2.1 Melanins

Melanins are brown to black colored complex natural pigments which are widely distributed throughout life forms in nature with few exceptions including arachnids. Melanins have several biological functions which include thermoregulation, photo protection, acting as free radical sinks, cation chelators, acting as antibiotics etc. In plants melanin acts as cell wall strengtheners (Riley, 1997), while in animals it determines the skin color and also plays a crucial role in protecting the skin from damage caused by ultraviolet radiation (Huang et al., 2012). In the microbes, melanin acts against environmental stresses, and can make the bacteria resistant to antibiotics (Lin et al., 2005), while they are also involved in fungal pathogenesis (Butler and Day, 1998). In fungi some melanins act as a photosynthetic pigment which sequesters γ -radiation in order to generate energy for growth (Dadachova et al., 2007). These diverse biological roles of melanin make it suitable for the use in various medical, cosmetological and pharmacological applications.

2.2 Structure of melanin

Melanins structure constitute stacked polymer layers in which the monomer blocks are essentially derived from 5, 6-dihydroxyindole (DHI) and 5, 6-dihydroxyindole-2-carboxylic acid (DHICA) units in many possible oxidation states. These monomeric units were linked irregularly
Review of Literature

giving rise to a wide system of conjugated double bonds, although some preferential modes of polymerization have been reported. Catecholamine precursors that did not undergo cyclization can also be included in the polymer. The polymer layers are organized in randomly oriented local domains. It has been hypothesized that there may be covalent bonds present connecting different layers/domains, explaining why melanins are completely insoluble. Melanins show a 4'-7 linked substituted indoles indicating the high degree of conjugation of such domains. Carboxylic acid groups are attached to the C2 and the S6 functionalities are shown as carbonyls in the orthoquinone form, deprotonated hydroxyls in the catecholic form, and an equilibrium form of linked semiquinones (Fig. 2.1).

The three major functions are indicated as electronic reactions, which makes the polymer a potential free radical scavenger, photon absorption and metal ion binding through the carboxyl groups (Riley, 1997).

![Fig.2.1. Structure of melanin (Adapted from Riley, 1997)](image)
In several melanins of biological source, including melanin from human hairs, the true melanin polymer is tightly bound to protein-like material, which may be utilizing the remarkable binding affinity for metal ions, and indeed melanins usually contain a noticeable amount of metals (Ghiani et al., 2008).

2.2.1 Limitations in determining melanin structure

There are many reasons that limit the determination of melanin structure. These include the following

1. There are no standard methods available for the study of melanins, similar to the ones which are designed to study the structure of other biological macromolecules - proteins, nucleic acids or even membranes which can be considered macromolecules (taking the dispersive interactions as binding ones). For example, proteins are structured in a standard way (the primary, secondary, tertiary and quaternary structure), which repeats in every proteins, and proteins are extremely important, so there is a huge number of methods which are standardized in every way. ORD, CD, NMR, X-ray dispersion, EPR, molecular modelling, mass spectroscopy and many, many others. You put a sample and you get the elaborated results. In the case of melanin, there are no such standard methods, and everything must be generated from the very roots for each experiment (the method, theory, software, statistics etc.) (Sarna and Plonka, 2005).

2. And the second reason, which is actually also the reason for the 1st reason - the atypical hierarchic structure of melanin. The case is rather about microscopic, mesoscopic and macroscopic structural organisation, than about primary, secondary, tertiary structures. There must be a special
model generated, and every structural level corresponds to a different level of organization. While on the macroscopic level you can say about even organelles (melanosomes) and packing of melanin inside them - as agglomerates, and the microscopic structure represents the molecular level (biochemical) in which the primary and secondary, and to some degree even the tertiary structure is set, there are no examples of molecular organization of biological molecules corresponding to the mesoscopic structure, and this is probably the most characteristic and responsible for the unusual properties of melanin resembling the inorganic, solid body-like substance. There are no typical, periodical bonds (e.g. peptide bond, phosphodiester bond) in the amorphic structure of melanin. Limitation necessitates building the whole theory of structural organization of the polymer, from scratch (Meredith and Sarna, 2006).

2.3 Melanin Biosynthetic Pathways

Depending on the metabolic pathway by which it is synthesized, melanins can be broadly divided into three main types- Eumelanin, Pheomelanin and Allomelanins.

The best-understood melanization pathway is the classic Mason-Raper pathway (Raper, 1927; 1928), in which tyrosinases yield melanin via the intermediate dihydroxyphenylalanine (DOPA). This results in the formation of eumelansins, the most common type of melanin. Eumelanin biosynthesis starts with either tyrosine or DOPA as the precursor. The enzyme tyrosinase oxidizes DOPA and tyrosine to form DOPAquinone. DOPAquinone is a highly reactive molecule. Intramolecular nucleophilic addition by the amino group produces cyclodopa. Cyclodopa is then oxidized to form DOPAchrome (a red colored compound). DOPAchrome is
a relatively stable molecule, but will spontaneously decompose to form DHI (5, 6-dihydroxyindole), giving off CO$_2$. If the enzyme dopachrome tautomerase (Dct) is present, DOPAchrome will instead tautomerise to give DHICA (5,6-dihydroxyindole-2-carboxylic acid) as the predominant monomer, retaining the carboxylic acid group. So the availability of Dct determines the relative amounts of DHI and DHICA produced, which will surely affect the ratio of these components in the final eumelanin macromolecule (Ito, 2003). Several other environmental factors can also determine the DHI and DHICA ratio in eumelanin, and hence varies widely depending upon the source of the eumelanin under study (Pezzella et al., 1997).

![Fig.2.2. Biosynthetic pathways of eumelanin and pheomelanin (Adapted from Solano, 2014)](image-url)
Highly reactive DOPAquinone in the absence of thiol compounds, undergo intramolecular cyclization, leading to the formation of eumelanin. But if thiols such as glutathione and cysteine are present, DOPAchrome will react with them and give rise to thiol adducts of DOPA and cysteinylDOPAs, among which 5-S-cysteinylDOPA (5-S-CD) is the major isomer. Further oxidation of the thiol adducts results in pheomelanin production via benzothiazine intermediates. Most melanin pigments present in pigmented human tissues appear as mixtures or copolymers of eumelanin and pheomelanin (Solano, 2014) (Fig. 2.2).

Fig. 2.3. Biosynthesis pathway of Neuromelanin (Adapted from Solano, 2014)
The other classes of melanins include allomelanins and neuromelanins. Neuromelanin is found in the brain stem and inner ear of humans and higher primates. The function of neuromelanin is still unknown. Although it is thought to have some biological significance; neuromelanin is decreased or absent in individuals with Parkinson’s disease but it is not clear whether there is any relationship between neuromelanin and Parkinson’s disease (Fitzpatrick et al., 1987; Chen and Chavin, 1965) It is suggested that the pigment might modulate neurotoxic processes through interaction through iron, binding of drugs or reaction with free radicals and free radical producing species (D’Ischia and Prota, 1997). Additionally, albinism often leads to deafness, suggesting its biological functionality (Nicolaus, 2005).

Neuromelanin is a mixture of pheomelanin and eumelanin, derived from dopamine and 5-S cysteinyldopamine units. According to Bush et al. (2006) neuromelanin granules have a pheomelanin core and eumelanin covering the core, which is compatible with an occasional exhaustion of the glutathione or cysteine reduction system during neuromelanin formation (Ito et al., 1986).

The precursor of neuromelanin is L-tyrosine, which is hydroxylated to L-DOPA by neuronal tyrosine hydroxylase. The high amino acid decarboxylase activity in catecholaminergic neurons yields dopamine and this is oxidized to dopamine quinone. This reaction is catalyzed by a peroxidase or it occurs spontaneously by the action of reactive oxygen species. Similarly to DOPAquinone in the Raper-Mason pathway, this quinone is pivotal in the route, giving place to 5-S-cysdopamine or DHI depending on the presence or the absence of L-cysteine during the
dopamine quinone formation. (Fig. 2.3) Neuromelanin is usually a mixed melanin, as both indole and benzothiazine units are incorporated to its structure (Fodorow et al., 2005)

Allomelanins are the least studied and most heterogeneous group of melanins which includes DHN-melanin, homogentisic acid (pyomelans), γ-glutaminyl-4-hydroxybenzene (GHB-melanin), catechols, 4-hydroxyphenylacetic acid etc. Although the Raper-Mason pathway is the major route for melanin biosynthesis in higher organisms, the biosynthesis of allomelanins are the predominant ones in lower organisms like bacteria, fungi etc. and takes place from precursor different from L-tyrosine. (Plonka and Grabacka, 2006).

Fig. 2.4. Biosynthesis pathway of DHN-melanin (Adapted from Solano, 2014)
DHN-melanin is formed by the pentaketide pathway. 1,8-dihydroxynaphthalene is formed from acetyl-CoA through a carboxylation to malonyl-CoA which is the substrate of the pentaketide synthase system. In this route, a tetrahydroxy naphthalene is first formed, and then that compound is transformed in 1,8-dihydroxynaphthalene (DHN) through scytalone and vermelone as stable intermediates. This dihydroxy derivative (DHN) is the characteristic substrate of fungal polyphenol oxidases (laccase), to form naphthalene quinone (Fig. 2.4). Similar to other melanin forming pathways, those dihydroxy and quinonic forms polymerize in the final phase to form DHN-melanin (Eisenman and Casadevall, 2012).

Mainly plant melanins belongs to catechol-melanin are formed by the catalytic action of catechol oxidases (enzyme similar to tyrosinases), giving rise to quinones. No cysteine is added to the pigment and no indolic units are formed in agreement with the sulfur and nitrogen economy in plants. The o-quinones formed by the action of catechol oxidases on catechol can react with the solvent water as a redox system to yield 1,2,4-trihydroxybenzene. Some other studies propose that catechol and o-quinone can undergo a dismutation reaction to a semiquinone radical. This radical can dimerize to a biphenol structure or react through the oxygenated groups to yield oxygenated heterocycles (Fig. 2.5). The positions for cross linking of these possible intermediates and the structure of the final polymer still remain unidentified (Mason, 1949).
\(\gamma\)-glutaminyl-4-hydroxybenzene (GHB-melanin) is the predominant melanin produced by mushrooms by the action of tyrosinase enzyme. The precursor of GHB-melanin is chorismate, which is converted to p-aminophenol and conjugated with a glutamyl residue to form GHB. This monophenol is oxidized to the o-diphenol \(\gamma\)-glutaminyl-3,4-dihydroxybenzene (GDHB) and subsequently \(\gamma\)-glutaminyl-3,4-dihydroxybenzo-quinone (GBQ). Then, glutamyl residues are mostly removed of the final pigment, as understood from the nitrogen content of the melanin, but the distal phase of melanization is again an undefined set of redox reactions among several intermediates (Fig. 2.6). The melanin thus formed is GHB-melanin, but sometimes also called PAP-melanin as the initial substrate is p-aminophenol and the glutamyl moiety participates in the pathway but it is removed before polymerization (Bisko et al., 2007).
Mushroom tyrosinase shows greater affinity for glutaminyl hydroxybenzene than for p-aminophenol. The characteristics, chemical structures, and biological properties of GHB-melanin from Agaricaceae are important because of their relevance in the study of mushroom browning during development and post-harvest storage (Jolivet et al., 1998).

Predominant melanin subclass produced by bacteria belongs to pyomelanins (HGA-melanin) which are produced by the oxidation of homogentisate (HGA). This pathway is a part of the tyrosine catabolism which involves transamination and formation of HGA by 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27). The absence or very low homogentisate 1,2-dioxygenase (EC 1.13.11.5) activity of this enzyme, catalyzing the rate-limiting step of L-tyrosine catabolism, allows
the accumulation of homogentisate; and a change in the catabolic route of L-tyrosine occurs, to form melanin as a sub-product (Solano, 2014).

**Fig. 2.7.** Biosynthesis pathway of Pyomelanin (HGA-melanin) (Adapted from Solano, 2014)

As in other melanin producing pathways, the rate limiting step is the oxidation of the accumulated diphenol to the corresponding quinone, which in this particular system is a p-quinone (Fig. 2.7). Subsequent redox reactions between mixed diphenols and quinones give rise to an ill-defined polymer. The final steps of this polymerization pathway are also undefined and poorly studied, but, as in other systems, a phenol oxidase or laccase can be involved in the oxidation of HGA to the corresponding p-quinone (Solano, 2014).

This form of pyomelanin is reported in several fungal species like *Aspergillus fumigatus* (Schmaler-Ripcke et al., 2009), in bacteria such as *Vibrio cholerae* (Coyne and al-Harthi, 1992); and even in humans during
alkaptonuric conditions. In some bacteria such as *Serratia marcescens* (Trias *et al.*, 1989) and *Pseudomonas* sp. (Yabuuchi and Ohyama, 1972), similar dark yellowish pyomelanin can also be formed from other \( p \)-dihydroxyphenols different from HGA.

Though many enzymes are involved in melanin biosynthesis the presence of tyrosinase is predominant in most of the biosynthetic pathways of all melanin types. So this enzyme can be considered as the principal factor which control melanogenesis (Claus and Decker, 2006) as the polymerization process of melanin is still unknown.

### 2.4 Key enzymes involved in melanogenesis

The characteristic enzymatic system related to melanin formation can be collectively termed as the phenolase system. Phenolases are mixed oxygenases involved in the incorporation of one atom of the atmospheric oxygen into the phenolic substrates (Mason *et al*., 1955). The system shows several forms in different organisms. They are always copper containing proteins which is able to oxidize phenols (monophenols, o-diphenols, and p-diphenols). In general, they do not show high substrate specificity. Usually, they oxidize aminophenols to o-diphenols, and those are further oxidized to o-quinones. Laccases show more affinity for p-diphenols, and they are involved in the synthesis of microbial melanins (Solano, 2014).

#### 2.4.1 Tyrosinase

The most common phenolase system in nature is the tyrosinase (EC 1.14.18.1) system. It is involved in melanin biosynthesis, in animals, fungi, yeasts, and bacteria. The enzyme shows high affinity towards the amino acid L-tyrosine. It catalyses two enzymatic reactions, (i) the ortho-
hydroxylation of monophenols to o-diphenols (monophenolase, cresolase activity) and (ii) the oxidation of o-diphenols to o-quinones (diphenolase, catecholase activity). These activities enable the enzyme to convert tyrosine to DOPA and then to DOPAquinone. The enzyme belongs to hemocyanin like family of proteins which reveals a conserved position of Cu-coordinating histidine residues which form the CuA and CuB domains (Burmester and Scheller, 1996).

![Fig. 2.8] Types of bacterial tyrosinases (Adapted from Fairhead and Meyer, 2012)

Bacterial tyrosinases can be divided up into five main types. Type I tyrosinase was first reported in *Streptomyces* sp. where the tyrosinase required a caddy protein (CP) for copper incorporation (Chen *et al.*, 1993). Caddy protein is also responsible for the secretion of melanin. Type II tyrosinases are similar to those in *Streptomyces* sp. in terms of size, but do not require a caddy protein, but may also be secreted. An example for this
is the *Bacillus megaterium* tyrosinase (Fishman, 2009). Type III tyrosinases are similar to fungal tyrosinases where the tyrosinase domain is followed by a C-terminal (CT) extension, which must be removed for the enzyme to be fully active, for example, *Verrucomicrobium spinosum* tyrosinase (Fairhead and Meyer, 2012). Type IV tyrosinases are smaller in size than others and are reported to be active only as homodimers, for example, tyrosinase from *Bacillus thuringiensis* (Liu et al., 2005). Type V enzyme are more similar to laccases and do not have the signature copper A and B binding motifs or the oxygen binding motifs common to tyrosinases (Fig. 2.8). The tyrosinase-like activity is suggested to be due to two extra copper binding motifs present in the N-terminus of the protein which complement the four copper binding sites typical of laccases, for example, multipotent polyphenol oxidase from *Marinomonas mediterranea* (Sanchez et al., 2001).

### 2.4.2 Catechol oxidase

The common form of phenolase in plants is the catechol oxidase (EC 1.10.3.1). The name is due to the absence of monophenol hydroxylase activity in these phenolases, and the preferred substrates are nitrogen-devoid o-diphenols, such as catechol. They are responsible for the browning of fruits and leaves during injury, as the catechols contained in the vegetal tissue are exposed to oxygen. As browning is an undesirable process in fruit handling and after harvest marketing, the inhibition of these enzymes has important biotechnological applications (Eleftherianos and Revenis, 2010).
2.4.3 Laccases

Plants and fungi contain phenolase called laccases (EC 1.10.3.2) which are generally more active on p-diphenols than on o-diphenols. They are generally involved in several processes specific to the plant or fungal species, and, only in few cases, they are just involved in melanin synthesis. The enzyme belongs to the class oxidoreductases which contain one to four copper atoms in their active site. But it does not resemble tyrosinases, they belong to the family of blue copper-containing oxidases, together with ascorbate oxidase and ceruloplasmin (Messerschmidt and Huber, 1990; Valderrama et al., 2003). Some microorganisms show both tyrosinase and laccase activities. For example, in Sinorhizobium meliloti, a plasmid-encoded tyrosinase and a laccase have been demonstrated (Mercado-Blanco and Toro, 1996; Castro-Sowinski et al., 2002). In addition to a typical tyrosinase, a ‘multipotent’ phenol oxidase with both tyrosinase and laccase activities were reported from Marinomana mediterranea. The enzyme has extra histidine-rich copper-binding domains that are very likely related to its unique properties (Sanchez et al., 2001). The phenoxazinone synthase of Streptomyces antibioticus which is involved in the biosynthesis of actinomycin is a multi-copper enzyme with laccase activity (Freeman et al., 1993). More laccases have been isolated and characterized from Streptomyces cyaneus (Arias et al., 2003), Streptomyces griseus (Endo et al., 2002), and Streptomyces lavendulae (Suzuki et al., 2003) to name a few.

2.4.4 Polyketide synthases

Polyketide synthases (PKS) catalyzes the condensation of activated primary metabolites (acetyl-CoA and malonyl-CoA) to form β-ketoacetyl
polymers linked to the enzyme by thioester bonds. The enzyme belongs to a multi domain proteins related to fatty acid synthases involved in the biosynthesis of DHN melamins. The PKS family is highly diversified and plentiful. Numerous microorganisms utilize these enzymes to produce pigments, antibiotics, toxins and other products of intermediate metabolism (Hutchinson, 2003; Snyder et al., 2003). There are only few DHN-melanin producing PKS enzymes, with PKS type I responsible for producing aromatic, not reduced polyketides like melanin (Kroken et al., 2003).

2.4.5 p-hydroxyphenylpyruvate hydroxylase

p- hydroxyphenylpyruvate hydroxylase (HPPH) (EC 1.13.11.27) is an Fe(II)-containing non-heme oxygenase that catalyzes the formation of pyomelanin or homogentisic acid (alkapton) from 4-hydroxyphenylpyruvate. This enzyme which belongs to the phenylalanine and tyrosine degradation pathway is ubiquitous among living organisms. Deregulation of this metabolic pathway can lead to the retention of toxic alkapton in the cells and tissues (Menon et al., 1991).

2.4.6 4-hydroxyphenylacetic acid 3-hydroxylase

4-hydroxyphenylacetic acid 3-hydroxylase (HPA) (EC 1.14.13.3) is a key enzyme involved in the microbial degradation of phenylalanine, tyrosine and many aromatic amines. The enzyme belongs to a separate family of hydroxylases which catalyzes the formation of allomelanin-like polymers (Gibello et al., 1997). Tyrosine is also a substrate of HPA, but (unlike tyrosinase) this enzyme does not contain copper, which does not increase its enzymatic activity.
2.5 Genetic background of melanin biosynthesis

A large number of pathways and organisms involved in melanin synthesis are discussed so far. But very less information is available about the molecular genetics underlying melanogenesis.

*Streptomyces* is the microorganism in which genetics of melanin biosynthesis is mostly revealed. In *Streptomyces antibioticus* and *Streptomyces glaucescens*, the melC operon (Chen *et al.*, 1993) consists of the tyrosinase gene (melC2) preceded by the melC1 gene encoding a conserved protein essential for the expression of melanin in a polycistronic operon. Two structural genes are encoded by melC1 in the extra chromosomal part of the genome. The first open reading frame encodes MelC1 protein, and the second gene encodes apotyrosinase (Bernan *et al.*, 1985). The MelC1 protein forms a heterodimer with tyrosinase, acting as its trans-activator (Chen *et al.*, 1993). It is demonstrated that this interaction is necessary to incorporate two copper ions crucial for the enzymatic activity of the enzyme.

In *Streptomyces avermitilis*, four melanin gene clusters are identified which can be classified into three types: (i) melanin pigment formation involving tyrosinase, (ii) hydroxyphenylpyruvate dioxygenase, and (iii) type-II PKS. Two melanin gene clusters involving tyrosinase were found in the genome. Both clusters composed of two genes, tyrosinase cofactor (MelC1) and tyrosinase (MelC2) (Fig.2.9). Other gene clusters includes one which is involved in pyomelanin biosynthesis and the genes involving melanin biosynthesis by the aromatic polyketide route (Ômura *et al.*, 2001).

Whole genome sequencing of a high-melanin-yielding *Aeromonas media* reveals that protein coincident with typical bacterial tyrosinase has
not been found in the organism. In contrast, the genome of the bacteria carries multiple genes potentially involved in melanogenesis, such as genes encoding phenylalanine 4-monoxygenase and pterin-4-alpha-carbinolamine dehydratase. Genes responsible for pyomelanin synthesis were also found which includes 4-hydroxyphenylpyruvate dioxygenase, homogentisate 1,2-dioxygenase, outer membrane lipoprotein, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase, and ABC transporter ATP-binding protein (Chai et al., 2012).  

**Fig. 2.9.** Gene clusters for melanin biosynthesis in *Streptomyces avermitilis* (1) tyrosinase gene clusters (2) hydroxyphenylpyruvate dioxygenase (3) type-II PKS (Abbreviations of gene symbols: acp, acyl carrier protein; clf, chainlength factor; cyc, cyclase; hpd, 4-hydroxyphenylpyruvate dioxygenase; hyd, hydroxylase; ks,b-ketoacyl synthase; melC1, tyrosinase cofactor; melC2, tyrosinase; omt, O-methyltransferase; reg, regulatoryprotein.) (Adapted from Ōmura et al., 2001)  

*Marinomonas mediterranea* genome has a two-cistron operon, ppoB, responsible for melanogenesis (Lopez-Serrano et al., 2004). The ppoB1 gene encodes apotyrosinase and ppoB2 a chaperone incorporating copper ions into the active site of the enzyme. While ppoB2 product does not reveal any significant similarity with MelC1 of *Streptomyces antibioticus*,
M. mediterranea also contains another melanogenetic enzyme — laccase (ppoA gene) (Sanchez et al., 2001). Regulation of ppoA and ppoB expression proves the evolutionary importance and strongly adaptive character of melanin production for this species.

Shewanella colwelliana with Mel+ phenotype, a monocistronic, 1.3 kb melA operon has been identified. The melA encoded 39.5 kDa protein was identified as HPPD found also in Vibrio cholerae, Hypomonas sp. Aeromonas media, and Pseudomonas sp. (Fuqua et al., 1991). The Klebsiella pneumoniae 4-phenyloacetic acid hydroxylase activity is determined by two proteins encoded by separate cistrons — hpaA and hpaH, with hpaA encoding a flavoprotein with the enzymatic activity, while the hpaH product is a helper protein, necessary to achieve a high catalytic efficiency of HPA (Gibello et al., 1997).

2.6 Biological roles of melanin

2.6.1 Photoprotective role of melanin

The photo protective nature of melanin, especially eumelanin, is determined from its ability to serve as a physical barrier that scatters UV radiation (UVR), and act as an absorbent filter that reduces penetration of UVR. The efficacy of melanin as a sunscreen was assumed to be about 1.5-2.0 sun protective factors (SPF); possibly as high as 4 SPF, implying that melanin absorbs 50% to 75% of UVR. An SPF of 2 means the doubling of protection of the skin against sunburn (Brenner and Hearing, 2008).

2.6.2 Radio protective nature of melanin

Melanin absorbs almost all types of radiations; it is a good protector for very stressful conditions, as evidenced by the exposure to gamma
radiation. For instance, in Chernobyl, black highly melanized fungal species have responded to the deathly ionizing radiation with their enhanced growth. Those fungi are able to adapt morphologically to extreme conditions due to the excessive eumelanin production (Dadachova and Casadevall, 2008). This protective function of eumelanin is similar to higher animals, as, for example, bird population with pheomelanin in feathers which have declined due to the exposure to the radiation as results of the poorer protective properties of the latter type of melanin (Galván et al., 2011).

2.6.3 Melanin as electron acceptor in bacteria

Melanin contains various groups that are able to donate and accept electrons. Thus it can act as a final acceptor in the electron exchange with insoluble compounds of iron (Menter and Willis, 1997). A Shewanella algae producing pyomelanin, reduced it simultaneously with the oxidation of gaseous hydrogen (Turick et al., 2002). Having accepted numerous electrons, such ‘reduced’ melanin serves the bacteria as a reductor of insoluble ferric (III) oxides to the ferrous (II) state. As S. algae are unable to carry out fermentation, its survival in the conditions of variable oxygen concentration strongly depends on the presence of appropriate electron acceptors (Turick et al., 2002).

Another melanogenic bacteria Legionella pneumophila, the causative agent of Legionnaires' disease is known to produce pyomelanin pigment which confers ferric reductase activity (Chatfield and Cianciotto, 2007).
2.6.4 Melanin in nitrogen fixation by bacteria

Soil bacterium *Azotobacter chroococcum* has an active polyphenol oxidase which can produce melanin from catechol. This microorganism produces particularly large amounts of melanin when cultured under aerobic conditions. This is easy to explain, as oxygen is one of the substrates for polyphenol oxidase, but it turns out that this process is intensified in the absence of a nitrogen source in the medium. Although the intensity of melanogenesis does not seem to be directly correlated with the activity of nitrogenase (the key enzyme of atmospheric nitrogen fixation), it is possible that *Azotobacter* employs melanogenesis to enhance utilization of oxygen and to maintain reducing conditions necessary for binding atmospheric nitrogen (Shivprasad and Page, 1989).

2.6.5 Melanin as a virulence factor in bacteria

Pathogenic bacteria are known to produce considerable amount of melanin. Free-living strains of *Vibrio cholerae* are usually amelanotic or they produce pyomelanin (Kotob *et al.*, 1995). Meanwhile, under stress (hyperthermia, hyperosmotic medium, starvation) they induce synthesis of eumelanin. Production of pyomelanin has been reported in many species of bacteria, like *Pseudomonas aeruginosa*, *Hypomonas* sp., *Shewanella colwelliana*, some of which consist of both free-living (marine) and pathogenic strains (Shivprasad and Page, 1989). *Legionella pneumophila* may serve as another example of the latter group. It causes the so called Legionnaire’s disease (LD) and Pontiac fever (Wintemeyer *et al.*, 1994; Plonka and Grabacka, 2006). One of the factors responsible for the pathogenicity of this microorganism is legiolysin (Lly), a protein responsible for the fluorescent properties, pigment production and
hemolytic ability of the pathogen (Wintermeyer et al., 1991). It reveals over 80% similarity with HPPD, crucial for melanin production in *Shewanella colwelliana* and in *Pseudomonas* sp. (Fuqua et al., 1991; Fuqua and Weiner, 1993; Wintermeyer et al., 1994; Steinert et al., 2001).

This key enzyme for pyomelanogenesis confers hemolytic activity as well (Croxatto et al., 2002). The expression of HPPD is regulated in a similar way to other genes responsible for the virulence factors, such as production of metalloproteinases and extracellular lipopolysaccharides, used to create biofilms and necessary for adhesion to biological surfaces like fish skin (Croxatto et al., 2002).

The pathogenic bacterium *Burkholderia cepacia* serves as an example of how melanin production increases virulence. This microorganism causes dangerous lung infections, which often develop into sepsis, mainly due to the presence of lipopolysaccharide (LPS) which strongly enhance production and release of proinflammatory cytokines (Zughaier et al., 1999). Although LPS does not trigger an oxygen burst directly, it stimulates the immunological system to an accelerated oxidative response to other stimuli. Melanin isolated from *B. cepacia* reveals a dose-dependent ability to sweep $O_2^-$ produced by leukocytes during oxygen burst. However, melanin does not influence the release or kinetics of the production of reactive oxygen species (ROS) (Zughaier et al., 1999). The ability to remove superoxide anion allows *B. cepacia* to survive phagocytosis, so the host phagocytes are not able to eliminate the pathogen.

In *Proteus mirabilis*, an important cause of community-acquired infections of the urinary tract, tyrosinase was identified as the enzyme responsible for melanization. As it has been shown in this species, melanin
decreases the level of ROS, which probably makes the pathogen more resistant to the oxygen burst connected with the immunological response of the host (Agodi et al., 1996).

*Klebsiella pneumoniae*, responsible for a particularly dangerous form of pneumonia, produces melanin from 4-hydroxyphenylacetic acid using HPA, which is an unusual pathway of melanogenesis (Gibello et al., 1997). However, the importance of HPA and melanin for the virulence of this pathogen remains to be determined.

### 2.6.6 Enhancing antibiotic resistance

Enhanced production of melanin can increase the resistance of pathogenic microbes through binding with potential antibiotics such as tetracycline and vancomycin (Ikeda et al., 2003; Lin et al., 2005; Nosanchuk and Casadevall, 2006).

### 2.6.7 Metal binding ability of melanin

Melanins are able to bind heavy metals found commonly in the environment (Zunino and Martin, 1977; Rizzo et al., 1992; Fogarty and Tobin, 1996). The carboxyl, phenolic, hydroxyl, and amine groups of melanin serve as the numerous potential binding sites for metal ions (Fogarty and Tobin, 1996). Melanized *Cryptococcus neoformans* are more resistant to silver nitrate, a compound highly toxic to bacteria and fungi, than non-melanized ones (Garcia-Rivera and Casadevall, 2001).

### 2.6.8 Melanin as a thermoregulatory agent

Effective absorption of UV rays by melanin results in dissipating 90% of the absorbed energy as heat. This absorbed energy and conversion of
Chapter-2

heat can contribute to thermoregulation of melanized organisms, which is especially important for cold-blooded animals (Goodman and Bercovich, 2008)

2.7 Applications of melanin

2.7.1 Cosmetic formulations

Melanin is used as an additive in sun screen lotions and creams because of their immense UV absorptive and radical scavenging properties. These melanin containing cosmetics were claimed to protect the cells from high energy blue/violet visible light that may induce premature ageing. Dark color of melanin can be converted to lighter one by chemical conversions (without losing their peculiar properties) which minimizes the undesirable aesthetic impact when added to light colored cosmetics. Excellent wound healing property has been shown by a formulation supplemented with melanin. A melanin-based composition has proved to strengthen the hair. It is also used in photo protective eye glasses too. (Riley, 1997; Nosanchuk and Casadevall, 2006).

2.7.2 Preventing UV mediated degradation of bio-insecticides

The endotoxin produced by *Bacillus thuringiensis* which having bio-insecticide potential can be easily inactivated by solar radiation in nature. Ultra violet region of the sunlight, especially the UV-A (400 nm to 320 nm) and UV-B (320 nm to 290 nm) are responsible for the inactivation of microbial insecticides (Pusztai *et al.*, 1991). The melanin produced from *Aeromonas media* and *Bacillus cereus* supplementation in bio-insecticidal preparations results in the protection of the insecticidal crystal proteins
from degradation caused by UV radiation (Wan et al., 2007; Zhang et al., 2007).

2.7.3 Melanin as vaccine against melanoma

The melanin can be used as vaccine against melanoma (Human melanocyte cancer). Melanoma is a malignant tumour of melanocytes which are found predominantly in skin, bowel and eyes. The lymphocytes of melanoma patients can be restimulated in vitro with autologous tumour cells to generate antitumor cytolytic T lymphocytes (CTL). Such antitumor CTLs are shown to recognize melanin as an antigen. Also when blood lymphocytes of melanoma patients are stimulated in vitro with tumour cells of the same patient, one often observes the proliferation of T lymphocytes that exert cytolytic activity on the autologous melanoma cells (Brichard et al., 1993). The antibody response to fungal melanin showed that melanin can be immunogenic, and the humoral immune response is T cell independent. The melanin antigen may therefore constitute a useful target for specific immunotherapy of melanoma. This clearly indicates that melanin can be used as vaccine against the melanoma (Nosanchuk et al., 1998).

2.7.4 Bioremediation of radioactive waste

Bacterial melanin production was shown to enhance uranium immobilization in contaminated soil. In order to develop in situ uranium bio-immobilization technology, one-time addition of tyrosine to soil was exploited. The indigenous microbes produced pyomelanin, which in turn resulted in uranium immobilization. Thus melanin producing bacteria can be used effectively in the bioremediation of radioactive wastes (Turick et al., 2008).
Chapter 2

2.7.5 Melanin biosynthetic genes as reporter genes

The genes responsible for the melanin biosynthesis in bacteria can be used as reporter gene to screen the recombination in host bacteria as an alternative to conventional blue-white screening method in *E. coli*. The production of melanin on tyrosine agar indicates the wild type while the absence of melanin confirms the recombination. (Tseng *et al*., 1990; Adham *et al*., 2003).

2.7.6 Melanin as anti-venom

Melanin extracted from black tea has shown anti venin activity in mice. Melanin was injected immediately after the venom administration in dose of 3 mg per mouse in the same place of venom injection. Melanin demonstrated neutralization effect against all venoms tested. The greatest antivenin effect was found against Japanese mamushi snake venom. Low toxicity of melanin in combination with its antagonistic activity against different venoms may allow effective life-saving treatment against snakebites (Hung *et al*., 2004).

2.7.7 Melanin inhibits Human Immunodeficiency Virus (HIV) replication

Montefiori and Zhou (1991) reported that synthetic soluble melanins can inhibit replication of human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) in two human lymphoblastoid cell lines (MT-2 and H9) and in phytohemagglutinin-stimulated human T cells. Effective concentrations of 0.15-10 μg/mL had no cell toxicity. Melanin prevented the infection by cell-free virus and interfered with HIV-induced syncytium formation and cytopathic effects when fusion-susceptible, uninfected cells
were mixed with chronically infected cells. Melanin also blocked the HIV-1 envelope surface glycoprotein, and T cell specific monoclonal antibody leu-3a (CD4), but not leu-5b (CD2), from binding to the surface of MT-2 cells.

2.7.8 Antioxidant activity of melanin

It was found that the scavenging or chelating properties of melanin are mainly involved in their antioxidant activity. These properties can be utilized at the stage of initiating chain reactions by trapping of transitional metals or inactivating superoxide radicals. However, free-radical scavengers are not effective in preventing propagation of chain reactions due to their inability to react with peroxyl radicals (Denisov and Khudyakov, 1987). Nevertheless, the strong antioxidant activity of melanin indicates that it is probably able to terminate chain propagation. This possibility has not been investigated. Melanin from *Aspergillus nidulans* (Goncalves and Pombeiro, 2005), *Pleurotus cystidiosus* (Selvakumar et al., 2008), tea (Hung et al., 2002b), *Cryptococcus neoformans* (Jacobson and Tinnell, 1993) and *Ophiocordyceps sinensis* (Dong and Yao, 2012) has been proved to be powerful antioxidant.

2.7.9 Antimicrobial property of melanin

Melanin pigment from *Streptomyces* sp. had shown antibacterial activity against *Escherichia coli*, *Lactobacillus vulgaris* (Vasanthabharathi et al., 2011), *Pseudomonas aeruginosa*, *Vibrio parahaemolytics* (Sivaperumal et al., 2014a) and ornamental fish pathogens like *Vibrio* sp. and *Aeromonas* sp. (Sivaperumal et al., 2014b).
2.7.10 Effect of bacterial melanin on brain plasticity

Water soluble bacterial melanin injected intramuscularly on the day after the unilateral ablation of the sensorimotor cortex was demonstrated in rats using the example of the recovery of operant conditioned reflexes (OCR) and movement of a paralyzed hind leg. The injections of low concentrations of melanin results in the clinical recovery after CNS lesion possibly entail a large number of trophic mechanisms that provide regeneration. It was shown that these cells were undamaged in animals treated with melanin (Gevorkyan et al., 2008).

Petrosyan et al. (2014) reported that bacterial melanin increases vascularization, dilates the capillaries in nervous tissue and stimulates the process of sprouting which in turn favours recovery after motor tract and peripheral nerve damage. Bacterial melanin stimulates regeneration and microcirculation in Substantia Nigra pars compacta (SNc) after unilateral destruction. Bacterial melanin could be a potential biologic medical product for the treatment of Parkinson's disease (PD). Biological compensatory action of bacterial melanin (positive allosteric modulator) and its immuno-modulatory effects can ameliorate manifestations of the neurodegenerative disorder. A pharmacokinetic study with isotope labelling has confirmed the ability of bacterial melanin to cross the blood-brain-barrier. The study with radiolabeled melanin confirmed that bacterial melanin is eliminated through liver and kidneys and has a favourable pharmacokinetic profile for use as a therapeutic and neuro-protective agent (Petrosyan and Hovsepyan, 2014).
2.7.11 Melanin in nanoparticle synthesis

Apte et al (2013) had employed L-DOPA-melanin for synthesis of silver and gold nanostructures. The biopolymer act as a reducing and stabilizing agent for the synthesis of silver and gold nanostructures. The induced pigment reduced silver nitrate and chloroauric acid to silver and gold nanostructures, respectively. The silver nanoparticles were smaller in size (7 nm) and displayed excellent anti-fungal properties towards an *Aspergillus* sp. isolated from a wall surface. An application of these nanoparticles as effective paint-additives has been demonstrated.

2.7.12 Melanin as Magnetic Resonance Imaging (MRI) contrast enhancer

In Gastrointestinal imaging, the oral administration of a complex containing melanin from tea leaves (*Thea sinensis* Linn.) at a concentration of 0.1 mM and MRI contrast agent gadolinium (Gd) provides essential enhancement to longitudinal relaxation times (T(1))-weighted spin echo image. The required contrast and delineation of the stomach wall demonstrated uniform enhancement of MRI with melanin complex (Hung et al., 2002a).

2.7.13 In waste water treatment plants

Heavy water is a class of highly contaminated waste water, even if the concentration is small, it can cause harm, and the toxicity is long-term sustainability. Melanin secreted by *Aureobasidium pullulans* was proved to be an efficient adsorber of Chromium (VI) (Cr$^{6+}$) ions present in waste water (Yu et al., 2011). Melanin could even eliminate Lead and Copper.
contaminants (Sono et al., 2012) from waste water making it an effective innovation in water purification systems.

2.7.14 Anti-inflammatory activity of melanin

Grape melanin showed potent inhibitory effect on adjuvant induced disease (AID) in rat, suppressing significantly the primary inflammation and almost totally the secondary lesions of arthritis. The serum proinflammatory cytokines IL-1, IL-6, TNF-α and the serum globulin fraction were elevated in AID rats, which were normalised by melanin treatment. (Avramidis et al., 1998).

2.7.15 Anti-cancer activity of melanin

Imbalanced expression of cytokines has been implicated in the progression of many diseases like cancers. Herbal melanin extracted from Nigella sativa L. modulates the expression of TNF-α, IL-6 and VEGF by the human monocytes, total peripheral blood mononuclear cells (PBMC) and THP-1 cell line. This finding suggests that N. sativa melanins may have an immunoregulatory activity that could contribute to therapeutic interventions relating to diseases associated with imbalanced cytokine production and cancer (El-Obeid et al., 2006).

2.7.16 As photon harvesting units in solar cells

Ruthenium charge transfer complexes are currently used as the photon harvesting components in “dye sensitized solar cell” (DSSCs). They produce a relatively broad band UV and visible response, but have long term stability problems and are expensive to manufacture. Melanins can be suitable replacements for the ruthenium complexes due to its peculiar properties such as strong broad band absorption, chemically and
photochemically very stable, can be cheaply and easily synthesized, and are also bio-available and bio-compatible. (Meredith et al., 2005).

2.7.17 In Sodium ion batteries

Aqueous sodium-ion charge storage devices combined with biocompatible electrodes are ideal components to power next-generation biodegradable electronics. Melanins of natural (derived from *Sepia officinalis*) and synthetic origin are evaluated as anode materials in aqueous sodium-ion storage devices. Na\(^+\) loaded melanin anodes exhibit specific capacities of 30.4 ± 1.6 mAhg\(^{-1}\). Full cells composed of natural melanin anodes and \(\lambda\)-MnO\(_2\) cathodes exhibit an initial potential of 1.03 ± 0.06 V with a maximum specific capacity of 16.1 ± 0.8 mAhg\(^{-1}\). Natural melanin anodes exhibit higher specific capacities compared with synthetic melanins due to a combination of beneficial chemical, electrical, and physical properties exhibited by the former. Taken together, these results suggest that melanin pigments may serve as a naturally occurring biologically derived charge storage material to power certain types of medical devices. (Kim et al., 2013).

2.8 Melanin producing bacteria

Pathogenicity of bacteria is generally associated with increased production of melanin. The melanin biosynthesis via homogentisic acid was first reported in *Vibrio cholerae, Hyphomonas* species and *Shewanella colwelliana* (Kotob et al., 1995). The synthesis of melanin and its characterization such as solubility, radical scavenging property was initially studied in *Proteus mirabilis* (Agodi et al., 1996). A novel marine bacterium *Alteromonas* strain MMB-1, was isolated from the Mediterranean Sea and its melanin producing ability was studied using L-tyrosine as a precursor
previously (Solano et al., 1997). The melanin pigment from *Burkholderia cepacia* was reported to escape monocyte respiratory burst activity by scavenging superoxide anion (Zughaier et al., 1999).

The extra cellular melanin from *Shewanella algae* BrY was reported to serve as the sole terminal electron acceptor. *Shewanella* melanin reduces insoluble hydrous ferric oxide in the absence of bacteria, and melanin was proved as a soluble Fe (III)-reducing compound (Turick et al., 2002).

Melanin production was studied on UV-resistant mutant of *Bacillus thuringiensis* subsp. kurstaki and its UV-protection ability for insecticidal crystals was tested (Saxena et al., 2002). The thermo tolerant strains of *Bacillus thuringiensis* were also reported for melanin production (Ruan et al., 2004). A wild strain of *Bacillus thuringiensis* subsp. *dendrolimus* L-7601 produced melanin efficiently and the UV-protection efficacy of melanin on insecticide formulations following UV irradiation were also investigated (Chen et al., 2004). A hexa-hydroxy-perylene-quinone melanin was produced earlier from *Streptomyces griseus* through polyketide synthase (PKS) catalyzed mechanism was explored (Funa et al., 2005).

The purification of water-soluble melanin was reported for the first time from *Bacillus thuringiensis* subsp. galleriae strain K1 which was carried out using different sorbants like activated charcoal, CM cellulose, silica gel C-25I and Dowex (Aghajanyan et al., 2005). The induction of melanin synthesis in *Cryptococcus neoformans* by *Klebsiella aerogenes* was observed, in which colorless colonies of *C. neoformans* grown near to *K. aerogenes* colonies were shown to produce melanin. This study concluded that precursor for melanin synthesis was produced by *C.*
**Review of Literature**

*neoformans* (Frases et al., 2006). The optimization of physico-chemical parameters for the melanin production was studied formerly in *E. coli* W3110 (Lagunas-Munoz et al., 2006). The melanin biosynthesis was also reported earlier from *Streptomyces* species (Dastager et al., 2006). The synthesis of pyomelanin was reported previously from *Burkholderia cepacia* C5424 by using a homogentisate intermediate and the antioxidant properties of melanin were also studied in this strain (Keith et al., 2007).

The high level of melanin was produced earlier from novel species of *Aeromonas media* and offers effective photo protection of a commercial bioinsecticide against UV radiation (Wan et al., 2007). The production of water-soluble melanin was reported recently from recombinant deep Sea sediment meta-genomic clone of *E. coli* (Huang et al., 2009).

The production of melanin was reported recently from *Klebsiella* sp. GSK (Sajjan et al., 2010).

An economical fruit waste extract was used for melanin production by a garden soil isolate, *Bacillus safensis*. The melanin produced was confirmed by UV–visible spectroscopy, FTIR and XRD analysis. *B. safensis* melanin had shown significant photo-protective, radical scavenging and metal chelating activities. (Tarangini and Mishra, 2014).

A first time report on melanin production by psychrotolerant *Bacillus weihenstephanensis* came from North-eastern Poland. Physicochemical properties of the pigment and the mechanism of its synthesis in relation to *B. weihenstephanensis* genotypic and phenotypic characteristics was evaluated. Several biochemical tests showed that melanin biosynthesis by *B. weihenstephanensis* was associated with laccase activity. The
presence of the gene encoding laccase was confirmed by the next generation whole genome sequencing of one \textit{B. weihenstephanensis} strain (Drewnowska \textit{et al.}, 2015).

Melanins produced by different bacteria, their type and the yield are depicted in table 2.1 below.

<table>
<thead>
<tr>
<th>Melanin producer</th>
<th>Type of melanin</th>
<th>Concentration</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Streptomyces lusitanus}</td>
<td>-</td>
<td>4.2 g/L</td>
<td>Madhusudhan \textit{et al.}, 2014</td>
</tr>
<tr>
<td>\textit{Streptomyces sp.}</td>
<td>-</td>
<td>21.13 g/L</td>
<td>Vasanthabharathi \textit{et al.}, 2011</td>
</tr>
<tr>
<td>\textit{Brevundimonas sp.}</td>
<td>DOPA melanin</td>
<td>6.811 g/L</td>
<td>Surwase \textit{et al.}, 2013</td>
</tr>
<tr>
<td>\textit{Klebsiella sp.}</td>
<td>DOPA melanin</td>
<td>0.13 g/L</td>
<td>Sajjan \textit{et al.}, 2010</td>
</tr>
<tr>
<td>\textit{Pseudomonas sp.}</td>
<td>DOPA melanin</td>
<td>5.35g/L</td>
<td>Tarangini and Mishra, 2013</td>
</tr>
<tr>
<td>\textit{Actinoalloteichus sp.}</td>
<td>-</td>
<td>85.37 μg/L</td>
<td>Manivasagan \textit{et al.}, 2013</td>
</tr>
<tr>
<td>\textit{Streptomyces kathirae}</td>
<td>-</td>
<td>13.7 g/L</td>
<td>Guo \textit{et al.}, 2014a</td>
</tr>
<tr>
<td>\textit{Shewanella algae}</td>
<td>Pyomelanin</td>
<td>0.173 g/L</td>
<td>Turick \textit{et al.}, 2002</td>
</tr>
<tr>
<td>\textit{Nocardiopsis alba}</td>
<td>-</td>
<td>3.4 g/L</td>
<td>Kiran \textit{et al.}, 2014</td>
</tr>
<tr>
<td>\textit{Bacillus safensis}</td>
<td>-</td>
<td>6.96 g/L</td>
<td>Tarangini and Mishra, 2014</td>
</tr>
</tbody>
</table>

Melanin from \textit{Streptomyces sp.} MVCS13 has shown potential antibacterial activity against ornamental fish pathogens \textit{Vibrio sp.} FPO5 (15±0.01 mm) followed by \textit{Aeromonas sp.} FPO6 (12±0.02 mm) which was
isolated from *Carassius auratus* infected fish. The minimum inhibitory concentration (MIC) ranges were observed between 18±0.01 and 27±0.03 μg/mL. The study suggests that *Streptomyces* sp. melanin can be used as an effective anti-bacterial agent for ornamental fish culture (Sivaperumal *et al.*, 2014b).

Bacterial melanins are advantageous over melanin from animals and plants. Bacteria do not have the problems of seasonal variations and are selected arsenals as they modify themselves according to the medium and conditions provided to them. Targeting melanogenesis in microbes may help to discover antimicrobial drugs. For example, melanins produced by *Cryptococcus neoformans* and *Burkholderia cepacia* offer virulence and contribute to the resistance of these pathogenic bacteria towards antibiotics. The melanin can even remove radioactive wastes like uranium confirms its metal chelating ability. Therefore, all these properties of bacterial melanin make them unique and are widely used in cosmetic, sunscreen protection creams, eye glasses, pharmaceuticals, and food industries (Tarangini and Mishra, 2014).