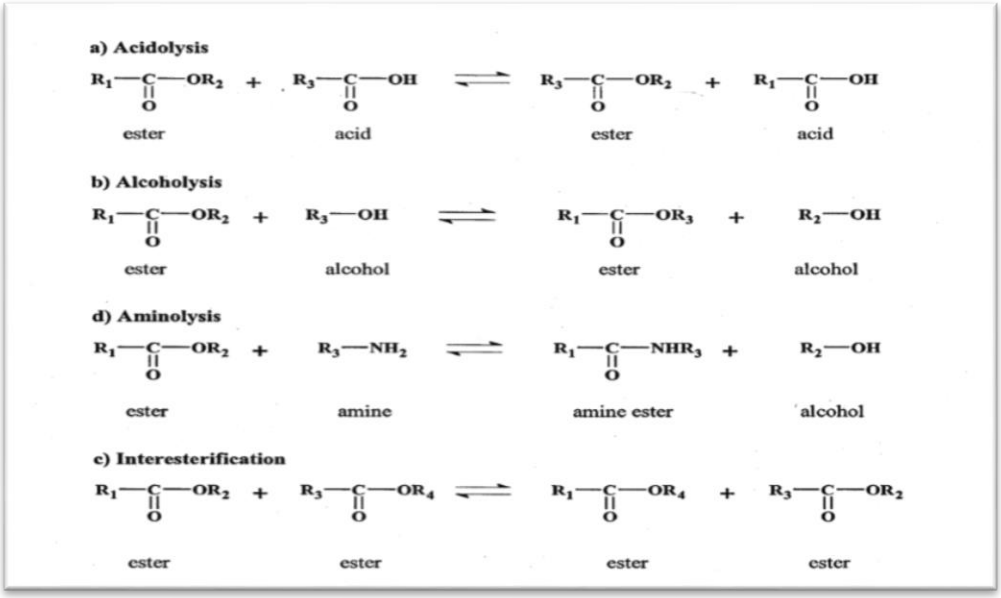


Fig 2.4: Lipase-catalysed transesterification reactions

acidolysis, alcoholysis, amino lysis, and interesterification (where two acyl moieties are exchanged between two acylglycerols at different positions on the TAG backbone) to produce acid, alcohol, amine or ester respectively instead of water (in esterification) as by-products, and esters of the different acyl group. Lipase-promoted acyl migration has been used to modify the physical and nutritional properties of TAGs by either randomly or specifically distributing FA residues on the glycerol backbone (Xu et al.,2000).

2.1.2 Occurrence of Lipases

Lipases are ubiquitous enzymes (Brady et al., 1990; Beisson et al., 2000), and have been found in animals, plants, bacteria, yeast and fungi (Mukherjee and Hill, 1990; Jaeger et al., 1994). However, most industrial lipases are derived from fungi, and bacteria. Lipases from Candida, Penicillium and Streptomyces species have been extensively studied and these lipases differ from each other in their molecular and catalytic properties (Iwa et al., 1975; Rua et al., 1993; Abramic et al., 1999). Microbial lipases have attracted considerable attention and are preferred for commercial applications due to its relative abundance, easy extraction procedures, good stability and many stereo-specific properties (Macrae and Hammond, 1985; Margolin and Klibanov, 1987). Conversely, the source, physical and chemical conditions or properties of the enzyme has been said to determine the extraction efficiency, purification, stability and ultimately cost of the enzyme. Plants and animal sources were formally considered ideal for bulk enzyme production intended for the food industry, since they were assumed to be devoid of problems associated with toxicity or contamination, tedious extraction procedures and cost. However, the growing demand for enzymes have overwhelmed plant or animal. sources in addition to international politics, the wide seasonal variation in yields (for plant), and the mercy of the weather (which is unpredictable). The search for new enzyme sources is thus a key element to the growth and sustainability of the enzyme technology.

2.1.3 The Lipase Gene Family

The lipase gene family (LoF) is a member of the super family of enzymes of which esterase and thioesterases are members (Hide et al., 1992; ollis et al., 1992). This classification; the super family, is based on tertiary structural similarities rather than amino acid sequence, while the classification of members of the LoF is based on gene organization and amino

acid sequence homology. The LoF originally consist of pancreatic lipase (PL), hepatic lipase (HL) and lipoprotein lipase (LPL) (Rader and Jaye, 2000). More recently new members have been added to the LoF, including; pancreatic lipase related protein 1 and 2 (PLRP1 and PLRP2), sub members of PL (oiller et al., 1992), phosphatidylserine phospholipase Al (PS-PLAI) (van Groningen et al., 1997), endothelial lipase (EL) (Hirata et al., 2000; Rader and Jaye, 2000) and lipase H (UP H) (Jin et al., 2002). Members of the LoF encode proteins that hydrolyse circulating dietary TAos and phospholipids and aid in the assimilation and distribution of the products of hydrolysis among central and peripheral tissues. Earlier studies failed to detect these new members of the LoF and it seems there is still a long way to go in terms of elucidating their physiological role in diseases (Jin et al., 2002).

2.2 LIPASE PRODUCTION

Today, lipases stand amongst the most vital biocatalysts completing novel reactions in aqueous as well as nonaqueous media. It is one of the universal enzymes that have broad applications in various industries such as dairy, cosmetics, food, textiles, agricultural and medical industry. This is because of their capability to utilize a broad spectrum of substrates, high stability towards extremes of pH scale, temperature and organic solvents, and chemo- region and enantioselectivity. Because of the great potential of lipase, various sources have been discovered for its production. Moreover, the production of the enzyme is modified in order to produce maximum amount of product with minimal input. Finally, the product is extracted, purified and immobilized (if required) to increase its stability. Except this, enzyme can be engineered to get specific results to respective industry. Enzyme from a particular organism can be useful in a particular industry than the same enzyme from some other organism.

2.2.1 Source of lipase production

Lipase is produced from various organisms including bacteria, fungi, yeast, plants, etc. Most commonly used organism for lipase production is bacteria because they are more selective, stable and have a broad range of substrate. Also, they are easy to operate as a result of the arrival of a wide variety of gene manipulation technologies. Most of the

enzymes are produced in extracellular media. However, some productions are intercellular. Some microbial sources are listed below:

Bacteria

- *Bacillus subtilis*
- *Bacillus pumilus*
- *Pseudomonas aeruginosa*
- *Burkholderia cepacia*
- *Staphylococcus sp.*

Fungi

- *Rhizopus sp.*
- *Penicillum sp.*
- *Aspergillus sp.*
- *Mucor sp.*
- *Geotrichus sp.*
- *Rhizomucor sp*

Yeast

- *Candida cylindraceae*
- *Candida rugosa*
- *Candia deformans*
- *Yarrowia lipolytica*
- *Pichia xylosa*

2.2.2 Types of Fermentation

Microbial lipase is generally produced by submerged fermentation (Ito et al., 2001) but we can also use solid state culture method for lipase production (Chisti, 1999a). Enzymes can

be immobilized in some cases because of several advantages of immobilization (Hemachander et al., 2001).

1) Submerged Fermentation (SmF)

Submerged fermentations are conventional method used for the production of microbially derived enzymes. Submerged fermentation involves submersion of the microorganism in an aqueous medium having all the essential nutrients, vitamins, carbon, nitrogen sources, inducers etc. required for the growth of microorganisms and maximum production of enzymes.

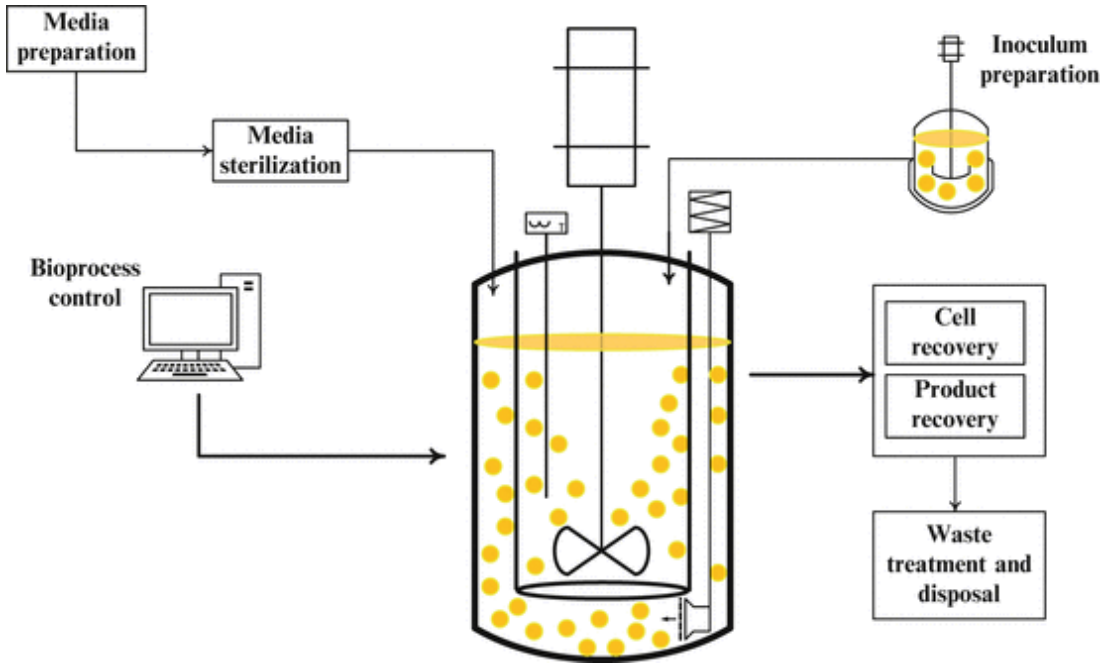


Fig 2.5: Submerged Fermentation

Submerged fermentation takes place in the big fermenter with up to 1,000 cubic meters. The fermentation media sterilizes nutrients which support renewable raw materials (Hermansyah et al., 2017). Most of the industrial enzymes are produced by microorganisms into the fermentation medium for the breakdown of carbon and nitrogen sources. Continuous fermentation and batch-fed fermentation are common. In the fed-batch fermentation, sterilized nutrients are added to the fermenter during the production of the biomass. In the continuous process, sterilized liquid nutrients are added into the fermenter with a similar flow rate as the fermentation broth leaving from the system. Physical parameters like temperature, pH, carbon dioxide formation and oxygen

consumption are calculated and controlled to optimize the fermentation method (ukessay, 2018).

Further, harvesting the enzymes from fermentation medium remove insoluble products, i.e. microbial cells. This removal is generally done by centrifugation. Most of the industrial enzymes are extracellular in nature. Enzymes remain in the fermented broth after the biomass has been extracted. The enzymes in the broth are then concentrated by methods like evaporation, crystallization, etc depending on their application. If pure enzymes are required, they are normally isolated by gel chromatography or by ion exchange chromatography.

2) Solid State Fermentation (SSF)

Solid State Fermentation is used for the production of bioproducts from microbes under low moisture condition for growth. The medium used for SSF is generally a solid substrate, and these substrates requires no processing. In order to optimize water and activity requirements, which are most important for the growth, it is necessary to take into account the water absorption properties of solid substrates during the fermentation. The power requirements for solid state fermentation are lower than submerged fermentation. Insufficient mixing, constrain of nutrient diffusion, metabolic heat accumulation renders SSF usually applicable for low value products with less observation and control (Pandey, 2003).

It offers following advantages over SmF:

• Higher fermentation productivity

• Higher end-product concentration

• Higher product stability

• Lower catabolic repression

SSF provides economical method when compared to SmF. The unitary lipase cost was 47% lower than the selling price in SSF, while 68% higher than that in SmF. SSF is basically a biomolecule manufacturing method used in pharmaceutical, food, textiles, cosmetic and fuel industries.

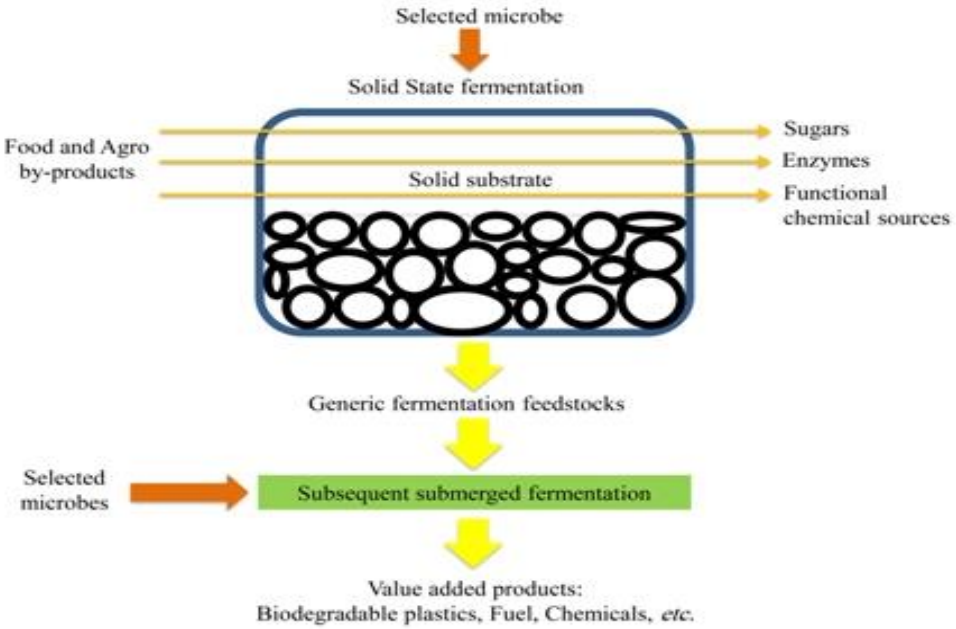


Fig 2.6: Solid State Fermentation

2.3 PURIFICATION OF LIPASE

After the production and optimization of enzyme, the product is purified to a certain extent. Lipases have been purified from the animal, plant, fungal and bacterial sources by different methods involving ammonium sulphate precipitation, gel filtration, and ion exchange chromatography. Recently, affinity chromatography techniques come into use because this technique decreases the number of necessary steps for lipase purification as well as increases the enzyme's specificity. Presently, reversed micelle and two-phase systems, membrane processed and immune-purification is being employed for lipase purification.

Industries look for purification strategies which are inexpensive, fast, high yielding and amenable for large-scale operations. The main constraints in traditional purification strategies include low yields and long time periods. Alternative new technologies such as membrane processes, aqueous two-phase systems, and immune-purification are gradually coming to the forefront for the purification of lipases. Aqueous two-phase system (ATPS) is an alternative bio separation method used in the purification of lipase from microbial sources over traditional purification techniques.

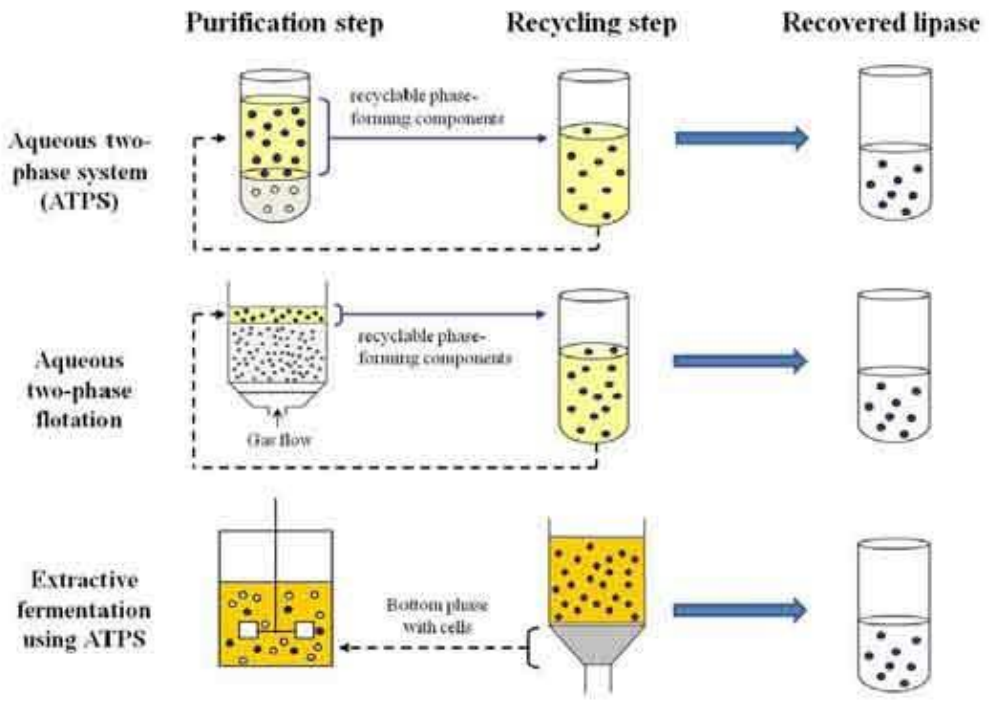


Fig 2.7: Aqueous two-phase system for purification

2.4 IMMOBILIZATION OF LIPASE

The operational stability of enzymes has been improved steadily over the years through the use of genetic engineering, immobilization, or process alterations. Enzyme immobilization is an important strategy to impart the desirable features of conventional heterogeneous catalysts on to biological catalysts. Besides enhancing stability, enzymes can acquire additional advantageous properties via immobilization:

(a) immobilized enzymes can be used repeatedly or continuously in a variety of reactors,

(b) can be easily separated from soluble reaction products and unreacted substrate, thus simplifying work-up and preventing protein contamination in final products.

Immobilization often stabilizes an enzyme, thereby, allowing their applications under harsh environmental conditions, such as pH, temperature, and organic solvents. The basic concept for enzyme immobilization is either to covalently attach or entrap the protein in

support materials; this prevents the enzyme from leaving while allowing substrates, products, and co-factors to permeate to the enzyme.

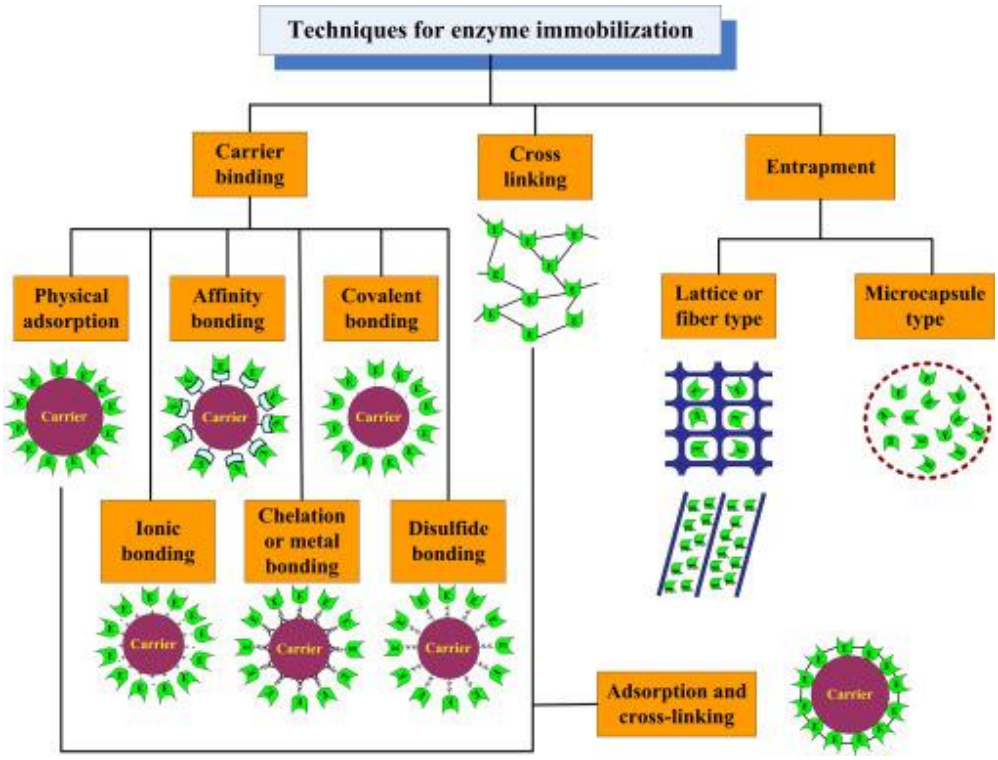


Fig 2.8: Types of Immobilization of enzymes

Entrapment, one of the immobilization techniques, can be defined as physical restriction of enzyme within a confined space or network. Gelation of polyanionic or polycationic polymers by the addition of multi- valent counter-ions is a simple and common method of enzyme entrapment. Alginates are one of the most frequently used polymers due to their mild gelling properties and non- toxicity. Alginate is an anionic linear copolymer composed of 1.4'-linked β-D-mannuronic acid and α-L-guluronic acid in different proportions and sequential arrangements. Enzymes are entrapped by drop-wise addition of an aqueous solution of sodium alginate and a biocatalyst to a hardening solution of a Ca2+ salt. The cation acts a cross linking agent towards the alginate biopolymer and the droplets precipitate as beads with biocatalysts entrapped within the network.

2.5 APPLICATIONS OF LIPASE

Microbial lipases biotechnologically valuable enzymes, primarily owing to the flexibility of their applied properties and simple production. Microbial lipases are wide heterogeneous in their protein properties and substrate specificity, that creates them

terribly engaging for industrial applications. In the industrial segment, lipases and cellulases are anticipated to post the best gains. It is expected that within the next few years lipases can get advantage from their adaptability and continuing penetration into the detergent and cosmetics markets (Hasan et al., 2006). Major application of lipase is summarized in table.

Industry	Action	Product or application
Detergents	Hydrolysis of fats	Removal of oil stains from fabrics
Dairy foods	Hydrolysis of milk fat, cheese ripening, modification of butter fat	Development of flavoring agents in milk, cheese, and butter
Bakery foods	Flavor improvement	Shelf-life prolongation
Beverages	Improved aroma	Beverages
Food dressings	Quality improvement	Mayonnaise, dressings, and whippings
Health foods	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish products; fat removal
Fats and oils	Transesterification; hydrolysis	Cocoa butter, margarine, fatty acids, glycerol, mono-, and diglycerides
Chemicals	Enantioselectivity, synthesis	Chiral building blocks, chemicals
Pharmaceuticals	Transesterification, hydrolysis	Specialty lipids, digestive aids
Cosmetics	Synthesis	Emulsifiers, moisturizers
Leather	Hydrolysis	Leather products
Paper	Hydrolysis	Paper with improved quality
Cleaning	Hydrolysis	Removal of fats

Table 2.2: Industrial applications of microbial lipases (Vulfson, 1994)

Lipases in organic synthesis

Use of lipases in organic chemical synthesis is turning more and more vital. Lipases catalysed various chemo-, regio-, and stereoselective transformations (Rubin and Dennis, 1997b; Kazlauskas and Bornscheuer, 1998; Berglund and Hutt, 2000). Majority of lipases used as catalysts in chemistry is of microbic origin. These enzymes work at the hydrophilic-lipophilic interface and tolerate organic solvents in the reaction mixtures. Use of lipases within the synthesis of enantiopure compounds has been mentioned by Berglund and Hutt (2000). The enzymes turn the chemical reaction of water-immiscible triglycerides at the water–liquid interface. Under given conditions, the number of waters within the reaction mixture can confirm the direction of the lipase-catalyzed reaction. When there's very little or no water, only esterification and transesterification are favored (Klibanov, 1997). Hydrolysis is that the favored reaction once there's excess water (Klibanov, 1997).

Lipase-catalyzed reactions in critical solvents are represented (Rantakyla et al., 1996; Turner et al., 2001; King et al., 2001).

Lipases in the detergent industry

Due to their ability to hydrolyses fats, it finds major use as additives in laundry industry and household detergents. Lipases are selected for detergent to meet the following requirements: (1) a low substrate specificity, i.e., an ability to hydrolyse fats of various compositions;

(2) its capability to resist relatively harsh washing conditions i.e. pH 10–11 and temp 30–60˚C;

(3) its ability to resist damaging enzymes and surfactants, which are essential ingredients of many detergent formulations (sharma et al, 2001).

Lipases in food industry

Lipases allow us to change the properties of lipids by modifying the location of fatty acid chains in the glyceride and replacing (Kazlauskas and Bornscheuer, 1998). Lipases are also used to take away fat from meat and fish merchandise (Kazlauskas and Bornscheuer, 1998).

Lipases in pulp and paper industry

Lipases are used to eliminate the pitch of the pulp produced for paper manufacturing. Nippon Paper Industries, Japan, have developed a pitch management technique which uses the Candida rugosa fungal lipase to hydrolyse approx. 90% of the wood triglycerides.

Bioremediation

Bioremediation is the process employed to decontaminate samples from oil spills, oil-wet soils, industrial wastes, and wastewater tinged with lipids that can otherwise have hazardous consequences when they enter natural resources without prior treatment. Lipase-catalysed bioremediation steps can be adopted to treat the wastes effectively from lipid processing factories and restaurants.

2.6 MELANIN STUDY

Now coming to Melanin study,

Melanins are macromolecules, formed by oxidative polymerization of phenolic or indolic compounds (El-Naggar, N.E.A. and El-Ewasy, S.M., 2017) It is a polymer of various groups able to donate or to 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 the ferrous (II) state. (Figure.2.1.)

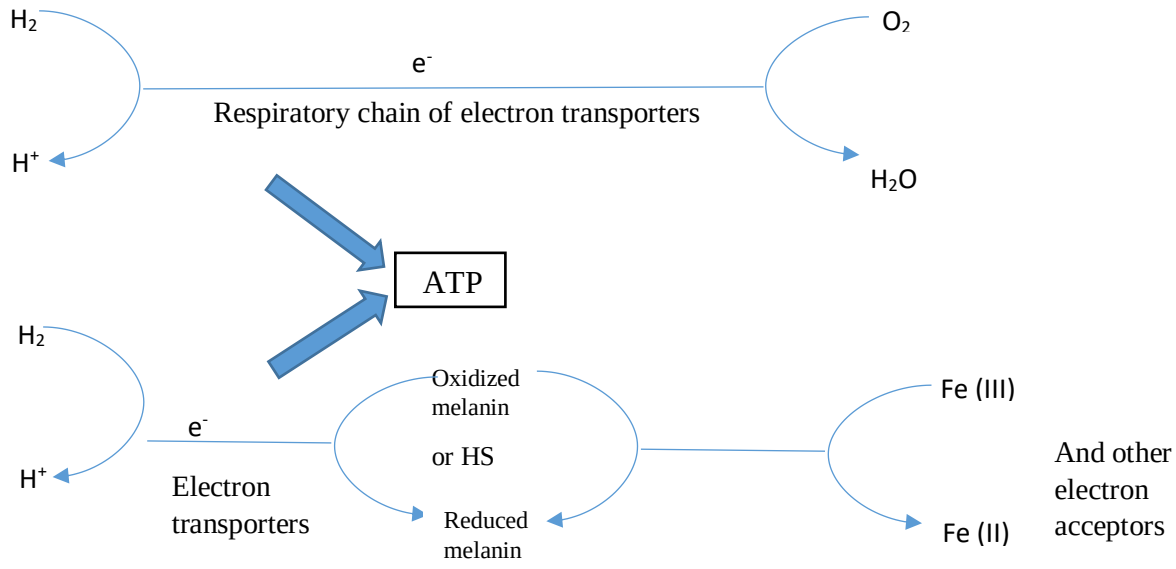


Fig 2.9: Function of melanin as an electron acceptor in the respiratory chain of marine bacteria, in comparison to the aerobic variant of the process. (Plonka and Grabacka, 2006)

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

- **Eumelanin**, are black to brown colour pigments produced by melanisation by classic Mason-Rapper pathway (Figure.2.10.), which produce tyrosine intermediates or metabolites by the action of tyrosinases (Figure.2.11.).

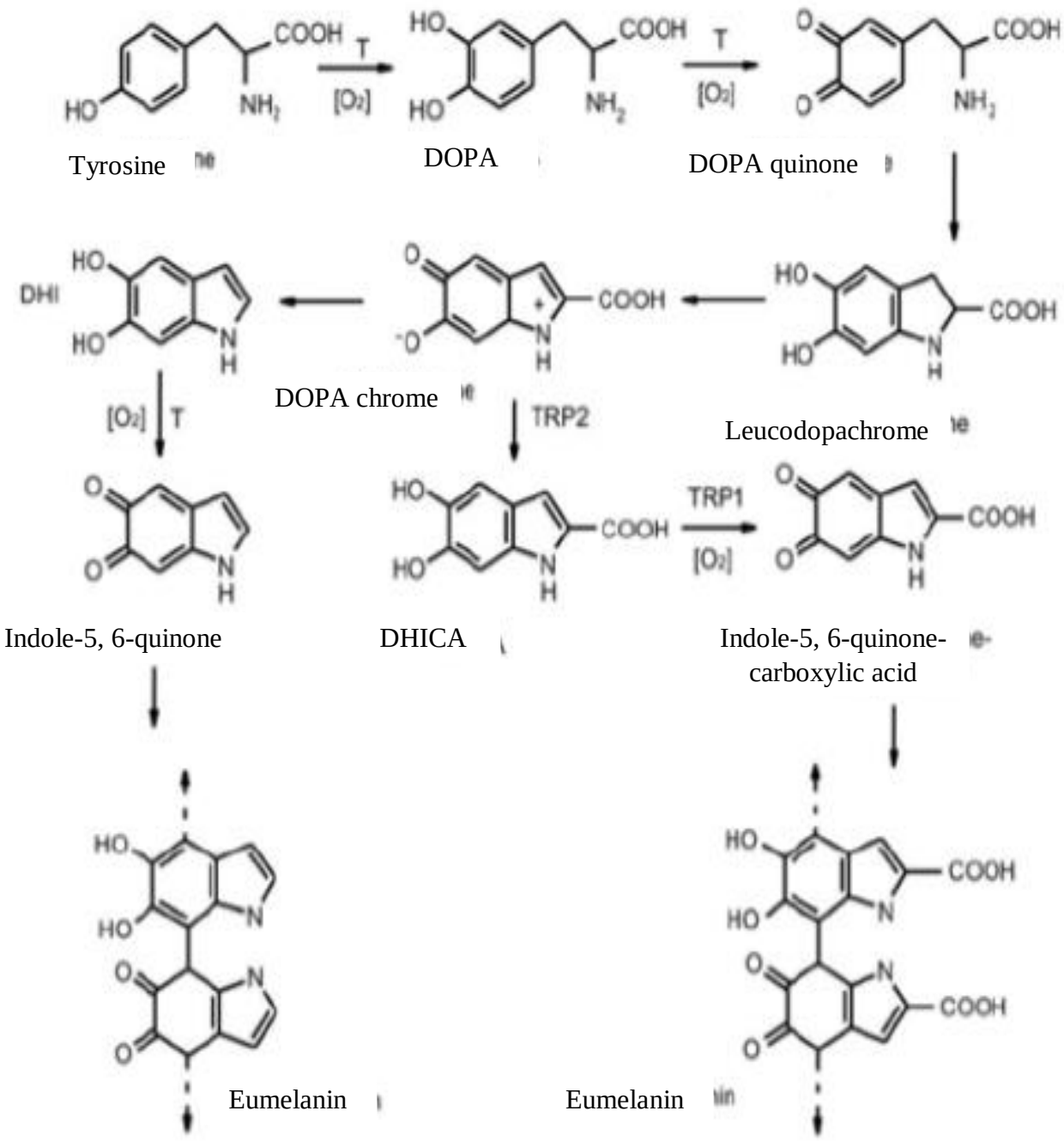


Fig 2.10. Mason Rapper pathway (Adapted from Plonka and Grabaka, 2006)

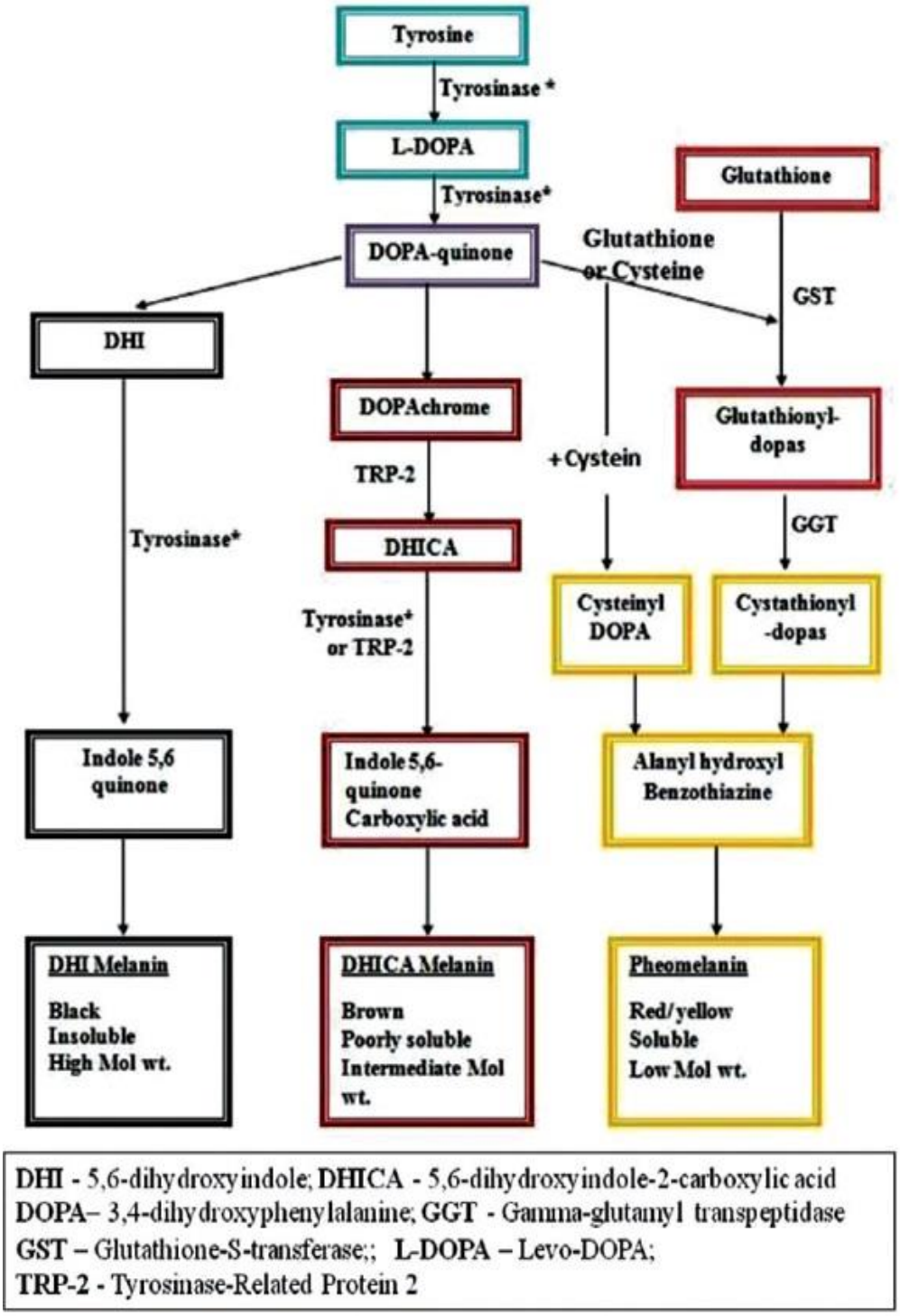


Fig 2.11. Eumelanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

- **Pheomelanin**, are brown, red or yellow colour pigments which are produced in course of oxidation of tyrosine and/or phenylalanine to dihydroxyphenylalanine (DoPA) and dopaquinone (Tarangini, . Pheomelanin results from cysteinylation of DoPA and these are sulphur containing compounds. (Figure.2.4.)

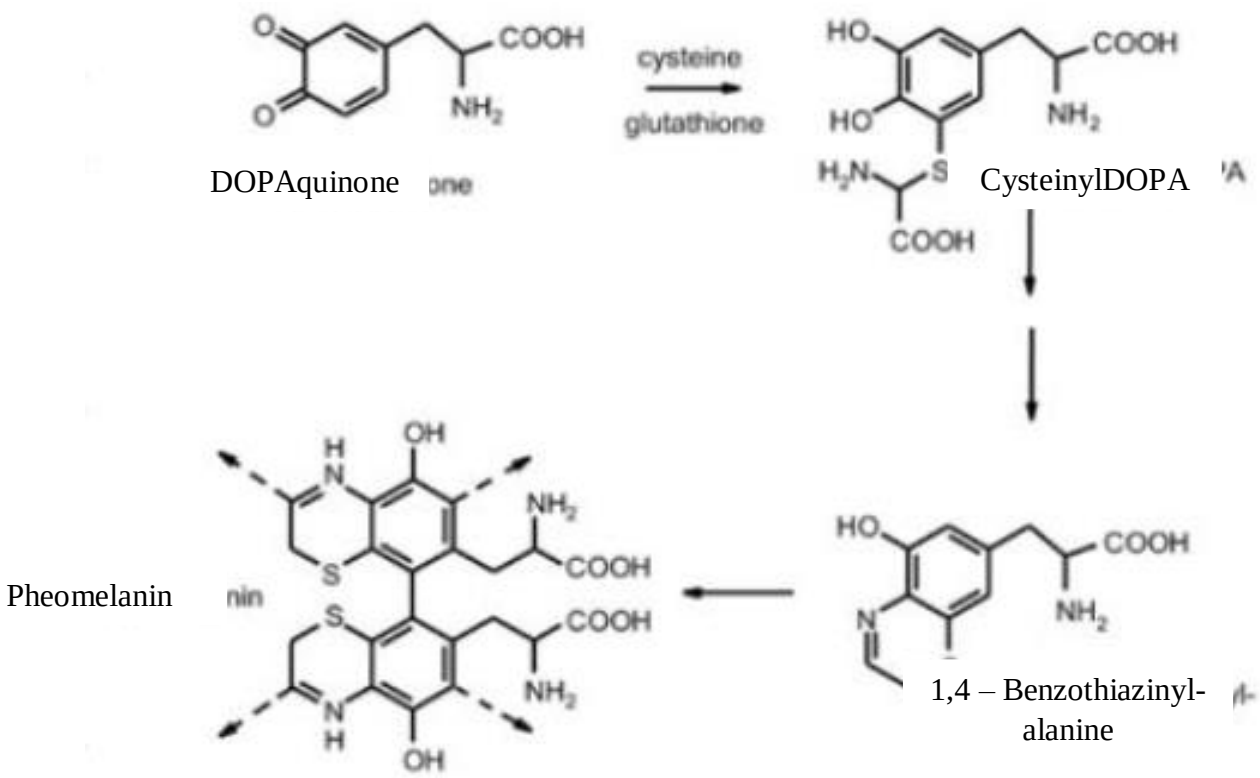


Fig 2.12. Pheomelanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

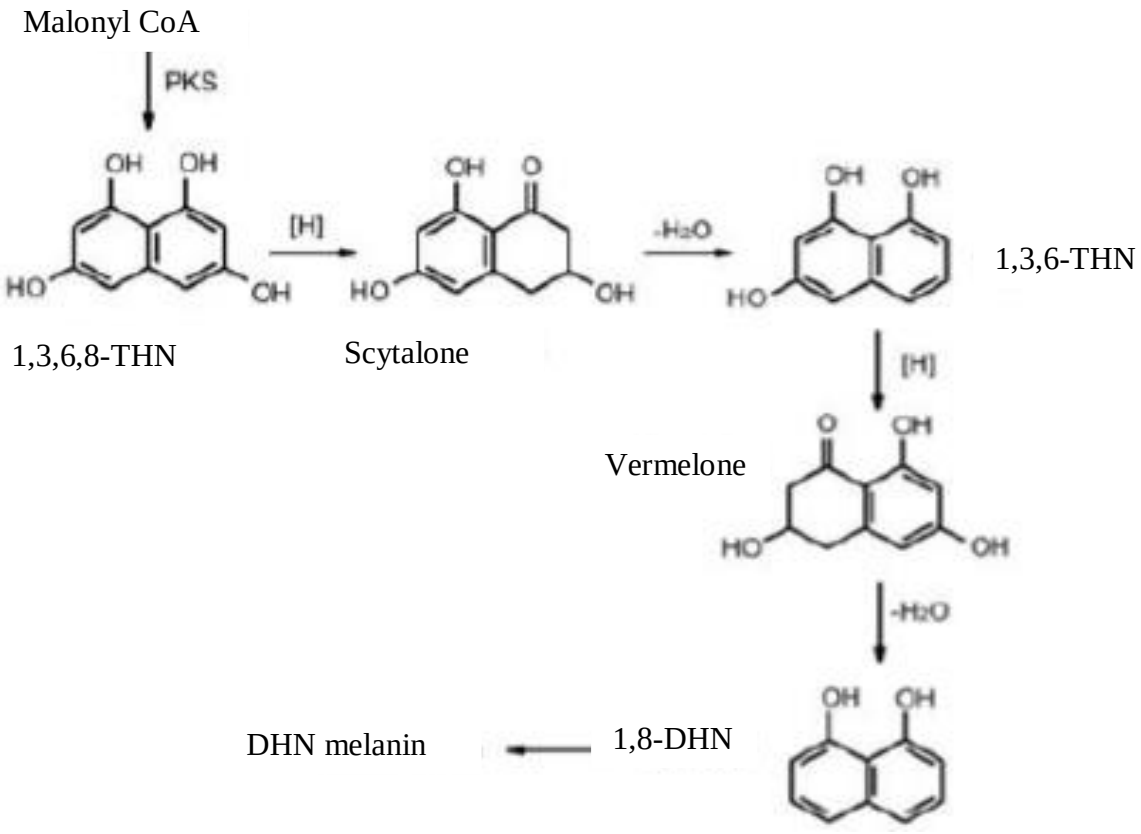


Fig 2.13. DHN-melanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

- **All**o**melanin**, include nitrogen free heterogeneous group of polymers formed from catechol precursors. These melanins are least studies and most heterogeneous group of melanins which are formed 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.

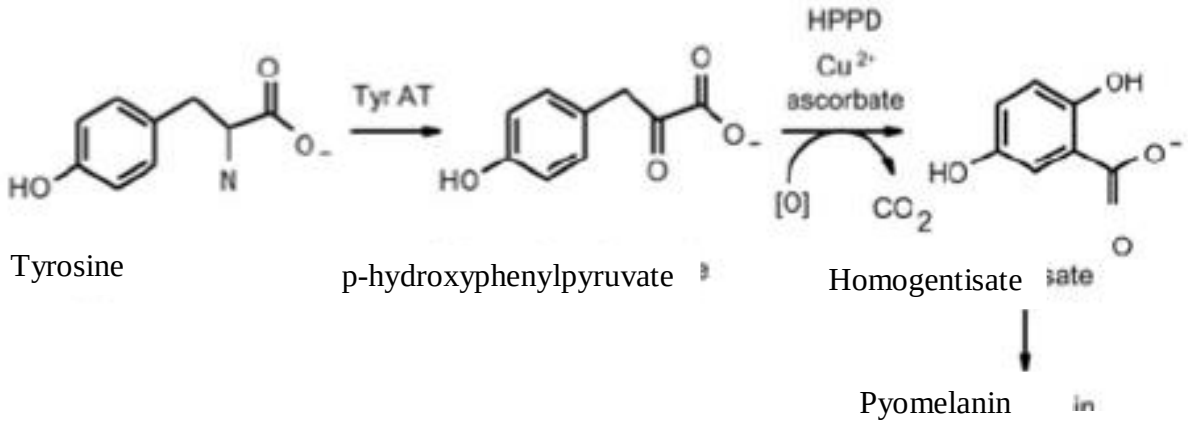


Fig 2.14. Pyomelanogenesis pathway (Adapted from Plonka and Grabaka, 2006)

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 been reported by a wide variety of microorganisms, which include: fungus such as *Cryptococcus neoformans, Sporothrix schenckii, Sepia officinalis, Aspergillus niger, Penicillium marneffei, Paracoccidioides brasiliensis, Histoplasma capsulatum, C. neoforman*. Also in bacteria, for example, bacterial melanin pigments were found in different bacterial genera, such as *Rhizobium sp*. (Cubo *et al.,* 1988), *Bacillus thuringiensis* (Aghajanyan *et al.*, 2005), *Pseudomonas aeruginosa* (Rodríguez *et al.*,2009), *Klebsiella sp*. (Sajjan *et al.*, 2010) and *Modestobacter versicolor* (Reddy *et al.*,2007), etc., but at insufficient yields for large-scale applications.

Hence, isolating a high melanin producer and identifying a suitable promotional medium is essential for enhancing biological melanin production. However, produced with conventional technology, 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).

2.7 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 vitr*o. Melanin is also used to generate monoclonal antibodies for the treatment of human metastatic melanoma (Montefiori and Zhou, 1991).

2.8 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.

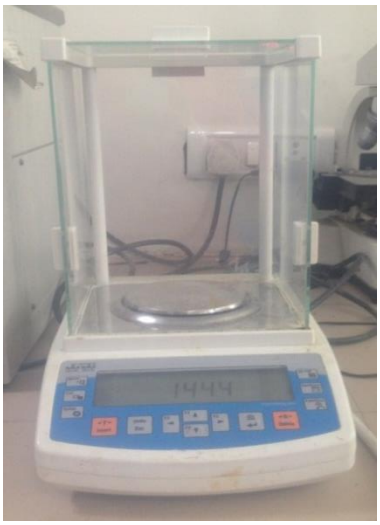


Fig 2.15. Radwag Weighing Balance

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.

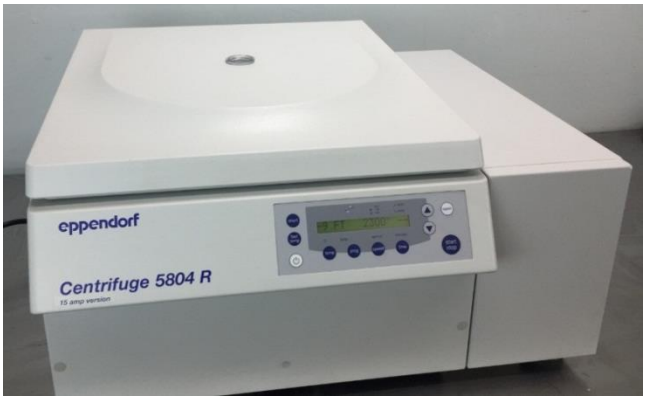


Fig 2.16. Eppendorf Centrifuge 5804 R

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.

ULTRASONICATOR

Sonication works on the principle of using sound waves to agitate particles in a solution. Ultrasonication typically uses ultrasound waves with frequencies of 20kHz or higher. These frequencies are above what you can hear, but ear protection is still recommended during sonication because the process creates a loud screeching noise. The greater the frequency, stronger the agitation of particles.

UV-VIS SPECTROPHOTOMETER

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.

FOURIER TRANSFORM INFRARED (FTIR) 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.

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 also known as high performance liquid chromatography. It 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.

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 of 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

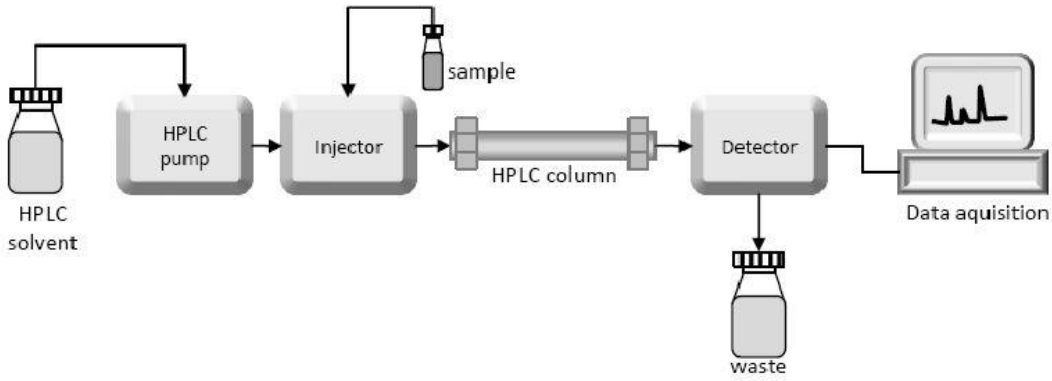


Fig 2.17. HPLC block diagram

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.

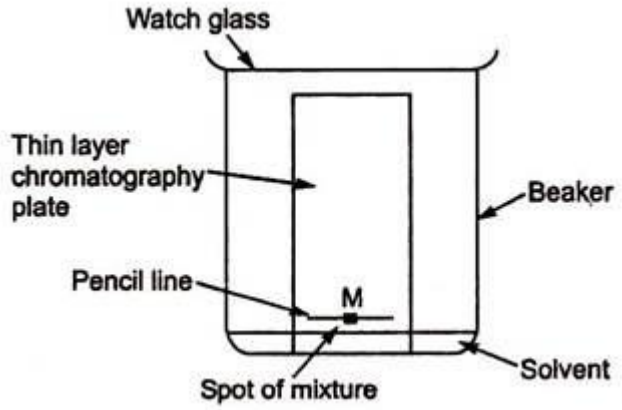


Fig 2.18 Thin Layer chromatography

In order to standardize the best solvent for melanin, TLC was performed using different solvent system.

CHAPTER - 3

METHODOLOGY

3a.1 PRODUCTION OF LIPASE

3a.1.1 Collection of Samples

For the production of lipase water samples from DTU lake was collected in plastic bottles and six cultures was acquired from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.

S. No	MTCC ID	Genus	Species	Incubation Time
1	10661	Aeromonas	sp.	24 hours
2	1908	Candida	cylindracea	48 hours
3	2488	Pseudomonas	aeruginosa	24 hours
4	7098	Pseudomonas	fluorescens	16-18 hours
5	740	Staphylococcus	aureus subsp. Aureus	12 hours
6	109	Klebsiella	pneumoniae subsp. pneumoniae	48 hours

Table 3.1: Details of cultures acquired from MTCC

3a.1.2 Isolation of Producer Strains

Various 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 four days. Selective colonies then were separated out for sub culturing and characterization.

3a.1.3 Revival of Freeze-Dried Cultures

Culture acquired from MTCC was in freeze-dried form, these cultures was revived according to the revival protocol provided by MTCC with its incubation time. Procedure of revival are as follows:

1. Ampoule should be opened with care as the contents are in a vacuum.
2. Mark on the ampoule near the middle of the cotton wool with a sharp file.
3. Disinfect the ampoule surface with alcohol around the mark.
4. Wrap cotton around the ampoule and break at the marked area with ampoule cutter.
5. Gently take away the pointed part of the ampoule. Snap opening will draw the cotton plug to one end; hasty opening will release fine particles of dried organisms into the air of the laboratory.
6. Remove the cotton plug with care and add approx. 0.3 to 0.4 ml of medium specific to each culture to make a suspension of the culture. For fungal cultures, suspension should be made in about 0.4 ml sterile water and let it stand for 20 minutes before transferring on to solid medium.
7. Streak some drops of the suspension to specified liquid medium in a Petri dish.
8. Incubate at recommended temperature and under appropriate conditions for the culture.
9. If instructions are properly followed, growth of the culture should be obtained within a few days. Some of fungi require more time for incubation even up to 15 days.
10. All the remains in the original ampoule should be sterilized before discarding.

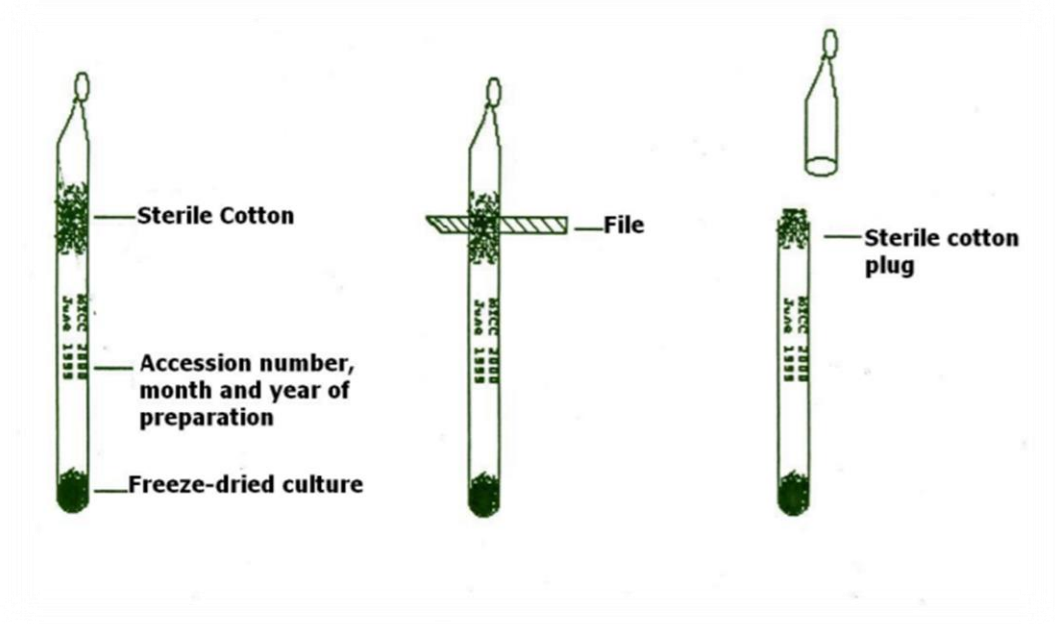


Fig 3.1: Instructions for opening the freeze-dried ampoule

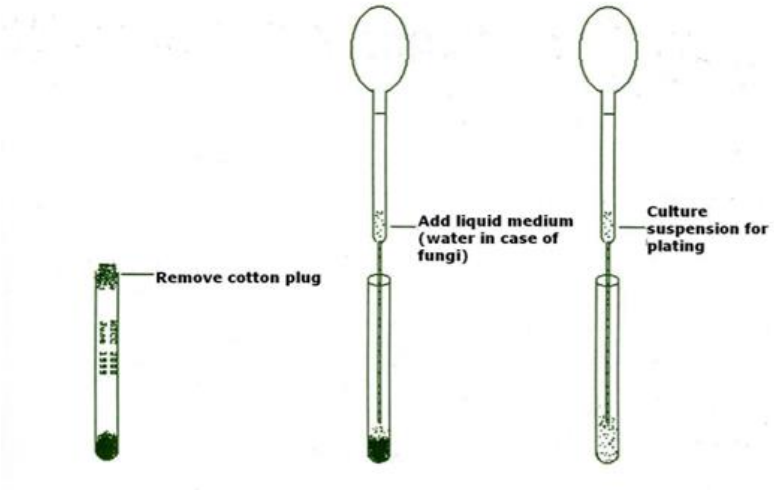


Fig 3.2: Instructions for revival of culture from ampoule

To obtain single colonies of the culture was plated on Nutrient agar tributyrin plate. Nutrient agar provided essential media for microbes to grow. on the other hand, tributyrin (oil) enabled differentiation between lipolytic and non-lipolytic strains. The agar tributyrin media contained 2% tributyrin, 3% agar and 1% gum acacia, used as emulsifying agent. All the components were mixed using sonicator to get a homogenous solution. Pure cultures were isolated and further sub-cultured through transfer between various nutrient agar plates and finally to nutrient broth.

3a.2 CHARACTERIZATION OF LIPASE

Enzyme characterization was done by performing pNPP based lipid assay at different temperatures (20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C) and different pH (3, 4, 5, 6, 7, 8, 9, 10, 11) to obtain optimum temperature and pH for lipase activity.

3a.3 QUALITATIVE ANALYSIS OF LIPASE PRODUCTION

Qualitative analysis of Lipase production was done by using Tween 20 agar plate assay. Tween 20 agar plate show the zone of precipitation around the well due to the formation of insoluble calcium salts by the released free fatty acids due to the action of the lipase enzyme added to the well, which forms complexes with the calcium salts present in the medium.

Tween 20 medium is composed of (g/L):

Pepton**e**: 10 grams; **NaCl:** 5 grams;

CaCl2.2H2o: 0.1 grams; **Agar-agar:** 20 grams;

Tween 20: 10 mL (v/v).

Tween 20 agar plate showing the zone of

precipitation around the well due to the formation of insoluble calcium salts by the released free fatty acids due to the action of the lipase enzyme added to the well, which forms complexes with the calcium salts present in the medium.

The strain under consideration is inoculated in the well created on respective plates and then the plates are left for incubation at 37ºC for 1-2 days. Results are analysed thereafter.

3a.4 GRAM STAINING OF STRAINS

Gram staining is microbiological differential staining technique. It determines whether the organism is gram positive or gram negative by the structure of cell wall of organisms. When the cultured organisms were stained with primary stain, some bacteria retain the strain where others get decolourized by decolourizer. Those bacteria that retain the first stain square measure known as Gram-positive and people bacterium that gets decolorized and so get counterstained square measure known as Gram-negative. All the isolated colonies on the nutrient agar media were screened for its gram behaviour and observed for analysis.

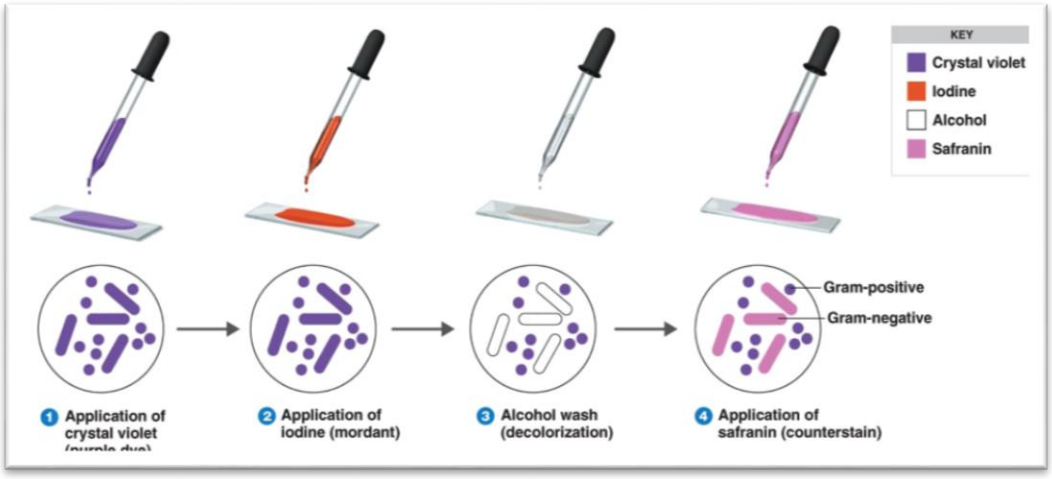


Fig 3.3: Procedure of Gram Staining

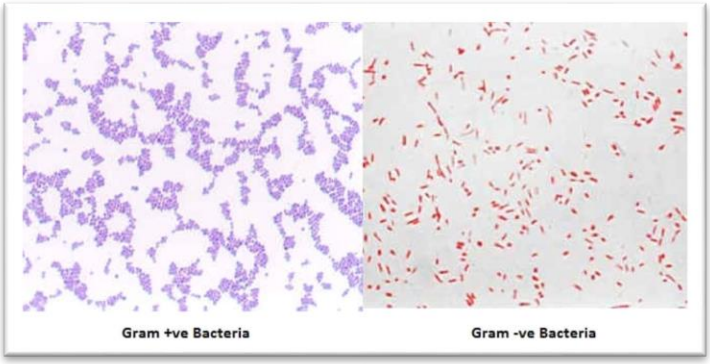


Fig 3.4: Gram staining Interpretation

3a.5 LIPASE ASSAY USING P-NITROPHENOL PALMITATE

- pNPP acts as a substrate for lipase and gets hydrolysed to yellow coloured p-nitrophenol and palmitic acid. 10mg pNPP is dissolved in 10ml of isopropanol and vortex it so that pNPP get completely dissolved in it and no precipitate remains.

- Now, add this substrate solution (10 ml) dropwise to 90ml of (pH8 100 mM) potassium phosphate buffers to obtain a milky white solution.

- Transfer 2.4 ml of this solution to test tubes and put test tubes in water bath at 37°C for priming. Also place enzymes in water bath at same temperature.

- Now, add 200 µL of enzyme to all the test tubes having substrate solution except control.

- Keep the solution for approximately 2 minutes or till pale yellow colour appears.
- Stop reaction by removing enzyme substrate solution from water bath.
- Add 100 µL of 100 Mm CaCl2 solutions to precipitate out p-nitrophenol.
- Centrifuge at 4°C for 5 minutes at 10,000 g.
- Take absorbance of supernatant at 540 nm.

For melanin study

3b.1 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.

3b.2 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.

3b.3 GRAM STAINING OF BACTERIAL STRAINS

All the isolated colonies on the nutrient agar media were screened for its gram behaviour.

3b.4 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.

3b.5 PIGMENT EXTRACTION AND PURIFICATION

- To precipitate the melanin, the pH of the supernatant was adjusted to 2.0 using 6 M HCl and was allowed to stand for overnight.
- Suspension was boiled for 1h to avoid the formation of melatinoidin.
- After cooling, the precipitate was then collected by centrifugation at 9000 g for 15 min.
- Deep brown color pellet was washed properly with 1ml of 0.1 N HCl followed by sterile distilled water. Ethanol added to the pellet, was placed in the ultrasonicator for 10 minutes and then was kept in water bath for 10 minutes.
- Purified pigment was left to dry at room temperature for complete evaporation of ethanol and was stored at − 20 °C until further use.

3b.6 UV-VIS SPECTROSCOPIC 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.

3b.7 FTIR SPECTROSCOPIC 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 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 spectrometer.

3b.8 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.

3b.9 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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 of 10 µg/ml of standard melanin in selected solvents were recorded individually. The spectrums were superimposed to get overlay spectrums. From this overlain 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 to 1 ml/min.

CHAPTER – 4

RESULTS AND DISCUSSIONS

4.1 ISOLATED PURE CULTURES

Pure cultures of thirteen unknown strains were successfully isolated from DTU lake water and it is further tested for lipase production by tween 20 plate analysis. only four strains out of six acquired from MTCC cultures were revived successfully. Further studies were performed on both known and unknown strains. Known strains are *Aeromonas sp, Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa* and unknown strains which are isolated from DTU are named KT1, KT2, KT3, KT4, KT5, KT6, KT7, KT8, KT9, KT10, B1, B2, A1.

4.2 OPTIMAL TEMPERATURE AND pH FOR LIPASE ACTIVITY:

Temperature(ºC)	O.D. Lipase	Lipase units
20	0.082	0.1068
30	0.195	0.568
40	0.229	0.6948
50	0.0754	0.081
60	0.0675	0.0488
70	0.0634	0.0456
80	0.059	0.012
90	0.041	0

Table 4.1: Lipase Assay readings for different temperature

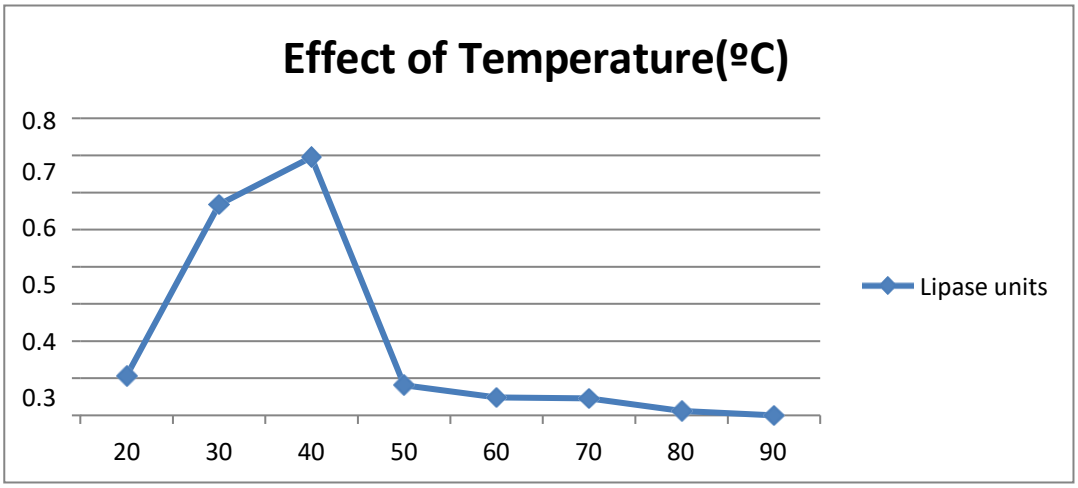


Fig 4.1: Effect of temperature on enzyme activity

pH	O.D. Lipase	Lipase units
3	0.059	0.012
4	0.066	0.044
5	0.098	0.172
6	0.167	0.446
7	0.233	0.71
8	0.175	0.478
9	0.103	0.19
10	0.078	0.09
11	0.067	0.0468

Table 4.2: Lipase Assay readings for different pH

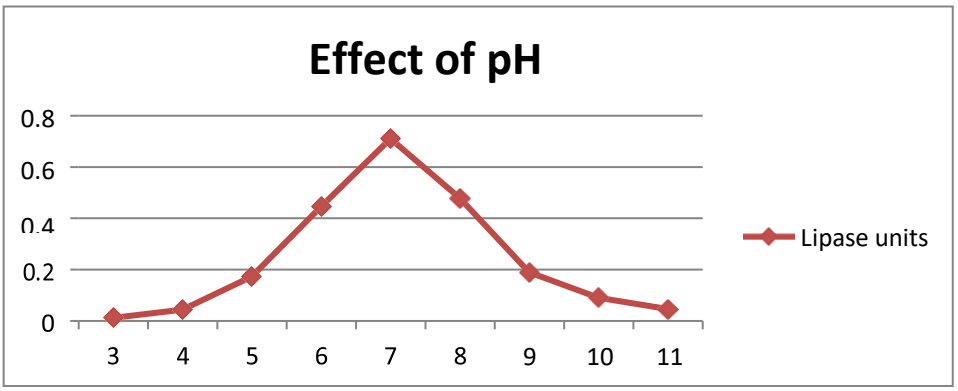


Fig 4.2: Effect of pH on enzyme activity

Lipase activity were calculated at various pH and temperature values. The study revealed that maximum enzyme activity was seen between 30-40ºC at pH 7.

Following tables 2 and 3 provide lipase units produced at each temperature and pH values. Corresponding to the tables are graphs showing the production trend.

4.3 QUALITATIVE ANALYSIS BY TWEEN 20 AGAR

Qualitative analysis of unknown wild strains was done by tween 20 agar plate to identify lipase producing bacteria. out of thirteen wild strain only eight strains produce lipase identified by tween 20 agar plate analysis. Some bacteria are producing lipase in good quantity, while other strains are producing lipase in less amount. Results are illustrated below in pictures.

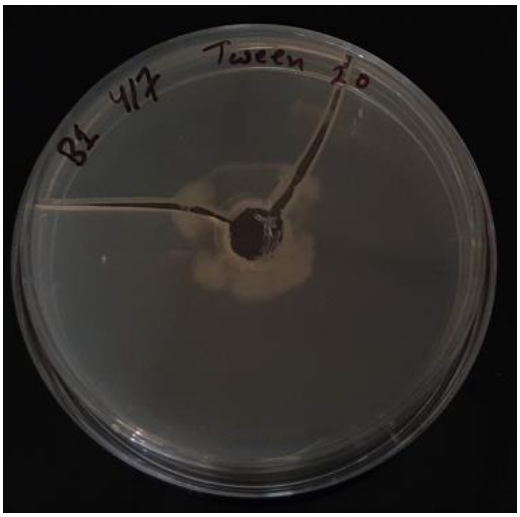


Fig 4.3 : B1 result plate

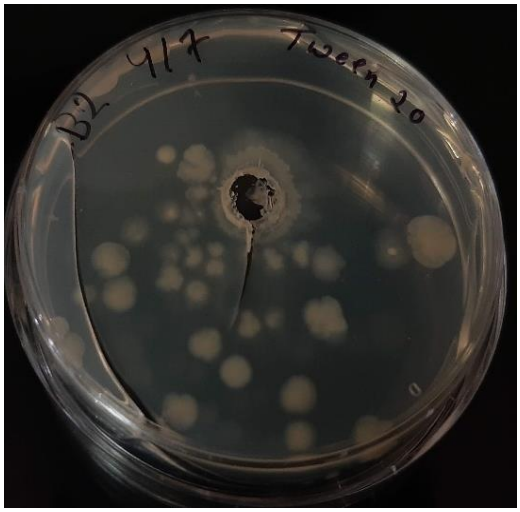


Fig 4.4: B2 result plate

Fig 4.5: A1 plate result

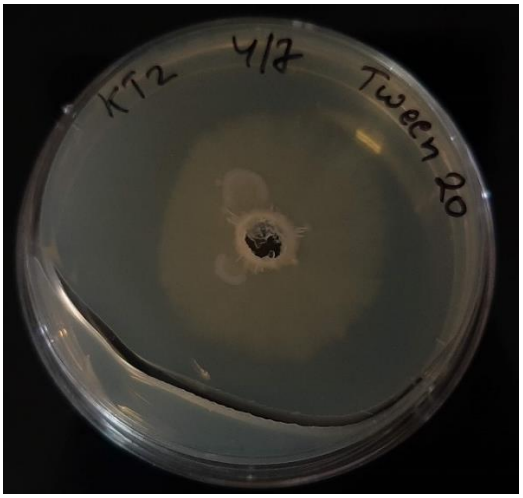


Fig 4.6: KT2 plate result

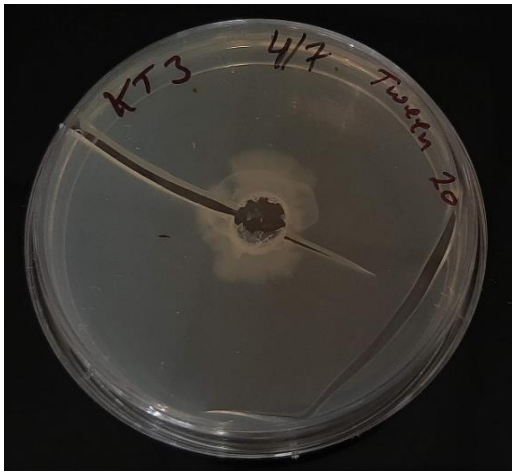


Fig 4.7: KT3 plate result

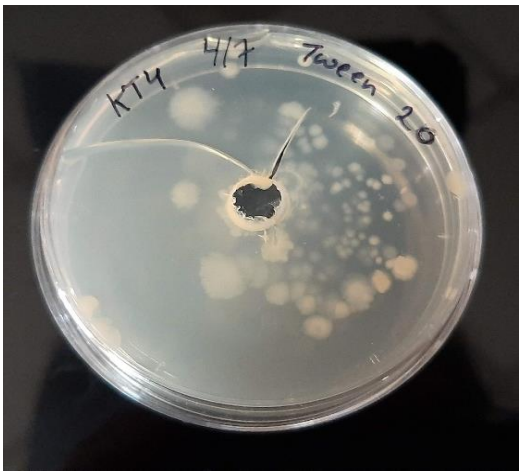


Fig 4.8: KT4 plate result

Fig 4.9: KT5 plate result

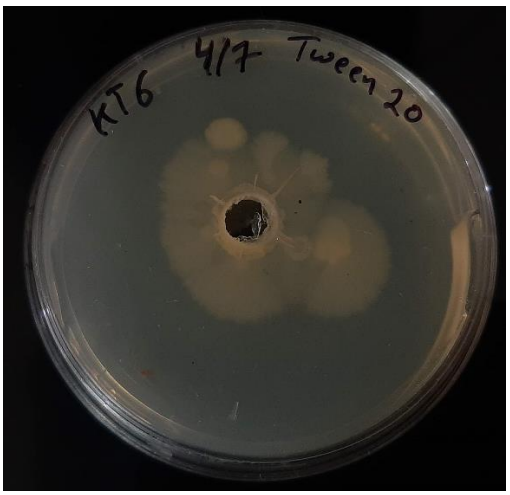


Fig 4.10: KT6 plate result

It is clear from the above results that lipase is a ubiquitous compound, it is present in a variety of organisms. Amongst the tested organisms, KT2 produced the greatest amount of lipase followed by KT6. This is evident from the dense and large precipitation zone formed by their strain. The remaining strains showed less lipase production. The isolation of lipolytic organisms from DTU Lake also suggests that there is substantial amount of oil and fat contamination in water.

4.4 GRAM STAINING RESULTS

All the strains isolated from DTU lake and successfully activated cultures acquired from MTCC are gram negative in nature except Staphylococcus aureus, identified by gram

staining. These gram-negative bacteria have a thin layer (10% of cell envelope), that's why do not retain the purple stain and are counter-stained pink by safranin. Staining results are:

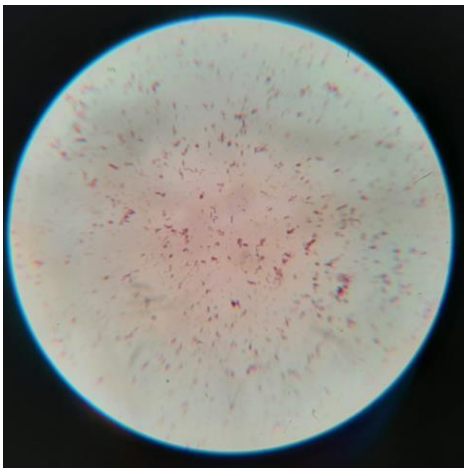


Fig 4.11: B1 staining result

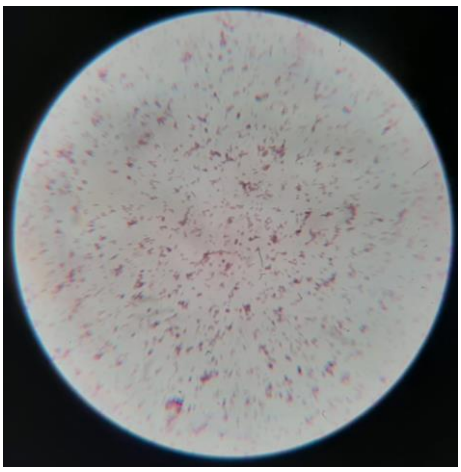


Fig 4.12: B2 staining result

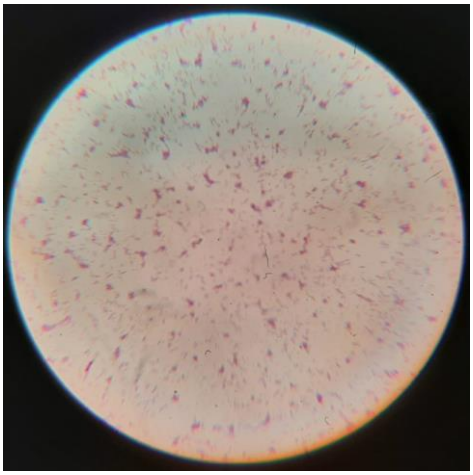


Fig 4.13: KT 2 staining result

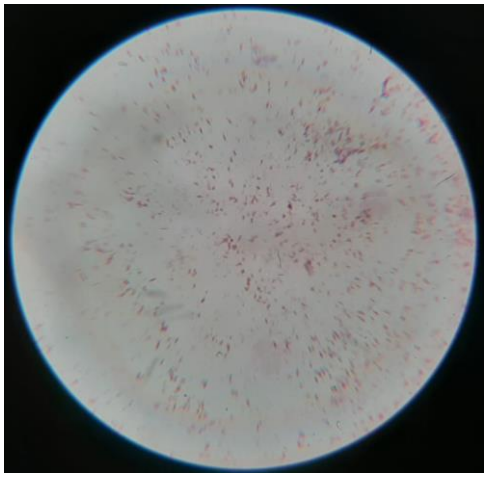


Fig 4.14: KT 3 staining result

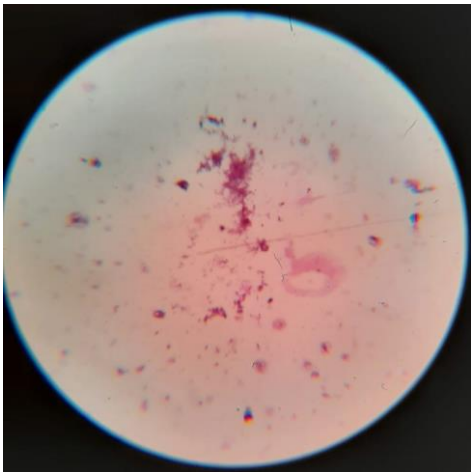


Fig 4.15: KT 4 staining result

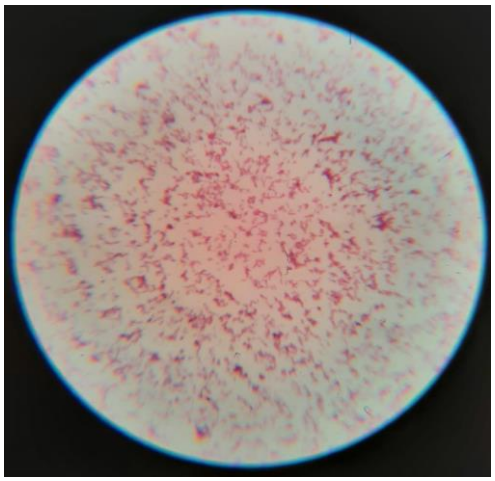


Fig 4.16: KT 5 staining result

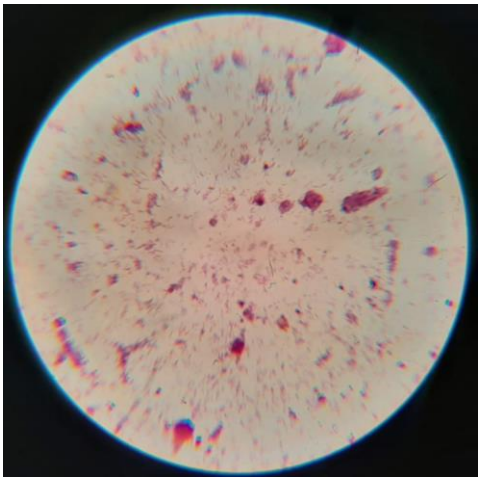


Fig 4.17: KT 6 Staining result

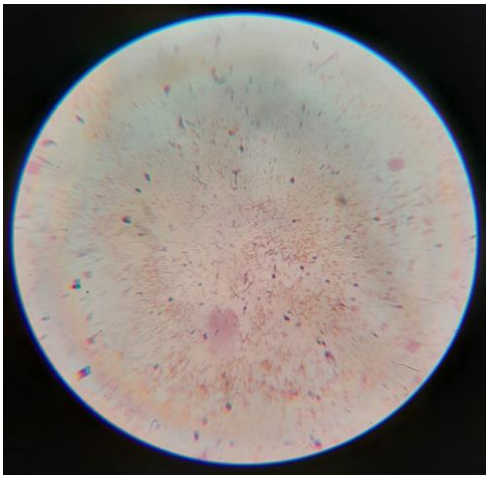


Fig 4.18: A1 staining result

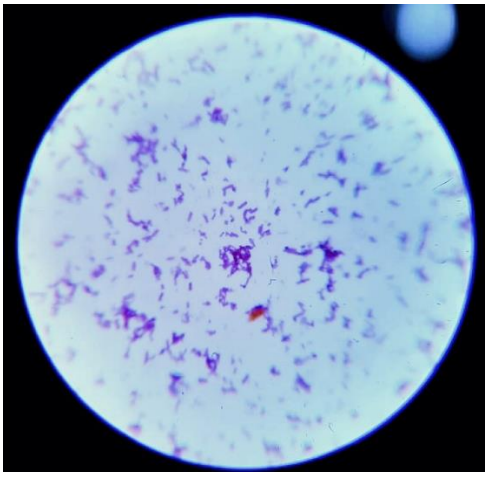


Fig 4.19: *S. aureus* staining result

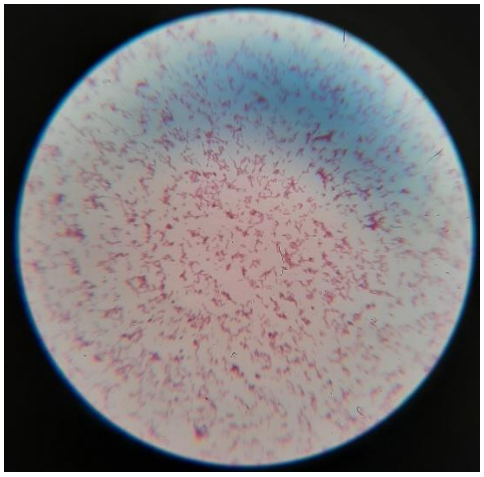


Fig 4.20: *Aeromonas* staining result

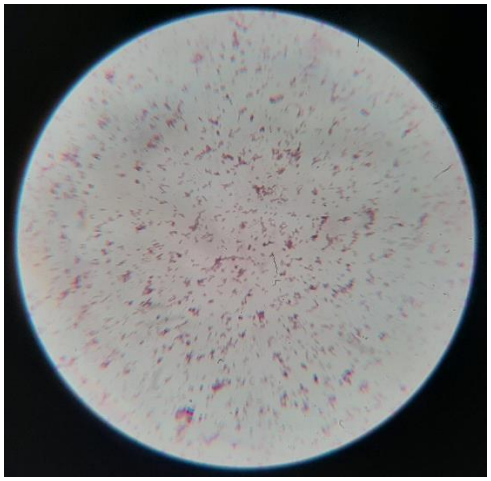


Fig 4.21: *P. aeruginosa* staining result

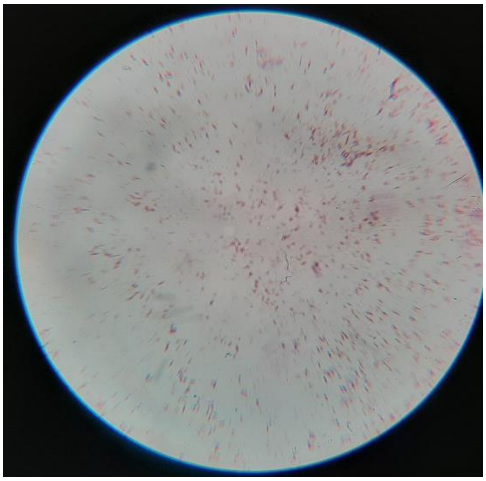


Fig 4.22: *K. pneumoniae* staining result

4.5 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 excepted 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.

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 colourless	Turns colourless

Table 4.3 Chemical characterization of test samples of melanin

4.6 UV SPECTROSCOPIC ANALYSIS

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. (a)

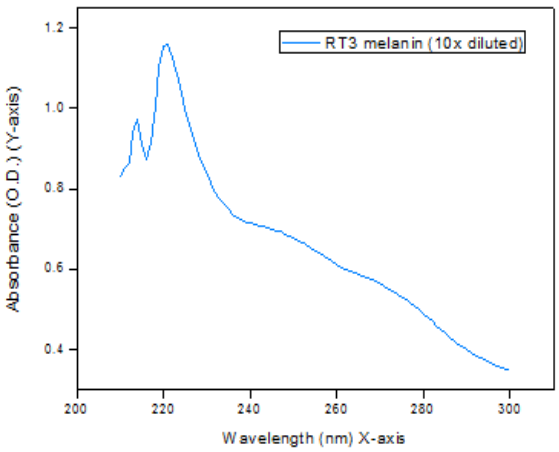


Figure 4.23. (b)

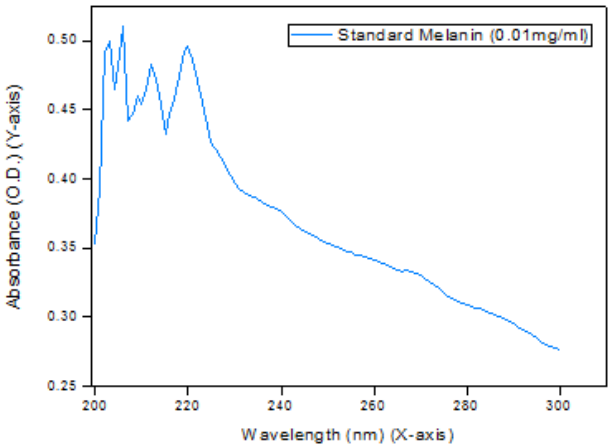


Fig 4.23. UV spectrum of melanin from 200 nm to 300 nm (a) For melanin pigment extracted from bacterial strain RT3 (b) For synthetic melanin (10 mg/L concentration)

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.**)

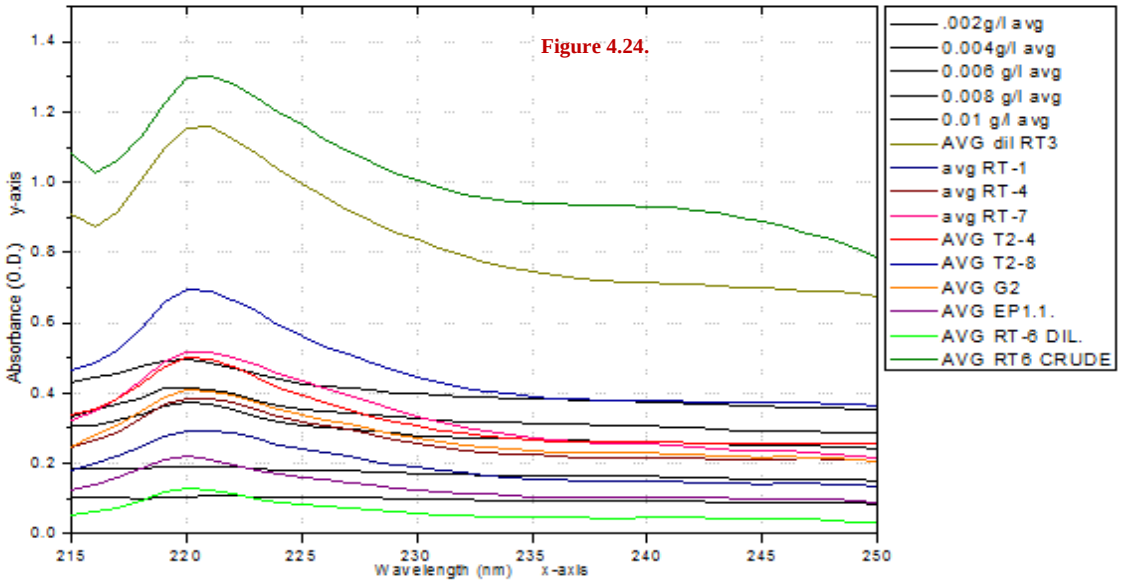


Figure.4.24. UV-Vis spectrum of the melanin sample from bacterial cultures vs standard melanin (in concentrations ranges of 0.002 – 0.01 g/l), with strong peaks at 220nm.

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.

4.7 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 were 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

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

Table 4.4: Results of TLC analysis of melanin pigment

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 run 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.

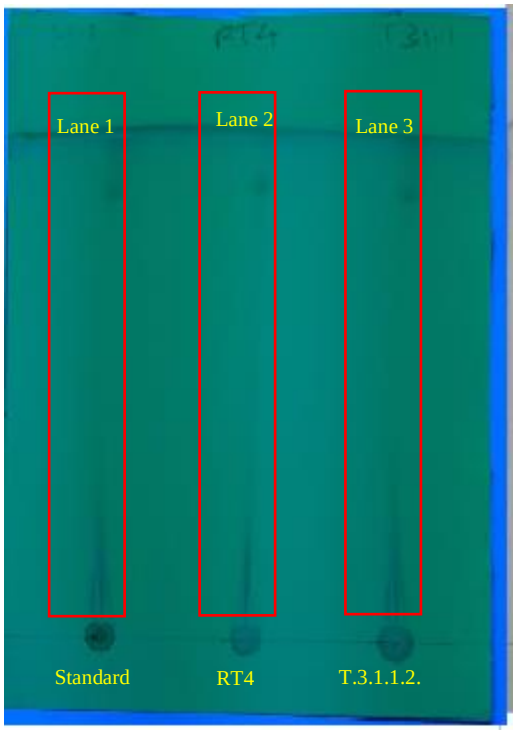


Figure.4.25.

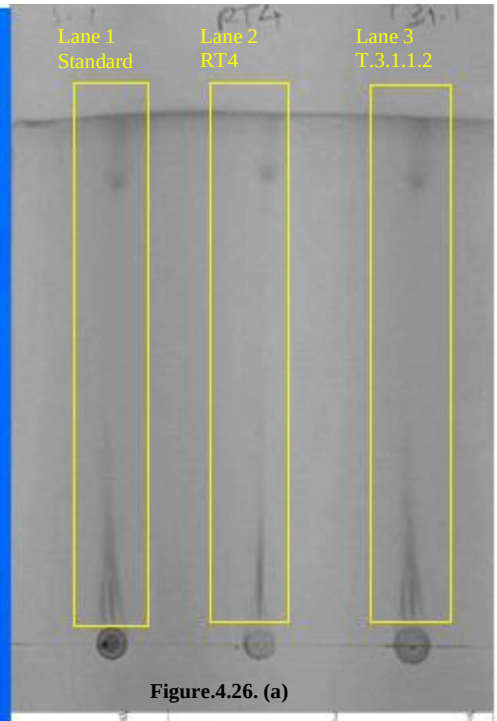


Figure.4.26. (a)

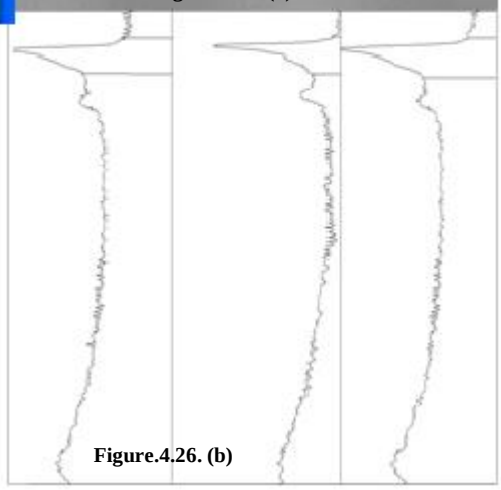


Figure.4.26. (b)

Figure.4.25. TLC analysis of melanin pigment after plate development

Figure.4.26. (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.

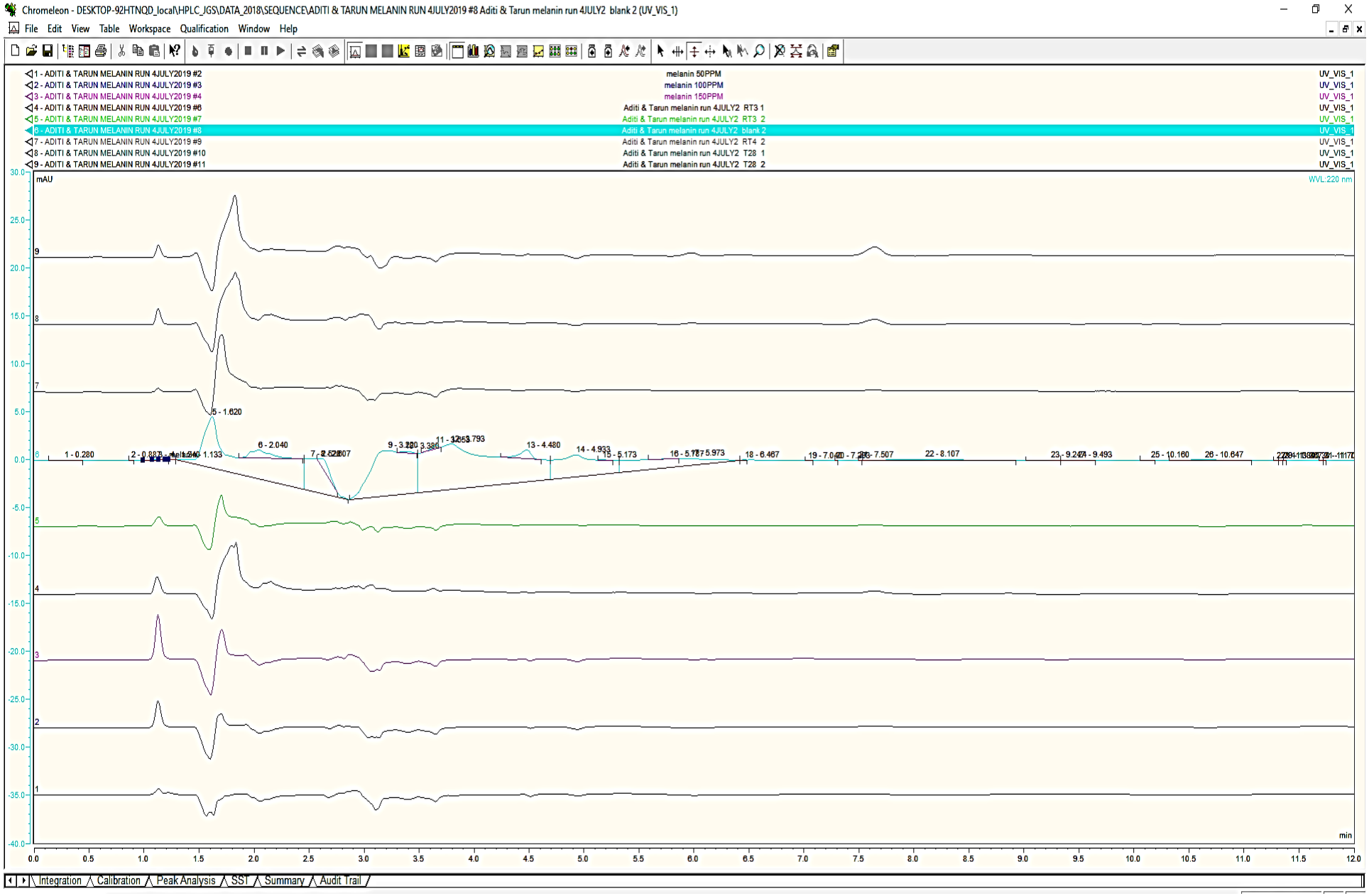
A significant characteristic chromatogram peak was observed against the run of each sample and the standard of the melanin pigment.

4.8 HPLC 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 melanin, 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.


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
min
Integration Calibration Peak Analysis SST Summary Audit Trail

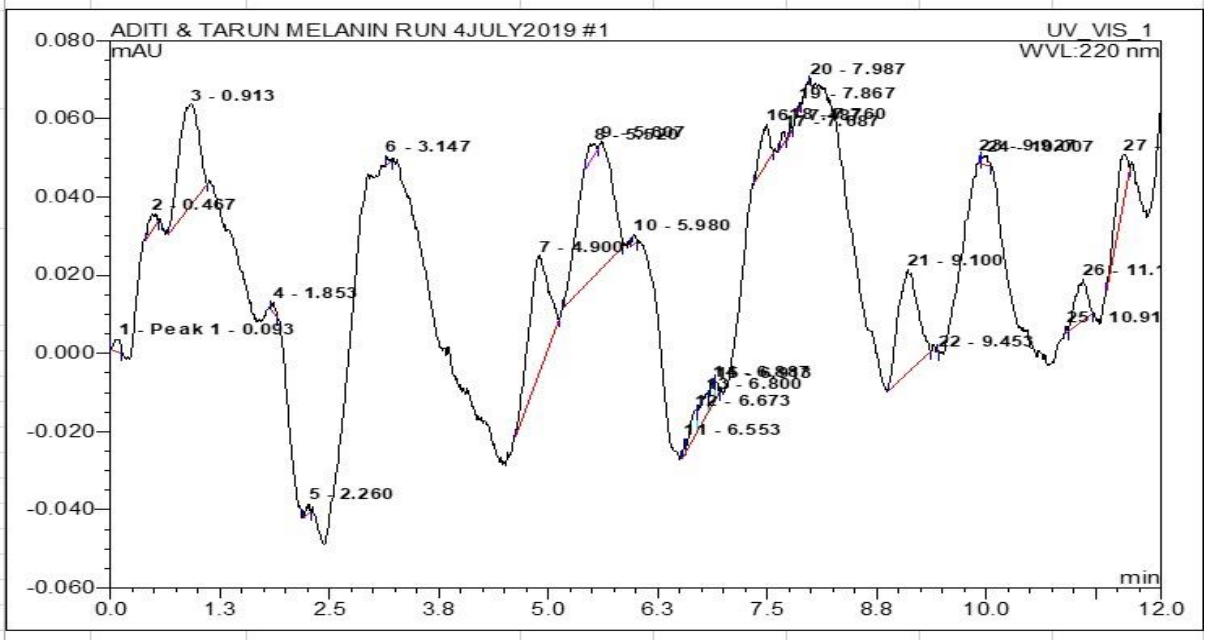
Upon looking at the chromatogram above and analysis of peak heights at different retention times, we couldn't find any trend which could be used for the analysis of the peaks in order to quantify them. Upon further investigation we infer that in order to analyse melanin we need to further improvise our 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.

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., the obtained pigment should be stored in dried state at – 20 degree Celsius in order to maintain stability, 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. Detailed results are given in APPENDIX 1.

APPENDIX-1

1 BLANK

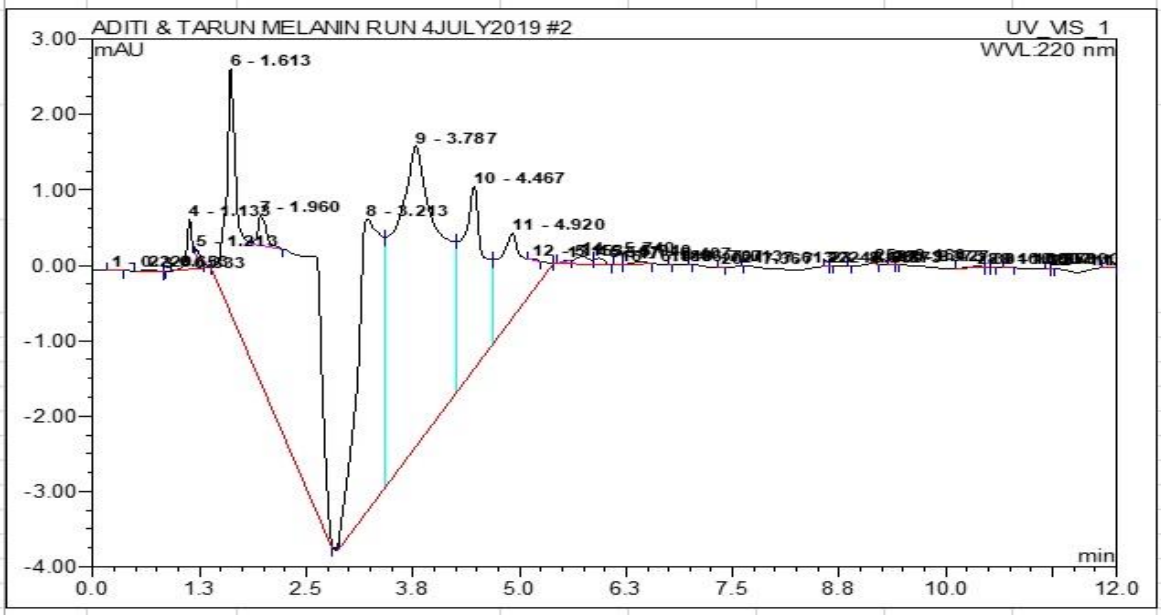
Sample Name:	**BLANK**	*Injection Volume:*	**10.0**
Vial Number:	**RA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**blank**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
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	

2 melanin 50PPM

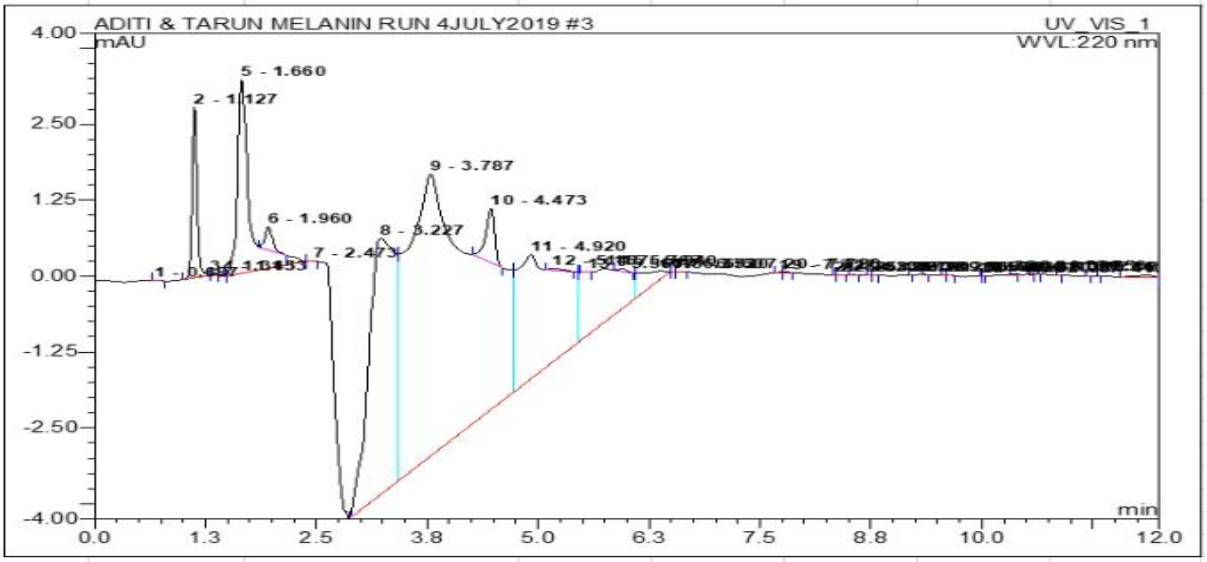
Sample Name:	**melanin 50PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA2**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**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

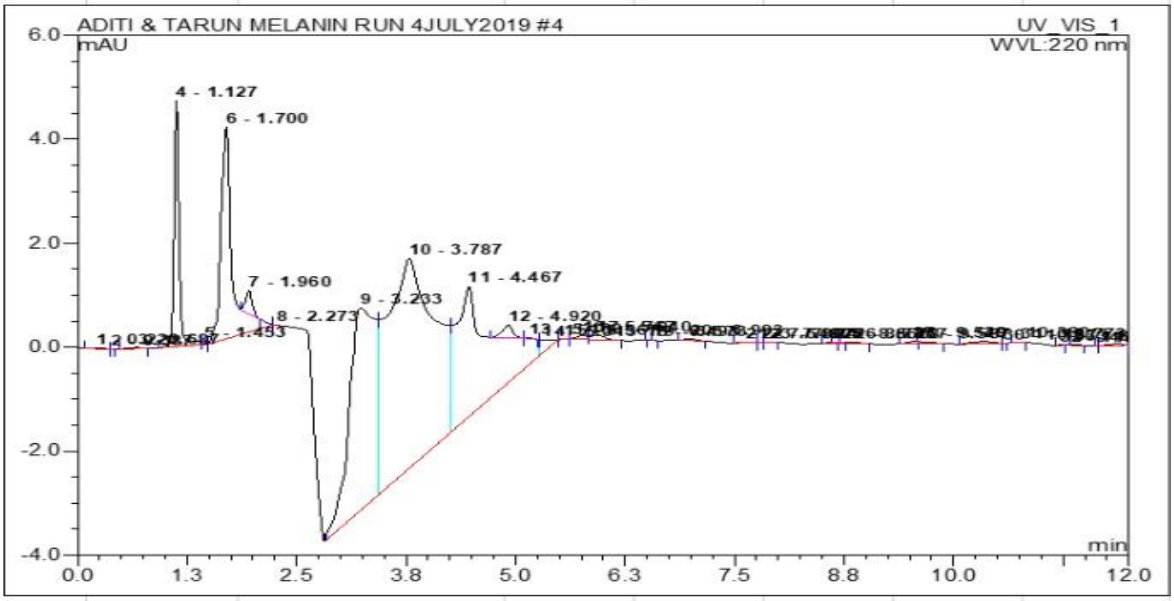
Sample Name:	**melanin 100PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA3**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**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

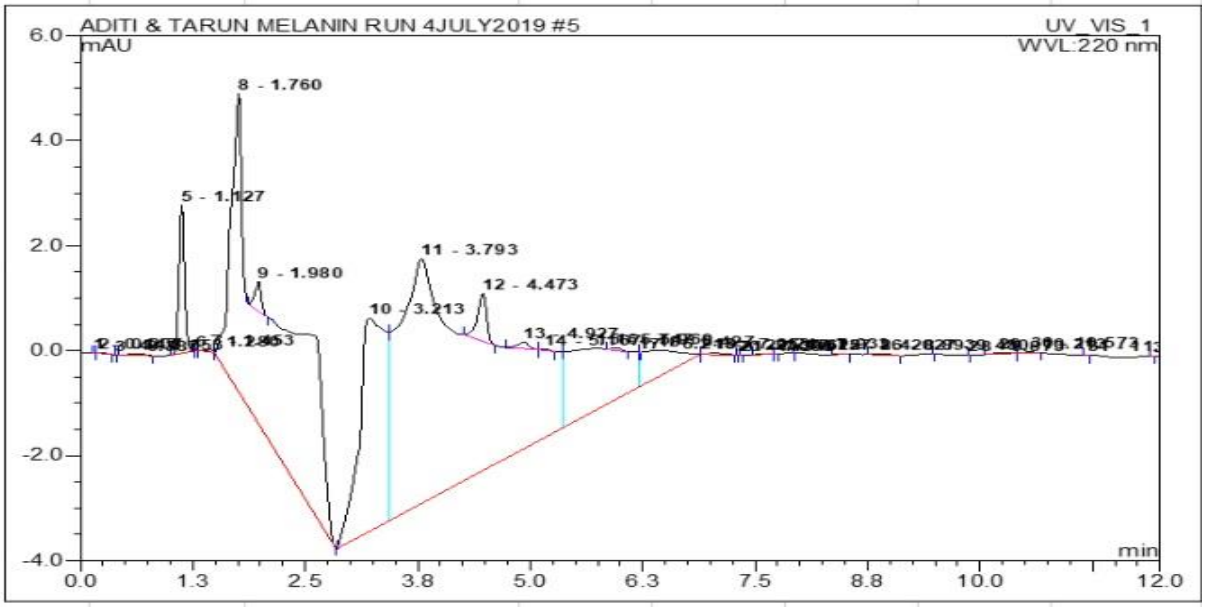
Sample Name:	**melanin 150PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA4**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**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

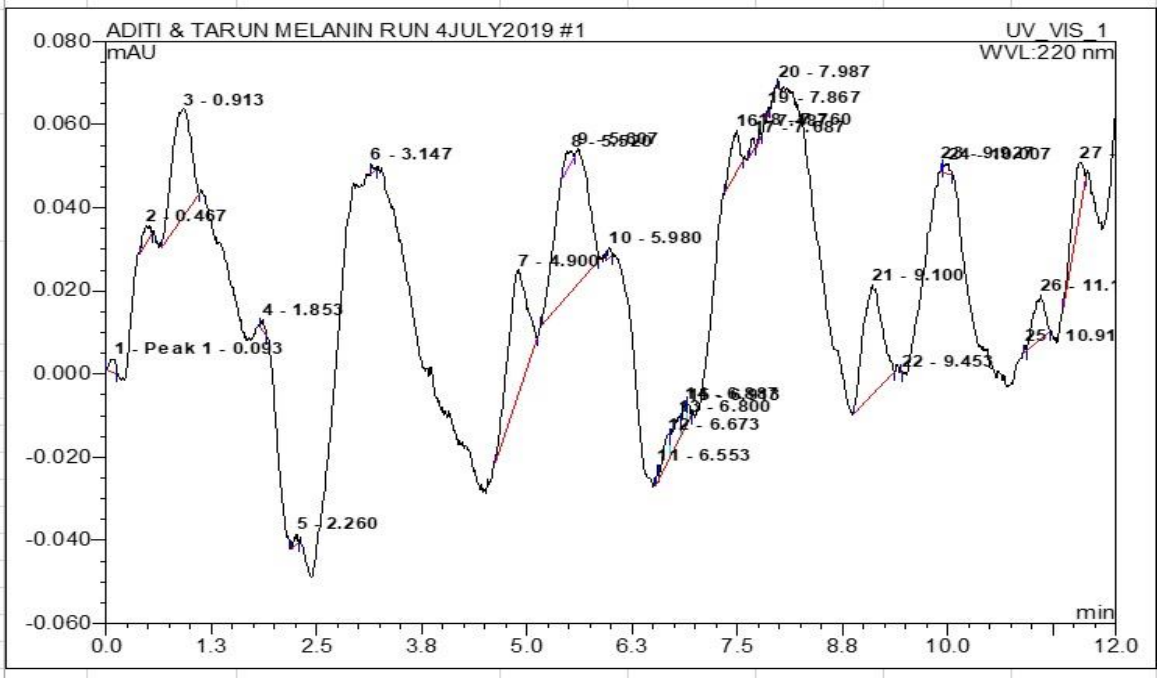
Sample Name:	**melanin 200PPM**	*Injection Volume:*	**10.0**
Vial Number:	**RA5**	*Channel:*	**UV_VIS_1**
Sample Type:	**standard**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**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

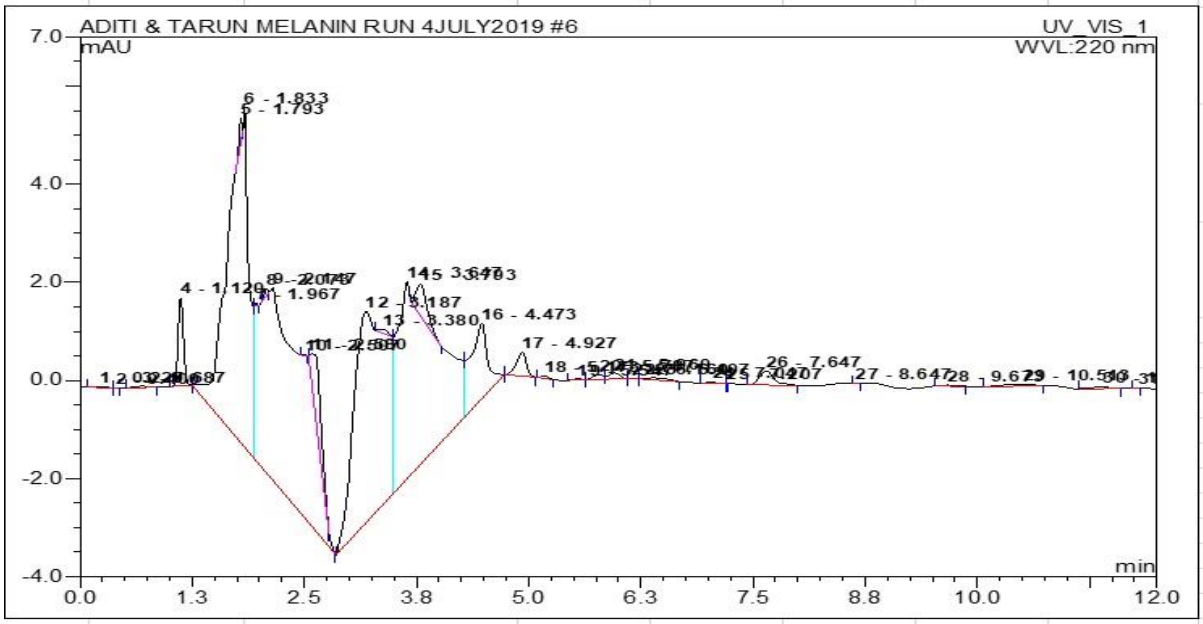
Sample Name:	**BLANK**	*Injection Volume:*	**10.0**
Vial Number:	**RA1**	*Channel:*	**UV_VIS_1**
Sample Type:	**blank**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 4JULY2019**	*Dilution Factor:*	**1.0000**
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

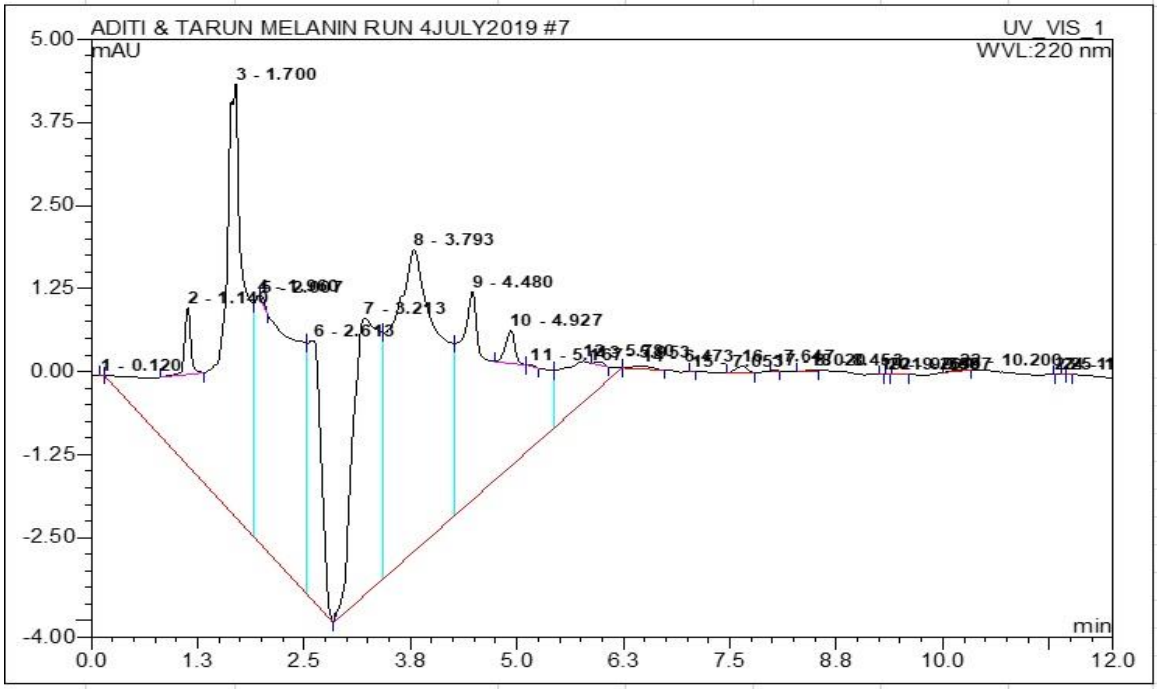
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT3 1**	*Injection Volume:*	**10.0**
Vial Number:	**RA6**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 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

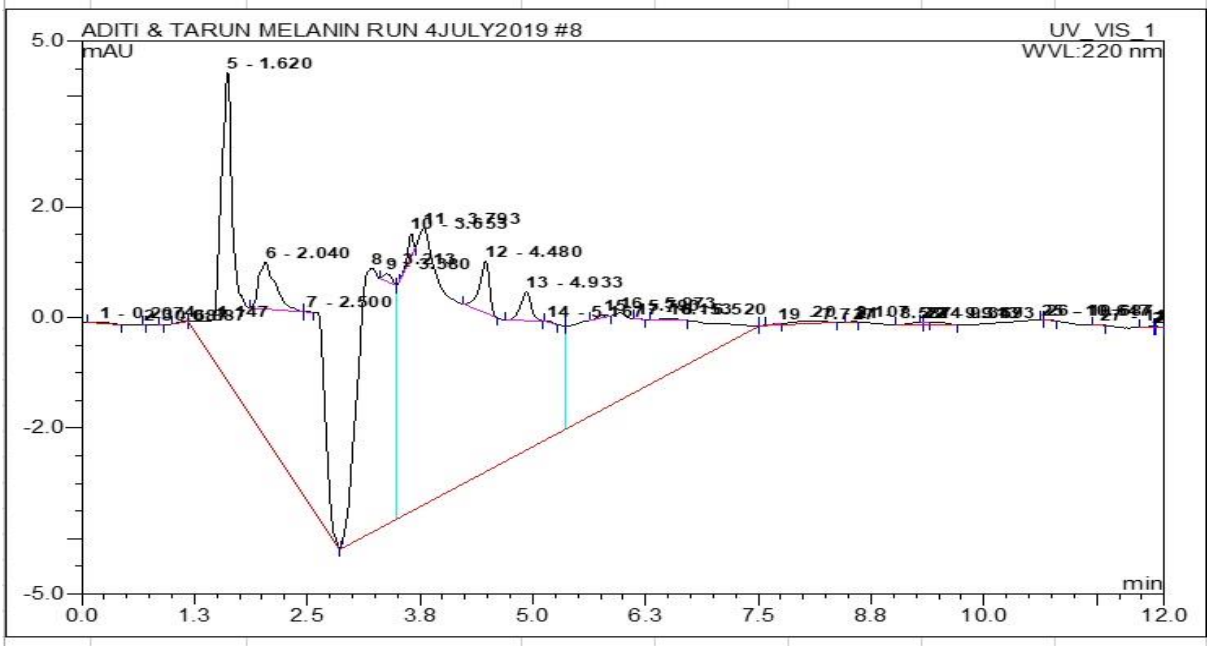
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT3 2**	*Injection Volume:*	**10.0**
Vial Number:	**RA7**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 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

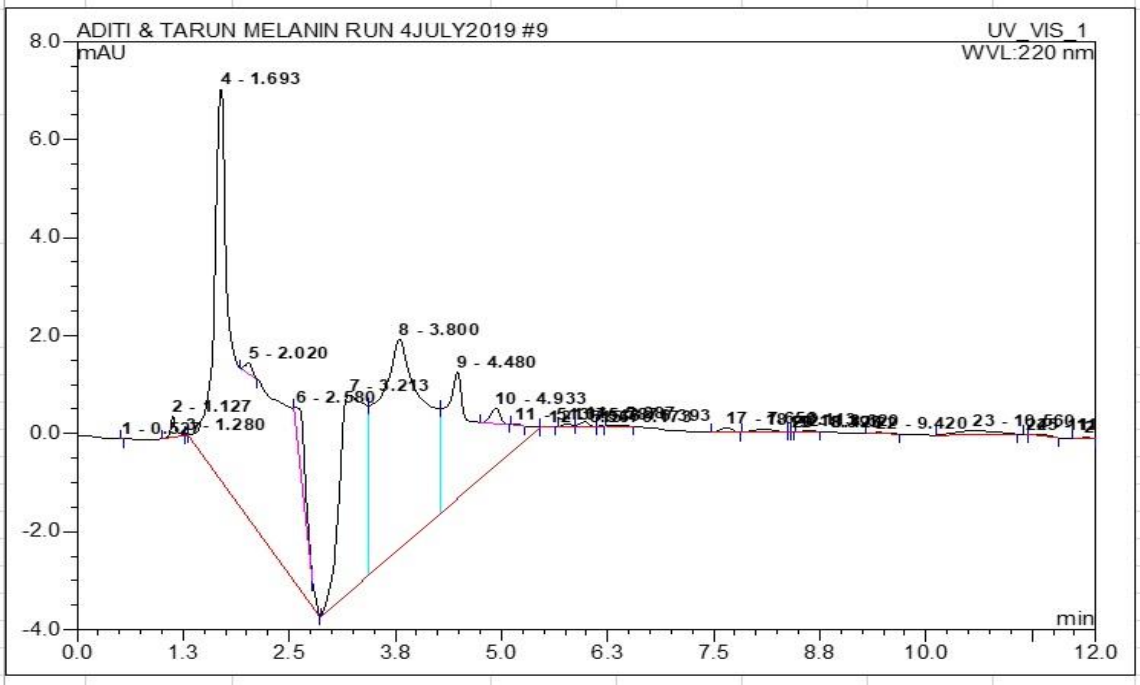
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT4 1**	*Injection Volume:*	**10.0**
Vial Number:	**RA8**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 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 min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
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

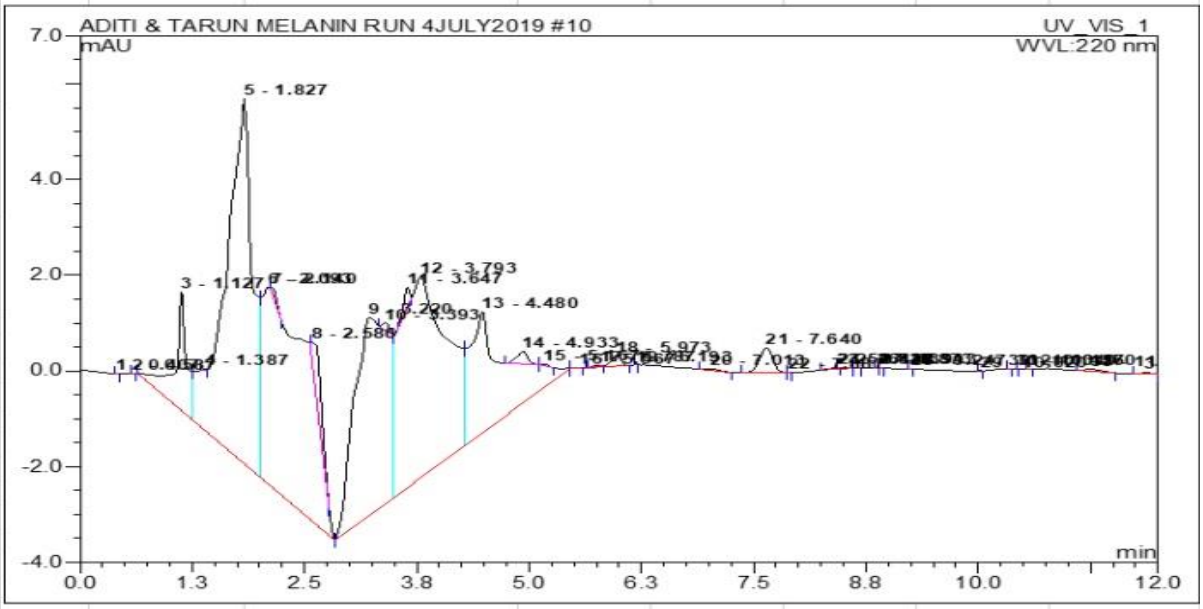
Sample Name:	**Aditi & Tarun melanin run 4JULY2 RT4 2**	*Injection Volume:*	**10.0**
Vial Number:	**RB1**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 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

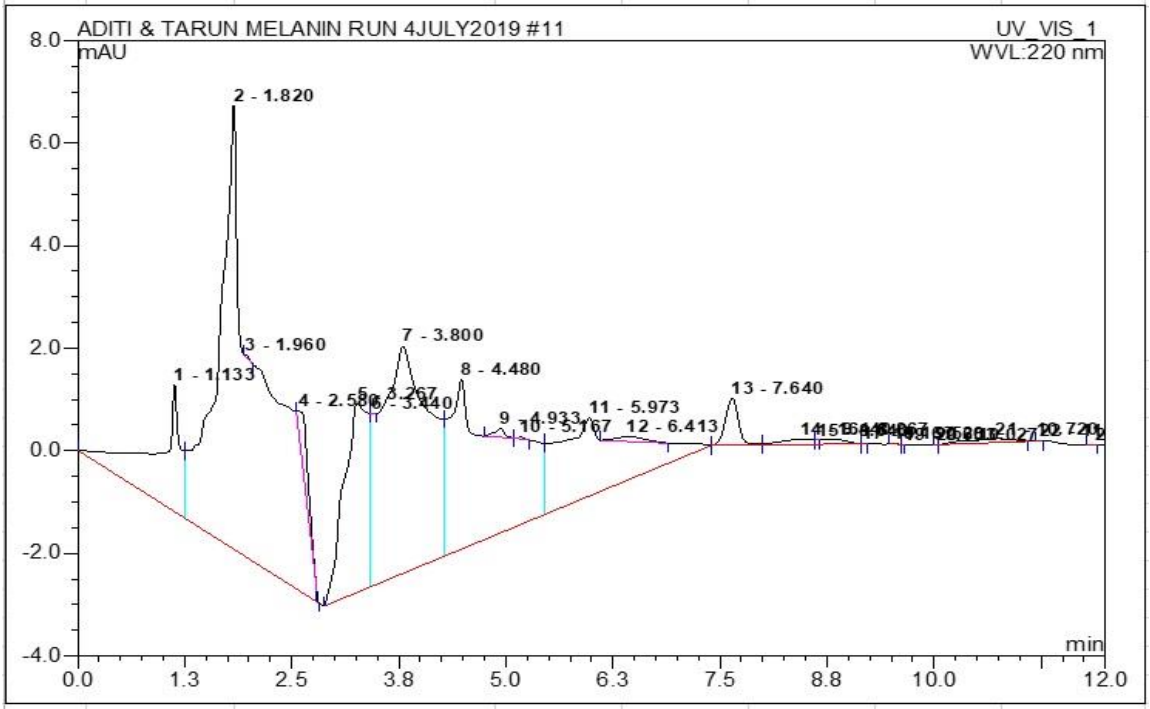
Sample Name:	**Aditi & Tarun melanin run 4JULY2 T28 1**	*Injection Volume:*	**10.0**
Vial Number:	**RB2**	*Channel:*	**UV_VIS_1**
Sample Type:	**unknown**	*Wavelength:*	**220**
Control Program:	**Aditi & Tarun melanin run 4JULY2019**	*Bandwidth:*	**n.a.**
Quantif. Method:	**Aditi & Tarun melanin run 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 min	Peak Name	Height mAU	Area mAU*min	Rel.Area %	Amount	Type
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	

CONCLUSION

- The importance of lipase in industries has long been realized. It has left a mark on various industries such as food industry, oil and fat industry, detergent industry, pulp and paper industry, leather industry, textile industry etc. Therefore, optimal production of enzyme with high activity and specificity is still an important field of study.
- Comparative study of lipase production amongst different microbial strains was successful in opening new windows for isolation of producer strain. It suggested that lipolytic organisms not only reside in conventional oil-contaminated areas, but also in aging water bodies such as DTU Lake. This can also be viewed from a different perspective that will help us to point out level of oil contamination in water. Hence, researchers can work together with greater efficiency and can devise methods for water treatment accordingly.
- 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.

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