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# Computational model for pathway reconstruction to unravel the evolutionary significance of melanin synthesis

**Sudha Singh[1], Anvita Gupta Malhotra[1], Ajay Pandey[2] & Khushhali M Pandey[1]***

[1]Department of Chemical Engineering and Biotechnology, MANIT, Bhopal (M.P.) - 462051; [2]Department of Applied Mechanics, MANIT, Bhopal (M.P.) – 462051; Khushhali M Pandey - E-mail: menaria.khushhali@gmail.com; *Corresponding author

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**Abstract:**
Melanogenesis is a complex multistep process of high molecular weight melanins production by hydroxylation and polymerization of polyphenols. Melanins have a wide range of applications other than being a sun - protection pigment. Melanogenesis pathway exists from prokaryotes to eukaryotes. It has evolved over years owing to the fact that the melanin pigment has different roles in diverse taxa of organisms. Melanin plays a pivotal role in the existence of certain bacteria and fungi whereas in higher organisms it is a measure of protection against the harmful radiation. We have done a detailed study on various pathways known for melanin synthesis across species. It was divulged that melanin production is not restricted to tyrosine but there are other secondary metabolites that synthesize melanin in lower organisms. Furthermore the phylogenetic study of these paths was done to understand their molecular and cellular development. It has revealed that the melanin synthesis paths have co-evolved in several groups of organisms. In this study, we also introduce a method for the comparative analysis of a metabolic pathway to study its evolution based on similarity between enzymatic reactions.

**Keywords:** Pathway alignment, Melanogenesis, Tyrosinase, PPO, Laccase, Phylogenetic study, Melanogenesis.

**Background:**
A metabolic pathway represents a topical description of a network metabolites and enzymes. These enzymes are coded by genes. Thus the advent of post-genomic era has enhanced our understanding on the function and importance of the enzymes; further several genome-scale efforts are underway to reconstruct metabolic networks for a variety of organisms such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Homo sapiens*. The mammon of information pertaining to the biochemical reactions that take place within a cell and the enzymes responsible to catalyze these reactions are being stored in several online public databases like KEGG **[1]**, Eco-Cyc **[2]**, etc. These repositories collect and maintain information about complex cellular processes, such as metabolism, signal transduction and cell cycle by storing the corresponding networks of interacting molecules in digital forms, often as graphical pathway diagrams.

There has been exponential increase in availability of pathway information. This has enabled the cross species comparison at the functional level. As the basis for such studies, pathway alignment has been proven to be an effective technique that provides a clear indication of specific differences between pathways **[3].**

Pathway alignments is performed at two levels; firstly between the enzymes that participate in the aligned pathways, and secondly, the orientation between their topologies **[4].** Comparing analogous pathways reveals intricate insights into individual functional differences among species. For implementing this study, the pathway under study from

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different organism should be brought under unified framework, Further; an appropriate strategy needs to be built to study their co-evolution.

Comparative analysis of metabolic pathways in different genomes yields important information on their evolution, on pharmacological targets and on biotechnological applications. Sequence alignment is a well-established tool for investigating and comparing proteins from different species and for identifying characteristic gaps, insertions and dissimilarities. The availability of full genomic sequences and the increasing body of biochemical data open up higher-order possibilities for comparative analysis **[5].** The alignment of biochemical pathways from different species is an important step towards a more global comparison from a physiological viewpoint **[6, 7].**

Here we present a computational approach for pathway reconstruction and studying its evolution. This approach can be used for building phylogenies and for pointing out specific differences that can then be analyzed in depth. Also, this approach can be used for detecting co-evolutionary relationships between metabolic pathways.

For the current study, melanogenesis pathway is taken as example and the study is carried out taking Mammals, Plants, Fungi, Bacteria and Archea as representative taxa. Melanogenesis is a pathway for melanin synthesis. Melanin polymers are high molecular weight formed by oxidative polymerization of phenolic or indolic compounds. These reactions are catalysed by copper based enzymes **[8, 9].** Production of melanin is one of the most universal phenomena, but at the same time there exist enigmatic adaptations of living organisms to the variable environmental conditions **[10].** In a number of fungi, pigments related or identical to melanin are produced which are either located on cell walls or exist as extracellular polymers. Melanins have very diverse roles and functions in various organisms. The role of melanin in human is photo protection **[11],** whereas in plants they are essential for pigmentation and are important factors in browning reaction. For pathogens and herbivores melanin production is related to wound healing, stress and primary immune response **[12].** Melanized microorganisms inhabit some remarkably extreme environments including high altitude, Arctic and Antarctic regions. Melanins also protect microorganisms against cell damage by solar UV radiation or generation of reactive oxygen species. Further it also guards from high temperature, chemicals (e.g. heavy metals and oxidizing agents) and biochemical (e.g., host defences against invading microbes) stresses. Therefore, in many pathogenic microbes (for example, in *Cryptococcus neoformans*, a fungus) melanins appear to play important roles in virulence and pathogenicity by protecting the microbe against immune responses of its host.

The presence of various kinds of melanins in representatives of almost every large taxon suggests an evolutionary importance of melanogenesis. The use of melanin pigment differ in different taxa, hence the evolution of this pathway can be described in correlation with the role of this pigment in various organism. The phylogenetic analysis of melanogenesis pathway was performed by using alignment tools for metabolic pathway's enzymes sequences. Phylogenetic alignment study for analysing evolutionary relationship among groups of organisms was done with the help of Clustal W and Phylip tools. This study of pathway evolution gives an overview on the application of the melanin in different taxa.

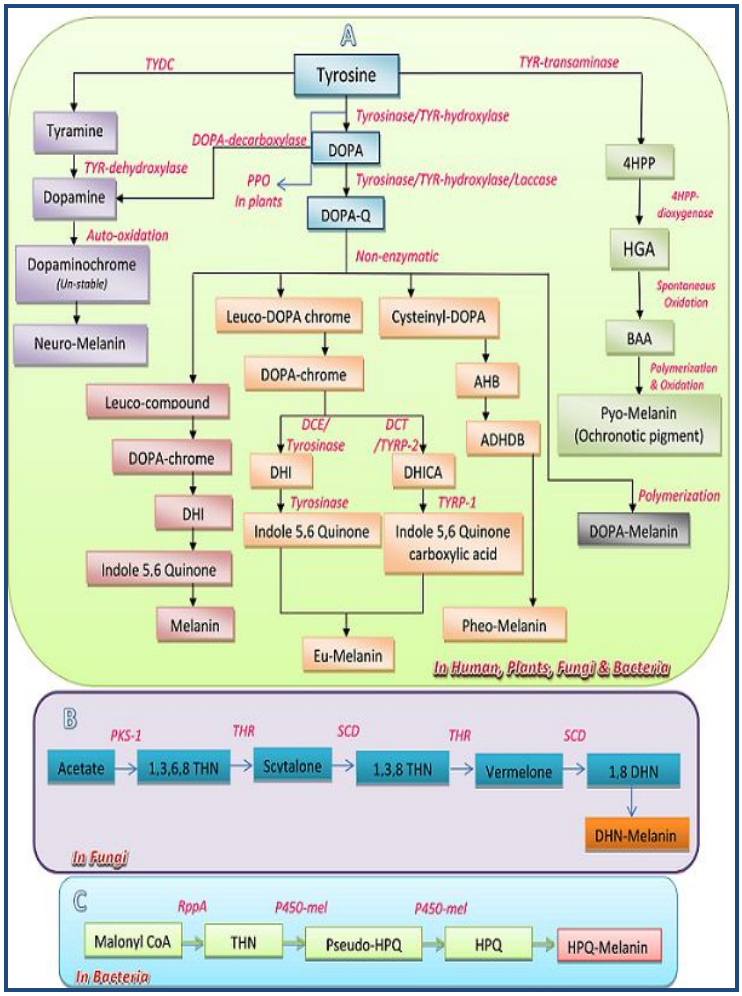


**Figure 1: (A)** represents the melanin synthesis in human, plants, fungi and bacteria starting from tyrosine whereas; **(B & C)** shows melanogenesis from secondary metabolites in fungi and bacteria respectively.

**Methodology:**

There is plenty of information available pertaining to melanin synthesis in different species. The first and the most important step for the study was to collate this data and reconstruct a comprehensive pathway incorporating variations from different taxa. Further the evolution of the melanogenesis path was studied under the following heads: (1) Reconstruction of the pathway; (2) Collection of enzyme details; (3) Phylogenetic study for each enzyme involved in the process; (4) Pathway evolution.

***Reconstruction of pathway***

The term "melanin" originates from *melanos*- a Greek word for black. Melanin is a high molecular weight pigment, ubiquitous in nature, with a variety of biological functions **[13].** The melanin production can begin from either L-tyrosine or secondary metabolite. Melanogenesis begins with the production of DOPA-Quinone, a highly reactive o-Quinone. Path from tyrosine to DOPA-Quinone is common among mammals, plants and some fungi. However these first two steps


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are catalyzed by enzyme tyrosinase in case of mammals and Polyphenol oxidase (PPO) enzyme in plants and fungi.

The most commonly available melanogenesis pathway proceeds from the amino acid L-tyrosine though a series of enzymatic and chemical steps initiated by tyrosine hydroxylation to yield DOPA and DOPA oxidation to DOPA-Quinone. Both the reactions are catalyzed by tyrosinase (monophenol monooxygenase, EC 1.14.18.1, a melanocyte-specific copper-containing glycoprotein) in mammals and PPO in plants **[14].** In the absence of low molecular weight thiolic compounds, DOPA-Quinone cyclizes rapidly and is converted to DOPA-chrome. This latter compound is a relatively unstable intermediate that undergoes spontaneous decarboxylation to yield 5,6-dihydroxyindole (DHI) which, in turn, can be oxidized either spontaneously or enzymatically by tyrosinase, ultimately leading to the formation of the melanin polymer. This is the common Mason-Raper-derived scheme of melanogenesis **(Figure 1A).** However, it has recently been proved that mammalian melanocytes contain a second melanogenic enzyme, termed DOPA Chrome Tautomerase (DCT) (EC 5.3.3.12, that can efficiently catalyze the tautomerization of DOPA-chrome to the highly stable carboxylated product DHI-2-Carboxylic Acid (DHICA). At this level, the synthesis of Eu-Melanin can be carried out according to two pathways. DHI is oxidized, under the action of tyrosinase or of a peroxidase, to indole-5,6-quinone, while DHICA, under the action of TYRP-1, gives 5,6-dihydroindole-2-carboxylic acid. The indole-5,6-quinone and the 5,6-dihydroindole-2-carboxylic acid polymerize so as to form melanochromes and then Eu-Melanin. In mammals this process occurs in highly specialized cells called melanocytes that are present in the bottom layer (the stratum basale) of the skin's epidermis **[15].**

Pheo-Melanin synthesis proceeds in three distinctive steps. The initial step is the production of sulfur compounds (Cysteinyl-DOPA) by the rapid addition of glutathione and of cysteine to DOPA-Quinone, which continues as long as cysteine is present. The second step is the oxidation of Cysteinyl-DOPA to alanylhydroxybenzothiazine and then to Pheo-Melanin **(Figure 1A).** Pheo-Melanin is orange-yellow melanin whereas Eu-Melanin is darkbrown in color. The ratio of Eu- to Pheo-Melanin is determined by the tyrosinase activity and cysteine concentration **[16].**

There are alternative pathways for melanin production present in fungi and bacteria beginning from tyrosine. In bacteria, tyramine (4-hydroxyphenethylamine) is produced by tyrosine decarboxylation. Further, tyramine is converted to dopamine which is then auto-oxidised to highly unstable dopaminochrome and finally to Neuro-melanin **[17].** Another path occurs in fungi and some bacteria wherein phenylalanine forms tyrosine which is further utilised for catabolism to acetoacetic & fumaric acids or biosynthesis of melanin and ochronotic pigment. This ochronotic pigment or Pyo-Melanin is produced by degradation of L-tyrosine to HGA that can polymerize to Pyo-Melanin **(Figure 1A) [18, 19].**

Many fungi are able to synthesize black or brown pigments derived from L-tyrosine via DOPA. The DOPA-Melanin pathway, in which tyrosinases or laccases hydroxylate tyrosine via DOPA to DOPA-Quinone, which then auto-oxidizes and polymerizes, is the best-characterized melanization pathway from L-tyrosine. However, brown pigments may also be produced from L-tyrosine via a pathway involving the accumulation and auto-oxidation of intermediates of tyrosine catabolism. For instance, Pyo-Melanins are synthesized from tyrosine through *p*-hydroxyphenylpyruvate (HPP) and homogentisic acid (HGA). Pyo-Melanin and Alkapto-Melanin are merely different designations for the same pigment. However, the pigment produced by microbes is often referred to as Pyo-Melanin, and the pigment produced by humans is called Alkapto-Melanin. The term Pyo-Melanin was first introduced by Yabuuchi and Ohyama, who described a water soluble brown pigment produced by the bacterium *Pseudomonas aeruginosa* **[20].**

All brown and black fungi can also synthesize their pigments through the dihydroxynaphthalene (DHN)-Melanin pathway, in which hydroxynaphthalene (HN) and tetralone polyketides are the common intermediates. DHN-Melanin biosynthesis starts with polyketide synthase using acetate as a precursor **(Figure 1).** An HN reductase then converts 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN) to scytalone. Dehydration of scytalone forms 1,3,8-trihydroxynaphthalene (1,3,8-THN), which is converted to 1,8-DHN after an additional reduction and dehydration step. Finally, oxidative polymerization of 1,8-DHN gives the end product, DHN(1,8-dihydroxynaphthalene)-Melanin **(Figure 1B) [21].**

In bacteria, HPQ (1,4,6,7,9,12-hexahydroxyperylene- 3,10-quinone) melanin is synthesized by the condensation of malonyl-CoA to yield THN by the action of RppA. Subsequent aryl coupling of THN occurs to yield unstable HPQ by the action of P-450mel. HPQ then autopolymerizes to form HPQ-Melanin (dark green pigment) **(Figure 1C) [22].**

***Collection of Enzymes details***

In this study, protein sequences of enzymes were considered since they lead to a much greater sensitivity. All the enzymes involve in melanin production (starting from tyrosine or secondary metabolite) were taken for study. List of all the enzymes considered along with their availability in different taxa are shown in **Table 1 (see supplementary material).** To search for accurate enzyme sequences, firstly corresponding exact E.C numbers were extracted from BRENDA **[23]** database. Further, using these E.C numbers, protein sequences from different taxa were taken from Uniprot database **[24]. Table 2 (see supplementary material)** illustrates information on the details of protein sequences along with the species of the taxa of which the sequence was dug out of the database. Subsequently, presence of all the listed enzymes were checked in all 5 taxa considered and the corresponding results are elucidated in **(Table 1)**.

***Phylogenetic analysis of the enzymes***

For each enzyme involved in melanin synthesis, its sequences (if available) from 5 different families were extracted. For example - tyrosinase enzyme is present in all 5 taxa, thus giving us 5 sequences of tyrosinase from different taxa. Later, Multiple Sequence Analysis (MSA) for these enzymes was performed via Clustal W **[25].** Following MSA evolution of individual enzyme was studied using Phylip tool **[26].** In Phylip the executable

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packages *seqboot* was used for bootstrapping, *prodist* for distance computation, *neighbour.exe* for tree prediction and *consensus.exe* was employed for consensus tree generation using neighbour-joining method.

Bootstrapping is a statistical estimate for testing the reliability of the tree produced. Here bootstrapping process with (1000) replicates was used for tree evaluation. These replicates support the data for each node of the tree under consideration. For viewing trees generated by phylip, MEGA **[27]** application was used.

***Pathway Evolution***

The final step of the study involves the prediction of pathway evolution model. On the basis of evolutionary analysis of individual enzyme, pathway phylogeny was analyzed.

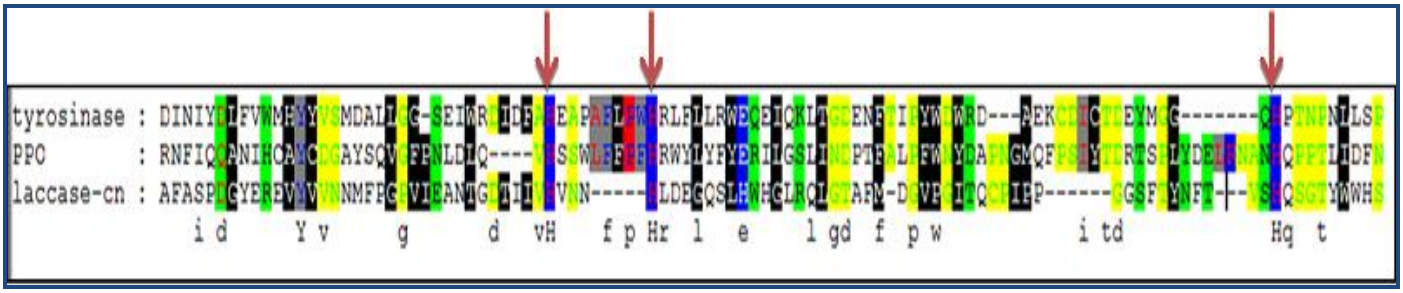


**Figure 2:** Alignment of active site with three histidine residues conserved of tyrosinase, PPO and laccase

**Discussion:**

Various pathway databases were searched and online literature was studied to arrive at a detailed pathway for all types of melanin synthesis in humans, plants, fungi and bacteria. In humans, there are two extra enzymes DCT and TYRP1 present for synthesis of Eu-Melanin. Further Pheo-Melanin is also produced depending on cysteine or gluthione concentration in the body. Moreover, Alkepto-Melanin is also produced which is generally known as Pyo-Melanin in fungi. This is because all the enzymes responsible for this melanin production are present in humans. In case of plants, function of tyrosinase is performed by PPO enzymes. These two enzymes show high sequence similarity. However, other than this the DOPA melanin synthesis pathway is similar to other taxa. Fungi have three kinds of melanin production namely DOPA melanin (from Tyrosine), DHN-Melanin (from secondary metabolite) and Pyo-Melanin (from tyrosine). Other than this, fungal melanin is also produced from dopamine using laccase enzyme. Laccase is third enzyme having tyrosinase domain other than tyrosinase and PPO. These three enzymes show similar functions in different species which are also represented by their sequence similarity. These enzymes show similar domain for tyrosinase activity and are extremely important for melanin production. In bacterial species, melanin is produced by either tyrosine via tyramine path or HPA or by secondary metabolite like malonyl-CoA. The melanogenesis pathways in bacteria are usually derived from fungal paths. However, presence of melanin in Archea is an issue of ongoing debate, but our study reveals that the presence of tyrosinase and TYDC enzymes will aid in production of melanin pigment which can help the organism for protection against radiation and extreme climatic conditions.

***Comparing tyrosinase, PPO and laccase enzymes***

Tyrosinase is a copper monooxygenases that catalyzes the hydroxylation of monophenols and the oxidation of o-diphenols to o-quinols. This enzyme, found in prokaryotes as well as in eukaryotes, is involved in the formation of pigments such as melanins and other polyphenolic compounds. Tyrosinase binds two copper ions (CuA and CuB). All tyrosinases have this binuclear type 3 copper centers within the active site. Each of the two copper ions has been shown to be bound by three conserved histidines residues. It is the rate – limiting enzyme for controlling the production of melanin **[28, 29].** On a similar note, the PPO and Laccase enzyme have three histidine residues conserved. Alignment of these three enzymes w.r.t the histidine conservation is shown in (**Figure 2).** It was thus evident that tyrosinase, PPO and laccase perform similar functions but are activated under different condition in different species.

***Pathway evolution***

There has been considerable evolution in melanogenesis from bacteria to humans as indicated by tyrosinase enzyme that is present in all the taxa. The phylogenetic analysis suggests the evolution of this enzyme from bacteria to humans. However, tyrosinase of Archea showed considerable similarity with plant PPO **(Figure 3).** In all species, other than human melanin, production is solely handled by tyrosinase but in humans there are TYRP 1 and 2 enzymes that help tyrosinase. In plants and humans there is one main path for melanin synthesis, whereas in fungi and bacteria there is more than one pathway for melanin production; depending on its use and application.

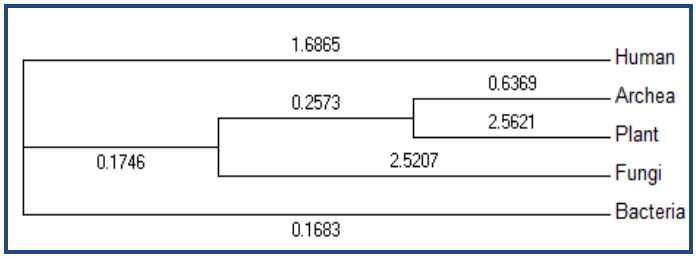


**Figure 3:** Phylogeny of tyrosinase enzyme.

**Conclusion:**

We demonstrate that distance measures that are based on the topology and the content of metabolic networks are useful for studying evolution and co-evolution. In addition, such studies allow us to further improve pathway alignment techniques by pathway reconstruction strategies, besides; they also help us study co-evolution of metabolic pathways. The availability of complete genome sequences has enhanced our understanding


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of ancestral relationships. Further progress in protocols for sensitive database searches, combined with cross-genome sequence comparisons and phylogenetic analysis, should significantly improve our ability to decipher functions of individual proteins encoded in these genomes. All this data can then collectively use to decode the mechanisms of cell function. Classification of protein families and domains as per INTERPRO **[30]** database provides a useful tool for such cross-genome sequence analysis and metabolic pathway reconstruction at enzyme level. We assume that the exponential increase in the number of completely sequenced genomes will result in improved functional assignments to hypothetical proteins and will ultimately result in a reliable evolutionary system of protein classification **[31].**

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## Supplementary material:

**Table 1:** List of enzymes considered in study along with their reaction and availability in representative organisms.

| S.no | Enzymes | Reaction | Human | Plant | Fungi | Bacteria | Archea |
|---|---|---|---|---|---|---|---|
| 1. | Tyrosinase | (i)DHI → Indole 5,6 Quinone | ✓ | ✓ | ✓ | ✓ | ✓ |
| 2 | P-450mel | (i)THN → Pseudo-HPQ | ✓ | ✓ | ✓ | ✓ | ✓ |
| 3 | Tyrosine decarboxylase | (i)Tyrosine → Tyramine | × | ✓ | ✓ | ✓ | ✓ |
| 4 | Tyrosine transaminase | (i)L-Tyrosine → 4HPP | ✓ | ✓ | ✓ | ✓ | × |
| 5 | DOPA decarboxylase | (ii)L-DOPA → Dopamine | ✓ | ✓ | ✓ | ✓ | × |
| 6 | HPP dioxygenase | (i)4HPP → Benzoquinone acetic acid | ✓ | ✓ | ✓ | ✓ | × |
| 7 | Laccase | (i)L-DOPA → DOPA-Quinone<br>(ii)Dopamine → Dopamine-Quinone | × | ✓ | ✓ | ✓ | × |
| 8 | Tyrosine hydroxylase | (i)Tyrosine → DOPA<br>(ii)DOPA → DOPA-Quinone | ✓ | ✓ | × | ✓ | × |
| 9 | PKS-1 | (i)MalonateCoA → 1,3,6,8 THN | × | ✓ | ✓ | ✓ | × |
| 10 | Polyphenol oxidase | (i)Tyrosine → O-Quinone | × | ✓ | ✓ | ✓ | × |
| 11 | Phenylalanine hydroxylase | (i)Phenylalanine → Tyrosine | ✓ | ✓ | × | ✓ | × |
| 12 | THR | (i)1,3,6,8 THN → Scytalone<br>(ii)1,3,6,8 THN → Vermelone | × | × | ✓ | ✓ | × |
| 13 | Dopachrome decarboxylase | (i)DOPA-chrome → DHI | ✓ | × | × | × | × |
| 14 | DCT/TYRP-2 | (i)DOPA-chrome → DHICA | ✓ | × | × | × | × |
| 15 | TYRP-1 | (i)DHIC → Indole 5,6 Quinone | ✓ | × | × | × | × |
| 16 | Scytalonedehydrotase | (i)Scytalone → 1,3,8THN | × | × | ✓ | × | × |
| 17 | RppA | (i)MalonylCoA → THN | × | × | × | ✓ | × |

**Table 2:** Enzymes with their E.C number, Uniprot id and species from which sequences were extracted

| | Enzymes | E.C number | Human-ID | Plant | ID | Fungi | ID | Bacteria | ID | Archea | ID |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | Tyrosinase | 1.14.18.1 | P14679 | *Medicagotruncatula* | A2Q4Q4 | *Agaricusbisporus* | C7FF04 | *Streptomycesantibioticus* | P07524 | *CandidatusNitrosopumilus* | - |
| 2 | P-450mel | - | P15538 | *Vitisvinifera* | F1T282 | *Agaricusbisporus* | Q8WZH5 | *Actinomycessp. oral* | F9EGM9 | *Haladaptatuspaucihalophilus* | E7QSM9 |
| 3 | Tyrosine decarboxylase | 4.1.1.25 | - | *Arabidopsisthaliana* | Q8RY79 | *Aspergilluskawachii* | G7XA98 | *Streptomycessviceus* | B5HRY3 | Methanocaldococcusjannaschii | Q60358 |
| 4 | Tyrosine transaminase | 2.6.1.5 | P17735 | *Arabidopsisthaliana* | Q9LVY1 | *Aspergillusniger* | A2QUB8 | *Asticcacauliscentricus* | E8RTW2 | - | - |
| 5 | DOPA decarboxylase | 4.1.1.25 | P20711 | *Medicagotruncatula* | G7L1B0 | *Beauveriabassiana* | J5K5Q1 | *Escherichiacoli* | F4TWT9 | - | - |
| 6 | HPP dioxygenase | 1.13.11.27 | P32754 | *Arabidopsisthaliana* | P93836 | *Yarrowialipolytica* | Q6CDR5 | *Streptomycesavermitilis* | Q53586 | - | - |
| 7 | Laccase | 1.10.3.2 | - | *Oryza sativa subsp. japonica* | Q10ND7 | *Cryptococcusneoformans* | Q5KEA0 | *Streptomyceslavendulae* | Q8GB87 | - | - |
| 8 | Tyrosine hydroxylase | 1.14.16.2 | Q2M3B4 | *Musa acuminata* | D2CVP2 | - | - | *Streptomycesachromogenes* | C0LTT0 | - | - |
| 9 | PKS-1 | - | - | *Musa acuminata* | D5KZJ8 | *Aspergillusochraceus* | A3KLM2 | *Streptomycesclavuligerus* | B5GU50 | - | - |
| 10 | Polyphenol oxidase | 1.14.18.1 | - | *Vitisvinifera* | P43311 | *Agaricusbisporus* | C7FF04 | *Streptomycesauratus* | J1S178 | - | - |
| 11 | Phenylalanine hydroxylase | 1.14.16.1 | P00439 | *Pinustaeda* | E5KBU3 | - | - | *Bacillusthuringiensis* | J4A963 | - | - |
| 12 | THR | 1.1.1.252 | - | - | - | *Magnaportheoryzae* | Q12634 | *Serratia sp.* | G0C2B7 | - | - |
| 13 | DOPA-chrome decarboxylase | 4.1.1.25 | P30046 | - | - | - | - | - | - | - | - |
| 14 | DCT/TYRP-2 | 5.3.3.12 | P30046 | - | - | - | - | - | - | - | - |
| 15 | TYRP-1 | 1.14.18.1 | Q6LES1 | - | - | - | - | - | - | - | - |
| 16 | Scytalonedehydrotase | 4.2.1.94 | - | - | - | *Magnaportheoryza* | P56221 | - | - | - | - |
| 17 | RppA | - | - | - | - | - | - | *Saccharopolysporaerythraea* | Q8KQM3 | - | - |


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**List of Abbreviation:**

| Abbreviations | |
|---|---|
| TYR | TYRosinase |
| DCE | DOPAChrome Conversion Enzyme |
| TYRP-1 | TYrosinase-Related Protein-1 |
| DCT | DOPAChrome Tautomerase |
| TYRP-2 | TYrosinase-Related Protein-2 |
| SCD | SCytalone Dehydratase |
| THR | HN Reductase |
| PKS-1 | PolyKetide Synthases-1 |
| PPO | PolyPhenol Oxidase |
| DOPA | 3,4-DihydrOxyPhenylAlanine |
| TYDC | TYrosine DeCarboxylase |
| DHIC | 5,6-DiHydroxyIndole-2-Carboxylic acid |
| HPP | 4-HydroxyPhenylPyruvate |
| DHI | 5,6-DiHydroxyIndole |
| HGA | HomoGentisic Acid |
| AHB | Alanyl-Hydroxy-Benzothiazine |
| ADHDB | Alanyl 5,6 DiHydro- Di-Benzothiazine |
| DHN | 1, 8-DiHydroxyNaphthalene |
| THN | 1,3, 6,8-TetraHydroxyNaphthalene |
| BAA | Benzoquinone Acetic Acid |
| HPQ | 1,4,6,7,9,12-HexahydroxyPerylene-3,10-Quinone |
| DOPA-Q | DOPA-Quinone |


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