ÇUKUROVA UNIVERSITY
INSTITUTE OF NATURAL AND APPLIED SCIENCES

PhD THESIS

Ebrahim VALIPOUR

ISOLATION AND CHARACTERIZATION OF A TYROSINASE ENZYME FROM NATIVE BACTERIAL STRAIN, CLONING OF THE RELATED GENE, CHARACTERIZATION OF THE RECOMBINANT ENZYME AND ANALYSIS OF ITS BIOTECHNOLOGICAL APPLICATIONS SUCH AS L-DOPA AND MELANIN PRODUCTION

BIOTECHNOLOGY DEPARTMENT

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BIOTECHNOLOGY DEPARTMENT

We certify that the thesis titled above was reviewed and approved for the award of degree of the PhD of Science by the board of juries on 29/07/2015.

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ABSTRACT

PhD THESIS

ISOLATION AND CHARACTERIZATION OF A NOVEL TYROSINASE ENZYME FROM NATIVE BACTERIAL STRAIN, PRODUCTION OF THE ENZYME BY CLONING, CHARACTERIZATION OF THE RECOMBINANT ENZYME AND ANALYSIS OF ITS BIOTECHNOLOGICAL APPLICATIONS SUCH AS L-DOPA AND MELANIN PRODUCTION

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Tyrosinase is a type 3 copper-containing enzyme that catalyzes the conversion of L-tyrosine to L-DOPA and finally to melanin. Melanin has a broad spectrum of biotechnological and biological functions and it is widely used in pharmacology, cosmetics and other industrial fields. L-DOPA is the preferred drug for treatment of Parkinson's diseases. In this study a *Bacillus* sp. having tyrosinase enzyme was isolated from native and the strain was identified by morphological, biochemical and molecular analysis. Production of the enzyme by the native isolate was optimized using classical and statistical (Box- Behnken design) methods and the enzyme was characterized. After that the tyrosinase coding gene was cloned in two expression system (*E.coli BL21 (DE3) pLysS* and *Rosetta-gami 2 (DE3) pLysS*) using pET22b as an expression vector and expression of the gene in the two expression system was compared. The gene fragment was sequenced using with Sanger sequencing method. The sequence was subjected to insilico analysis using different bioinformatics programs and websites. Conversion of l-tyrosine to L-DOPA and melanin was evaluated by TLC and HPLC methods. The isolated strain showed up to 99% homology with *Bacillus megaterium*. Production of the enzyme by the native M36 *Bacillus* sp. was determined as 0.05IU/ml, after optimization of culture condition it was reached to 0.38 IU/ml; also this product was reached to 31 IU/ml after optimization of expression of recombinant M36 tyrosinase enzyme. The optimum temperature for native and recombinant M36 tyrosinase activity was 40 °C and 45-50°C also; optimum pH for native and recombinant enzyme activity was 7.0 and 7.5 respectively. In SDS-PAGE analysis the native and recombinant M36 tyrosinase enzyme determined as 34kDa and 35 kDa, respectively. M36 melanin produced in this research, showed structural similarity with standard synthetic melanin from sigma according to the solubility, FT-IR and EPR analysis. Also it showed notable anti-bacterial, anti-UV and ant-oxidant effects. MTT assay showed the M36 melanin to have anti-cancer effect.

Key words: Melanin, L-Dopa, Tyrosinase, ,Cloning, pET22b.

ÖZ

DOKTORA TEZİ

DOĞAL BAKTERİ SUŞLARINDAN TİROZİNAZ ENZİM İZOLASYONU, KARAKTERİZASYONU, ÜRETİCİ GENİN KLONLANMASI, REKOMBİNANT ENZİMİN KARAKTERİZASYONU VE L-DOPA VE MELANİN ÜRETİMİ GİBİ BİYOTEKNOLOJİDE KULLANILABİLİRLİĞİ

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Tirozinaz, L-tirosin'in L-DOPA'ya ve son olarak melanin'e dönüşümünü katalize eden tip-3 bakır içerikli bir enzimdir. Melanin, biyoteknolojik ve biyolojik fonksiyonları ile geniş bir yelpazeye sahiptir ve ilaçbilim, kozmetik ve başka endüstri alanlarda yaygın olarak kullanılmaktadır. L-DOPA, Parkinson hastalıkları tedavisinde tercih edilen bir ilaçtır.

Bu çalışmada, tirozinaz enzimi bulunduran bir *Bacillus* türü doğadan izole edilmiştir ve bu suş morfolojik, biyokimyasal ve moleküler analiz ile tanımlanmıştır. Doğal izolattan enzimin üretimi klasik ve istatistiki metotlar (Box-Behnken dizaynı) ile optimize edilmiş ve enzim karakterize edilmiştir. dahasonra, tirozinaz kodlayan gen ekspresyon vektörü olarak pET22b kullanılarak iki ekspresyon sisteminde (*E.coli BL21 (DE3) pLysS* and *Rosetta-gami 2 (DE3) pLysS*) klonlanmıştır. İki sistemindeki gen ekspresyonları karşılaştırılmıştır. Gen fragmenti, Sanger sekanslama metoduyla selamlanmıştır. Sekans, farklı biyoinformatik programları ve web siteleri kullanarak insiliko analize tabi tutulmuştur. L-tirozin'in L-DOPA ve melanine dönüşmesi TLC ve HPLC yöntemleri ile saptanmıştır. Üretilen melanin incelenmiştir. İzole edilen suş *Bacillus megaterium* ile %99 homoloji göstermiştir. Doğal M36 *Bacillus* türünden enzimin üretimi 0.05 IU/ml olarak belirlenmiştir. Kültür koşullarının optimizasyonundan sonra 0.38 IU/ml'ye ulaşmıştır. Rekombinant M36 tirozinaz enziminin ekspresyonunun optimizasyonundan sonra ise 31 IU/ml'ye ulaşmıştır. Doğal ve rekombinant M36 tirozinaz enzimi için optimum sıcaklık sırasıyla 40 °C ve 45-50 °C; optimum pH sırasıyla 7.0 ve 7.5 idi. SDS-PAGE analizinde, doğal ve rekombinant M36 tirozinaz enzimi sırasıyla 34kDa ve 35 kDa olarak belirlenmiştir.

Bu çalışmada üretilen M36 melanin, FT-IR ve EPR analizine göre Sigma standart sentetik melanin ile yapısal benzerlik göstermiştir. Aynı zamanda, üretilen M36 melanin önemli anti-bakteriyel, anti-UV ve anti-oksidan etki göstermiştir. MTT deneyi, M36 melaninin anti-kanser etkisi olduğunu göstermiştir.

Anahtar Kelimeler: Melanin, L-Dopa, Tirozinaz, Kloanlama, pET22b

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I'm not that good in words and writing letters but in my simplest way; I want to say I love you and I want to thank you for all you have done for me. A million thanks to the best wife in the world!

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LIST OF ABBREVIATIONS AND NOMENCLATURE

μM	: Micromolar
AMPS	: ammonium persulfate
ATP	: adenosine triphosphate
bis	: N’-methylenebisacrylamide
bp	: Base pair
CBB	: Coomassie Brilliant Blue
D.D.W	: Double Distilled Water
EDTA	: Etilendiamin Tetra-acetic acid
kDa	: Kilodalton
LB	: Luria Broth
L-DOPA	: L-3,4-dihydroxyphenylalanine
M	: Molar
mL	: Milliliter
mM	: Millimolar
nm	: Nanometer
OD	: Optical Density
PAGE	: Polyacrylamide gel Electrophoresis
rpm	: Revolutions per minute
SOC	: Super Optimal broth with Catabolite repression
sp.	: Species
SDS	: Sodium dodecyl sulfate
SDS-PAGE	: Sodium dodecyl sulfate Polyacrylamide gel Electrophoresis
TBE	: Tris/Borate/EDTA
TCA	: Trichloroacetic acid
TE	: Tris EDTA
Tris	: 2-Amino-2-Hidroxi Methylpropan-1,3-diol
TEMED	: N,N,N’,N’-Tetrametil etilendiamin
UV	: ultraviolet
U/mg	: Unite/miligram

1. INTRODUCTION

1.1. Enzymes

Enzymes, produced by living systems, are protein catalysts that can accelerate chemical reactions, the transfer of electrons, atoms, or functional groups, by several orders of magnitude while maintaining high fidelity of reaction trajectories and producing low levels of side-reaction products. They catalyze nearly all the reaction types known in organic chemistry. For catalytic activity, enzymes may require organic (e.g., coenzymes such as flavin mononucleotide and thiamine pyrophosphate) or inorganic (e.g., Cu^{+2}) cofactors that, like the enzyme itself, are not consumed in the catalyzed reactions. Over 4000 enzymes, each catalyzing different reactions, have been identified in nature for mediating biological processes. As such, although enzymes are often designated by using short trivial names, they also have been assigned longer systematic names and four-part classification numbers (EC number, 'enzyme commission number') by the enzyme nomenclature system maintained by the International Union of Biochemistry and Molecular Biology and the International Union of Pure and Applied Chemistry. Thus, depending on the type of reaction catalyzed, enzymes are divided into six main classes:

1. Oxidoreductases transfer of electrons from one substrate molecule to another (e.g., dehydrogenases, reductases, oxidases).

2. Transferases transfer of functional group, from one substrate molecule to another (e.g., glycosyl transferases, acetyl transferases, and aminotransferases).

3. Hydrolases transfer of functional group from substrate to water (e.g., glycoside hydrolases, peptidases, esterases).

4. Lyases eliminate the functional group from substrate with the formation of double bonds. Thus, bonds are cleaved using a different principle than hydrolysis (e.g., pectate lyases break glycosidic linkages by elimination).

5. Isomerases transfer groups from one position to another in the same molecule (e.g., glucose isomerase).

6. Ligases Ade a function group to substrate usually coupled with ATP hydrolysis (e.g., glycine– tRNA ligase). The major classes are further subdivided into subclasses (Berg et al., 2012).

tyrosinase enzyme (EC1.14.18.1) is belong to the Oxidoreductases group. The tyrosinase enzyme is known by different name as following: tyrosinase; monophenol monooxygenase; phenolase; monophenol oxidase; cresolase; monophenolase; yrosine-dopa oxidase; monophenol monooxidase; monophenol dihydroxyphenylalanine:oxygen xidoreductase; N-acetyl-6-hydroxytryptophan oxidase; monophenol, dihydroxy-L-phenylalanine oxygen oxidoreductase; o-diphenol:O2 oxidoreductase; phenol oxidase (Halaouli et al., 2005).

Tyrosinase enzyme is a type III copper protein found in a broad variety of bacteria, fungi, plants, insects, crustaceans, and mammals, which is involved in the synthesis of melanin. The enzyme, which is activated upon binding molecular oxygen, can catalyse both a monophenolase reaction cycle and a diphenolase reaction cycle. During the monophenolase cycle, one of the bound oxygen atoms is transferred to a monophenol (such as L-tyrosine), generating an o-diphenol intermediate, which is subsequently oxidized to an o-quinone and released, along with a water molecule.

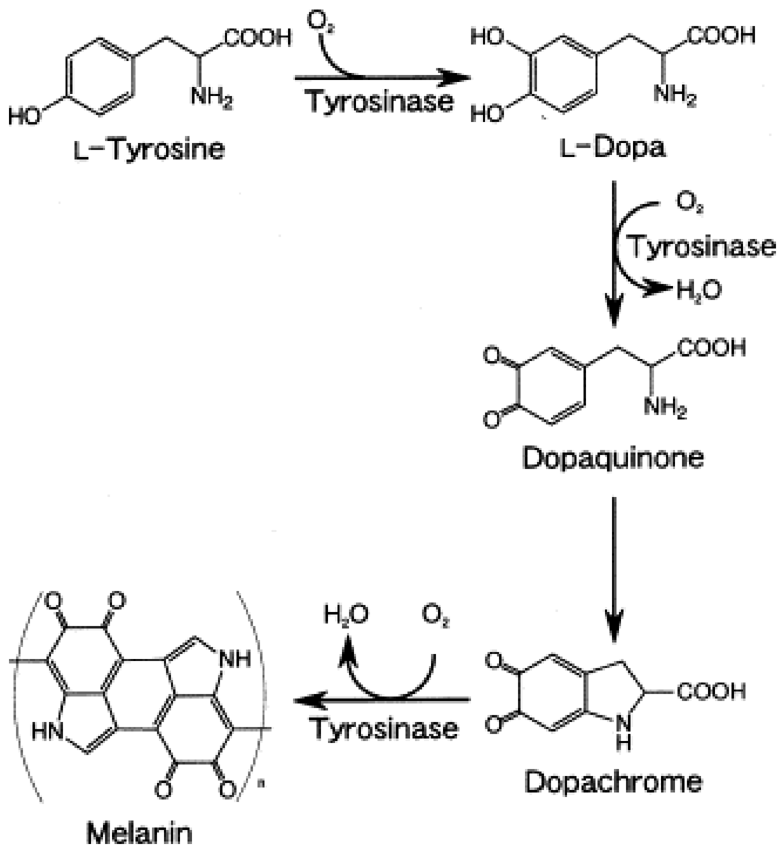


Figure 1.1. Melanin synthesis pathways

The enzyme remains in an inactive deoxy state, and is restored to the active oxy state by the binding of a new oxygen molecule. During the diphenolase cycle the enzyme binds an external diphenol molecule (such as L-dopa) and oxidizes it to an o-quinone that is released along with a water molecule, leaving the enzyme in the intermediate met state. The enzyme then binds a second diphenol molecule and repeats the process, ending in a deoxy state (Ramsden et al., 2014). The crystal structure of tyrosinase enzymes has demonstrated the presence of two copper ions (necessary for activity of the enzyme) in the catalytic core. In all tyrosinases of different origins and in the haemocyanins each of the copper ions is coordinated by three histidine residues that are found in a characteristic pattern in the primary structure. In the tyrosinase from Streptomyces glaucescens, for example, the key role of histidines at position 37, 53, 62, 189, 193 and 215 in the coordination of copper, and thus in catalytic activity, was confirmed by the decrease o of activity upon their substitution with other amino acids (faccio et al., 2012). Howard et al., 1948, elucidate the biosynthetic pathway for melanin formation by tyrosinase enzyme. In mammals, tyrosinase catalyzes the biosynthesis of melanin pigments, which contributes to a fundamental part of the protection of the skin, against UV radiation. It is also related to the browning reactions of fruit and vegetables. In fungi, the role of this enzyme is correlated with the differentiation of reproductive organs and spore formation, virulence of pathogenic fungi, and tissue protection after injury (Seo et al., 2003). In bacteria, tyrosinase enzyme is the key enzyme in initiating of melanin biosynthesis pathway and plays an important protective and survival role.

Due to the requirement to a caddie protein for activity, bacterial tyrosinases have been grouped in five groups (faccio et al., 2012). Type I tyrosinases are typical of Streptomyces sp. tyrosinases (Ty) where the tyrosinase requires a caddy protein (CP) for copper incorporation (Chen et al.,1992). These tyrosinases are typically secreted by the TAT signal pathway (Leu et al., 1992). Type II tyrosinases are similar to *Streptomyces* sp. tyrosinases in terms of size but do not require a caddy protein and may also be secreted, for example, *Bacillus megaterium* tyrosinase (shoster et al., 2009). Type III tyrosinases are similar to fungal tyrosinases where the main tyrosinase domain is followed by a C-terminal (CT) extension that must be removed (via proteolysis) for the enzyme to be fully active, for example, *Verrucomicrobium spinosum* tyrosinase (Farihead et al., 2010). Type IV tyrosinases are purportedly smaller in size than other examples and are reported to be active only as homodimers, for example, *Bacillus thuringiensis* (Liu et al., 2004). Although they have tyrosinase-like activities, type V are more similar to laccases in structure and do not have the signature copper A and B binding motifs or the oxygen binding motifs common to tyrosinases. 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-Amat et al., 2001).

Tyrosinases have several biotechnological applications relying on the ability of the enzymes to oxidise both small phenolic molecules and protein-associated phenolic groups, i.e. the side chain of the amino acid tyrosine. The biotechnological and industrial applications of Tyrosinases are as following:

1- One of their applications is bioremediation of contaminant soil and wastewater containing phenolic compound (Marino et al., 2011; El. Shora et al., 2008; Ensuncho et al., 2005; Battaini et al., 2002) and dyes. (Saratale., 2011; Franciscon et al., 2012; Saratale et al., 2009).The phenolic compounds are transformed by the enzyme to quinines which auto-oxidize to form insoluble polymeric compounds that precipitate from water

2- Biosynthesis and medical application such as production of L-DOPA, the preferred drug for treatment of Parkinson's disease (Xu et al., 2012; Rani et al., 2007), production of hydroxytyrosol as a food additive (Allouche et al.,2006; Espin et al,2001; Brooks et al., 2006), production of estrogenic compound (Pandey et al., 1989), production of melanin for therapeutic uses (Baranowitz et al., 2003), treatment of neurological disease (Berliner, 1997), and production of antibiotic linkomaycin (Michaliket al.,1975), cosmetic application as a self-tanning agent (Herlihy,2002), and production of dye (Warner et al., 2010).

3- Food application such as production of theaflavins which are a major group of polyphenol compounds in black tea and have strong antioxidant, anticancer and other bioactive properties (Tu et al., 2005), improving of dairy product texture and cross-linking of meat protein (Thalmann et al., 2002; Ercili-Cura et al., 2008).

4- In developing of biosensors to monitor, measure and eliminate of fenolic component in fruit juices, jams, musts and red wines (Gu et al., 2009; Syvitel et al., 1998) which can elevate the quality and safety of this important products in Turkey.

5- Material engineering such as modification of wool (Jus et al., 2009; Lantto et al., 2005), modification of silk fibroin (Freddi et al., 2006) production of polyphenolic polymer (de Faria et al., 2007), production of water-resistant adhesives (Yamada et al., 2008) treatment of textiles (Franciscon et al., 2012), and immobilization of various enzymes on chitosan (Barbari et al., 2006).

6- Production of melanins which are dark-brown to black pigments of macromolecules formed by oxidative polymerization of phenol and/or indolic compounds, which widely exist in animals, plants and microorganisms.

1.2. Melanin

Melanin is an irregular light-absorbing polymer containing indoles and other intermediate products derived from the oxidation of tyrosine. It is the major pigment present in the surface structures of vertebrates. The critical step in melanin biogenesis is the oxidation of tyrosine by the enzyme tyrosinase. In vertebrates this enzyme is active only in specialized orgauelles in retinal pigment epitbellum and melanocytes. In mammals melanin is formed as intracellular granules. Melanin granules are transferred from melanocytes to epithellal cells and form tbe predomluaut plgmeut of hair and epidermis. Generally, melanosomes in animal materials are distributed in a biologically environment. The isolation of melanin from such tissues has commonly been achieved by using concentrated acid and sodium hydroxide. Extraction of natural melanin is not a reliable method because of employing harsh procedure which can modify their physical and chemical properties. Also, chemical synthesized melanins cannot fully represent the nature of melanins because they do not contain any proteins as associated in vivo. In recent studies, new enzymatic isolation methods were developed which provided more homogenous and intact products than chemical extraction. These protocols often involve consecutive enzymatic treatments with proteinase K, collagenase, and other kinds of protease to separate the melanin from proteins and other biologic materials (Liu et al., 2004) (Chen et al., 2008).

Melanins show a broad spectrum of biotechnological and biological functions, including antioxidant activity (Kumar et al., 2011), antitumor activity (El-Obeid et al., 2006), antivenin activity (Hung et al., 2004), anti-virus (Garger et al., 2001), liver protecting activity (Sava et al., 2003) and radio protective (Dadachova et al., 2007) etc. Melanins are widely used in medicine, pharmacology, cosmetics and other fields. Also, there is a strong consumer demand for melanin as a natural colourant in food and cosmetics, particularly as a component of photo-protective creams and as substitute for synthetic dyes (Kumar et al., 2011; Dong et al., 2012).

1.3. L-dopa

Parkinson's disease (PD) has been a serious challenge to public health around the world. 3,4-dihy- deoxy phenyl-L-alanine (L-DOPA) is an amino acid analogue and drug of choice in the PD-a degenerative neurological disorder. In the United States alone, about 1 million people are affected by PD and worldwide about 5 million. PD occurs among 1% of individuals aged 60 years while 4% of those 80 years old. The disease (PD) is characterized by progressive death of dopamine producing neurons in the basal ganglia of the brain. Loss of the neurotransmitter dopamine can cause a number of effects, including rigidity (muscles resistant to movement), akinesia (inability to initiate movement), bradykinesia (slowness of movement), and rest tremor [4]. Many brain cells of PD patients contain Lewy bodies which are unusual deposits or clumps of the protein alpha synuclein, along with other proteins. Researchers are unable to find out formation of Lewy bodies or their role in development of the disease. For many decades, there were no effective treatments for PD, and it was thought to be a terminal illness. But in the 1940s and 1950s, neurosurgeons began to perform surgery on the basal ganglia of brain which gave little cure in PD symptoms. Though surgery was effective, it was risky, with about 12 percent of patients dying as a result of operation. the biggest advancement in Parkinson's treatment came in the 1960s, when researchers identified low levels of dopamine in brain among diseased persons. This research revolutionized the treatment of PD, led to the development of levodopa, also called L-DOPA (Larodopa, Dopar), which is been used as medication (Davie, C. A. 2008). L-DOPA can easily cross the blood–brain barrier and gets converted into dopamine; it can be administered orally to relieve the symptoms of PD (Surwase et al., 211) Levodopa is still the cornerstone of Parkinson's treatment today.

Interest in L-DOPA therapy for Parkinson's disease has been considerably enhanced since the recent release of this drug to all medical practitioners. Both experimental and clinical studies have suggested that the depletion of dopamine can be corrected by the administration of L-DOPA either orally or intravenously. A level of homovanillic acid (the main breakdown product of dopamine) in the cerebrospinal fluid of patients before and after the administration of oral L-DOPA proves its applicability.

Parkinson's disease affects a part of brain called the "basal ganglia", which controls movement. Cells in the basal ganglia begin to degenerate as a result of the condition, and loss of their ability to produce a neurotransmitter (a chemical that carries messages between brain and nerve cells) called dopamine. As dopamine levels drop, the production of another neurotransmitter, called acetylcholine, increases. The balance between these two is critical, because they have opposite effects; acetylcholine stimulating muscle contraction, and dopamine damping it down. When the balance shifts in favour of acetylcholine, muscles become rigid with increasing jerky movements which are difficult to control this is often accompanied by tremors in hands. Whilst conventional drugs can be administered to control symptoms, they have a range of unpleasant side effects and cannot limit its progression. During the early stages of the disease, conventional drugs, called anticholinergics, can reduce symptoms of muscle rigidity and excess salivation by blocking the action of acetylcholine. However, they can cause dry mouth, constipation, anxiety, drowsiness and blurred vision. Whereas, direct administration of L-DOPA also has dangerous side-effects, including nausea, internal blee- ding, palpitations, dizziness and depression because of it is being converted to dopamine before it reaches to brain (Sushama et al., 2013).

Several investigators have studied biological routes for production of L-DOPA. For example, systems involving whole cells of Escherichia coli, Erwinia herbicola or Aspergillus oryzae have been used to transform L-tyrosine into L-DOPA in the laboratory. However, the specific activities in these processes are low and both the initial substrate (L-tyrosine) and the desired product (L-DOPA) suffer side reactions that produce other metabolites. The poor performance of whole-cell systems stimulated researchers to use tyrosinases in cell-free systems. So far all studies have involved mushroom tyrosinase obtained from Sigma. The productivities of L-DOPA that have been obtained in batch reactors using immobilized tyrosinase range from 1.44 to 54 mg/(L·h). Unfortunately, these productivities are still relatively low, which is due to two factors. Firstly, the conversion of the added L-tyrosine is incomplete, with less than 30 % of it being consumed during the process. Secondly, although side reactions with L-tyrosine are avoided in these enzymatic systems, some of the L-DOPA that is produced is lost in a second tyrosinase-catalyzed reaction that produces dopaquinone. This dopaquinone isomerizes spontaneously to leukodopachrome, which in turn reacts with a second dopaquinone mole-cule, regenerating one molecule of L-DOPA but also producing a dopachrome molecule, which then polymerizes spontaneously to produce melanin. L-ascorbate is added to the reaction medium in an attempt to minimize melanin production. It acts by reducing dopaquinone back to L-DOPA, in a non-enzymatic reaction. However, since high concentrations of L-ascorbate not only inhibit the cresolase activity responsible for L-DOPA synthesis but also irreversibly inactivate the tyrosinase, the L-ascorbate is added in the same concentration as L-tyrosine. The problem is that the L-ascorbate is consumed continuously during the process. Although it could be continuously fed into the medium in order to maintain its concentration constant, the problem would not be solved because the continued accumulation of diphenols would activate the melanin formation pathway anyway (de Faria et al., 2007).

1.4. Cloning in Molecular Biology

In biology, cloning is the process of producing similar populations of genetically identical individuals that occurs in nature when organisms such as bacteria, insects or plants reproduce asexually. Cloning in biotechnology refers to processes used to create copies of DNA fragments (molecular cloning), cells (cell cloning), or organisms. Molecular cloning is a set of experimental methods in molecular biology that are used to assemble recombinant DNA molecules and to direct their replication within host organisms. The use of the word cloning refers to the fact that the method involves the replication of one molecule to produce a population of cells with identical DNA molecules. Molecular cloning generally uses

DNA sequences from two different organisms: the species that is the source of the DNA to be cloned, and the species that will serve as the living host for replication of the recombinant DNA. Molecular cloning methods are central to many contemporary areas of modern biology and medicine (Green et al., 2012).

Genes encoding many microbial enzymes have been cloned and the enzymes expressed at levels hundreds of times higher than those naturally produced. Over 60% of the enzymes used in the detergent, food and starch processing industry are recombinant proteins.181 Recombinant DNA technology has been used beneficially in the enzyme industry in the following ways: 182,183

(a) To produce industrial organisms; enzymes obtained from microbes which are difficult to grow or modify genetically;

(b) To increase enzyme productivity by use of multiple gene copies, strong promoters, and efficient signal sequences;

(c) To produce in a safe host; useful enzymes obtained from a pathogenic or toxin-producing microorganism; and

(d) To improve the stability, activity or specificity of an enzyme by protein engineering.

Recombinant DNA technology makes a revolution in enzyme industry because by this way it was possible to manipulate the stability, activity, and amount production of the enzymes. For example, *P. pastoris*, the methanol-utilizing yeast, was engineered to produce *S. cerevisiae* invertase and to excrete it into the medium at 100 mg l^{-1} (Van Brunt et al., 1986).

By cloning of *B. amyloliquefaciens* α-amylase gene in *B. subtilis* using plasmid pUB110 , the Production was 2,500-fold greater than that in wild-type *B. subtilis* and five-fold that of the *B. amyloliquefaciens* donor (Palva et al., 1982).

expression of exoglucanase gene from cellulolytic *Cellulomonas fimi* was increased to a level of over 20% of cell protein by cloning in *E. coli (*O'Neill et al., 1986).

a xylanase gene of *S. lividans* was overproduced after Self-cloning uo to six-fold (Mondou et al.,1986).

Cloning of the benzylpenicillin acylase gene of *E. coli* resulted in a 45-fold increase (Mayer et al. 1980).

Aspartase production was increased 30-fold by cloning in *E. coli* (Komatsubara et al., 1986).

Captopril esterase of *P. putida*, used in preparing the chiral captopril sidechain, was cloned in *E. coli* with a 38-fold increase in activity (Elander et al., 1995).

A 1,000-fold increase in phytase production was achieved in *A. niger* by use of recombinant technology (Van et al., 1993).

The properties of many enzymes have been altered by genetic means. “Brute force” mutagenesis and random screening of microorganisms over the years led to changes in pH optimum, thermostability, feedback inhibition, carbon source inhibition, substrate specificity, Vmax, K_m and K_i. This information was later exploited by the more rational techniques of protein engineering. Single changes in amino acid sequences have yielded similar types of changes in a large variety of enzymes. Today, it is no longer necessary to settle for an enzyme’s natural properties; these can be altered to suit the needs of the investigator or the process. For example, a protease from *Bacillus stearothermophilus* was increased in heat tolerance from 86°C to 100°C, being made resistant to boiling. The enzyme was developed by site directed mutagenesis. Only eight amino acids had to be modified. Temperature stability at 100°C was increased 340-fold without a decrease in activity at lower temperatures. All eight mutations were far from the enzyme’s active site (Van et al., 1998).

1.5. Expression Systems

Today, there is a wide selection of expression systems available for large-scale recombinant protein production (*Bacteria*, baculovirus-mediated insect cell expression, yeast, and several mammalian based systems). Each has its own respective advantages in relation to cost, ease of use, and their post-translational modification profiles. Here, we review several of the most popular systems (with specific emphasis on *E. coli* and baculovirus- mediated expression systems) and their adaptability to multi-parallel protein expression applications.

1.6. *E. coli*

E.coli is one of the earliest and most widely used hosts for the production of heterologous proteins. There are some advantages and disadvantages. These include rapid growth, rapid expression, and ease of culture and high product yields (Terpe et al., 2006). It is used for massive production of many commercialized proteins. This system is excellent for functional expression of non-glycosylated proteins. E. coli genetics are far better understood than those of any other microorganism. Recent progress in the fundamental understanding of transcription, translation, and protein folding in E. coli, together with the availability of improved genetic tools, is making this bacterium more valuable than ever for the expression of complex eukaryotic proteins. Its genome can be quickly and precisely modified with ease, promotor control is not difficult, and plasmid copy number can be readily altered. This system also features alteration of metabolic carbon flow, avoidance of incorporation of amino acid analogs, formation of intracellular disulfide bonds, and reproducible performance with computer control. E. coli can accumulate recombinant proteins up to 80% of its dry weight and survives a variety of environmental conditions. The E. coli system has some drawbacks, however, which have to be overcome for efficient expression of proteins. High cell densities result in toxicity due to acetate formation; however, this can be avoided by controlling the level of oxygen. Proteins which are produced as inclusion bodies are often inactive, insoluble and require refolding. In addition, there is a problem producing proteins with many disulfide bonds and refolding these proteins is extremely difficult. The E. coli system produces unmodified proteins without glycosylation which is the reason why some produced antibodies fail to recognize mammalian proteins (Demain et al., 2009).

The pET Systemis the most powerful system yet developed for the cloning and expression of recombinant proteins in E. coli. Based on the T7 promoter-driven system originally developed by Studier and colleagues , the pET System has been greatly expanded and now includes over 35 vector types, 11 different *E.coli* host strains and many other companion products designed for efficient detection and purification of target proteins .

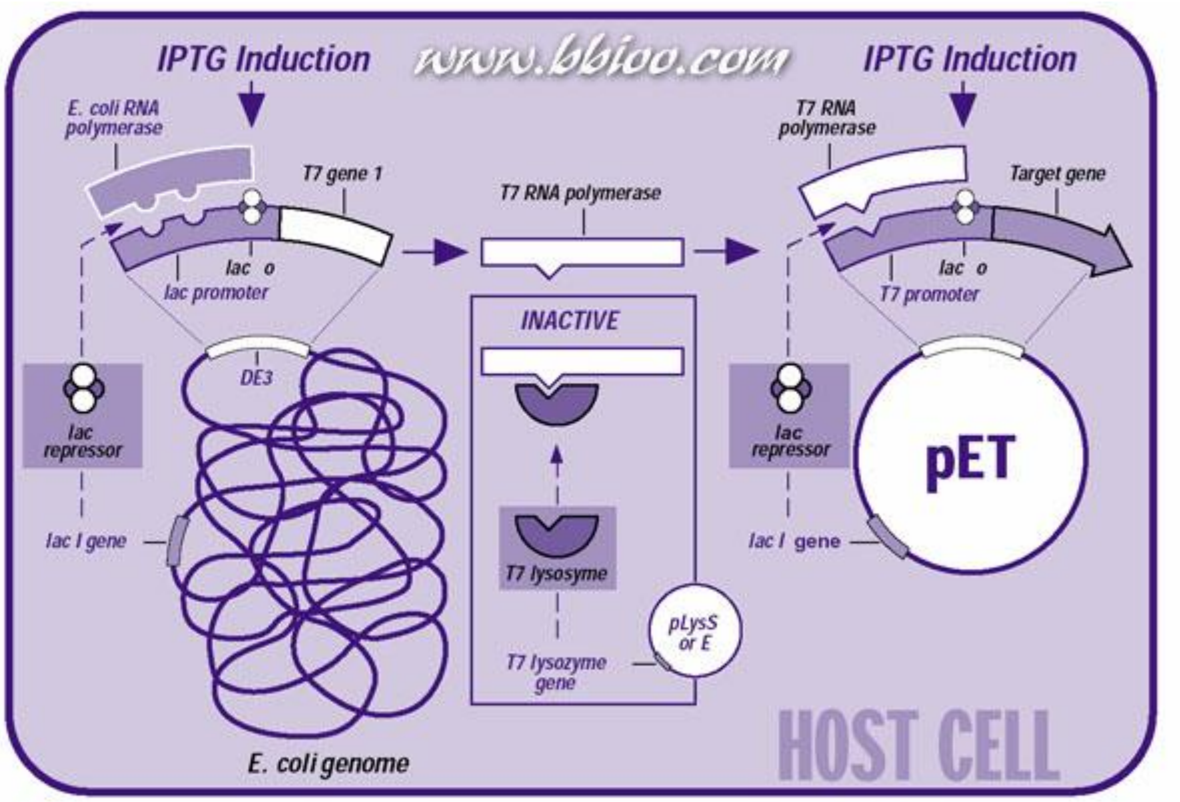


Figure 1. 2 Control elements of the pET system

Several different host strains are available as λDE3 lysogens. The most widely used host is BL21, which has the advantage of being deficient in both *lon* and *ompT* proteases. Novagen has introduced two derivatives of BL21 designed for special purposes. The B834 series is methionine deficient and, therefore, enables high specific activity labeling of target proteins with ^{35}S-methionine or selenomethionine (Studier et al., 1990). The BLR strain is a *recA*- derivative that improves plasmid monomer yields and may help stabilize target plasmids containing repetitive sequences. The AD494 strains are thioredoxin reductase (*trxB*) mutants that enable disulfide bond formation in the *E.coli* cytoplasm. This allows for the potential production of properly folded, active proteins. Other available strain backgrounds include the K-12 strains HMS174 and NovaBlue, which are *recA*, like BLR. These strains may stabilize certain target genes whose products may cause the loss of the DE3 prophage. NovaBlue is potentially useful as a stringent host due to the presence of the high affinity *lacI*^{q} repressor encoded by the F episome. In addition, Novagen offers the λDE3 Lysogenization Kit for making new expression hosts with other genetic backgrounds.

An alternative for expressing extremely toxic genes or preparing a new λDE3 lysogen is to provide T7 RNA polymerase by infection with λCE6. Although not as convenient as inducing a λDE3 lysogen with IPTG, this strategy may be preferred for certain applications (Tesfai et al., 2012).

1.7. Rosetta-gami 2(DE3)pLysS

Rosetta-gami 2 host strains combine features of Origami 2 and Rosetta 2, allowing for enhanced disulfide bond formation and enhanced expression of eukaryotic proteins that contain codons rarely used in E. coli. These strains are derived from Origami 2, a kanamycin-sensitive K-12 strain carrying the trxB and gor mutations for disulfide bonds formation in the cytoplasm. The cells carry the chloramphenicol-resistant plasmid, pRARE2, which supplies tRNAs for seven rare codons, AUA, AGG, AGA, CUA, CCC, GGA, and CGG under the control of their native promoter. The gor mutation is selectable on tetracycline.

Rosetta-gami 2(DE3)pLysS strains have many advantages for recombinant protein production specifically the starins supply tRNAs for the codons AUA, AGG, AGA, CUA, CCC, GGA and CGG. The tRNA genes are driven by their native promoters. Also, they are deficient in the *lon* protease and lack the *ompT* outer membrane protease that can degrade proteins during purification. Thus, some target proteins may be more stable in these strains than in host strains containing these proteases (Choi et al., 2004).

The genotype of the strain as following:

Δara–leu7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F ’[lac+ lacIq pro] gor522 ::Tn10 trxB pLysSRARE27 (Cam^R, Str^R, Tet^R)

1.8. *E.coli* BL21(DE3)pLysS

E.coli BL21(DE3) pLysS Competent Cells allow high-efficiency protein expression of any gene that is under the control of a T7 promoter and has a ribosome binding site. BL21(DE3)pLysS is lysogenic for λ-DE3, which contains the T7 bacteriophage gene I, encoding T7 RNA polymerase under the control of the lac UV5 promoter. BL21(DE3)pLysS also contains a plasmid, pLysS, which carries the gene encoding T7 lysozyme. T7 lysozyme lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. For researchers doing more than one transformation, competent cells are available in standard format (200µl aliquots). For added convenience, single-use competent cells (50µl aliquots) also are offered (Davanloo et al., 1984).

Genotype: F^-, ompT, hsdSB (rB^-, mB^-), dcm, gal, λ(DE3), pLysS, Cm^r.

1.9. pET22b Vector

The pET-22b (+) vector (Cat. No. 69744-3) carries an N-terminal pelB signal sequence for potential periplasmic localization, plus optional C-terminal His•Tag® sequence. Unique sites are shown on the circle map. Note that the sequence is numbered by the pBR322 convention, so the T7 expression region is reversed on the circular map. The cloning/expression region of the coding strand transcribed by T7 RNA polymerase is shown below. The f1 origin is oriented so that infection with helper phage will produce virions containing single-stranded DNA that corresponds to the coding strand. Therefore, single-stranded sequencing should be performed using the T7 terminator primer (Cat. No. 69337-3). The map of the vector as following:

pET 22B vector contains pectate lyase B (PelB) ' N-terminal leader sequence' composed from 22 amino acids and almost 2.2 kDa which can direct target protein to periplasmic space of the host cell by Sec pathway. Also it contains ampicillin resistance gene which make the host cell to be resistance for ampicillin (novagen pET system manual, 2012).

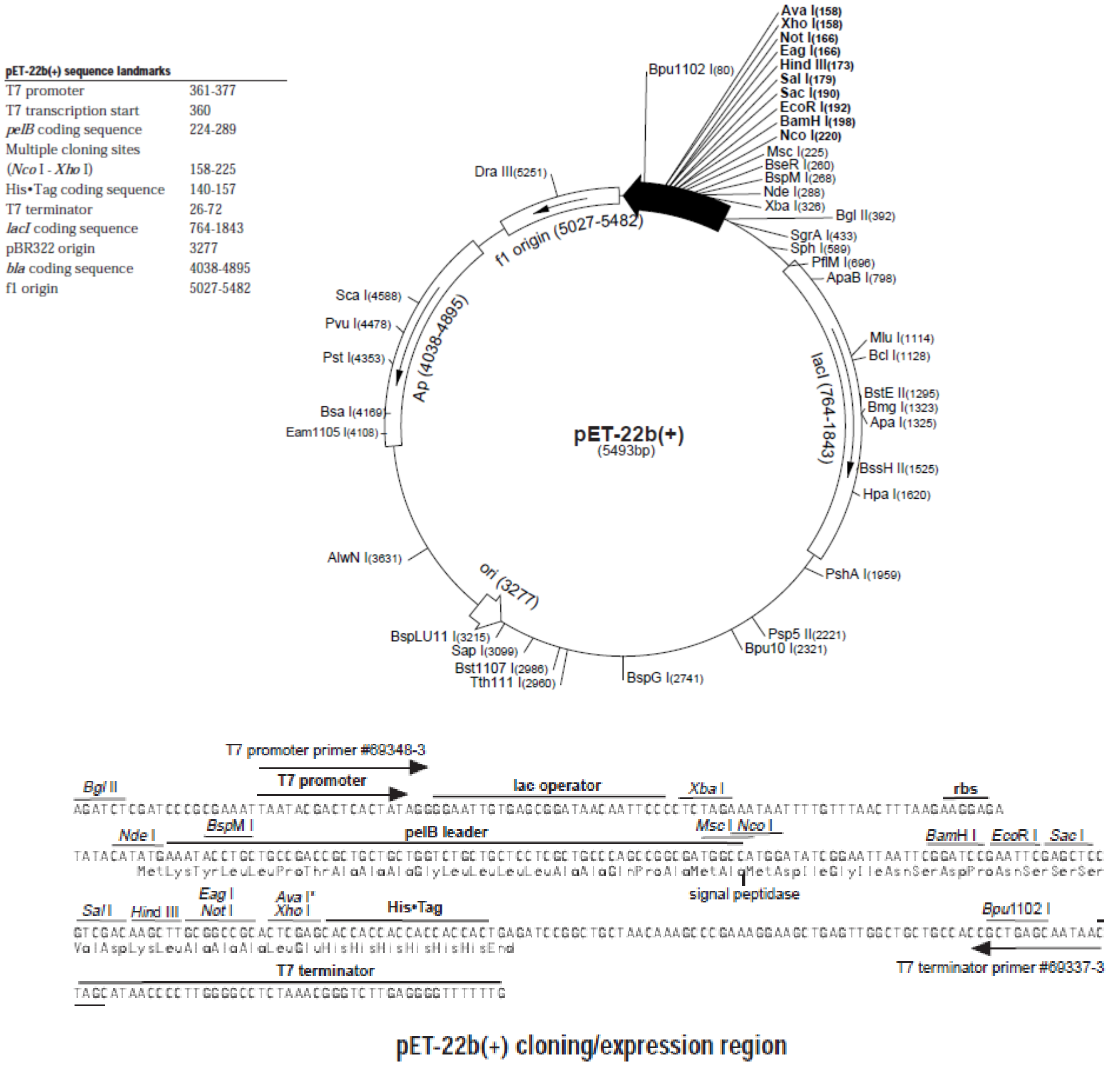


Figure 1. 4. The map of pET22B vector.

1.10. Codon Usage

Codon usage may also affect the level of protein expression. If the gene of interest contains codons not commonly used in *E. coli*, low expression may result due to the depletion of tRNAs for the rarer codons. When one or more rare codons is encountered, translational pausing may result, slowing the rate of protein synthesis and exposing the mRNA to degradation. This potential problem is of particular concern when the sequence encodes a protein >60kDa, when rare codons are found at high frequency, or when multiple rare codons are found over a short distance of the coding sequence. For example, rare codons for arginine found in tandem can create a recognition sequence for ribosome binding (e.g., AGGAGG) that closely approximates a Shine- Dalgarno sequence UAAGGAGG. This may bind ribosomes nonproductively and block translation from the bona fide ribosome binding site (RBS) at the initiator codon further upstream. Nonetheless, the appearance of a rare codon does not necessarily lead to poor expression. It is best to try expression of the native gene, and then make changes if these seem warranted later. Strategies include mutating the gene of interest to use optimal codons for the host organism, and co-transforming the host with rare tRNA genes. In one example, introduction into the *E. coli* host of a rare arginine (AGG) tRNA resulted in a several-fold increase in the expression of a protein that uses the AGG codon. In another case, substitution of the rare arginine codon AGG with the *E. coli*-preferred CGU improved expression. Other work has shown that rare codons account for decreased expression of the gene of interest in *E. coli*. Rare codons may have an even more dramatic effect on translation when they occur close to the initiator codon. While codon usage is not the only or most important factor, be aware that it may influence translation efficiency.

1.11. Importance of the Study

Phenolic contaminants are found in wastewaters of various industries including pulp paper, coal conversion, petroleum refining, beverage (beer factory wastewaters), textile and non-textile dye and other synthetic compounds. They are heavily found in paper mill effluents and olive oil mill wastewaters (OMW) and also released into the environment by the degradation of pesticides. Many of these phenolic compounds are easily absorbed by animals and plants and have a broad spectrum of toxic effects (Oktem et al., 2012).

Phenols, and particularly chlorophenols, are toxic, and potentially carcinogenic, and they can affect the taste and odor of drinking water with concentrations as low as a few $\mu g.L^{-1}$.Therefore, the presence of phenols in wastewater strictly regulated in many countries, e.g. the European Community Directive (80/778/EEC), sets a maximum concentration of 0.5 $\mu g \cdot L^{-1}$ in aquatic environments and their individual concentration should not exceed 0.1 $\mu g \cdot L^{-1}$ (Santana et al., 2009).

One of the most important industrial activities in Turkey with its magnitude of economy and provided employment is the textile industry while the wastewater originated from activities in this industry has more phenolic contaminants in different concentrations. Due to many published academic papers it is sensible to use tyrosinase enzyme for bioremediation of toxic phenolic compounds from wastewaters (Ensuncho et al., 2005) and for progression and improvement of Turkish textile industry by using of cross-linking characteristic of this enzyme (Shuster et al., 2010).

In Turkey, there is a good potential to development of silk industry which is economy source of many family in Ankara, Antalya, Sakarya, Bolu, Eskişehir and specifically in Bursa. The tyrosinase enzyme can provide modification and advancement in silk industry of Turkey. Also, this enzyme can be useful in recovering of sericin protein in silk industry which has wide applications in medical, pharmaceutical and cosmetic (Freddi, 2006).

L-DOPA is considered as the most potent drug available in the market for the treatment of Parkinson's disease. The world market for L-DOPA amounts to about 250 tons $year^{-1}$, and the total market volume is about \$101 billion $year^{-1}$.Tyrosinase catalyses the conversion of L-tyrosine to L-DOPA (Surwase et al., 2012)

Due to the report published in Parkinson Hastalığı Derneği in July 29, 2011, it is estimated to be almost 200,000 parkinson's patients in Turkey and 10,000,000 in world (Parkinson Hastalığı Derneği, 2011).

Biotransformation of L-tyrosin in to L-DOPA catalysed by tyrosinase enzyme is the one of the main and safe method in L-DOPA production, also, the value of L-DOPA in Sigma Aldrich company is 160 €/gr and of L-tyrosin is 0.52 €/gr meaning that L-DOPA is almost 320 times more expensive than L-tyrosin. 3-hydroxytyrosol, an excellent antioxidant and expensive (6680 €/gr) food additive is also synthesized by tyrosinase enzyme.

So, the tyrosinase enzyme can be useful and beneficial in medical and even in economy of Turkey by production of L-DOPA and 3-hydroxytyrosol.

In conclusion, tyrosinase enzyme can be very useful and beneficial in bioremediation, medical activity such as biosynthesis of L-DOPA and another anti-oxidands, food industry, textile industry and melanin synthesis in worlds and specifically in Turkey.

So it was decided to isolation of native *Bacillus* sp. having tyrosinase enzyme, cloning of the regarding gene in *E.coli*, production of L-DOPA and melanin by both native and the recombinant tyrosinase enzyme and characterization of the native *Bacillus* sp. isolate, native tyrosinase enzyme, recombinant tyrosinase enzyme, produced L-DOPA and melanin by various tests. Also analysis of biotechnological usage of the products was done.

2. LITERATURE REVIEW

Howar and Mason, in 1948, elucidate the biosynthetic pathway for melanin formation by tyrosinase enzyme. And in 1949, Lerner, et al. presented the preparation and properties of mammalian tyrosinase from the Harding-Passey mouse melanoma and showed that the enzymatic activity is associated with cytoplasmic particles and is not in true solution, at least in the type of tissue extracts ordinarily prepared.

In 1972, Lerch and Ettlincer carried out the Purification and Characterization of a Tyrosinase enzyme from *Streptomyces gzaucescens*, the enzyme contains an average molecular weight of 29100 Da and one atom of copper in the cuprous form and is strongly inhibited by KCN and 4-nitro-catechol.

In 1973, the enzyme causing black and brown color in mushroom was identified as tyrosinase enzyme, the active site of which contains a binuclear copper cluster, by Schoot-Uiterkamp and et al.

In 1983, Katz et al. reported the cloning of a DNA fragment containing the tyrosinase gene from *S. antibioticus* into the *Streptumyces* plasmid vectors, pIJ37 and pIJ41. Also they presented the preliminary structural and biochemical characterization of the clones and use of the tyrosinase gene in vector construction.

In 1996, Ikeda et al. reported the Cloning and sequence analysis of the highly expressed melanin synthesizing gene operon from *Streptomyces castaneoglobisporus* and suggested that the gene contributing to the high expression of the tyrosinase activity in *S. castaneoglobisporus* was ORF378, rather than TY*RC*.

Kong et al., in 2000, carried out the purification and characterization of a highly stable tyrosinase from *Thermomicrobium roseum*. The activity of the enzyme was optimal at pH 9.5 and 70 °C, and was increased by addition of 1 mM Mg^{2+}, K^{+} or Cu^{2+}.

In 2002, Solano et al. carried out cloning and molecular characterization of a SDS-activated tyrosinase from *Marinomonas mediterranea*. In this research intrinsic fluorescence and kinetic properties has indicated that the activation was caused by an

SDS-dependent conformational change facilitating the substrate accessibility to the dicopper active site.

In 2004, Brooks et al. published the biotransformation of halophenols using crude cell extracts of *Pseudomonas putida* F6 And showed that the rate of substrate consumption decreased with increasing substituent size (F>Cl>Br>I).

In 2004, Lie et al. A heat-inducible tyrosinase of a wild strain of *Bacillus thuringiensis* identified and partially characterized and showed that the properties of the tyrosinase are important to the application of *Bacillus thuringiensis* as a biology pesticide.

In 2005, Armen et al. performed the isolation, purification and physicochemical characterization of water-soluble *Bacillus thuringiensis* melanin. The molecular weight of the purified melanin determined by SDS-PAGE was 4 kDa and the concentration of paramagnetic centers in melanin was 0.21×10^{18} spin/g.

In 2005, Roan et al cloned the tyrosinase-encoding gene (mel) from *B. thuringiensis* 4D11, using PCR techniques and expressed in *Escherichia coli* DH5α and showed a DNA fragment with 1179 bp which contained the intact mel gene in the recombinant plasmid pGEM1179 imparted the ability to synthesize melanin to the *E. coli* recipient strain.

In 2005, Ito and Kuniyo reported the catalytic properties of an organic solvent–resistant tyrosinase from *streptomyces* sp. REN-21 and its high-level production in *E. coli*. The enzyme was shown to oxidize Gly-L-Tyr most effectively among peptide substrates tested in the research.

In 2005, lantto et al. reported that tyrosinase has capability to activate tyrosine residues of wool fibres and to catalyze crosslink formation between peptides derived from a wool protein hydrolyzate resulting in enzymatic modification of wool.

Ensuncho et al. in 2005 carried out the removal of aqueous phenol using immobilized enzymes in a bench scale and pilot scale three-phase fluidized bed reactor.

In 2006, Khajeh et al. performed the isolation and biochemical characterization of laccase and tyrosinase activities in a novel melanogenic soil bacterium and showed that melanin protects bacterial strain against UV lights and the oxidant

In 2007, McMahon et al. performed biochemical characterisation of the coexisting tyrosinase and laccase in the soil bacterium *Pseudomonas putida* F6. The purified tyrosinase was a monomer with a relative molecular mass of approximately 39,000 while the purified laccase had a relative molecular mass of approximately 59,000.

In 2008, a new tyrosinase was isolated from *Aeromonas media* strain WS and purified to homogeneity by wan et al. The purified tyrosinase, termed TyrA, had a molecular mass of 58 kDa and an isoelectric point of 4.90.

Hamed et al., in 2008, used tyrosinase enzyme from *Bacillus thuringiensis* for the decontamination of water polluted with phenols.

In 2009, jus et al. reported the potential of tyrosinases to activate tyrosine residues of wool protein fibers for cross-linking with different materials like collagen, elastin and gelatin was assessed.

Shuster and fishman, in 2009, described the isolation of a tyrosinase producing bacterium from soil, the cloning of the tyrosinase gene into *Escherichia coli* and the subsequent purification and characterization of the enzyme. The activity of the purified enzyme was increased in the presence of water miscible organic solvents.

Fairhead and Thony-Meyer, in 2010, reported that the tyrosinase from the bacterium *Verrucomicrobium spinosum* has a C-terminal extension, that must be removed (via proteolysis) to be fully active.

In 2012, Sambasiva Rao et al. reported the production of laccase- and peroxidase-free tyrosinase enzyme by isolated *Streptomyces antibioticus*.

Raval et al., in 2012, reported the identification of the isolate producing tyrosinase and confirmation of the production of L-DOPA as well as optimization of the environmental parameters for maximum production of tyrosinase and L-DOPA.

Surwase et al., in 2012, carried out the Optimization of L-DOPA production by *Brevundimonas* sp. SGJ using response surface methodology.

3. MATERIAL AND METHOD

3.1. Materials

3.1.1. LB Medium

Yeast extracts	5g
NaCl	10g
Triptone	10g

add D.D.W to 1000 ml

3.1.2. T Medium

tryptone	1.5g
NaCl	5g
yeast extract	1.5g

add D.D.W to 1000 ml

3.1.2. TB Medium

tryptone	1.2g
yeast extract	2.4 g
glycerol	0.4%

add potassium phosphate buffer 89 m M to 1000 ml

3.1.3. SOC Media (15 ml)

Peptone	2%
Yeast Extract	0.5%
NaCl	0.05%
MgCl2	10 mM
MgSO4	10mM

Glucose 20mM

Solubilize in 15 ml ddH2O, autoclave and aliquot and store at -20o C

3.1.4. TE (for 100 ml)

Tris-Hcl	pH=8 10mM (0.12 gr)
EDTA	1mM (0.0322 gr)

add D.D.W to 100 ml

3.1.5. TBE Buffer pH=8 (10 X) for 1000 ml

Tris base	890 mM	107.812 gr
Boric Acid	890 mM	55.0 gr
EDTA	25mM	9.25 gr

add dd H_2O to 1000 ml adjust pH to 8

3.1.6. 10X Agarose Gel Loading Buffer

Ficol 15%	1.5gr
Bromophenol blue 0.2%	0.02g
Xylene cyanole FF 0.2%	0.02g

add dd H2O to 10ml (Stor at -20°C)

3.1.7. Solutions for SDS-PAGE:

Solution A for 100 mL

Acrylamide 30% w/v	29.2gr
Bis-acrylamide 8% w/v	0.8gr

add DDW to 100 ml and Stir until completely dissolved.

Solution B (4 X separating Gel Buffer) for 100 mL

Tris-HCL 2M (pH:8.8)	75ml
SDS 10%	4ml
H_2O	21ml

Solution C (4 X Stacking Gel Buffer 100 ml)

Tris –HCL 1M (pH:6.8)	50ml
SDS 10%	4ml
H_2O	46ml

Ammonium Persulfate 10%

0.5 gr of Ammonium persulfate in 5ml H_2O

Electrophoresis Buffer: 1 liter

Tris-base	3gr	25mM
Glycine	14.4gr	192mM
SDS	1gr	0.1%

add H_2O to make 1 litter pH=8.3

Coomassie Gel Stain 1 litter

Coomassie Blue R-250	1.0gr
Methanol	450 ml
H_2O	450 ml
Glacial acetic acid	100 ml

Coomassie Gel Destain : 1 litter

Methanol	100ml
Glacial acetic acid	100ml
H_2O	800ml

Electroblotting Buffer for 1 litter

Tris-base	2.224 gr	20mM
glycine	11/26gr	150mM
Methanol	200ml	20%
Add D.D.W	800ml	

3.1.8. Bacterial Strains and Plasmids

Rosetta-gami 2(DE3) pLysS, E,coli Bl21DE3 PlysS from Novagen and JM109 from promega. pET22-b from nova gene and pGEM'-Teasy vector from promega were used in this research.

3.1.9. Kit Used

Wizard® SV Gel and PCR Clean-Up System, Wizard®Plus SV Minipreps DNA Purification System Wizard® Genomic DNA Purification Kit and MagneHis™ Protein Purification System were purchased from promega.

3. 1. 10. Enzymes

NcoI, NotI, GoTaq® Flexi DNA Polymerase, DNA ligase and RNase H purchased from promega.

3.1.11. Chemicals

All chemicals and solutions used were supplied by MERCK (GERMANY) or SIGMA (USA) or DIFCO (USA) unless stated otherwise in the text. DNA and protein size markers were supplied by PROMEGA.

3.1.12. Standard Graphs Designed for Melanin, L-DOPA and L-Tyrosine

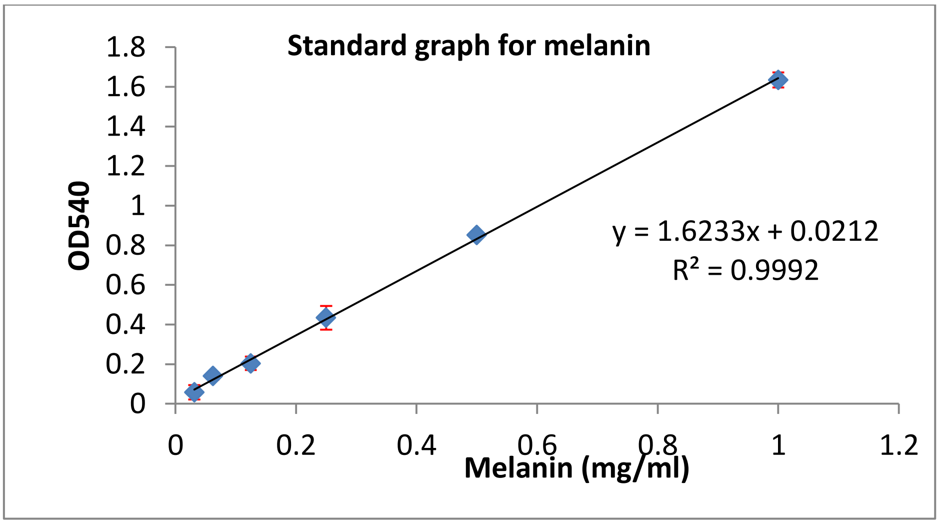


Figure 3.1. Standard graph for melanin

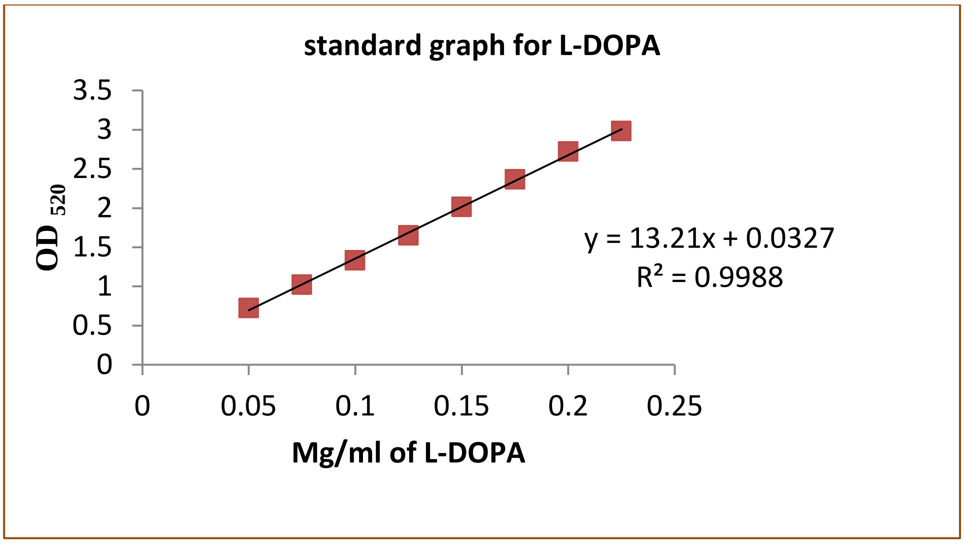


Figure 3.2. Standard graph for L-DOPA

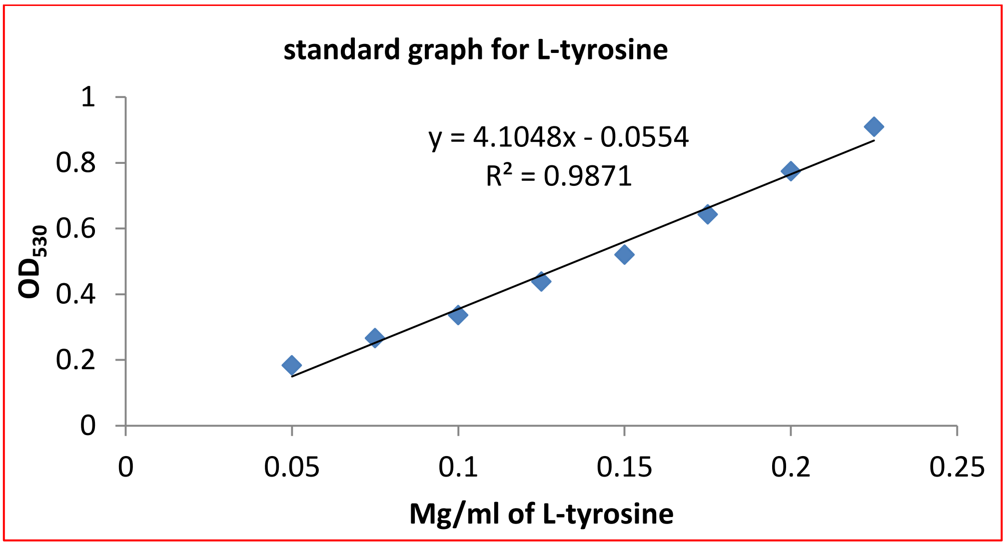


Figure 3.3. Standard graph for L-tyrosine

3.2. Methods

3.2.1. Isolation of Tyrosinase Enzyme Producing *Bacillus* sp.

Soil samples were gathered from different regions of Turkey. Almost 2 g of the soil sample was solved in 10 ml sterile water and subjected to heat –shock for isolating of *bacillus* sp. the isolated bacterial strains were cultured on T medium (1.5g tryptone, 1.5.0g yeast extract and 5.0g NaCl per liter with a final pH of 7.0) supplemented by l-tyrosine (1%) and CuSO4 (100mM) and incubated at 37°C for 72 hour. Colony which formed black or black-brown zone on medium containing tyrosine while it made clear zone in medium without tyrosine was selected as a prospective colony containing tyrosinase activity (Dalfard et al. 2006; Sambasiva et al. 2012). Further confirmation studies are carried out by inoculating a loopful of each colony in liquid media at 37^{o}C for 72 h at 200 rpm (above medium without agar) and enzyme activity was quantified by the amount of the coloured intermediate dopachrome present in supernatant determined by using a spectrophotometer at 475 nm. Dopachrom is an intermediate of melanin biosynthesis that is made from o-quinones by nonenzymatic oxidation. The extinction coefficient (ε) for the product dopachrome is 3600 L/mol.cm (Sambasiva et al. 2013). Among 12 positive strains, the *bacillus sp*. M36 with higher activity was selected for further study. To determine whether the observed brown color was due to the presence of manganese oxide, small pieces of Whatman paper dipped in 5M $MnCl_2$ were then placed at one end of the plates and monitored for one week (Dalfard et al. 2006; Shuster and Fishman 2009; Sambasiva et al. 2012).

3.2.1.1. Identification of Isolated Bacterial Strain

Identification of the strain was carried out by studding its morphological and biochemical characteristics according to the methods described in Bergey's Manual of Systematic Bacteriology (Caf et al. 2009; Zanjani et al. 2012).

In order to analysis of 16S rDNA, wide range of *bacillus* strains 16s rDNA sequences were extracted, aligned and analyzed by http://www.ncbi.nlm.nih.gov/ , http://www.ebi.ac.uk/ and bioinformatics software such as MEGA5. By considering the conserved regions of the blasted sequences, primer pairs were designed by Clon Manager.9 and Oligo.7 programs in such a way that they could bind and work with the large number of *bacillus* sp. and could amplified ≥ 1400 bp of 16s rDNA.

The primers were synthesized by "integrated DNA Technologies, Inc. (IDT)". After that *bacillus* sp. M36 genomic DNA was extracted and purified using Wizard® Genomic DNA Purification Kit (Promega) and used as a template to amplify 16s rDNA sequence using the designed *bacillus* specific primers and GoTaq® Flexi DNA Polymerase by PCR. A DNA thermal cycler (Eppendorf) was used and programmed as follows: initial denaturation at 94°C for 4 min, melting at 94°C for 30 s, annealing at 50°C for 30 s, elongation at 72°C for 90 s; 30 cycles, followed by a final extension step at 72°C for 5 min.

After purification by Wizard® SV Gel and PCR Clean-Up System, the PCR product was ligated to pGEM'-Teasy vector and transformed to JM109 Competent Cells. By culturing on LB medium containing X-Gal, the transformed competent cells with recombinant vector (having 16s rDNA fragment) was screened. Then the screened colonies were analyzed by direct colony PCR using the *bacillus* sp. specific primers. Thereafter, the vector containing 16s rDNA fragment was extracted and purified by Wizard®Plus SV Minipreps DNA Purification System and utilized for sequencing of 16s rDNA using pUC/M13 Primers, ABI PRISM® BigDye Terminator Cycle Sequencing Kit and Applied Biosystems® Genetic Analysis system with Sanger sequencing method (Radjasa et al. 2007).

3.2.1.2. Phylogram Analysis

The obtained sequence was subjected to nucleotide blast, alignment analysis and construction of the phylogenetic tree by the neighbor joining method (Saitou and Nei 1987) using http://www.ncbi.nlm.nih.gov/ , http://www.ebi.ac.uk/ and bioinformatics software such as MEGA5 (Tamura et al. 2011). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.25). The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar, 2002).

3.2.1.3. Optimization of Culture Condition for Enzyme Production

In order to production of enzyme, culture condition such as carbon sources, nitrogen sources pH values, temperature and incubation time was optimized. At first the same medium used to screen bacterial strain having tyrosinase activity was opted for enzyme production. To determine the effect of temperature and pH on the growth and enzyme production, experiments were carried out at various temperatures (20°C-80°C) and pH (5-12), and optimum pH and temperature was selected (Dalfard et al., 2006; Anupama and Jayaraman, 2011). In order to optimize incubation time, the isolated strain was cultured in the medium and incubated at optimum temperature and pH followed by monitoring the enzyme activity (by measuring OD_{530}) for 24 hour. For selection of optimum ingredient of the medium, the effect of some nitrogen sources such as peptone, casein, gelatin and ammonium nitrate on enzyme production was studied by using each of these compounds as a nitrogen source instead of tryptone in medium. Then the best nitrogen source was selected. After that some carbon sources (starch, glucose, glycerol, maltose and fructose) were incorporated to the media (with optimum nitrogen source) and their effect on enzyme production were evaluated. Also, in this research by incorporating various concentrations (0.2-3mg/ml) of l-tyrosine to medium, the concentration of l-tyrosine which had maximum effect on enzyme production was selected. Finally, amount of yeast extract, nitrogen source, NaCl and $CuSo_4$ was optimized by Box-Behnken design from response surface method using Design Expert Software (trial version 9) (table 1).

Table 3.1. The levels of variables chosen for the Box-Behnken optimization experiments.

Variable	unite	Levels (-1)	Levels (0)	Levels (+1)
Yeast extract	gr/100ml	0.05	0.525	1
Tryptone	gr/100ml	0.05	0.225	1
NaCl	gr/100ml	1	3	5
$CuSo_4$	µM	50	175	300

An experimental design of 29 experiments with five central points were formulated and the experiments were conducted in three replicate in 100 ml Erlenmeyer flasks containing production medium prepared according to the design and inoculated with the *bacillus* sp. M36 and incubated for 16 hour at shaker incubator (175 rpm) at 36 °C. Result of the experiments was measured as international unit (IU) of enzyme by measuring of dopachrom amount using OD_{475} by spectrophotometry and submitted to ANAVA analysis by the design expert software. The IU of enzyme was calculated by eqn 1 (Bisswanger, 2001).

Eqn 1.

$$\mathrm{IU/ml} \sim \mu\mathrm{mol/\min/ml)} = \frac{\mathrm{absorption/\min} \cdot \mathrm{assay\ volume\ (ml)} \cdot \mathrm{dilution\ factor} \cdot 10\,000}{\varepsilon_{\mathrm{nm}}(\mathrm{l} \cdot \mathrm{mol^{-1}\ cm^{-1}}) \cdot 1\ \mathrm{cm} \cdot \mathrm{enzyme\ volume\ (ml)}}$$

A second order polynomial model which describes the relation between the response and the chosen variables was developed and given in Eqn 2.

Eqn 2.

$$Y = \alpha_0 + \sum_{j=1}^{k} \alpha_j x_j + \sum_{j=1}^{k} \alpha_{jj} x^2{}_j + \sum\sum \alpha_{,j}\ x_{,}\ x_j$$

Where Y is the response, α_0, α_j, α_{jj}, α_{ij} are the regression coefficients for the intercept, linear, quadratic and interaction effects, respectively and X_i and X_j are coded independent variables.

3.2.1.4. Enzyme Production and Partially Purification

The *bacillus* sp.M36 was cultured at optimized condition for 16 hours then In order to enzyme purification, to start with, the cell free extract was prepared then the extract was subjected to ammonium sulfate precipitation and dialysis.

For cell free extract preparation, the medium culture was centrifuged at 6000 g for 10 min at 4°C when OD_{530} of medium culture was 1.3. Then the obtained supernatant was stored at +4°C and the pellets were washed twice in ice-cold 50mM potassium phosphate buffer, pH 7.0. After that the pellets were resuspended in 0.1 M sodium phosphate pH 7.0 containing an inhibitory bacterial proteases cocktail (1: 4, µl: mg cell mass) and disrupted by sonication. The homogenate was centrifuged at 14000 g for 15 min. The supernatant achieved by the previous centrifuge at 6000 g along with the supernatant obtained by centrifuge at 14000 g were used as a cell free extract (Lopez-Serrano et al. 2002; McMahon et al. 2007; Michalik et al. 2012). The cell free extract was subjected to precipitation with ammonium sulfate (40, 50, 60, 70, 75, 80, 85 and 90% saturation) for 1 h with gentle stirring. After fractionation with ammonium sulfate the precipitated proteins are recovered by centrifugation at 12000g for 30 min. and are dialyzed against 50 mM sodium phosphate buffer, pH 6.8 with 0.02% sodium azide, 0.01 mM CuSO4. The fractions were tested to tyrosinase activity and active fractions were stored at –20 ° C without loss of activity (El-Shora rt al. 2008). Protein contents of the samples were determined by Bradford method using bovine serum albumin (BSA) as the standard (Kohashi et al. 2004).

3.2.1.5. Enzyme Assay

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme was determined before the enzyme activity was assayed and an aliquot of the enzyme solution is added to a 0.1M sodium phosphate buffer (pH 6.8) containing 1mM L-tyrosine and L-DOPA , and the formation of dopachrome is monitored by measuring the absorbance at 475 nm. Dopachrom "coloured intermediate" is an intermediate of melanin biosynthesis that is made from o-quinones by nonenzymatic oxidation. The extinction coefficient (ε) for the product dopachrome is 3600 L/mol.cm (Sambasiva Rao et al., 2013). The initial rate is used for the calculation of tyrosinase activity. One international unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 μmol of L-tyrosine to dopachrom per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome (3600 M^{-1} cm^{-1}) by the following equation:

$$\mathrm{IU/ml} \sim \mu\mathrm{mol/\,min\,/ml)}$$
$$= \frac{\mathrm{absorption/\,min} \cdot \mathrm{assay\ volume\ (ml)} \cdot \mathrm{dilution\ factor} \cdot 10\,000}{\varepsilon_{\mathrm{nm}}(\mathrm{l} \cdot \mathrm{mol^{-1}\,cm^{-1}}) \cdot 1\,\mathrm{cm} \cdot \mathrm{enzyme\ volume\ (ml)}}$$

3.2.1.6. Electrophoretic Study

The enzyme solution was loaded in several well of Non-denaturing PAGE (8% w/v) and after separating protein bands, a single lane of the gel was sliced out of the gel using a clean scalpel. The tyrosinase enzyme related band was stained by placing the gel slice in substrate solution (0.1 mg/ml l-tyrosine and 50 µM $CuSO_4$ in 0.1 M phosphate buffer, pH 7) for 60 min. The formation of a dark-brown band indicated the position of the tyrosinase enzyme. The remaining lanes of the gel were placed in 50mM phosphate buffer, pH 7.0. Using the activity stained lane as a guide to the location of tyrosinase, the corresponding band was sliced out of the unstained lanes. The gel slice was homogenized and resuspended in a 50mM phosphate buffer and left overnight at 4 ◦C. The gel suspension was centrifuged at 12000 g for 10 min to remove remaining gel fragments and the obtained supernatant was subjected to SDS-PAGE (12%) analysis for determination of the tyrosinase enzyme molecular weight (Arikan, 2008).

3.2.1.7. Effect of pH and Temperature on Enzyme Activity and Stability

For this purpose, 200µl of enzyme solution (protein content, 0.05 mg/ml) was added to 1800µl buffer containing 1mM of L-dopa for diphenolase and 1mm of l-tyrosinae for monophenolase activity and incubated for 45 min. The effect of pH on monophenolase activity was investigated by analyzing the activity at different pH values (pH 4, 5, 6, 7, 8, 9, 10, 11 and 12) and for diphenolase activity pH (4-7.5) were tested because L-DOPA spontaneously converted to dopachrome at pH values above 7.5. pH value in which the enzyme showed maximum relative activity was determined as optimum pH for the enzyme activity (Burhan et al., 2003).

Also, the enzyme activity was analyzed at a range of temperatures from 10 to 70 °C (10, 20, 30, 40, 50, 60 and 70) and the temperature showing maximum relative activity was determined as an optimum temperature for the enzyme activity. In order to ascertain of the temperature stability, the enzyme solutions in different tubes are incubated at various temperatures in the range from 0°C to 70oC for 2 hour then residual activity is assayed in enzyme assay condition (Liu et al., 2004).

3.2.1.8. Effect of Detergents on Enzyme Activity

To examine the effects of sodium dodecyl sulphate (SDS), ethylene diamine tetraacetic acid (EDTA), Urea, Tween-80, TritonX-100, β-Mercaptoethanol and PMSF are analyzed by incubating enzyme in the presence of these detergents and substrate (Aygan and Arikan, 2009).

3.2.1.9. Kinetic Study of m36 Tyrosinase Enzyme

The initial rate of enzyme reaction for l-tyrosine and L-DOPA was determined at various concentrations. The resulting data was analyzed and the Km and Vmax values are calculated by Michaelis–Menten and Hill equation $v_i = \frac{V_{max}[S]}{k_m+[s]}$ and Lineweaver-burk equation $\frac{1}{v_i} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}}$ [11]. After addition of 200 µl of enzyme solution (protein content, 0.05mg/ml) to potassium phosphat (50mM, pH, 7) containing varius concentrations ranging from 0.02 to 0.8 for tyrosine and 0.06 to 2.0 for L-DOPA, the reaction medium with L-DOPA and with l-tyrosine was incubated at room temperature for 30 min and 45 min, respectivly. After that the reaction medium with l-tyrosine was diluted 5 times and reaction medium with L-DOPA was diluted 10 time and both of them was subjected to study of OD_{475} by spectrofotometer. The obtained data was used to calculation of velocity.

3.2.1.10. Analysis of L-DOPA Production by the Enzyme

200µl of the enzyme solution was added to 50 mM potassium phosphate buffer pH 6.8 containing 4mM L-tyrosine, 5mM L-ascorbic acid and 5 µM $CuSo_4$ and incubated at optimum temperature for 45 min in shaker. The total volume of the reaction mixture was 5 ml. Control experiments without an enzyme were performed with the substrate.

3.2.1.11. Quantitatively Study

The amount of L-tyrosine used and L-DOPA produced was evaluated as following:

a) L-dopa

The L-DOPA produced in the reaction mixture was determined according to Arnow's method (Surwase et al., 2012). After adding one milliliter of HCl (0.5 N), 1.0 ml of nitrite molybdate reagent (10% w/v sodium nitrite and 10% w/v sodium molybdate) was added to 1.0 ml of the reaction mixture (a yellow coloration appeared). Then 1.0 ml of 1.0 N NaOH was added (a red coloration appeared) and total volume was adjusted to 5.0 ml with distilled water. OD_{520} of the mixture was measured by a double beam UV/VIS spectrophotometer. The amount of produced L-dopa was determined using standard curve. The standard curve was made using 0.05–0.225 mg/ml concentrations of L-DOPA in potassium phosphate buffer instead of reaction mixture. The regression equation obtained was $y = 13.21X + 0.0327$, $R^2 = 0.9988$.

b) L-Tyrosine

The L-tyrosine utilized in the reaction mixture was determined according to Arnow's method. One milliliter from the same reaction mixture was added with mercuric sulphate reagent, kept in a boiling water bath for 10 min, and cooled at room temperature. Then, 1 ml of nitrite reagent was added, the volume was adjusted to 5 ml with distilled water, and the absorbance was measured at 530 nm. The determination of tyrosine was based on the Millon reaction (Ali et al., 2007). The amount of l-tyrosine was determined using standard curve. The standard curve was made using 0.05–0.225 mg/ml concentrations of L-tyrosine in potassium phosphate buffer instead of the reaction mixture. The regression equation obtained was $y = 4.104X + 0.0554$, $R^2 = 0.9871$.

3.2.1.12. Thin Layer Chromatography and HPLC Analysis of the Reaction Mixture

The conversion of L-tyrosine to L-DOPA by M36 tyrosinase enzyme was analyzed by thin layer chromatography. For this purpose, phenol-water system (75:25) (w/v) was used as a mobile phase and 3% ninhydrin in n-butanol as spray and staining reagent. Besides of TLC analysis, transformation of L-tyrosine to L-DOPA was evaluated by High-performance liquid chromatography (HPLC). The HPLC analysis was carried out using a symmetry C18 column (4.6 × 250 nm) and Water/methanol (98/2)(V/V) containing 1% phosphoric acid as a mobile phase with a flow rate of 1 ml/min and UV detector at 280 nm. The standard L-tyrosine (0.05%) and L-DOPA (0.1%) were used (Raval et al., 2012).

3.2.2. Cloning

3.2.2.1. Growth Conditions and Maintenance of Bacterial Strains:

Host cells were grown in Luria Broth (LB) medium and stored on Luria agar (LA) plates. The cultures were stored at 4 °C and subcultured monthly. Cultures grown in Luria Broth until mid-log phase were covered with 20% glycerol for long term storage at –80°C. LB and LA media were supplemented with the appropriate antibiotics, whenever necessary.

3.2.2.2. Isolation of Genomic DNA from Gram Positive and Gram Negative Bacteria

Isolation of genomic DNA was performed using Wizard® Genomic DNA Purification Kites from promega as following instruction:

1. 1ml of an overnight culture was added to a 1.5ml microcentrifuge tube.

2. The culture was centrifuged at 13,000–16,000 × g for 2 minutes to pellet the cells and removed the supernatant.

3. The pellet was resuspended thoroughly in 480µl of 50mM EDTA.

4. Appropriate lytic enzyme(s) was added to the resuspended cell pellet in a total volume of 120µl.

5. The sample was incubated at 37°C for 30–60 minutes. Centrifuged for 2 minutes at 13,000–16,000 × g and removed the supernatant.

6. 600µl of Nuclei Lysis Solution was added and gently pipeted until the cells were resuspended.

7. The sample was incubated at 80°C for 5 minutes to lyse the cells; then cooled to room temperature.

8. 3µl of RNase Solution was added to the cell lysate and inverted the tube 2–5 times to mix.

9. The sample was incubated at 37°C for 15–60 minutes and cooled the sample to room temperature.

10. 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed vigorously at high speed for 20 seconds to mix the Protein Precipitation Solution with the cell lysate.

11. The sample was incubated on ice for 5 minutes and centrifuged at 13,000–16,000 × g for 3 minutes.

13. The supernatant containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of room temperature isopropanol.

14. The sample was gently mixed by inversion until the thread-like strands of DNA form a visible mass.

15. It was centrifuged at 13,000–16,000 × g for 2 minutes.

16. The supernatant was carefully poured off and drained the tube on clean absorbent paper and 600µl of room temperature 70% ethanol was added and the tube gently inverted several times to wash the DNA pellet.

17. The sample was centrifuged at 13,000–16,000 × g for 2 minutes and the ethanol was carefully aspirated.

18. The tube was drained on clean absorbent paper and allowed the pellet to air-dry for 10–15 minutes.

19. 100µl of DNA Rehydration Solution was added to the tube and rehydrated the DNA by incubating at 65°C for 1 hour. Periodically the solution was mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4°C.

20. The DNA was stored at 2–8°C.

3.2.2.3. Gel Electrophoresis

Agarose was solved in 1 x TBE-buffer and carefully boiled in the microwave oven. When completely melted, the liquid was cooled to about 60°C. The liquid gel was poured into the gel tray held in place by clamps. Air bubbles were removed, the comb was positioned and the gel was allowed to solidify (for about 45 minutes). The tray with the solid gel was placed into the electrophoresis unit with the slots facing the cathode and 1x TBE-buffer was added until the gel was fully submerged in buffer. The comb was removed carefully, preventing destruction of the sample wells. Samples were displaced with the 6x Loading Dye Solution (promega) and loaded onto the gel. Samples from miniprep digestions generally had a total volume of 60µL, those of PCR runs 30µL (including loading dye). For size determination, 5µL of the ready-to-use DNA Ladder Mix (promega) were employed. The lid was closed and a voltage of 90 V was applied for about one hour. As the bromphenol color front ran past the end of the gel, the electrophoresis was stopped and the gel was soaked to EtBr solution for 10 min and washed by distilled water for 15 min. After that the bands were visualized under UV-light and photographed.

3.2.2.4. Amplification of M36 Tyrosinase Gene by PCR

The forward primer containing *NcoI* restriction site (5-GTTCGCGATCGCCATGGGTAACAAGTATA GAGTTAG-3) and revers primer containing *NotI* restriction site (5-ATTTTTGCGGCCGCTGAGGAA

CGTTTTGATTTTCT-3) was designed and used to amplify the tyrosinase gene by GoTaq® Flexi DNA Polymerase using a thermal cycler (Eppendorf) programmed as follows: initial denaturation at 94°C for 5 min, melting at 94°C for 50 s, annealing at 60°C for 50 s, extension at 72°C for 55 s; 30 cycles, followed by a final extension step at 72°C for 5 min. ingredient of PCR reaction mixture was as following :

Component	Final Volume	Final Conc.
5X Green or Colorless GoTaq® Flexi Buffer	10 µl	1X
MgCl2 Solution (25mM)	1 0 µl	1.0–4.0mM
PCR Nucleotide Mix, (10mM each)	0.5µl	0.1mM
upstream primer (10 µM)	2.5 µl	0.5µM
downstream primer (10 µM)	2.5 µl	0.5µM
GoTaq® DNA Polymerase (5U/µl)	0.125µl	0.625U/50 µl
template DNA (0.08µg/µl)	5 µl	0.4µg/50µl
Nuclease-Free Water to	19.37µl	
Total volume	50 µl	

3.2.2.5. PCR Product Purification

PCR product was purified using Wizard® SV Gel and PCR Clean-Up System from promega by the following procedure:

1. Following electrophoresis, DNA band was excised from gel and placed gel slice in a 1.5ml microcentrifuge tube.

2. 10µl Membrane Binding Solution was Added per 10mg of gel slice and vortexed and incubated at 50–65°C until gel slice was completely dissolved.

3. An equal volume of Membrane Binding Solution was added to the PCR amplification.

4. SV Minicolumn was inserted into Collection Tube.

5. Dissolved gel mixture or prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute.

6. The sample was centrifuged at 16,000 × g for 1 minute and discarded flowthrough and reinserted Minicolumn into Collection Tube.

7. 700µl Membrane Wash Solution was added to the sample and centrifuged at 16,000 × g for 1 minute. The flowthrough was discarded and Minicolumn was reinserted into Collection Tube.

8. Step 4 was repeated with 500µl Membrane Wash Solution and centrifuged at 16,000 × g for 5 minutes.

9. The Collection Tube was emptied and the column assembly was recentrifuged for 1 minute with the microcentrifuge lid open (or off) to allow evaporation of any residual ethanol.

10. Carefully the minicolumn was transferred to a clean 1.5ml microcentrifuge tube.

11. 50µl of Nuclease-Free Water was added to the Minicolumn and Incubated at room temperature for 1 minute and centrifuged at 16,000 × g for 1 minute.

12. The Minicolumn was discarded and DNA was stored at 4°C or –20°C.

3.2.2.6. Plasmid Purification

Plasmid purification was done using Wizard®Plus SV Minipreps DNA Purification System by the following instruction:

1. 1–10ml of overnight culture was pelleted for 5 minutes.

2. The pellet thoroughly was resuspend with 250µl of Cell Resuspension Solution.

3. 250µl of Cell Lysis Solution was added to each sample and inverted 4 times to mix.

4. 10μl of Alkaline Protease Solution was added and inverted 4 times to mix and Incubated 5 minutes at room temperature.

5. 350μl of Neutralization Solution was added and inverted 4 times to mix.

6. The sample centrifuged at 13000 g for 10 minutes at room temperature.

7. Spin Column was inserted into Collection Tube.

8. The obtained cleared lysate was decanted into Spin Column and centrifuged at 13000 g for 1 minute at room temperature. The flowthrough was discarded and the column reinserted into Collection Tube.

10. 750μl of Wash Solution (ethanol added) was added to the sample and centrifuged at 13000 g for

1 minute. The flowthrough was discarded and the column reinserted into Collection Tube.

11. Step 10 was repeated with 250μl of Wash Solution.

12. The sample was centrifuged at 13000 g for 2 minutes at room temperature.

13. Spin Column was transfered to a sterile 1.5ml microcentrifuge tube.

14. 100μl of Nuclease-Free Water was added to the Spin Column and centrifuged at 13000 g for 1 minute at room temperature.

15. The column was discarded and DNA was stored at –20°C or below.

3.2.2.7. Digestion

Digestion of PCR product and vector was performed by the following reaction mixture as described by the manufacturer’s instruction.

Nuclease-Free Water	14μl
10X Restriction Buffer	2μl
Acetylated BSA (1mg/ml)	2μl
DNA (~1μg)	1μl
NcoI (10U)	1μl
NotI (10U)	1μl
Final Volume	20μl

The reaction mixture was mixed by pipetting and collected the contents at the bottom of the tube and Incubated at 37°C for 4 hours.

3.2.2.8. Ligation

In order to ligation of digested and purified PCR product and vector, the following reaction was assembled in a sterile microcentrifuge tube and Incubateed the reaction at 10°C temperature for overnight.

Vector DNA	100ng
Insert DNA	33ng
2X Rapid Ligation Buffer	5µl
T4 DNA Ligase (3u/µl)	1µl
Nuclease-free water to	10µl
Total reaction volume	50µl

3.2.2.9. Preparation of Competent Cell

Competent cells were prepared from host cells as the protocol of the molecular cloning manual book (Sambrook et al., 2012) as following:

1. 100 of LB media were inoculated with 1 mL starter culture and grow in 37°C shaker. The OD600 was measured every hour, then every 15-20 minutes when the OD got above 0.2.
2. When the OD600 reached 0.35-0.4, immediately the cells put on ice and the culture was chilled for 20-30 minutes.
3. The cells were harvest by centrifugation at 3000*g* for 15 minutes at 4°C.
4. The supernatant was decanted and gently resuspend each pellet in about 40mL of ice cold MgCl2.
5. The cells were harvested by centrifugation at 2000*g* for 15 minutes at 4°C.

6. The supernatant was decanted and the pellet was resuspended in about 40 mL of ice cold CaCl2. this suspension was kept on ice for at least 20 minutes.
7. The cells were harvested by centrifugation at 2000*g* for 15 minutes at 4°C.
8. The supernatant was decanted and the pellet was resuspended in ~20mL of ice cold 85 mM CaCl2, 15% glycerol.
9. The cells were harvested by centrifugation at 1000*g* for 15 minutes at 4°C.
10. The supernatant was decanted and the pellet was resuspended in 800µl of ice cold 85 mM CaCl2, 15% glycerol.
11. 100µl of the sample was aliquoted into sterile 1.5 mL microfuge tubes and the cells were stored in the -80°C freezer.

3.2.2.10. Transformation of Competent Cell

CaCl2 method was used to transform the competent cells (*Rosetta-gami 2(DE3)pLysS* and *E.coli Bl21DE3*) with the ligation mixture by the modified method described by Sambrook *et al*. (1989). 10 ml of LB was inoculated with 100 µl of overnight incubated *E. coli* cell culture, and incubated at 37°C until the OD600 reached at 0.5. 1 ml of the cell culture was centrifuged at 3000 rpm for 10 minutes and then the cell pellets were resuspended in 100 mM sterilized CaCl2. The cells were left on ice for 30 minutes. After that, 1-2µl of plasmid DNA was added to the competent cells. The competent cells were kept on ice for 1 hour, treated with heat shock at 42°C for 1 minute, and kept on ice for 10 minutes. 800 µl of LB medium was added; the competent cells were kept at 37°C with shaking, and then recentrifuged at 3000 rpm for 1-5 minutes. The cell pellets were resuspended in 100 µl of LB medium. Finally, the competent cells were spread on LB agar plates containing appropriate antibiotic and transferred to 37oC incubator for 16-20 hours.

3.2.2.11. Screening of Transformed Cell with High Tyrosinase Activity

The transformed cells were proved by culturing on medium containing ampicillin and direct colony PCR and some of transformed strains were cultured on

LB medium supplemented by agar (15%), ampicillin (100 µM), tyrosine (0.05%) and CuSo4 (100 µM) and incubated at 37 °C for overnight. Colony forming black or black-brown zone was selected as a colony containing active tyrosinase. One of the colonies with high activity was subjected to tyrosinase gene sequencing.

3.2.2.12. Sequencing

Recombinant pET22b vector (pET22b-tyr) was extracted from (*Rosetta-gami 2(DE3)pLysS*) and purified by "Wizard®Plus SV Minipreps DNA" and used as a templet to re-amplify the gene using the primer pairs mentioned above by PCR. After that the gene fragment ligated to pGEM'-Teasy vector and transformed to JM109 Competent Cells. Thereafter, the vector containing tyrosinase gene utilized for sequencing of the gene using pUC/M13 Primers, ABI PRISM® BigDye Terminator Cycle Sequencing Kit and Applied Biosystems® Genetic Analysis system with Sanger sequencing method. The sequencing process was performed in three repeats. After being insured from accuracy of the sequence, the sequence was analyzed by bioinformatics methods.

3.2.2.13. Bioinformatics Analysis

Homology searches of the obtained sequence (M36) in GenBank were done using the BLAST server (http://www.ncbi.nlm. nih.gov/BLAST/). Also the M36 sequence was converted to Amino acid sequence by web.expasy.org/translate . . Multiple alignments of protein sequences were done using the CLUSTALW program (http://www.ebi.ac.uk/clustalW/). The phylogenetic trees of proteins were constructed using clostalW and MEGA5 (Tamura et al. 2011). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 0.25). The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura et al. 2002).

The different physicochemical properties of the M36 tyrosinase were computed using ExPASy's ProtParam tool and these properties can be deduced from a protein sequence. The ProtParam includes the following computed parameters: Molecular weight (M.Wt), theoretical pI, instability index (II), aliphatic index (AI) and grand average of hydropathicity (GRAVY).

3.2.2.14. Enzyme and Protein Assay

Tyrosinase activity is assayed by using L-tyrosine and L-DOPA as substrates. The appropriate concentration of the enzyme was determined before the enzyme activity was assayed and an aliquot of the enzyme solution is added to a 0.1M sodium phosphate buffer (pH 6.8) containing 1mM L-tyrosine and L-DOPA , and the formation of dopachrome is monitored by measuring the absorbance at 475 nm. Dopachrom "coloured intermediate" is an intermediat of melanin biosynthesis that is made from o-quinones by nonenzymatic oxidation. The extinction coefficient (ε) for the product dopachrome is 3600 L/mol.cm (Rao et al ,2013). The initial rate is used for the calculation of tyrosinase activity. One unit (IU) of tyrosinase activity is defined as the amount of enzyme required to oxidize 1 μmol of L-tyrosine to dopachrom per minute under the above conditions, which was calculated using the molar extinction coefficient of dopachrome (3600 M^{-1} cm^{-1}) by the following equation:

$$\mathrm{IU/ml} \sim \mathrm{\mu mol/\min/ml)}$$
$$= \frac{\mathrm{absorption/\min \cdot assay\ volume\ (ml) \cdot dilution\ factor \cdot 10\,000}}{\varepsilon_{\mathrm{nm}}(\mathrm{l \cdot mol^{-1}\ cm^{-1}}) \cdot 1\ \mathrm{cm} \cdot \mathrm{enzyme\ volume\ (ml)}}$$

Protein contents of the samples were determined by Bradford method using bovine serum albumin (BSA) as the standard.

3.2.2.15. Optimization of Enzyme Expression

In order to enzyme production some important factors were optimized optimized by Box-Behnken design from response surface method using Design Expert Software (trial version 9) (table 2).

Table 3.2. The levels of variables chosen for the Box-Behnken optimization experiments for recombinant enzyme expression.

Variables	Unite	Levels (-1)	Levels (+1)
starting cell density of induction	OD_{600}	0.5	1
induction time	hour	6	20
IPTG	mM	0.4	1
Temperature	°C	20	37

An experimental design of 29 experiments with five central points were formulated and the experiments were conducted in three replicate in 100 ml Erlenmeyer flasks containing TB medium (tryptone 1.2%, yeast extract 2.4%, glycerol 0.4% and potassium phosphate buffer 89 m M) and incubated at shaker incubator (175 rpm). Result of the experiments was measured as international unit (IU) of enzyme by measuring of dopachrom amount using OD475 by spectrophotometry and submitted to ANAVA analysis by the design expert software. The IU of enzyme was calculated by eqn 1 (Bisswanger 2001).

A second order polynomial model which describes the relation between the response and the chosen variables was developed and given in Eqn 2.

3.2.2.16. Comparison of the Expression of M36 Tyrosinase Enzyme in Two Different Systems

The pET22b-tyr was transferred to E.coli BL21(DE3)pLysS and expression of the M36 tyrosinase enzyme under following optimum conditions was studied in both host cells (E.coli BL21(DE3)pLysS and Rosetta-gami B (DE3) pLysS).

Both of the host cells containing pET22b-tyr were cultured at the distinct optimum condition and after the end of induction times (6.5 hour for Rosetta-gami 2 (DE3) pLysS and 5 hour for E.cli BL21 (DE3)pLysS), the cell cultures were centrifuged and the cell pellets were re-suspended with 10 ml of ice-cold 0.1 M sodium phosphate pH 7.0 containing an inhibitory bacterial proteases cocktail (1: 4, µl: mg cell mass) and disrupted by sonication. The homogenates were centrifuged at 14000 g for 15 min and the supernatants were studied in aspect of enzyme activity. The obtained homogenates of the both host cells were analyzed by SDS–PAGE.

3.2 2.17. Expression and Purification

TB medium was inoculated with fresh and overnight culture of Rosetta-gami 2 (DE3) pLysS) having pET22b-tyr and subjected to expression under optimized conditions for 6.5 hours and extraction of protein and purification was done by the following promega manufacture’s instruction:

1. 1ml of bacterial culture was centrifuged at 10,000 × *g* for 2 minutes in a Microcentrifuge and the supernatant were removed completely.

2. The cell pellet was resuspended in 1X FastBreak™ Cell Lysis Reagent.

Example: For a 3 O.D.600 culture, 300µl of 1X FastBreak™ Cell Lysis **Reagent was used**

3. Lyophilized DNase I was added 1µl to the lysed bacterial culture and incubated with shaking for 10–20 minutes at room temperature on a rotary mixer or shaking platform.

4. 30µl of MagneHis™ Ni-Particles was added to cell pellet resuspended in 1X FastBreak™ Cell Lysis Reagent

5. The tube was inverted to mix (approximately 10 times), and incubated for 2 minutes atroom temperature.

6. The tube was placed in the magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles.

7. Using a pipette, the supernatant was carefully removed.

8. the tube was removed from the magnetic stand 150µl of MagneHis™ Binding/Wash Buffer was added to the MagneHis™ Ni-Particles and pipeted to mix.

9. The tube was placed in the magnetic stand for approximately 30 seconds to capture the MagneHis™ Ni-Particles. Using a pipette, the supernatant was carefully removed.

8. The wash step was repeated 2 times for a total of 3 washes.

9. The tube was removed from the magnetic stand and 100µl of MagneHis™ Elution Buffer was added and mixed.

10. The sample was incubated for 1–2 minutes at room temperature and placed in a magnetic stand to capture the MagneHis™ Ni-Particles. Using a pipette, the supernatant containing the purified protein was removed and the samples were analyzed by both SDS-PAGE and by functional assay.

3.2.2.18. Characterization of Recombinant M36tyrosinase Enzyme

3.2.2.18.1. Effect of pH and Temperature on Enzyme Activity and Stability

Effect of pH and temperature on enzyme activity and stability was done as mentioned for native tyrosinase enzyme.

3.2.2.18.2. Effect of Detergents on Enzyme Activity

To examine the effects of sodium dodecyl sulphate (SDS), ethylene diamine tetra acetic acid (EDTA), Urea, Tween-80, TritonX-100, β-Mercaptoethanol and PMSF are analyzed by incubating enzyme in the presence of these detergents and substrate (Aygan et al., 2009).

3.2.2.18.3. Kinetic Study of M36 Tyrosinase Enzyme

The initial rate of enzyme reaction for l-tyrosine and L-DOPA was determined at various concentrations. The resulting data was analyzed and the Km and Vmax values are calculated by Michaelis–Menten and Hill equation $v_i = \frac{V_{max}[S]}{k_m+[s]}$ and Lineweaver-burk equation $\frac{1}{v_i} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}$ (McMahon et al., 2007).

After addition of 100 µl of enzyme solution (0.31 IU) to potassium phosphat (50mM, pH, 7) containing varius concentrations ranging from 0.02 to 0.8 for tyrosine and 0.06 to 2.0 for L-DOPA, the reaction medium with L-DOPA and with l-tyrosine was incubated at room temperature for 30 min and 45 min, respectivly. After that the reaction medium with l-tyrosine was diluted 5 times and reaction medium with L-DOPA was diluted 10 time and both of them was subjected to study of OD_{475} by spectrofotometer. The obtained data was used to calculation of velocity.

3.2.3. Production of Melanin

Rosetta-gami 2 (DE3) pLysS) having pET22b-tyr was cultured on TB medium and incubated at 37°C until OD600 reach 0.85, then expression was induced by addition of 0.81mM IPTG and finally l-tyrosine (0.05%) and CuSo4 (100µM) was added to medium and incubated at 35.5 °C for 6 hours.

At the end of incubation, the culture medium was centrifuged to remove the cells, and melanin was precipitated from the supernatant by adjusting the pH to 3.0 with 5N HCl. The formed black pigment pellet was washed three times with HCl (0.1 N) and then with water. The pellet was washed with ethanol two times and then dried in air. The extracted pigment pellets were pooled for use in subsequent analyses.

3.2.3.1. FT-IR (Fourier- Transform Infrared) Spectroscopy Studies

The melanin pigment was grounded and the FT-IR spectrum was recorded at 4,000-400 cm-1 using a Perkin Elmer FT-IR spectrophotometer (USA) (Goncalves et al., 2012).

3.2.3.2. EPR Spectroscopy

One of the unique properties of melanin pigment is the presence of unpaired electrons in the polymer, which can be detected by EPR spectroscopy. EPR spectra were obtained with a Bruker EMX (X-Band) EPR spectrophotometer. (Beberok et al., 2010)

3.2.3.3. Antibacterial Effect

Some pathogenic bacteria such as Staphylococcus aureus, Acinetobacter baumannii, Enterobacter aerogenes, Pseudomonas aeruginosa, E.coli and Enterococcus faecalis that was resistance to many antibiotics was taken from balcali hospital. An overnight culture was prepared from the bacteria and the bacterial suspension was adjusted to the 0.5 McFarland standards. Then the number of colony forming unit (CFU) of the suspensions (their concentration was adjusted to 0.5 McFarland) was determined by serial dilution methods.

Mueller Hinton medium containing different concentration of melanin M36 (0, 5, 10, 15, 20, 30, 40, 50 and 60 μg/ml) was prepared and inoculated with 0.5 McFarland concentration of the bacteria and incubated at 37°C at 150 rpm for overnight. Then MIC is determined as the lowest dilution, which inhibited growth judged by both lack of turbidity in the tube and CFU counting.

After that MBC (Minimum Bactericidal Concentration) of the melanin was determined as a minimum concentration of melanin which can kill up to 90% of the CFU of the bacteria.

The standardization of the bacterial cell number used for susceptibility testing is of critical importance for obtaining accurate and reproducible results. The recommended final inoculum size for broth dilution is 5 * 10^5 (CFU) ml^{-1} (Lin et al., 2005).

3.2.3.4. Antioxidant Effect of the M36 melanin

For this purpose OH Scavenging and O2 -· Scavenging activity of the M36 melanin was studied as following:

3.2.3.4.1. OH Scavenging Assay

·OH scavenging activity was determined using a modified method of Wang et. Orthophenanthroline solution (5×l0-3 mol·L^{-1}) of 1.5 mL was taken and added with 2.0 mL of phosphate buffer (pH 7.4, 0.05 mol·L-1) and evenly mixed, then added with 1.0 mL of 7.5×10^{-3} mol·L^{-1} FeSO4 solution, evenly mixed immediately after the addition of each tube, and then added with 1 mL of H2O2 (0.1%), and finally supplemented with distilled water to a total volume of 10 mL. This reaction solution was kept at 37 °C for 1 h, and Adamaged was measured at 536 nm. The ·OH scavenging abilities of the melanin and Vc solutions were determined with the same method as described above. Melanin solutions or Vc solutions of different concentrations were added, and then added with H2O2. After being kept warm at 37 °C for 1 h, Asampled was measured. And the undamaged tubes were not added with H2O2, melanin solution or Vc solution (Ye et al.,2011).

·OH scavenging rate(%)＝[(Asampled - Adamaged) ／(Aundamaged - Adamaged)]×100.

3.2.3.4.2. O2 $^{-\cdot}$ Scavenging Assay

Pyrogallol oxidation method was adopted. Pyrogallol can be quickly oxidated by itself in alkaline conditions, generating series product that is absorbed at near 318 nm as well as releasing $O2 -\cdot$. Method: Tirs-HCl (pH=8.2, 50 mmol·L-1) of 3mL was taken and added with melanin solutions of different concentrations and evenly mixed, this solution was kept at 25 ℃ for 20 min then pyrogallol solution (7 mmol·L-1) of 0.3mL preheated at 25 ℃ was added to react exactly 4 min and HCl (10 mmol·L-1) of 1 ml was added to terminate reaction. A1 was measured at 318 nm.

O2 -·scavenging rate (%) ＝ [1-(A1-A1′)/ A0] ×100%

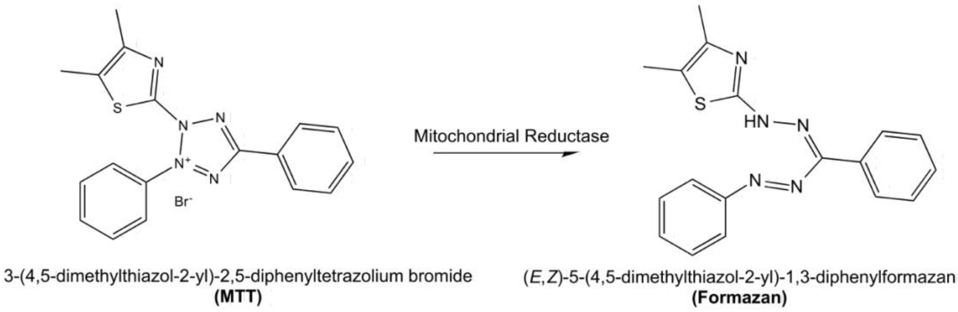
Where A1′ is the absorbance of water replacing reaction reagent; A0 is the absorbance of water replacing sample solution (Ye et al., 2011).

3.2.3.5. UV-Visible Spectroscopic Analysis of the Extracted Melanin

Different concentrations of purified melanin were prepared using the initial concentrations of 100 mg/l in 0.1 N NaOH, and diluted to 1:1, 1:2, and 1:3. Each alkaline solution was scanned from 180 to 900 nm wavelengths. A 0.1 N NaOH was used as the blank. The spectroscopic property of the melanin pigment obtained from Klebsiella sp. GSK was compared with synthetic melanin (Rani et al., 2013).

3.2.3.6. Anticancer Effect of the M36 Melanin

This assay requires cells that are actively able to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to an insoluble formazan precipitate. The assay is used as an indicator of mitochondrial function in live cells; however, loss of activity cannot be used to distinguish compromised cells from dead cells. As an indicator of cytotoxicity, MTT results have been reported to be comparable in sensitivity to those obtained using [3H] thymidine incorporation. The assay can be used with cultured cells or with tissues, as the colored product is solubilized and extracted from the test system. Because the dye needs to be extracted from the cells, this procedure cannot be used to provide cell counts (sambrook, 2012)



1. Plate cells (mouse fibroblastoma cell line) at 1×10^4 cells/well by adding 200 μl of a 5×10^4 cells/ml suspension to each well of a 96-well tissue culture plate. Include control wells with medium and no cells. Incubate overnight at 37°C and 5% CO2.

Test compounds (toxicant and vehicle) may be added before or after this incubation. Use plates with flat-bottomed wells for adherent cells and round-bottomed wells for suspension cells.

2. Carefully aspirate off the medium, avoiding removal of cells. If not previously incubated with toxicant, replace medium with fresh medium (200 μl) containing up to 0.1 ml of test substance. Include wells with medium without cells or test compound, medium without cells but with test compound, cells without test compound (but with test compound vehicle) as controls. Incubate at 37°C, 5% CO2, for predetermined times (e.g., several hours, overnight, and/or for several days).

3. After incubation with test compound, carefully aspirate off medium and replace with medium and 20 to 50 μl MTT solution for a total volume of 200 μl. Incubate for 4 to 6 hr at 37°C, 5% CO2.

4. Carefully remove the MTT-containing medium. Add 200 μl DMSO with 25 μl Sorensen’s buffer per well. Mix the plate until the formazan crystals are dissolved.

5. Read plate on microtiter plate reader at 550 to 570 nm.

6. Compare absorbance in wells containing the test substance with wells containing untreated control cells (sambrook, 2012)

3.2.3.7. Dyeing experiment

Lab scale dyeing experiments were carried out on cotton fabrics using the produced M36 melanin without mordanting. The melanin bath was prepared with the produced melanin 1% at H_2O; Dyeing was done at 100°C for 15 min. After dyeing the temperature was lowered to Room temperature, and then the dyed samples were rinsed and washed off in water and detergent. After that the cotton fabrics was rinsed and dried (Ali et al. 2011)

4. RESULT AND DISCUSSION

4.1.1. Isolation and Selection of *Bacillus Sp*. Producing Tyrosinase Enzyme

In this research 12 *Bacillus* strains having tyrosinase activity was isolated from soil samples. By comparison of them Figure 4.1, the strain with high tyrosinase activity (0.05 IU per milliliter of medium culture) was selected to enzyme production and named as *Bacillus* sp. M36. Figure4.2. According to the test by Whatman paper containing MnCl2, the pigmentation produced on the plate was independent of Mn^{2+}.

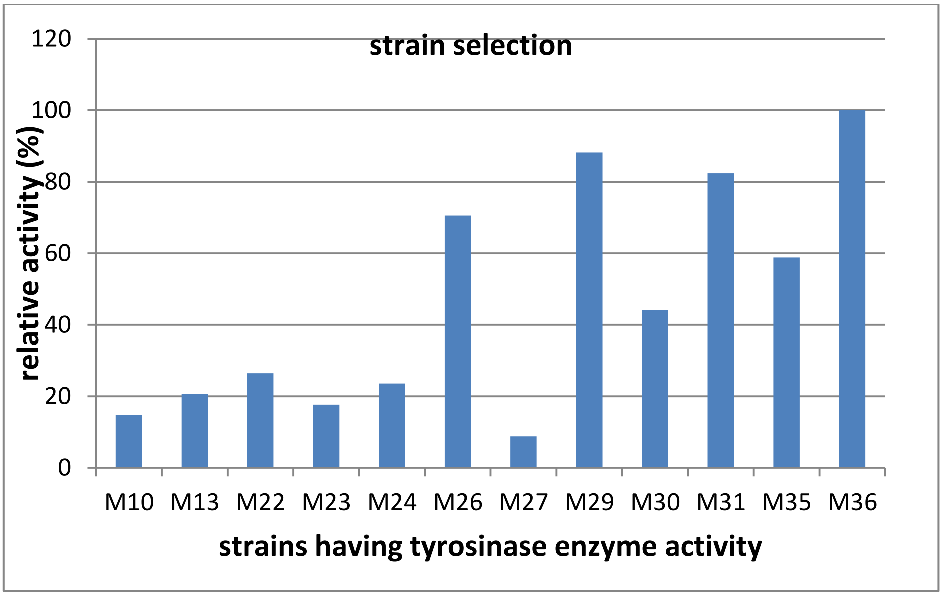


Figure 4.1. Comparison of the native strains producing tyrosinase enzyme and selection of the best of them.

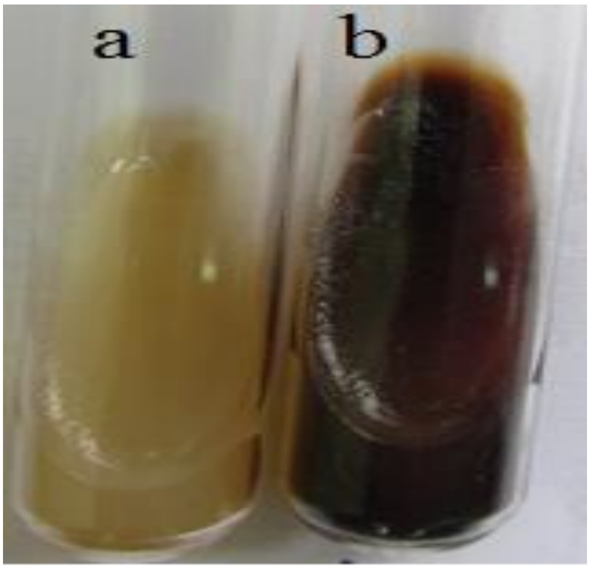


Figure 4.2. *Bacillus sp*.M36 in P medium without L-tyrosine (a) and with L-tyrosine (b) after 72 h incubation at 37°C.

4.1.2. Identification of the Isolated *Bacillus Sp.* M36

Morphological and biochemical characteristics of M36 strain (Table 4.1 and Figure 4.3) were done according to the Bergey's Manual and these characteristics demonstrated the strain to be *Bacillus* sp.

Primer pair's specific for *Bacillus* sp. was designed using oligo 7 and online internet systems in our laboratory in such a way that it was possible to amplify at least 1400 bp of the 16s rDNA sequence of *Bacillus* sp. the primer pairs was as following:

F=GCCTAATACATGCAAGTCGA,

R= TACGGYTACCTTGTTACGACT

16s rDNA sequence of *Bacillus* sp. M36 was amplified by the primers and analyzed in agarose gel (0.8%), which showed a bond of 1434 bp for the M36 *Bacillus* sp. (Figure 4.4). After that the gene fragment was sequenced by Applied Biosystems® Genetic Analysis system (Figure 4.5). BLAST analysis showed 99% homology of *Bacillus* sp. M36 with *Bacillus* m*egaterium* (Figure 4.6). Phylogram was created using MEGA 5.1 software (Figure 4.7). The optimal tree with the sum of branch length = 0.18290468 was shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown above the branches. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 1380 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Previously described criteria were used for this study: identification to the species level was defined as >99% similarity of the 16S rRNA gene sequence to the sequence of its closest bacterial relative in the GenBank database, and identification at the genus level was defined as >97% similarity of the 16S rRNA gene sequence to the sequence of its closest bacterial relative in the GenBank database (Nazia et al. 2012).This result was matching with the results obtained by Shuster and Fishman (2009) that isolated and studied tyrosinase enzyme from *Bacillus megaterium*.

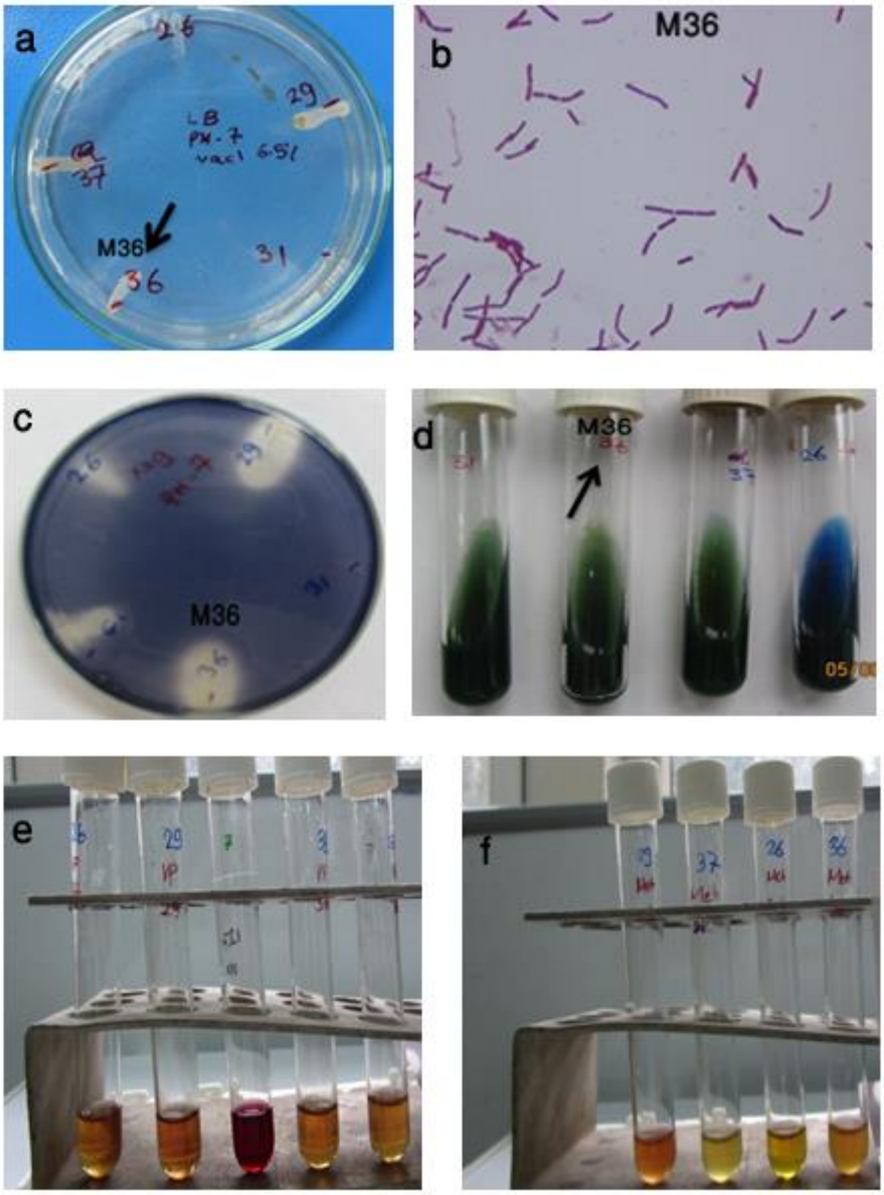


Figure 4.3. Morphological and some biochemical characteristic of the *Bacillus* sp. M36. A: represents ability of the strain for growth on medium with NaCl 6.5%, B: shows the gram and morphology of the strain, C: exhibits the hydrolysis of starch by the strain, D: shows the strain to be citrate positive, E: represents the strain to be VP negative and F: shows the result of methyl red test.

Table 4.1. Biochemical and morphological characteristics of the *Bacillus* sp. M36

Characteristic/biochemical test	Observation
α-hemolyse	Negative (-)
B-hemolyse	negative(-)
Catalase	Positive(+)
VP (voges-proskauer)	negative(-)
Methyl Red	negative(-)
Citrate	Positive(+)
starch	Positive(+)
Growth on medium containing 6.5% of NaCl	Positive(+)
Gram’s reaction	Positive(+)
Cell shape	Rod-like
Cell size	About 5 µm

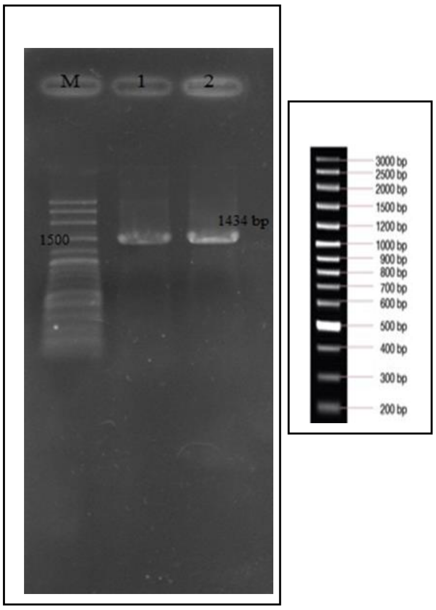


Figure 4.4. Amplified 16s rDNA (1434bp) of *Bacillus sp*. M36 in agarose gel 0.8%, lane M: DNA ladder from Vivantis lane 1 and 2: 16s rDNA of *Bacillus sp*. M36.

```
>16s rDNA of M36 Bacillus sp.
CAAGTCGAGCGAaCTGATTAGAAGCTTGCTTCTATGACGTTAGCGGCGGACGGGTGAGTA
ACACGTGGGCAACCTGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGGCTAATACCG
GATAGGATCTTCTCCTTCATGGGAGATGATTGAAAGATGGTTTCGGCTATCACTTACAGA
TGGGCCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGgCTCACCAAGGCAACGATGCATA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG
TGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACGAGAGTAAC
TGCTCGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGG
TTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGG
AACTTGAGTGCAGAAGAGAAAAGCGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATG
TGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGGCGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA
AGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
GAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAA
CTCTAGAGATAGAGCGTTCCCCTTCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGT
CAGCTCCTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGT
TGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAATCATCATGCCCCTTATNACCTGGGCTACCCCCGTGCTACAATGGATGG
TACAAAGGGCTGCCAGACCGCGAGGTCAAGCCAATCCCATAAAACCATTCTCAGTTCGGA
TTGTAGGCNGCAACTCGCCTCCCTGGAGCTGGAATCGCTAGGAATCGCGGATCAGCATGC
CGCGGTGAATAATTTCCCGGGCCTTGTACACACCGCCCGTCACACCCAGAGAGTTTGTAA
CACCCGAAGTCGGTGGAGTAACCGTAAGGAGCTAGCCGCCTAAGGTGGGACAGATG
```

Figure 4.5. Sequence of the M36 *Bacillus* sp. 16s rDNA

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus megaterium DSM319, complete genome	2569	28211	100%	0.0	99%	NC_014103.1
Bacillus sp. 1NLA3E, complete genome	2278	27237	100%	0.0	96%	NC_021171.1
Bacillus infantis NRRL B-14911, complete genome	2269	20422	100%	0.0	95%	NC_022524.1
Bacillus thuringiensis serovar konkukian str. 97-27 chromosome, complete genome	2201	30713	100%	0.0	94%	NC_005957.1
Bacillus pseudomycoides DSM 12442 chromosome, whole genome shotgun sequence	2199	2199	100%	0.0	94%	NZ_CM000745.1
Bacillus anthracis str. Sterne chromosome, complete genome	2197	24094	100%	0.0	94%	NC_005945.1
Bacillus anthracis str. Ames chromosome, complete genome	2197	24094	100%	0.0	94%	NC_003997.3
Bacillus cytotoxicus NVH 391-98, complete genome	2192	28419	100%	0.0	94%	NC_009674.1
Bacillus weihenstephanensis KBAB4, complete genome	2190	30642	100%	0.0	94%	NC_010184.1
Bacillus cereus ATCC 14579 chromosome, complete genome	2190	28417	100%	0.0	94%	NC_004722.1
Bacillus toyonensis BCT-7112, complete genome	2181	26133	100%	0.0	94%	NC_022781.1
Bacillus pumilus SAFR-032, complete genome	2174	15215	100%	0.0	94%	NC_009848.1
Bacillus pseudofirmus OF4, complete genome	2125	14872	100%	0.0	93%	NC_013791.2
Macrococcus caseolyticus JCSC5402 DNA, complete genome	2114	8459	100%	0.0	93%	NC_011999.1
Bacillus atrophaeus 1942, complete genome	2113	14753	100%	0.0	93%	NC_014639.1
Bacillus subtilis subsp. subtilis str. 168 chromosome, complete genome	2113	21036	100%	0.0	93%	NC_000964.3

Figure 4.6. The blast analysis showing up to 99% identity between *Bacillus sp.* M36 and *Bacillus megaterium* DSM 319.

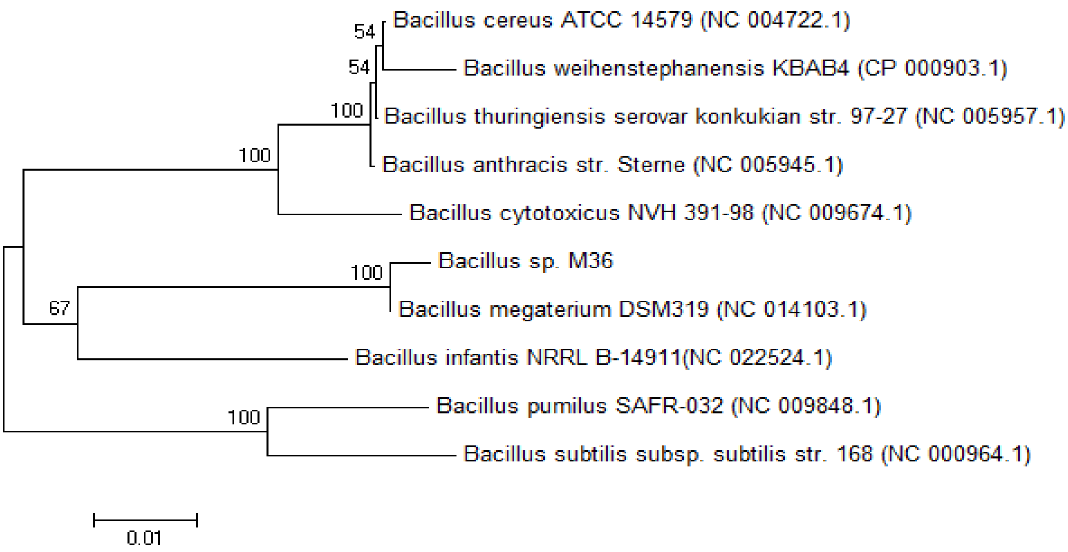


Figure 4.7. Neighbor-joining (NJ) tree showing the phylogenetic position of *Bacillus* sp. M36 and some related *Bacillus* species based on partial 16S rRNA gene sequences (1434NT). Sequence accession numbers are given in parentheses.

4.1.3. Optimization of Culture Condition for Enzyme Production

4.1.3.1. Classical Optimization

The pH range having role at the bacterial growth affects enzyme stability in the medium According to the earlier researches, there is an optimum pH range between 6.0 and 7.0 for the bacterial strains growth and enzyme production (Gupta et al. 2003; Deb and et al., 2013)). Findings of this research revealed an optimum pH of 7.0 for tyrosinase enzyme production by *Bacillus megaterium* M36 isolate (Figure. 4.8). Similarly, Parka and et al. have showed the optimum pH of 7.0 for enzyme production by *Bacillus megaterium* (park and et al., 2009). After pH (8.0) enzyme production was dropped drastically. It might be due to the fact that the enzyme was inactive in the alkali medium (Shuster and Fishman, 2012).

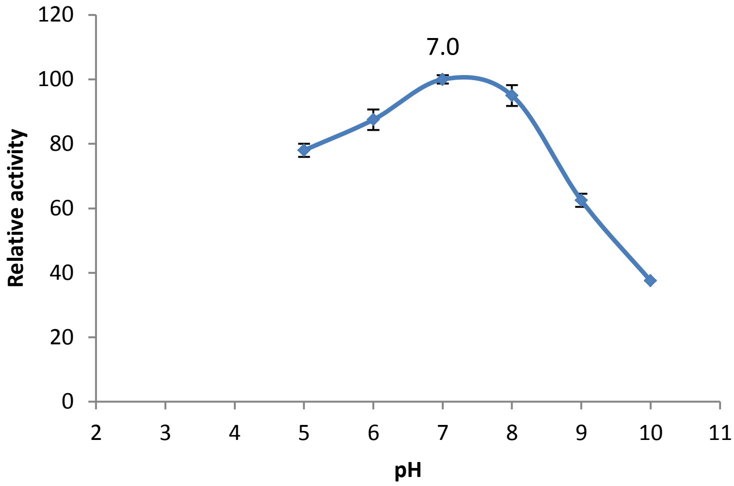


Figure 4.8. The effect of pH values on tyrosinase enzyme production. Maximum enzyme production was at pH= 7.0, after pH=7 the enzyme was decreased and reached to 37.5% at pH=10

An important parameter affecting growth and metabolites production by microorganisms is temperature which is usually varied from one organism to another (Kumar & Takagi 1999). Many of the enzyme production by *Bacillus* sp. have been carried at temperature of (30-40 °C). The *Bacillus megaterium* M36 showed maximum tyrosinase enzyme production at 36 °C which is in the range *Bacillus megaterium* optimum temperature (Figure. 4.9). The results of this paper were in line with the results that showed the optimum temperature of 37 °C for tyrosinase enzyme production from *Bacillus* sp (Dalfard and et al., 2006).

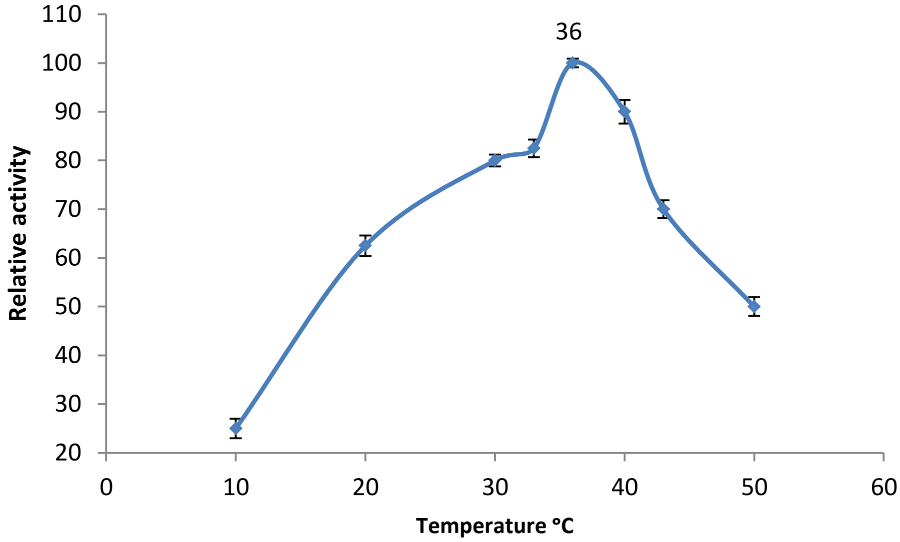


Figure 4.9. The effect of temperature on tyrosinase enzyme production by *Bacillus megaterium* M36 The yield was very low at temperature lower and higher than 36°C so that, it was decreased about 50% at 50°C and it was lowered about 75% at 10°C.

Bacillus megaterium M36 was cultured in liquid culture medium and OD_{530} of the culture was monitored for 19 h. The OD_{530} was increased until 10th hour followed by stationary phase until 16th hour (Figure 4.10.). In parallel with OD_{530} analysis, amount of biomass yield was also monitored for 19 hour which showed the same results. After 16th hour, the OD_{530} was re-increased exponentially which was accompanied with browning of the medium. As time passed by, the view of the medium was black- brown and finally black.

Maximum amount of tyrosinase enzyme was obtained at 16th of incubation. Although the value of OD_{530} and blackness (melanin) of the medium was increased, amount of active enzyme which can be obtained was decreased after 16th hour, as time went by. The decreases can be the result of proteolytic degradation.

The result of this result is matching with the results published by Surwase et al. (2011) who obtained maximum amount of tyrosinase enzyme at 18th hour of incubation. (Tomas and et al., 1980)

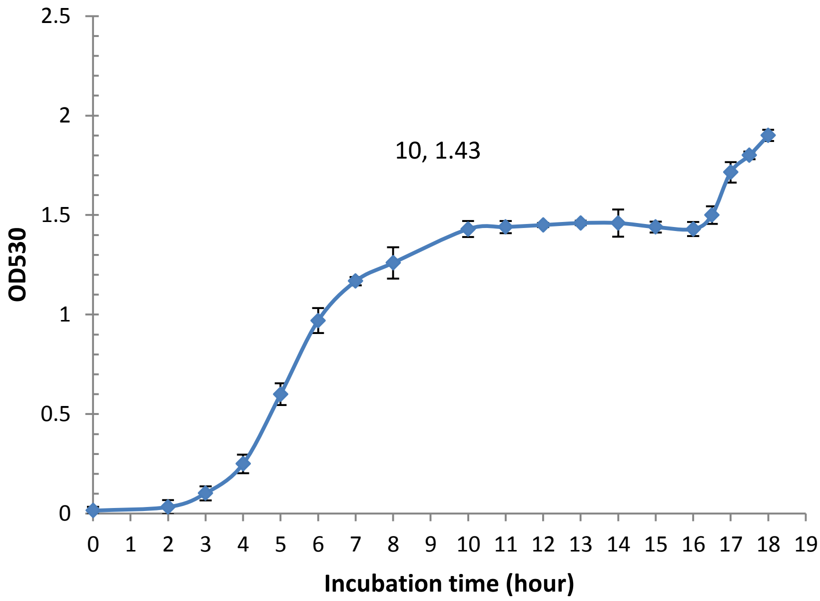


Figure 4.10. The effect of incubation time on tyrosinasee enzyme production by *Bacillus megaterium* M36.

The positive effect of L-tyrosine on tyrosinase enzyme production has been proved by many researches. So in this research by incorporating various concentrations (0.2-3mg/ml) of L-tyrosine to medium, the concentration of L-tyrosine which had maximum effect on enzyme production was selected. The result of this research pointed at 0.4 mg/ml of L-tyrosine as optimum concentration for enzyme production (Figure 4.11.). This result was in accordance with others findings (Survas et al , 2012, Survas and Jadhav, 2011)

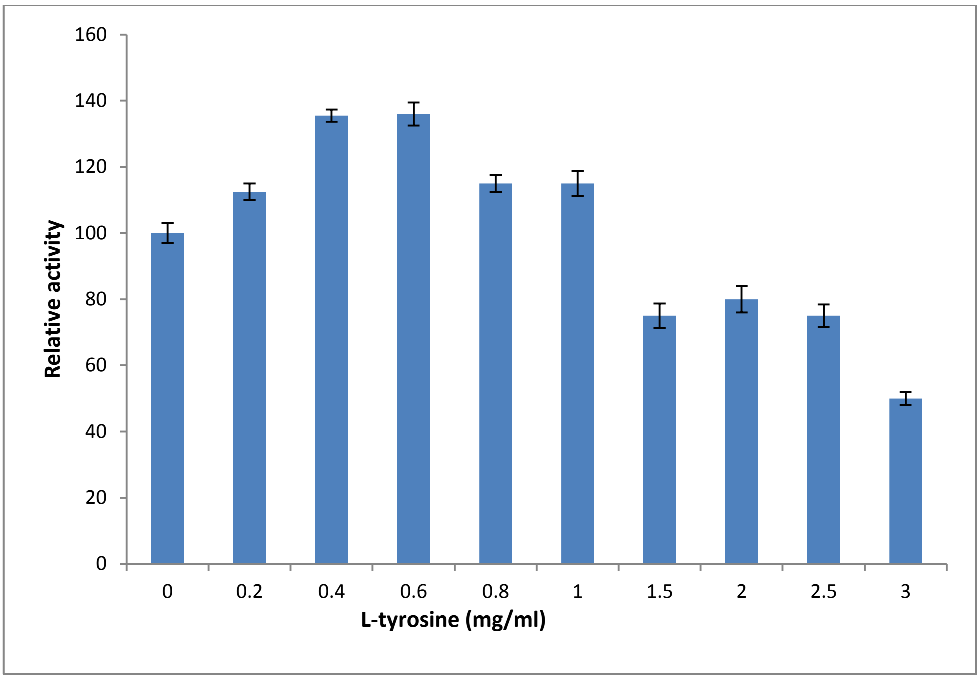


Figure 4.11. The effect of L-tyrosine concentration on tyrosinase enzyme production. Maximum yield was obtained at L-tyrosine concentration of 0.6 mg/ml.

Nitrogen and carbon sources are found to be playing prominent role in the growth and development of the bacteria. This research showed the tryptone as best nitrogen source for tyrosinase enzyme production, of course, the casein was also good for enzyme production (Figure 4.12).This can be resulted from that tryptone is an enzymatic digest of casein and both of them have the same composition. The presence of yeast extract was needed to use along with other compounds of medium. Without yeast extract, the strain didn’t produce detectable enzyme.

It is worthy of note that when cultured in mineral salt medium (without yeast extract) used by (Shuster and et al., 2009) for 52 h. *Bacillus megaterium* M36 could produce tyrosinase enzyme, nevertheless the produced enzyme was less than of that produced in medium mentioned above in 16 h. Incorporating of carbon sources such as glucose, starch, and etc didn’t positively affect enzyme production by the *Bacillus megaterium* M36.

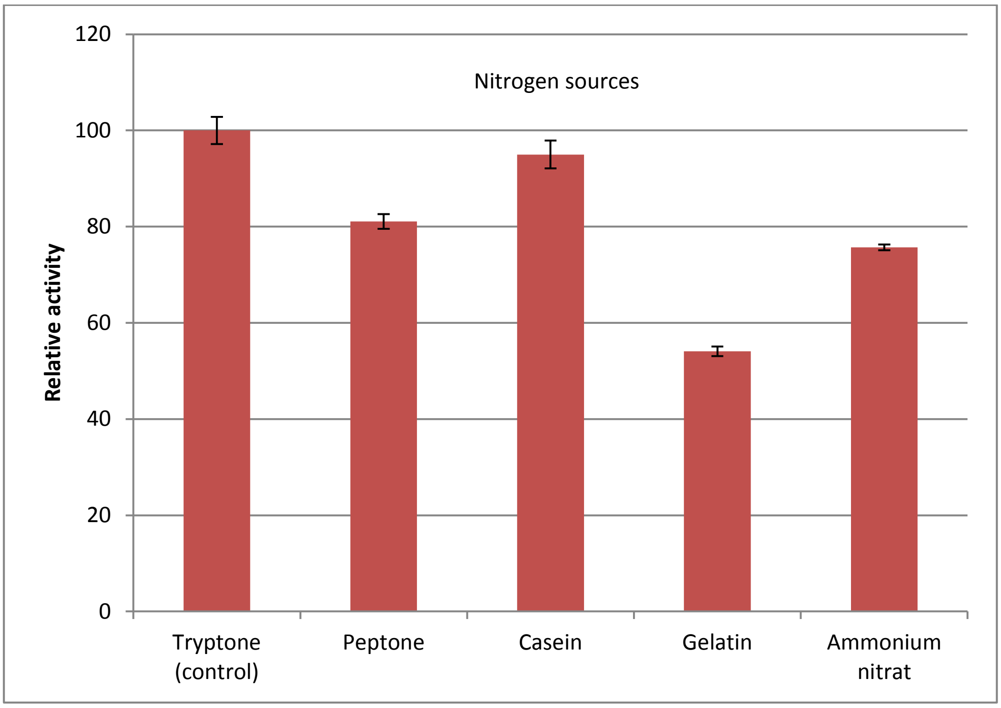


Figure 4.12. Effect of various protein sources on tyrosinase enzyme production. Trypton was the best nitrogen source for the enzyme production.

4.1.3.2. Statistical Optimization

Response surface methodology is one of the statistical techniques to evaluate the interaction between the experimental variables and the measured responses and to detect the optimal range of the variables within the design.

In this study, Box- Behnken design was used to determine the optimal level of the four variables (yeast extract, tryptone, NaCl and CuSo4) for tyrosinase enzyme production. ANOVA statistics results and the estimated regression coefficients are given in Table2. The Model F-value of 34.10 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A (yeast extract), B (tryptone), C (NaCl), D (CuSo4), CD, B2, D2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Lack of Fit F-value" of 0.97 implies the Lack of Fit is not significant relative to the pure error. There is a 56.42% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. The "Pred R-Squared" of 0.8709 is in reasonable agreement with the "Adj R-Squared" of 0.9430; i.e. the difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 21.353 indicates an adequate signal. This model can be used to navigate the design space.

Table 4. 2. Analysis of variance (ANOVA) for the fitted quadratic polynomial model of tyrosinase enzyme production.

Use your mouse to right click on individual cells for definitions.

Response 1 R1

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	0.044	14	3.117E-003	34.10	< 0.0001	significant
A-Yeast extr	*1.587E-003*	*1*	*1.587E-003*	*17.36*	*0.0010*	
B-Tryptone	*4.681E-003*	*1*	*4.681E-003*	*51.20*	*< 0.0001*	
C-NaCl	*5.333E-004*	*1*	*5.333E-004*	*5.83*	*0.0300*	
D-CuSo4	*1.302E-003*	*1*	*1.302E-003*	*14.24*	*0.0021*	
AB	*3.240E-004*	*1*	*3.240E-004*	*3.54*	*0.0807*	
AC	*1.690E-004*	*1*	*1.690E-004*	*1.85*	*0.1954*	
AD	*1.960E-004*	*1*	*1.960E-004*	*2.14*	*0.1652*	
BC	*1.600E-005*	*1*	*1.600E-005*	*0.18*	*0.6820*	
BD	*3.422E-004*	*1*	*3.422E-004*	*3.74*	*0.0735*	
CD	*9.610E-004*	*1*	*9.610E-004*	*10.51*	*0.0059*	
A^2	*1.025E-004*	*1*	*1.025E-004*	*1.12*	*0.3076*	
B^2	*0.024*	*1*	*0.024*	*267.05*	*< 0.0001*	
C^2	*2.719E-004*	*1*	*2.719E-004*	*2.97*	*0.1066*	
D^2	*0.013*	*1*	*0.013*	*142.72*	*< 0.0001*	
Residual	1.280E-003	14	9.142E-005			
Lack of Fit	*9.051E-004*	*10*	*9.051E-005*	*0.97*	*0.5642*	*not significant*
Pure Error	*3.748E-004*	*4*	*9.370E-005*			
Cor Total	0.045	28				

A second order polynomial equation given in Eqn. 2 was constructed by using the estimated coefficients (in coded units).

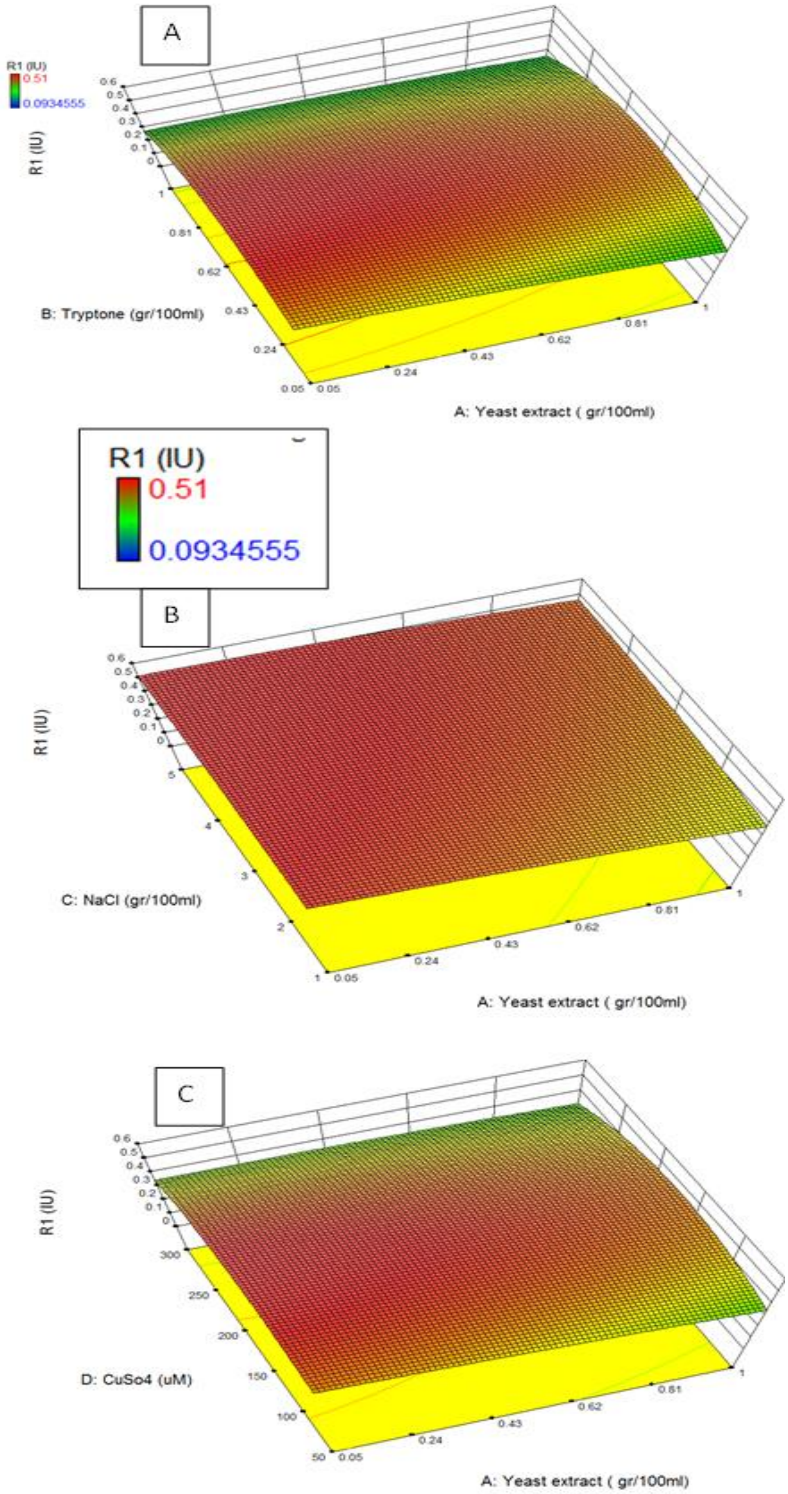
$$\text{Tyrosinase Enzyme (IU)} = 0.19 - 0.011A - 0.020B + 0.006667 - 0.010D + 0.009\,AB + 0.0065\,AC + 0.007AD + 0.002BC - 0.009250\,BD - 0.016CD - 0.003975A^2 - 0.061B^2 - 0.006475C^2 - -0.045D^2$$

The equation in terms of coded factors was used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients. The response surface curves represented in Figure4.13 A-F shows the interaction between variants. The mutual effect of yeast extract and tryptone was represented in Figure.4.13-A. The lower and higher levels of tryptone decreased the amount of tyrosinase enzyme, while middle levels enhanced enzyme production. Maximum yield was obtained at minimum level of yeast extract. When the concentration of yeast extract was increased the enzyme production was decreased steadily.

The result of this study was similar to findings of Survas et al. (2012) who showed the effect of tryptone on tyrosinase production by RSM methodology. Production of melanin using casein as a nitrogen source by *Bacillus thuringiensis* (Chen et al. 2004) and using tryptone as a nitrogen source by *Bacillus* cereus (Zhang et al. 2007) are in accordance with the result of this research. The effect of yeast extract was significant in this research. It is probably that this is because of vitamins complex in yeast extract. This finding was in contrast with the result of other scientists who showed yeast extract as a non-significant for tyrosinase enzyme production or tyrosinase activity (Survas et al. 2012). The Figure.4.13B Shows the effect of NaCl and yeast extract on tyrosinase enzyme production. When NaCl was in its low level, the increase of yeast extract made to decrease the enzyme production, while in high level of NaCl, the effect of yeast extract was decreased. When $CuSO_4$ was in low level, the increase of yeast extract caused to decrease of enzyme production. By steadily increase of $CuSO_4$ from 50µM to middle level, the enzyme production was increased whereas further increase in the levels resulted in the decrease in the yield. $CuSO_4$ increases tyrosinase activity at lesser concentration, because tyrosinase is a copper-containing enzyme. Higher levels of $CuSO_4$ might be toxic to the cells. In earlier reports, 0.25 mM of $CuSO_4$ is shown to be required for *Acremonium rutilum* (Krishnaveni et al., 2009) and 0.15 mM for *Pinguicula grandiflora* (Rani et al., 2007).

4.1.4. Validation of the Experimental Model

The optimum levels of yeast extract, tryptone, NaCl and $CuSo_4$ predicted by the model were 0.05%, 0.423%, 3.4% and 148.4 µM, respectively. Also, maximum amount of enzyme that can be produced under the optimum condition was predicted to be 0.527 IU. According to the results optimum culture condition for tyrosinase enzyme production by the *Bacillus megaterium* M36 was as follow: temperature (36°C), pH (7.0), incubation time (16 hour), L-tyrosine (0.4 mg/ml), yeast extract (0.05%), tryptone (0.423%), NaCl (3.4%) and CuSo4 (148.4 µM). Results of experiments under the optimized condition showed actual yield of 0.522 IU enzyme, while the result of experiment under initial condition using basal medium (before optimization) gave 0.0312 IU enzyme. A close correlation between the actual (0.522 IU) and predicted values (0.527 IU) was observed, which validates this model.


A
R1 (IU)
0.51
0.0934555
R1 (IU)
B: Tryptone (gr/100ml)
A: Yeast extract (gr/100ml)
R1 (IU)
0.51
0.0934555
B
R1 (IU)
C: NaCl (gr/100ml)
A: Yeast extract (gr/100ml)
C
R1 (IU)
D: CuSo4 (uM)
A: Yeast extract (gr/100ml)

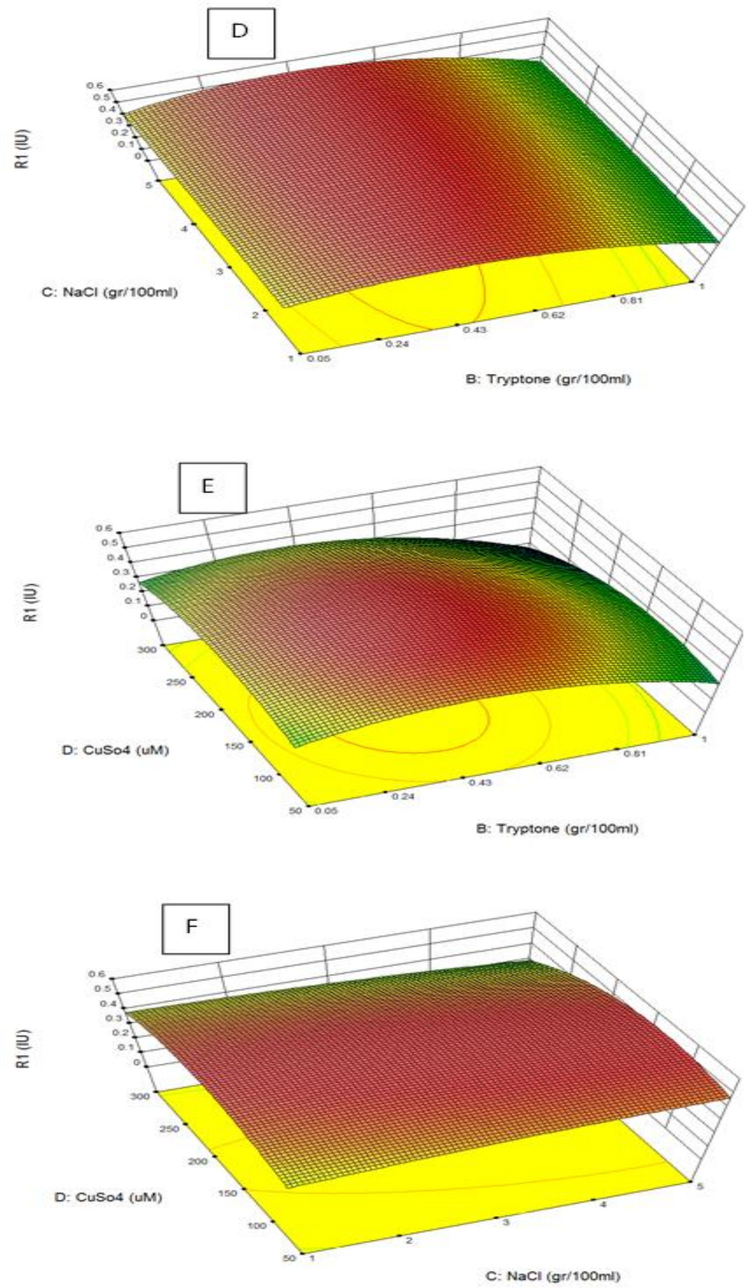


Figure 4.13. Response surface plot of the combined effects of yeast extract, tryptone, NaCl and CuSo4 on the tyrosinase enzyme production by *Bacillus megaterium* M36.

4.1.5. Characterization of the Native *Bacillus megaterium* M36 Tyrosinase Enzyme

The enzyme was precipitated by ammonium Sulfate 85% and centrifugation at 13000 g and dialyzed against 50 mM sodium phosphate buffer, pH 6.8 with 0.02% sodium azide, 0.01 mM CuSO4.

4.1.6. Effect of pH and Temperature on *Bacillus megaterium* M36 Tyrosinase Enzyme Activity and Stability

The result of this research showed that the *Bacillus megaterium* M36 tyrosinase enzyme had maximum activity at pH, 7.5 (Figure.4.14). This result was in accordance with tyrosinase enzyme originated from *Streptomyces* sp. REN-21 (pH 7.0), (Ito and Inouye 2005), *Rhizobium etli* CFN42 (pH 7.5) (Pinero et al. 2007), *S. glaucescens* (pH 6.8) (Lerch et al. 1972), and *Pseudomonas putida* F6 (pH 7.0) (McMahon et al 2007) notwithstanding, the tyrosinase enzyme from *Bacillus thuringiensis* (Liu et al., 2004) and *Trichonema roseum* (Kong et al., 2000) have shown to have maximum activity at 9.0 and 9.5, respectively

The tyrosinase enzymes have two cupper in its active site and each of the two metal atoms; Cu_A and Cu_B, of the active site are coordinated by three conserved histidines which are located in a ‘four a-helix bundle’. (Claus and Decker, 2006)

The a-helix is structured by hydrogen bonds. Generally when the pH change (extremely basic or acidic) causes changes in the charge of H-bond donor and acceptor groups, it can rearrange the H-bonds and change the conformation/folding of the protein. Before pH (6.5) and above pH (8.0) the activity of the enzyme was dropped (fig 2 and 3). These findings are similar to the finding of Shuster and Fishman (2009).

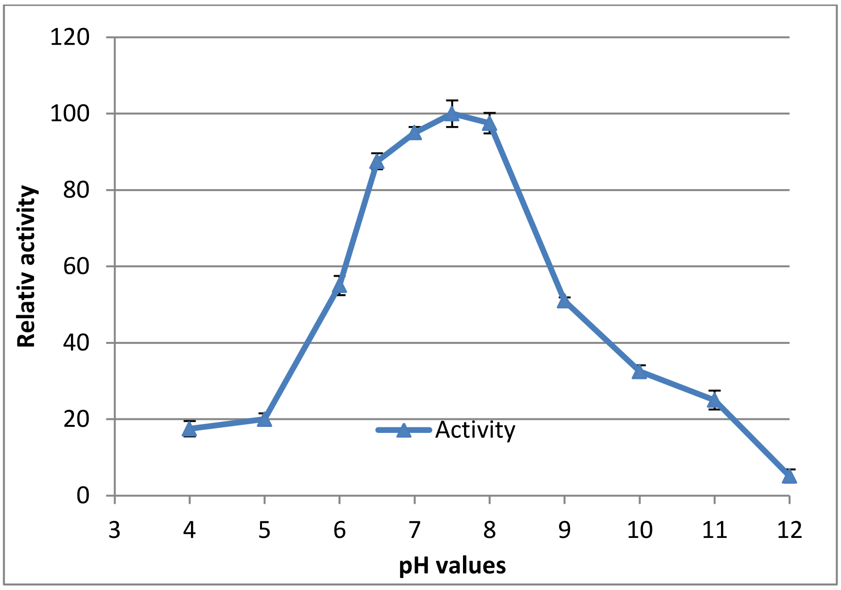


Figure 4.14. Effect of pH on activity and stability of the *Bacillus megaterium* M36 tyrosinase enzyme. The enzyme showed maximum activity at pH=7.5 and 97.5% of its maximum activity at pH=8.0. At pH lower than 6.5 and higher than 8.0 the activity of the enzyme was steeply decreased.

The *Bacillus megaterium* M36 tyrosinase enzyme showed optimum monophenolase and diphenolase activity at 40°C (Figure.4.15) also, the enzyme conserved 100% of its original activity at 4-45 °C (Figure.4.16). The monophenolase and diphenolase activity of the enzyme was deeply decreased at temperature below 30 °C and above 55 °C, probably this result was related to that, the tyrosinase enzyme has mostly composed from a-helix, at the other hand a-helix is more flexible than the other structures. This result was more or less closed to other investigations.

The *Bacillus megaterium* M36 tyrosinase enzyme showed up to 95% activity at temperature ranges from 35 °C-45°C, in contrast to this, the activity of tyrosinase enzyme from *P. putida* F6 (McMahon et al. 2007) has been decreased dramatically at temperature above 30°C and the enzyme of *Streptomyces michiganensis* DSM(Philipp et al. 1991) has showed optimum activity at 33°C. Moreover there is some reported tyrosinase enzymes whit higher optimum temperature. Also the *Bacillus megaterium* M36 tyrosinase enzyme was different with the tyrosinase from *Rhizobium etli* CFN42 (50°C) (Pinero et al. 2007), *Bacillus* (HR03) (55°C) (Dalfard et al. 2006), *Bacillus* thuringiensis (75°C) (El-Shora, Metwally, 2008) and *Thermomicrobium roseum* (70°C) (Kong et al. 2000).

The *Bacillus megaterium* M36 tyrosinase enzyme conserved its original activity at 45°C, contrary to this *Trichoderma reesei* (Cura et al. 2010) tyrosinase started to lose its activity relatively quickly at temperature above 30°C.

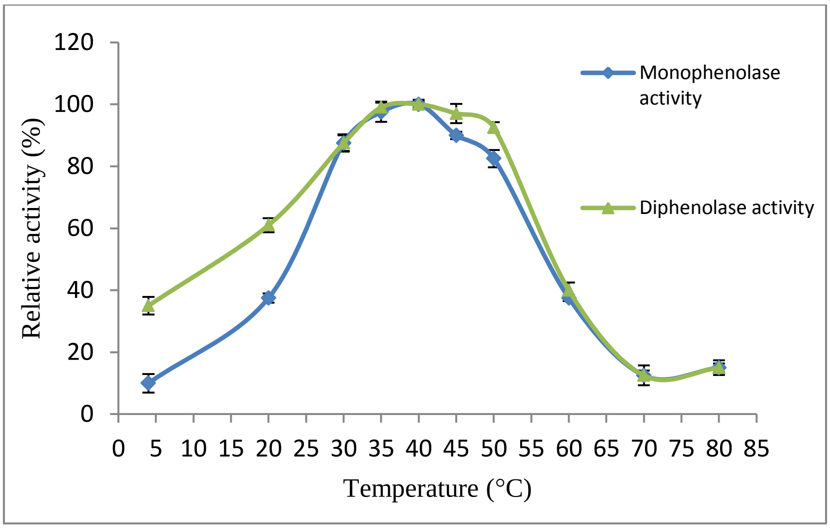


Figure 4.15. Effect of temperature on monophenolase (conversion of L-tyrosine to L-DOPA) and diphenolase activity (conversion of L-DOPA to dopa-quinone) of the *Bacillus megaterium* M36 tyrosinase enzyme. The enzyme showed maximum monophenolase activity at 40°C. Also, the enzyme showed maximum diphenolase activity at 40°C, 99% at 35°C and 92.5% at 50°C. At temperature lower than 30°C and higher than 55°C, both of the activities were severely decreased.

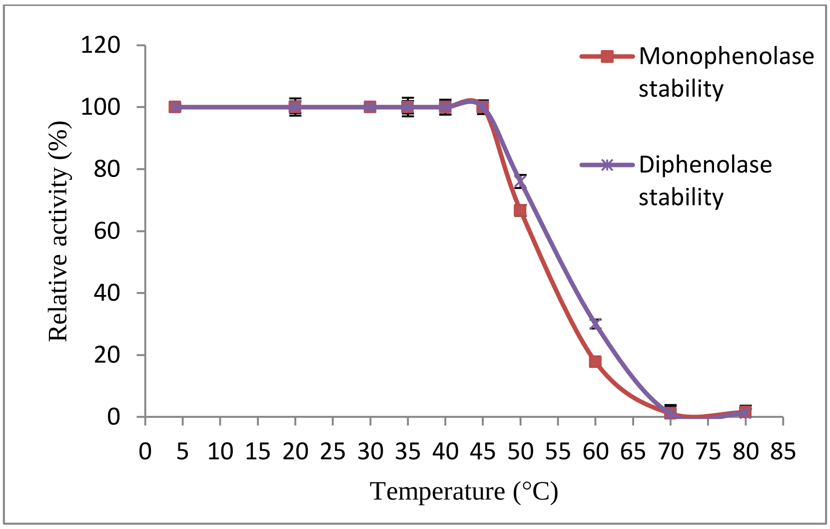


Figure 4.16. Effect of temperature on monophenolase and diphenolase stability of the *Bacillus megaterium* M36 tyrosinase enzyme. Both of the monophenolase and diphenolase activity were conserved 100% at temperature 0-45°C, after that the enzyme loosed its activity.

4.1.7. Electrophoresis and Zymagram analyses of *Bacillus megaterium* M36 Tyrasinase Enzyme

After dialysis, tyrosinase enzyme of *Bacillus megaterium* M36 was electrophoresed by using native polyacrylamide gel (8%), after specific staining with Comassie Brillant Blue R250, a distinct band was detected. by extracting of the tyrosinase enzyme from native gel using the method mentioned in material methods, and the enzyme was subjected to SDS-PAGE (12%) analysis (Figure 4.17). This analysis showed almost 34 kd single band of the enzyme. This result is similar to the result of Shuster and Fishman (2009) who have demonstrated the tyrosinase from *Bacillus* megaterium to be almost 35 kD.

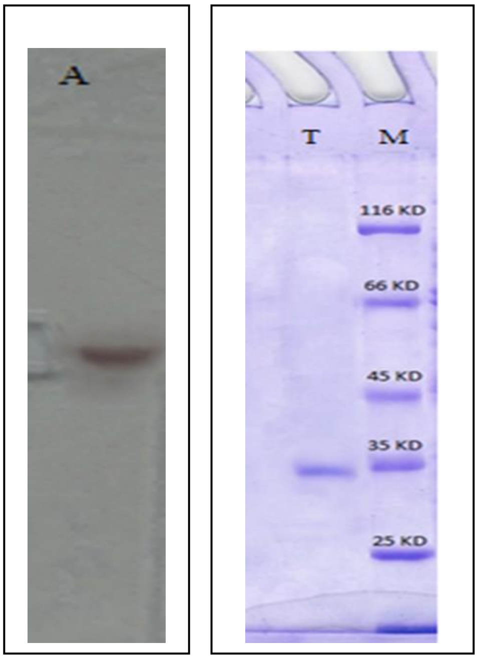


Figure 4.17. Electrophoresis analysis of the *Bacillus megaterium* M36 tyrosinase Enzyme. Lane (A) shows tyrosinase activity, lane (T) shows the enzyme molecular weight almost 34 KD, almost 15 µg of protein was loaded, lane (M) shows protein marker.

4.1. 8. Production of L-DOPA from L-tyrosine Using *Bacillus megaterium* M36 Tyrosinase Enzyme

In TLC and HPLC analysis, the transformation of L-tyrosine to L-DOPA was conspicuously detected. Ascorbic acid, used to prevent further oxidation of L-DOPA, did not give interfering spots (Figure.4.18). HPLC analysis showed differential rates of biotransformation depending on the reaction time (Figure. 4.19). The retention time for L-DOPA was 8.7 to 9.7 min and for L-tyrosine it was 14 to 14.5 min.

Also in quantitatively assay of L-tyrosine conversion to L-DOPA using both standard graph of L-tyrosine and L-DOPA, the following result was obtained: After 45 min incubation of the reaction mixture having 0.744 mg/ml (4mM) L-tyrosine, the remained L-tyrosine was 0.23mg/ml and obtained L-DOPA was 0.435 mg/ml.

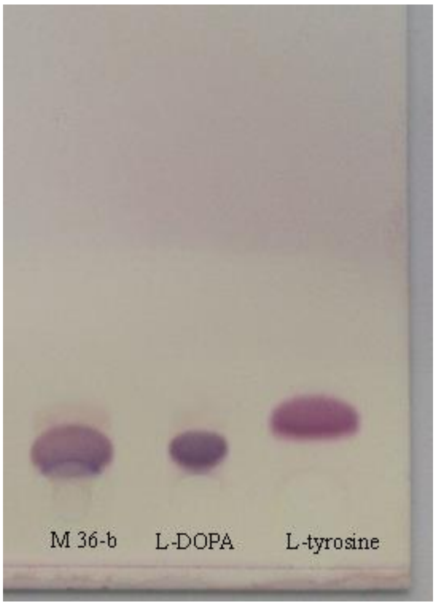


Figure 4.18. TLC analysis of L-tyrosine conversion to L-DOPA by *Bacillus megaterium* M36 tyrosinase enzyme.

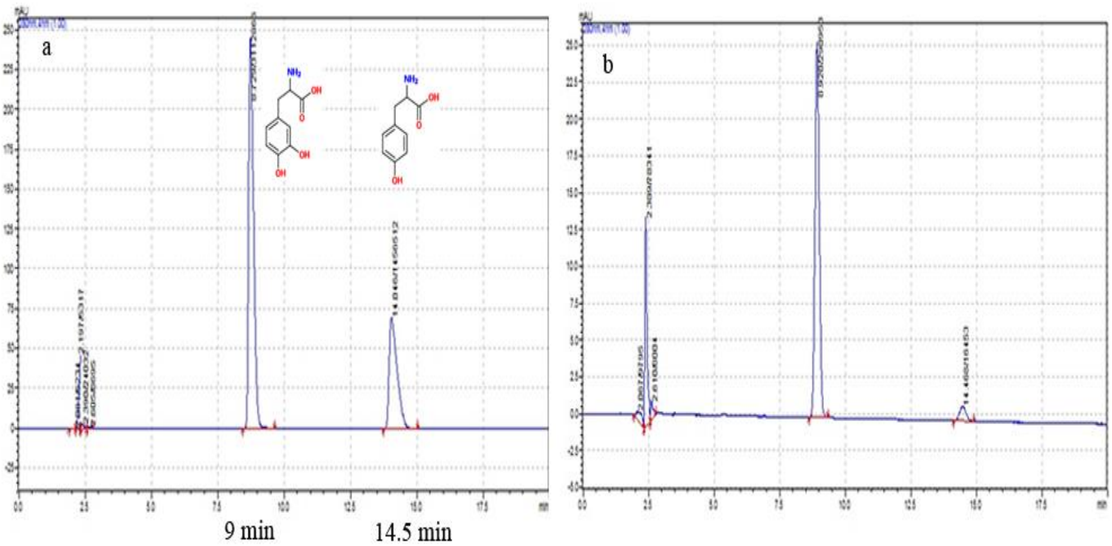


Figure 4.19. HPLC chromatogram for enzymatic transformation of L-tyrosine to L-dopa using *Bacillus megaterium* M36 tyrosinase enzyme under optimal conditions. The figure (A) shows the HPLC elution profile of standard L-DOPA and L-tyrosine and (B) shows the HPLC elution profile of reaction mixture with optimized reaction conditions.

4.2. Cloning of *Bacillus megaterium* M36 Tyrosinase Gene

4.2.1. Amplification of The *Bacillus megaterium* M36 Tyrosinase Gene Fragment

Genomic DNA *was* isolated from *Bacillus megaterium* M36 (Figure.4.20) and used for amplification of *Bacillus megaterium* M36 tyrosinase gene. After optimization of the PCR conditions such as different concentrations of primers, dNTPs, genomic DNA and $MgCl_2$, the gene fragment was amplified and analyzed by gel agarose (1%) (Figure.4.21). As it was shown in Figure.4. 21. The gene fragment was determined to have 918 bp long.

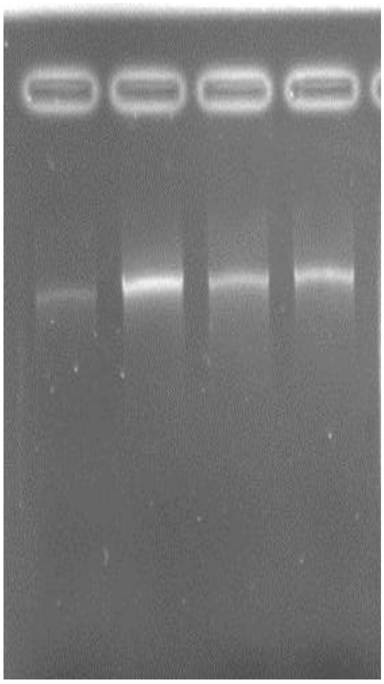


Figure 4.20. Electrophoresis analysis of the purified *Bacillus megaterium* M36 genomic DNA.

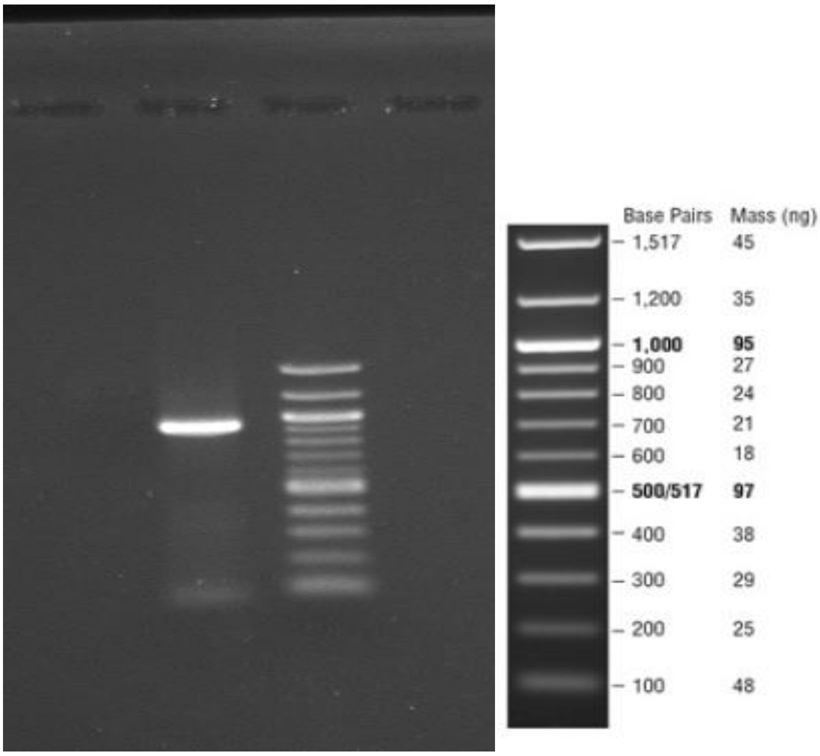


Figure 4.21. Amplified *Bacillus megaterium* M36 tyrosinase gene on agarose gel 1% with marker from promega.

4.2.2. Construction of Recombinant Expression Vector

After amplification, the gene fragment was extracted and purified by promega instruction (Figure. 4.22) then both of the purified PCR products and pET22b vector were digested by NotI and NcoI restriction enzymes followed by purification of the digested fragments. (Figure. 4.23)

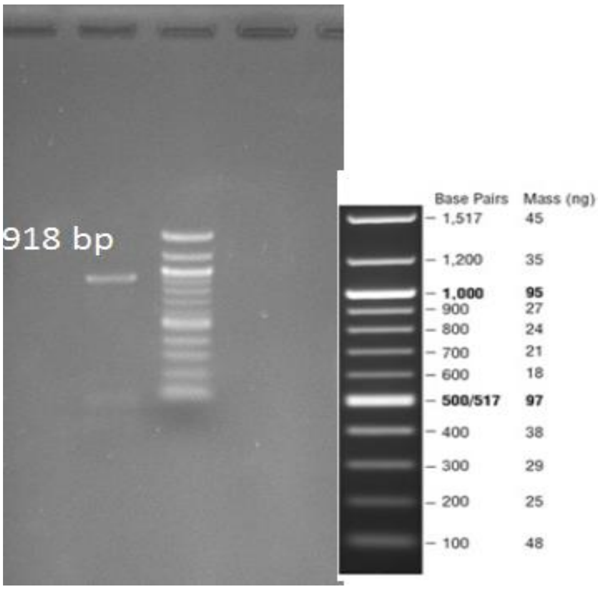


Figure 4.22. Electrophoresis of the purified PCR product

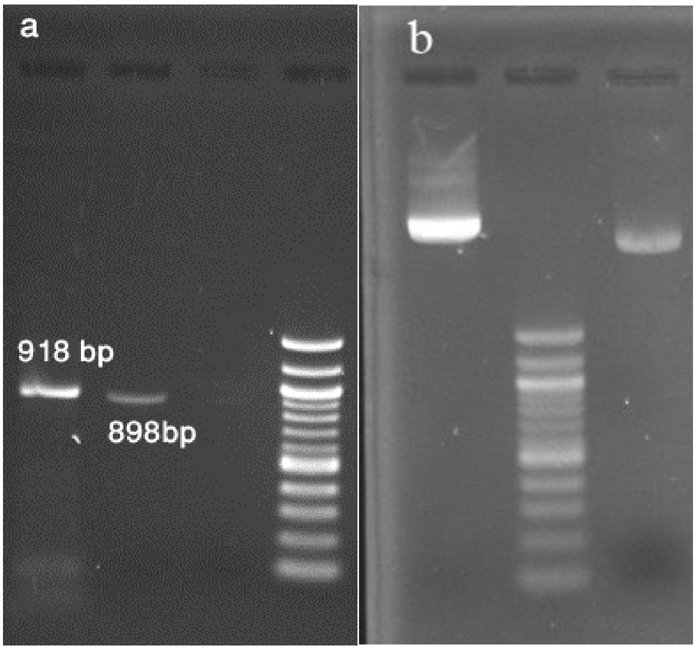


Figure 4.23. Electrophoresis of the digested and purified *Bacillus megaterium* M36 gene fragment (A) and pET22b vector (B).

The digested and purified *Bacillus megaterium* M36 gene fragment and pET22b vector were ligated together and transformed to *Rosetta gami 2 plysS* then the transformed strain was cultured on LB medium containing Ampicillin (50 µM) and incubated at 37°C for an overnight. In addition to this one control sample (regarding ligation mixture had all regent except gene fragment) was transformed to the host strain. (Figure. 4.24)

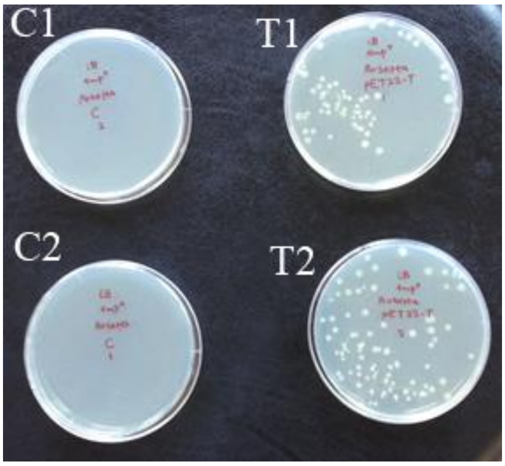


Figure 4.24. The growth of Rosetta gami 2 plys S transformed with control ligation product (C1-2) and test ligation product (T1-2).

Some of the colony grown on the medium (having Ampicillin) were taken and subjected to PCR analysis using the primers (which had been designed for *Bacillus megaterium* M36 tyrosinase gene) (Figure. 4.25).

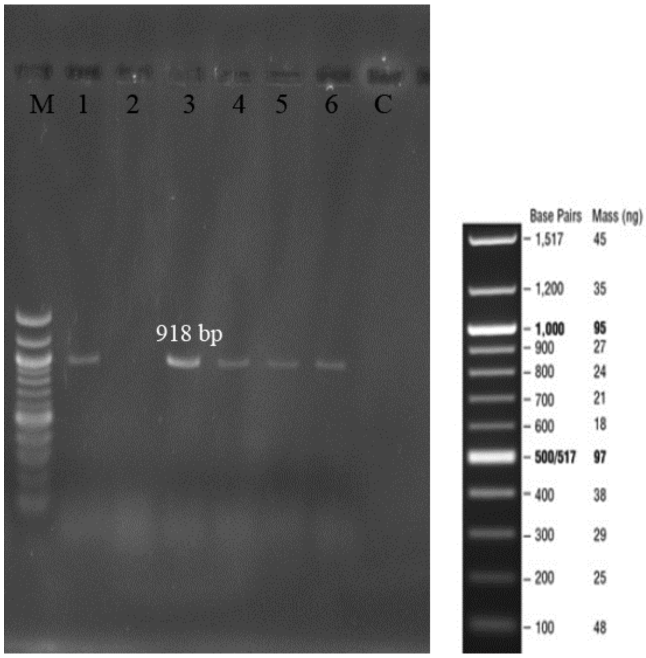


Figure 4.25. Electrophoresis of the direct colony PCR of the transformed strains (1, 2, 3, 4, 5, 6) along with nontransformed strain (C) and DNA marker (M).

Finally, the colony was cultured on LB medium containing ampicillin and incubated at 37°C, 150 rpm of shaker incubator and when OD_{600} of the culture reached to 1, the mediums were supplemented with L-tyrosine (0.05%), (Cuso4 100µM) and IPTG (1 mM) and continued to incubation for overnight.

The raised black-brown color in the medium cultures was considered as a sign of tyrosinase activity (Figure. 4.26).The most active one of the colonies was selected to further study and enzyme production.

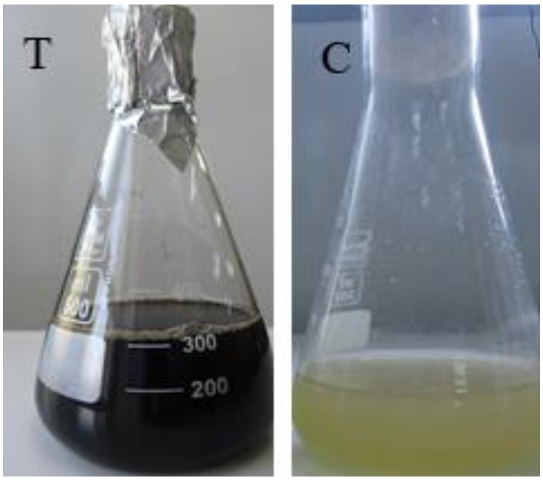


Figure 4.26. Tyrosinase activity of the transformed cell in LB medium containing L-tyrosine (T) and nontransformed cell (C) in the same medium.

4.2.3. Sequencing

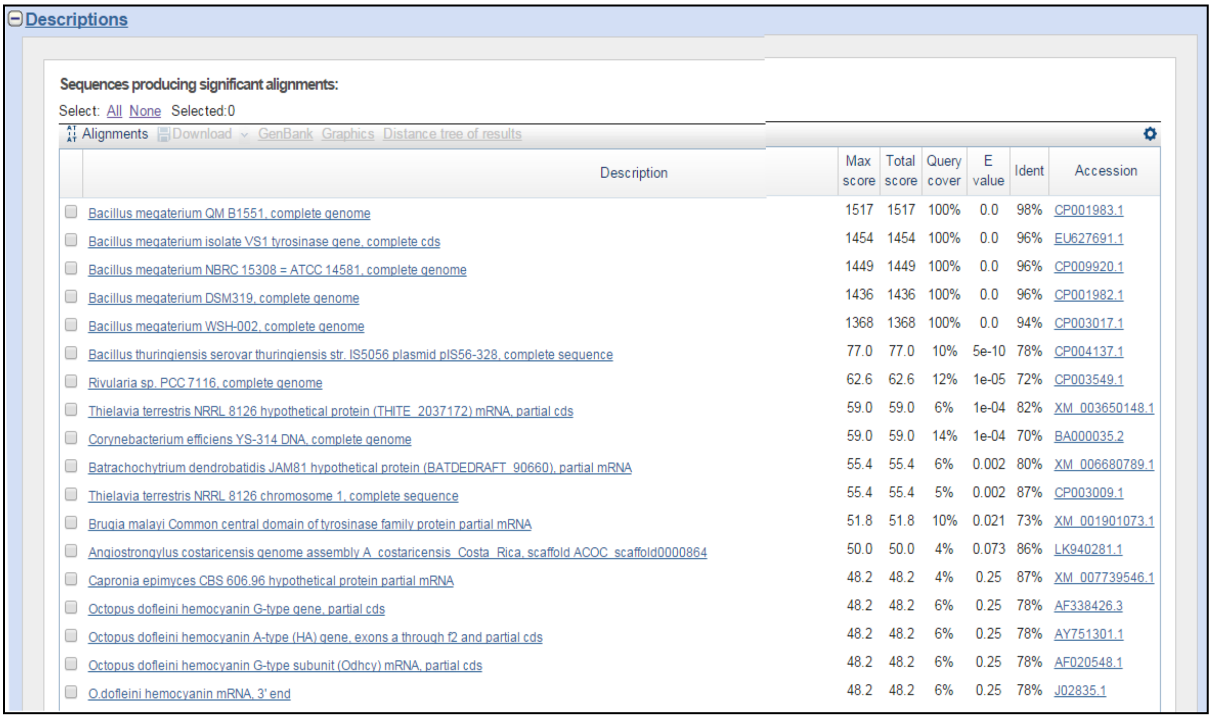
The recombinant vector (pET22b-tyr) was isolated from the selected colony and used for sequencing of the *Bacillus megaterium* M36 Tyrosinase gene fragment as described in methods (fig. 4.27).

```
>M36 tyrosinase (891bp)
ATGGGTAACAAGTATAGAGTTAGAAAAAACGTATTACGTCTTACGGACACGGAAAAAAGA
GATTTTGTTCGTACCGTGGCTATACTAGAGGAAAAAGGGATATATGACCGCTATATAGCT
TGGCATGGTGCAGCAGGTAAATTTCATACTCCTCCGGGCAGCGATCGAAATGCAGCACAT
ATGAGTTCTGCTTTTTTGCCGTGGCATCGTGAATACCTTTTACGATTCGAACGTGACCTT
CAATCAATCAATCCAGAAGTAACCCTTCCTTATTGGGAATGGGAAACGGACGCCCAGCTG
CAGGATCCCTCACAGTCACAAATTTGGAGTGCAGATTTTATGGGAGGAAACGGAAATCCA
AAAAAAGATTTTATCGTCGATACCGGGCCATTTGCAGCTGGGCGCTGGACGACGATCGAT
GAACAAGGAAATCCTTCTGGAGGGCTAAAACGTAATTTTGGAGCAACTAAAGAGGCACCT
ACATTGCCTACTCGAGATGATGTGCTCGATGCTTTAAAAATAACTCAATATGACACGCCG
CCTTGGGATATGACCAGCCAAAACAGCTTTCGTAATCAGCTTGAAGGATTTATTAACGGG
CCACAGCTTCACAATCGCGTACACCGTTGGGTTGGCGGACAGATGGGCGTTGTCCCTACT
GCTCCGAATGATCCTGTCTTCTTTTTACACCACGCAAATGTGGATCGTATTTGGGCTGCA
TGGCAAATTGTTCATCGCAATCAAAACTATCAGCCGATGAAAAACGGGCCATTTGGTCAA
AACTTTAGAGATCCGATGTATCCTTGGAATACAACCCCTGAAGACGTTATGAACCATCGA
AAGGCTGGGTACGTATACGATTTAGTATTGGGAAAATCAAAACGTTCCTCA
```

Figure 4. 27. Nucleotide Sequence of the *Bacillus megaterium* M36 tyrosinase gene fragment

4.2.4. Homology Analysis of the *Bacillus megaterium* M36 Tyrosinase Gene Fragment

The sequence was blasted and aligned by sequences with high homology (fig. 4. 28). The sequence showed the most homology (99%) to the sequences related to *Bacillus Megasterium*. This result is in agreement with the analysis of *Bacillus megaterium* M36. 16s rDNA sequence. After that some of *Bacillus megaterium* M36 tyrosinase sequences was extracted and aligned using Clone Manager Program (Figure 4.29).



Descriptions

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus megaterium QM B1551, complete genome	1517	1517	100%	0.0	98%	CP001983.1
Bacillus megaterium isolate VS1 tyrosinase gene, complete cds	1454	1454	100%	0.0	96%	EU627691.1
Bacillus megaterium NBRC 15308 = ATCC 14581, complete genome	1449	1449	100%	0.0	96%	CP009920.1
Bacillus megaterium DSM319, complete genome	1436	1436	100%	0.0	96%	CP001982.1
Bacillus megaterium WSH-002, complete genome	1368	1368	100%	0.0	94%	CP003017.1
Bacillus thuringiensis serovar thuringiensis str. IS5056 plasmid pIS56-328, complete sequence	77.0	77.0	10%	5e-10	78%	CP004137.1
Rivularia sp. PCC 7116, complete genome	62.6	62.6	12%	1e-05	72%	CP003549.1
Thielavia terrestris NRRL 8126 hypothetical protein (THITE_2037172) mRNA, partial cds	59.0	59.0	6%	1e-04	82%	XM_003650148.1
Corynebacterium efficiens YS-314 DNA, complete genome	59.0	59.0	14%	1e-04	70%	BA000035.2
Batrachochytrium dendrobatidis JAM81 hypothetical protein (BATDEDRAFT_90660), partial mRNA	55.4	55.4	6%	0.002	80%	XM_006680789.1
Thielavia terrestris NRRL 8126 chromosome 1, complete sequence	55.4	55.4	5%	0.002	87%	CP003009.1
Brugia malayi Common central domain of tyrosinase family protein partial mRNA	51.8	51.8	10%	0.021	73%	XM_001901073.1
Angiostrongylus costaricensis genome assembly A_costaricensis_Costa_Rica, scaffold ACOC_scaffold0000864	50.0	50.0	4%	0.073	86%	LK940281.1
Capronia epimyces CBS 606.96 hypothetical protein partial mRNA	48.2	48.2	4%	0.25	87%	XM_007739546.1
Octopus dofleini hemocyanin G-type gene, partial cds	48.2	48.2	6%	0.25	78%	AF338426.3
Octopus dofleini hemocyanin A-type (HA) gene, exons a through f2 and partial cds	48.2	48.2	6%	0.25	78%	AY751301.1
Octopus dofleini hemocyanin G-type subunit (Odhcy) mRNA, partial cds	48.2	48.2	6%	0.25	78%	AF020548.1
O.dofleini hemocyanin mRNA, 3' end	48.2	48.2	6%	0.25	78%	J02835.1

Figure 4.28. Blast analysis of the *Bacillus megaterium* M36 tyrosinase gene fragment.

```
                        10        20        30        40        50        60        70        80        90       100       110       120       130
                         *         *         *         *         *         *         *         *         *         *         *         *         *
gb|CP001983.1|    ATGAGTAACAAGTACAGAGTTAGAAAAAACGTATTACGGCTTACCGACACGGAAAAAAGAGATTTTGTTCGTACCGTGCTAATACTAAAGGAAAAAGGGATATATGACCGCTATATAGCCTGGCATGGTG
gb|CP001983.1|    ATGAGTAACAAGTATAGAGTTAGAAAAAACGTATTACATCTTACCGACACGGAAAAAAGAGATTTTGTTCGTACCGTGCTAATACTAAAGGAAAAAGGGATATATGACCGCTATATAGCCTGGCATGGTG
gb|EU627691.1|    ATGAGTAACAAGTACAGAGTTAGAAAAAACGTATTACATCTTACCGACACGGAAAAAAGAGATTTTGTTCGTACCGTGCTAATACTAAAGGAAAAAGGGATATATGACCGCTATATAGCCTGGCATGGTG
gb|CP009920.1|    ATGAGTAACAAGTATAGAGTTAGAAAAAACGTATTACATCTTACCGACACGGAAAAAAGAGATTTTGTTCGTACCGTGCTAACACTAAAGGAAAAAGGGATATATGACCGCTATATAGCCTGGCATGGTG
M36               ATGGGTAACAAGTATAGAGTTAGAAAAAACGTATTACGTCTTACGGACACGGAAAAAAGAGATTTTGTTCGTACCGTGGCTATACTAGAGGAAAAAGGGATATATGACCGCTATATAGCTTGGCATGGTG

                       140       150       160       170       180       190       200       210       220       230       240       250       260
                         *         *         *         *         *         *         *         *         *         *         *         *         *
gb|CP001983.1|    CAGCAGGTAAATTTCATACTCCTCCGGGCAGCGATCGAAATGCAGCACATATGAGTTCTGCTTTTTTGCCGTGGCATCGTGAATACCTTTTACGATTCGAACGTGACCTTCAATCAATCAATCCAGAAGT
gb|CP001983.1|    CAGCAGGTAAATTTCATACTCCTCCGGGCAGCGATCGAAATGCAGCACATATGAGTTCTGCTTTTTTACCGTGGCATCGTGAATACCTTTTACGATTCGAACGTGACCTTCAGTCAATCAATCCAGAAGT
gb|EU627691.1|    CAGCAGGTAAATTTCATACTCCTCCGGGCAGCGATCGAAATGCAGCACATATGAGTTCTGCTTTTTTACCGTGGCATCGTGAATACCTTTTACGATTCGAACGTGACCTTCAGTCAATCAATCCAGAAGT
gb|CP009920.1|    CAGCAGGTAAATTTCATACTCCTCCGGGAAGCGATCGAAATGCAGCACATATGAGTTCTGCTTTTTTACCGTGGCATCGTGAATACCTTTTACGATTCGAACGTGACCTTCAGTCAATCAATCCAGAAGT
M36               CAGCAGGTAAATTTCATACTCCTCCGGGCAGCGATCGAAATGCAGCACATATGAGTTCTGCTTTTTTGCCGTGGCATCGTGAATACCTTTTACGATTCGAACGTGACCTTCAATCAATCAATCCAGAAGT

                       270       280       290       300       310       320       330       340       350       360       370       380       390
                         *         *         *         *         *         *         *         *         *         *         *         *         *
gb|CP001983.1|    AACCCTTCCTTATTGGGAATGGGAAACGGACGCACAGCTGCAGGATCCCTCACAGTCACAAATTTGGAGTGCAGATTTTATGGGAGGAAACGGAAATCCCAAAAAAGATTTTATCGTCGATACCGGGCCA
gb|CP001983.1|    AACCCTTCCTTATTGGGAATGGGAAACGGACGCACAGATGCAGGATCCCTCACAATCACAAATTTGGAGTGCAGATTTTATGGGAGGAAACGGAAATCCCATAAAAGATTTTATCGTCGATACCGGGCCA
gb|EU627691.1|    AACCCTTCCTTATTGGGAATGGGAAACGGACGCACAGATGCAGGATCCCTCACAATCACAAATTTGGAGTGCAGATTTTATGGGAGGAAACGGAAATCCCATAAAAGATTTTATCGTCGATACCGGGCCA
gb|CP009920.1|    AACCCTTCCTTATTGGGAATGGGAAACGGACGCACAGATGCAGGATCCCTCACAATCACAAATTTGGAGTGCAGATTTTATGGGGGAAACGGAAATCCCAAAAAAGATTTTATCGTCGATACCGGGCCA
M36               AACCCTTCCTTATTGGGAATGGGAAACGGACGCCCAGCTGCAGGATCCCTCACAGTCACAAATTTGGAGTGCAGATTTTATGGGAGGAAACGGAAATCCAAAAAAAGATTTTATCGTCGATACCGGGCCA

                       400       410       420       430       440       450       460       470       480       490       500       510       520
                         *         *         *         *         *         *         *         *         *         *         *         *         *
gb|CP001983.1|    TTTGCAGCTGGGCGCTGGACGACGATCGATGAACAAGGAAATCCTTCTGGAGGGCTAAAACGTAATTTTGGAGCAACTAAAGAGGCACCTACATTGCCTACTCGAGATGATGTGCTCGATGCTTTAAAAA
gb|CP001983.1|    TTTGCAGCTGGGCGCTGGACGACGATCGATGAACAAGGAAATCCTTCCGGAGGGCTAAAACGTAATTTTGGAGCAACGAAAGAGGCACCTACACTCCCTACTCGAGATGATGTCCTCAATGCTTTAAAAA
gb|EU627691.1|    TTTGCAGCTGGGCGCTGGACGACGATCGATGAACAAGGAAATCCTTCCGGAGGGCTAAAACGTAATTTTGGAGCAACGAAAGAGGCACCTACACTCCCTACTCGAGATGATGTCCTCAATGCTTTAAAAA
gb|CP009920.1|    TTTGCAGCTGGGCGCTGGACGACGATCGATGAACAAGGAAATCCTTCCGGAGGGCTAAAACGTAATTTTGGAGCAACGAAAGAGGCACCTACACTCCCTACTCGAGATGATGTTCTCAATGCTTTAAAAA
M36               TTTGCAGCTGGGCGCTGGACGACGATCGATGAACAAGGAAATCCTTCTGGAGGGCTAAAACGTAATTTTGGAGCAACTAAAGAGGCACCTACATTGCCTACTCGAGATGATGTGCTCGATGCTTTAAAAA
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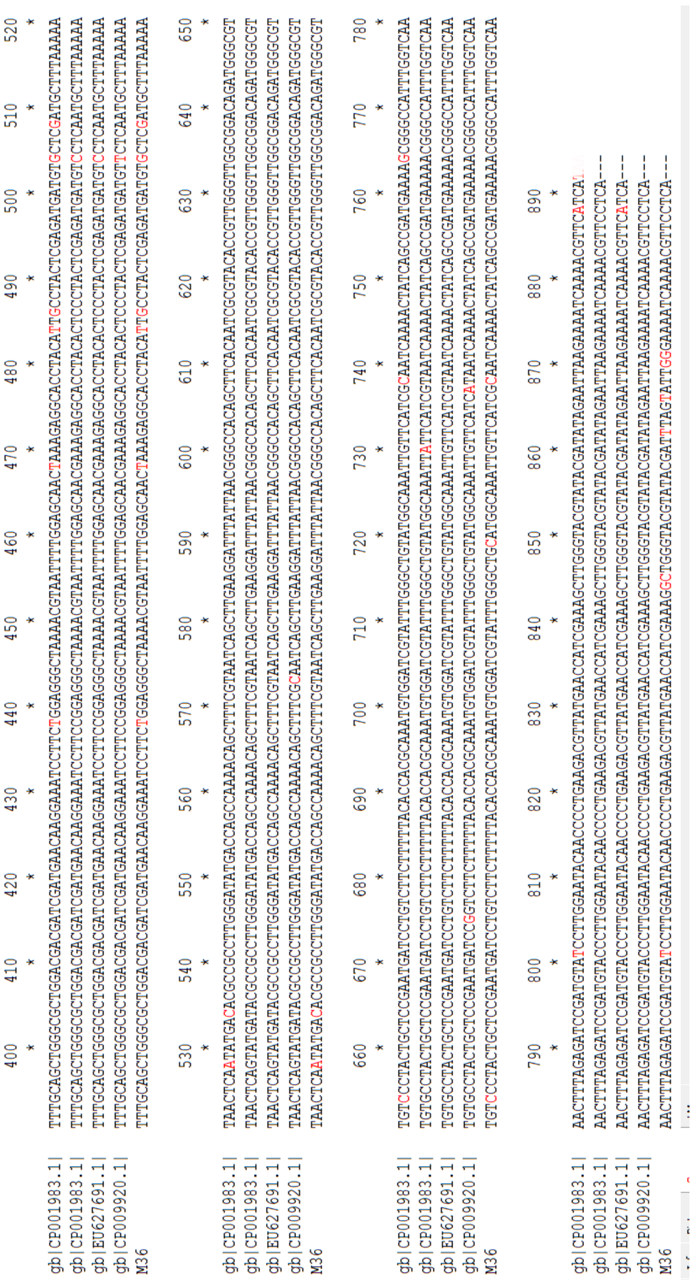


Figure 4.29. Alignment analysis of the *Bacillus megaterium* M36 tyrosinase gene fragment with the sequence showing high homology in figure 4.28.

After that the DNA sequences aligned in (Figure 4.29 were converted to amino acid sequences and again subjected to alignment analysis (Figure. 4.30). These analysis showed the positions where *Bacillus megaterium* M36 tyrosinase gene had mutations and it was different from the other tyrosinase gene sequences.

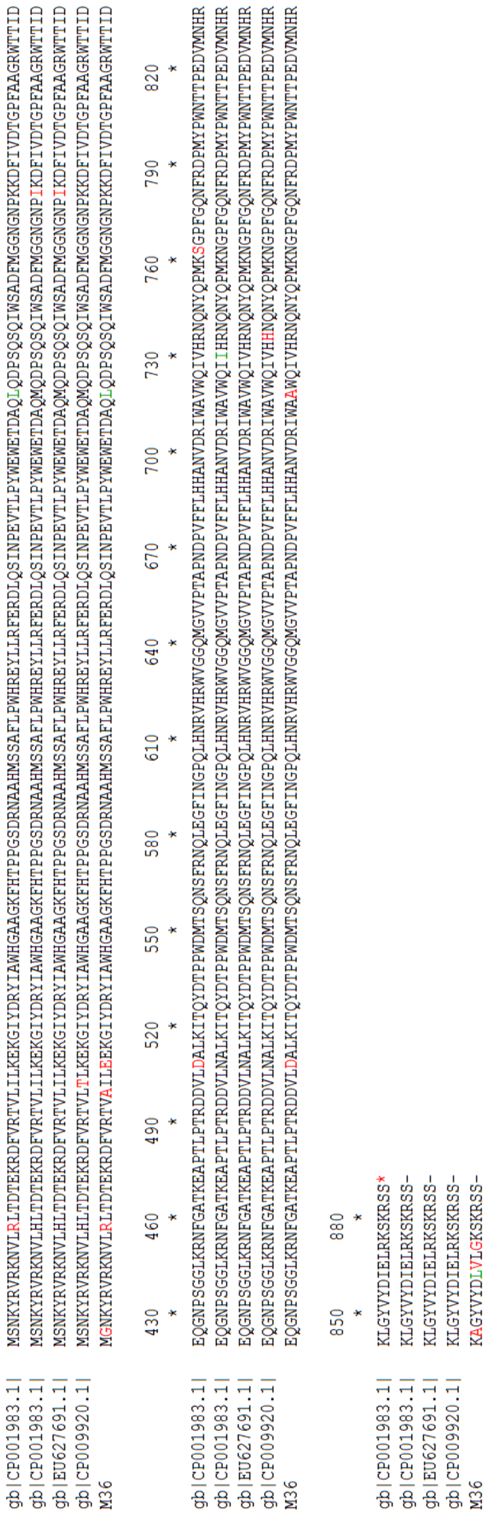
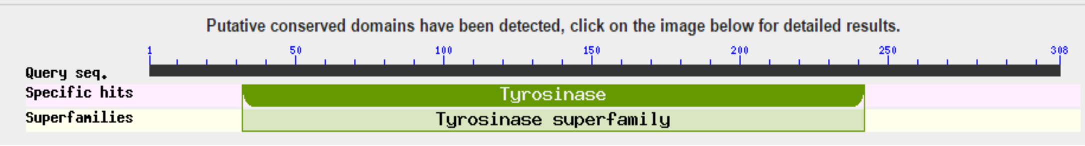


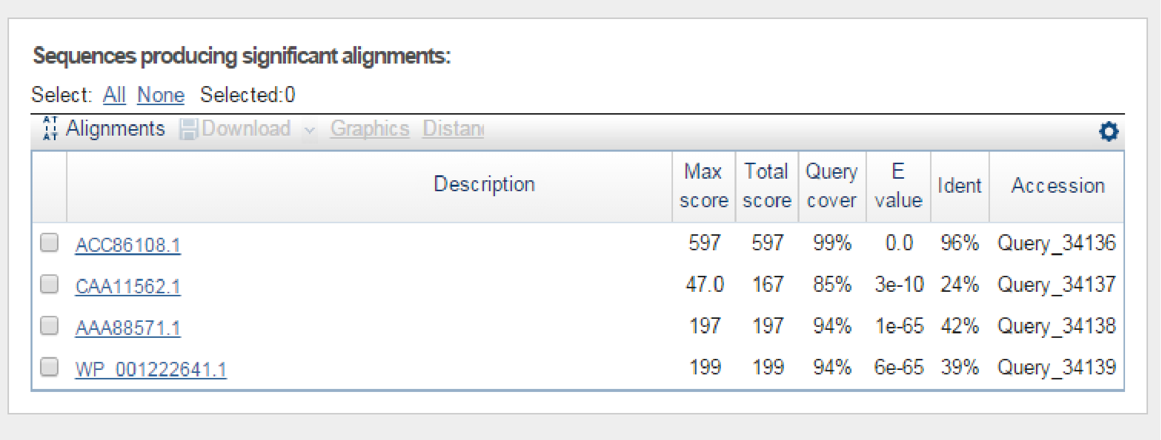
Figure.4.30. Alignment analysis of the amino acid sequence of the *Bacillus megaterium* M36 tyrosinase gene sequence with amino acid sequences related to the DNA sequences showed at Figure.4.29. alignment of the amino acid sequence of the *Bacillus megaterium* M36 tyrosinase

enzyme exhibited the substitution of G_{2}, R_{13}, A_{27}, E_{30}, L_{100}, D_{170}, A_{240}, A_{282}, L_{288}, V_{289} and G_{291} by S_{2}, H_{13}, L_{27}, K_{30}, M_{100}, N_{170}, V_{240}, L_{282}, I_{288}, E_{289} and R_{291}, respectively.

4.2.5. Analysis of the *Bacillus megaterium* M36 Tyrosinase Enzyme Conserved Domain

Inorder to study of conserved domain of the *Bacillus megaterium* M36 tyrosinase enzyme, the amino acid sequences was analysed by PSI-BLAST program (Figure. 4.31). Several tyrosinase enzyme homologs were extracted, aligned to study of CuA and CuB regions (conserved domain) (Figure. 4.32) and used to construct phylogenetic tree (Figure. 4.33). The *Bacillus megaterium* M36 tyrosinase enzyme contains the 6 conserved histidine residues involved in the binding of the copper pair (Hernàndez-Romero et al., 2006; Lopez-Serrano et al., 2002). Furthermore, the typical distance of H-x(8)-H in CuA and H-x(3)-H in CuB (Claus and Decker, 2006; Olivares et al., 2002) was kept. The closet homologues of the *M36* tyrosinase are the *Bacillus megaterium (96%), Agaricus bisporus(24%), Streptomyces antibioticus(42%), Bacillus thuringiensis(39%).*





Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download Graphics Distan

Description	Max score	Total score	Query cover	E value	Ident	Accession
ACC86108.1	597	597	99%	0.0	96%	Query_34136
CAA11562.1	47.0	167	85%	3e-10	24%	Query_34137
AAA88571.1	197	197	94%	1e-65	42%	Query_34138
WP_001222641.1	199	199	94%	6e-65	39%	Query_34139

Figure 4.31. PSI-BLAST analysis of the recombinant *Bacillus megaterium* M36 tyrosinase enzyme. ACC86108.1 (*Bacillus megaterium*), CAA11562.1 (*Agaricus bisporus*), AAA88571.1 (*Streptomyces antibioticus*), WP001222641.1 (*Bacillus thuringiensis*).

Acc. Number	CuA binding site
M36	AWHGAAGKFHTPPGSDRNAAHMSSAFLPWHREYLLRFERDLQSINPEVTLPYWEWETD-- 97
ACC86108.1	AWHGAAGKFHTPPGSDRNAAHMSSAFLPWHREYLLRFERDLQSINPEVTLPYWEWETD-- 97
AAA88571.1	TTHNAFILGDTDNGE--RTGHRSPSFLPWHRRFLLEFERALQSVDASVALPYWDWSAD-- 91
WP_001222641.1	YWHLMSMMDQTSTKP--GSAHGGPAFLPWHRYFINQLELDLQQIDSTVTLPYWDWTVN-- 102
CAA11562.1	FTEWAKERPSMNLYKAGYCTHGQVLFPTWHRTYLSVLEQILQGAAIEVAKKFTSNQTDWV 120
	. * * .*** :: :* ** *: : . .:
Acc. Number	**CuB binding site**
M36	QLEGFING-----------PQLHNRVHRWVG-----GQMGVVPTAPNDPVFFLHHANVDR 236
ACC86108.1	QLEGFING-----------PQLHNRVHRWVG-----GQMGVVPTAPNDPVFFLHHANVDR 236
AAA88571.1	HLEGWRG------------VNLHNRVHVWVG-----GQMATG-VSPNDPVFWLHHAYIDK 221
WP_001222641.1	RLEGWYGD-----------GRIHNQVHLWIAG-ATQGSMYWM-SSPNDPVFFLHHANVDR 266
CAA11562.1	AWERFSNHGISDDQHANSLESVHDDIHVMVGYGKIEGHMDHPFFAAFDPIFWLHHTNVDR 287
	* : . :*: :* :. * * :. **:*:***: :*:

Figure. 4.32. Alignment of the CuA and CuB regions of the *Bacillus megaterium* M36 tyrosinase. ACC86108.1 (*Bacillus* megaterium), CAA11562.1 (*Agaricus* bisporus), AAA88571.1 (*Streptomyces* antibioticus), WP001222641.1 (*Bacillus* thuringiensis).

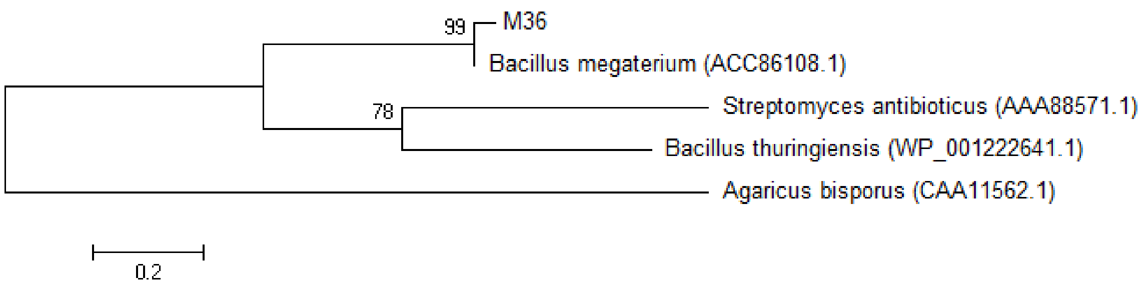


Figure 4.33. Phylogenetic tree for *Bacillus megaterium* M36 tyrosinase enzyme

4.2.6. Insilico Analysis of the Physiochemical Properties of the *Bacillus megaterium* M36 Tyrosinase Enzyme

The physiochemical properties of *Bacillus megaterium* M36 tyrosinase enzyme were assessed by ProtParam tool. The *Bacillus megaterium* M36 tyrosinase enzyme was predicted to have molecular weight of 35420.6 Daltons, amino acid numbers of 308 and the theoretical isoelectric point (pI) of 8.01, indicating that the protein is positively charged. The negative Grand average of hydropathicity (GRAVY) value of -0.795 for protein indicates that it is hydrophilic and soluble in nature. The instability index is computed to be 350.4 showing that the enzyme is stable. Amino acid composition of the *Bacillus megaterium* M36 tyrosinase enzyme was showed at Figure 4.34.

Number of amino acids: 308

Molecular weight: 35420.6

Theoretical pI: 8.01

Amino acid composition: CSV format

Ala	(A)	22	7.1%
Arg	(R)	21	6.8%
Asn	(N)	20	6.5%
Asp	(D)	21	6.8%
Cys	(C)	0	0.0%
Gln	(Q)	15	4.9%
Glu	(E)	13	4.2%
Gly	(G)	23	7.5%
His	(H)	16	5.2%
Ile	(I)	11	3.6%
Leu	(L)	19	6.2%
Lys	(K)	14	4.5%
Met	(M)	8	2.6%
Phe	(F)	14	4.5%
Pro	(P)	22	7.1%
Ser	(S)	13	4.2%
Thr	(T)	18	5.8%
Trp	(W)	11	3.6%
Tyr	(Y)	10	3.2%
Val	(V)	17	5.5%
Pyl	(O)	0	0.0%
Sec	(U)	0	0.0%
	(B)	0	0.0%
	(Z)	0	0.0%
	(X)	0	0.0%

Total number of negatively charged residues (Asp + Glu): 34
Total number of positively charged residues (Arg + Lys): 35

Figure 4.34. Amio acid composition of the *Bacillus megaterium* M36 tyrosinase enzyme

4.2.7. Secondary and Tertiary Structure Prediction for *Bacillus megaterium* M36 Tyrosinase Enzyme

Secondary and Tertiary structure of the *Bacillus megaterium* M36 tyrosinase enzyme was predicted by comparison with one from *Bacillus* megaterium using different bioinformatics analysis. (Figure 4.35)

Figure 4.35. Secondary structure of the *Bacillus megaterium* M36 tyrosinase enzyme

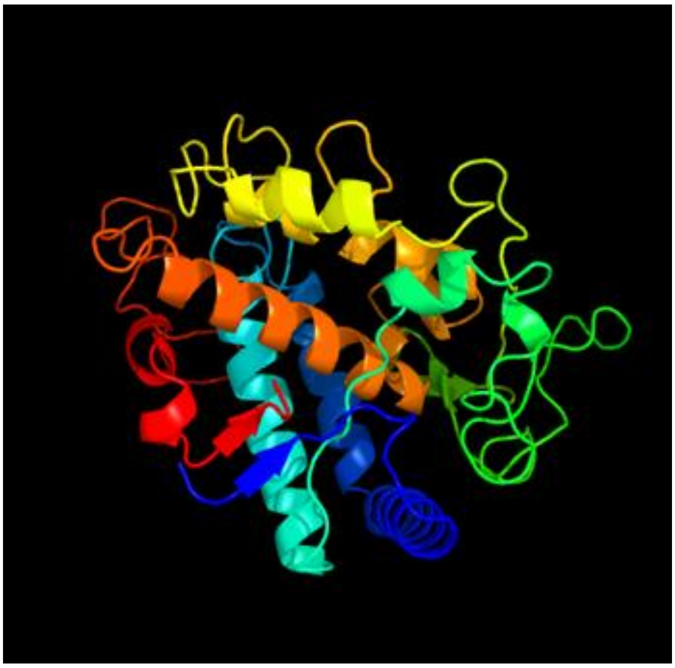


Figure 4.36. Predicted Tertiary structure of the *Bacillus megaterium* M36 tyrosinase enzyme

4.2.8. Codon Usage Analysis

Codons of *Bacillus megaterium* M36 tyrosinase gene fragment was analyzed and compared with codon usage of *E.coli* by *E.coli* Codon Usage Analyzer 2.1 from university of California (Figure. 4.37). The analysis showed that codons encoding to some amino acids such as prolin, serine, glycine, leucine, arginine and threonine are rare codon for *E.coli*. The CAI coefficient changes (the amount of similarity between codons of interest gene and codons of genes which have high level of expression in *E.coli*) of the *Bacillus megaterium* M36 tyrosinase gene was analyzed by http://www.genscript.com . Codon Adaptation Index (CAI) of the gene was 0.67 while a CAI of 1.0 is considered ideal while a CAI of >0.8 is rated as good for expression in the desired expression organism. The lower the number, the higher the chance, that the gene will be expressed poorly. Also, the percentage of low frequency (<30%) codons based on the *E.coli* was 8%. This can reduce the efficiency of translation or even disengage the translational machinery.

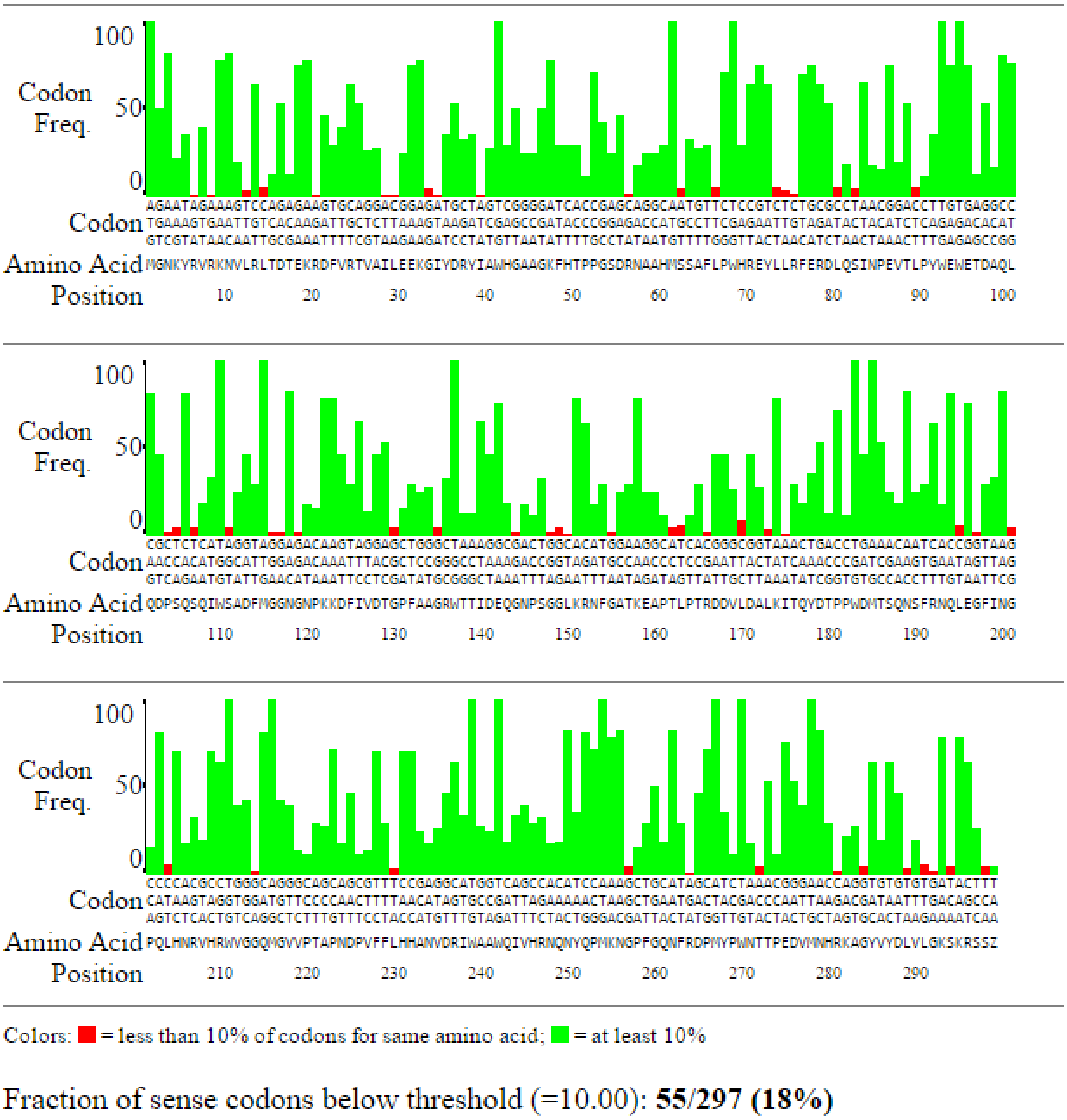


Figure 4.37. Codon usage analysis of the *Bacillus megaterium* M36 tyrosinase gene fragment

4.2.9. Optimization of *Bacillus megaterium* M36 Tyrosinase Gene Expression

In this study, Box- Behnken design was used to determine the optimal level of the four variables (starting cell density of induction, induction time, IPTG and induction temperature) for tyrosinase enzyme production. ANOVA statistics results and the estimated regression coefficients are given in Table 4.3

The Model F-value of 20.90 implies the model is significant. There is only a 0.01% chance that an F-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, D, BD, A2, B2, C2, D2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model. The "Lack of Fit F-value" of 25.56 implies the Lack of Fit is significant. There is only a 0.34% chance that a "Lack of Fit F-value" this large could occur due to noise. Significant lack of fit is bad -- we want the model to fit.

The "Pred R-Squared" of 0.7399 is in reasonable agreement with the "Adj R-Squared" of 0.9087; i.e. the difference is less than 0.2. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 17.271 indicates an adequate signal. This model can be used to navigate the design space.

Table 4.3 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of recombinant *Bacillus megaterium* M36 tyrosinase enzyme production.

Use your mouse to right click on individual cells for definitions.

Response **1** **R1**

ANOVA for Response Surface Quadratic model

Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1193.29	14	85.24	20.90	< 0.0001	significant
A-starting cell density of induction	*89.87*	*1*	*89.87*	*22.03*	*0.0003*	
B-induction time	*323.34*	*1*	*323.34*	*79.27*	*< 0.0001*	
C-IPTG	*31.72*	*1*	*31.72*	*7.78*	*0.0145*	
D-induction temperature	*148.83*	*1*	*148.83*	*36.49*	*< 0.0001*	
AB	*0.048*	*1*	*0.048*	*0.012*	*0.9148*	
AC	*0.56*	*1*	*0.56*	*0.14*	*0.7159*	
AD	*6.25*	*1*	*6.25*	*1.53*	*0.2361*	
BC	*7.59*	*1*	*7.59*	*1.86*	*0.1941*	
BD	*91.78*	*1*	*91.78*	*22.50*	*0.0003*	
CD	*14.06*	*1*	*14.06*	*3.45*	*0.0845*	
A^2	*77.78*	*1*	*77.78*	*19.07*	*0.0006*	
B^2	*41.88*	*1*	*41.88*	*10.27*	*0.0064*	
C^2	*180.26*	*1*	*180.26*	*44.20*	*< 0.0001*	
D^2	*195.53*	*1*	*195.53*	*47.94*	*< 0.0001*	
Residual	57.10	14	4.08			
Lack of Fit	*56.22*	*10*	*5.62*	*25.56*	*0.0034*	*significant*
Pure Error	*0.88*	*4*	*0.22*			
Cor Total	1250.40	28				

A second order polynomial equation given in Eqn. 3 was constructed by using the estimated coefficients (in coded units).

$$\text{Tyrosinase Enzyme (IU)} =$$
$$18.40+2.74A-5.19B+1.63C+3.52D-0.11AB-0.38AC+1.25AD-1.38BC$$
$$-4.79BD+1.87CD-3.46A^2-2.54B^2-5.27C^2-5.49D^2$$

The equation in terms of coded factors was used to make predictions about the response for given levels of each factor. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

The plot between predicted vs. actual (Fig. 38) is a good tool to study the significance of suggested model. The responses predicted from the empirical model are in agreement with the observed values in the range of the operating variables.

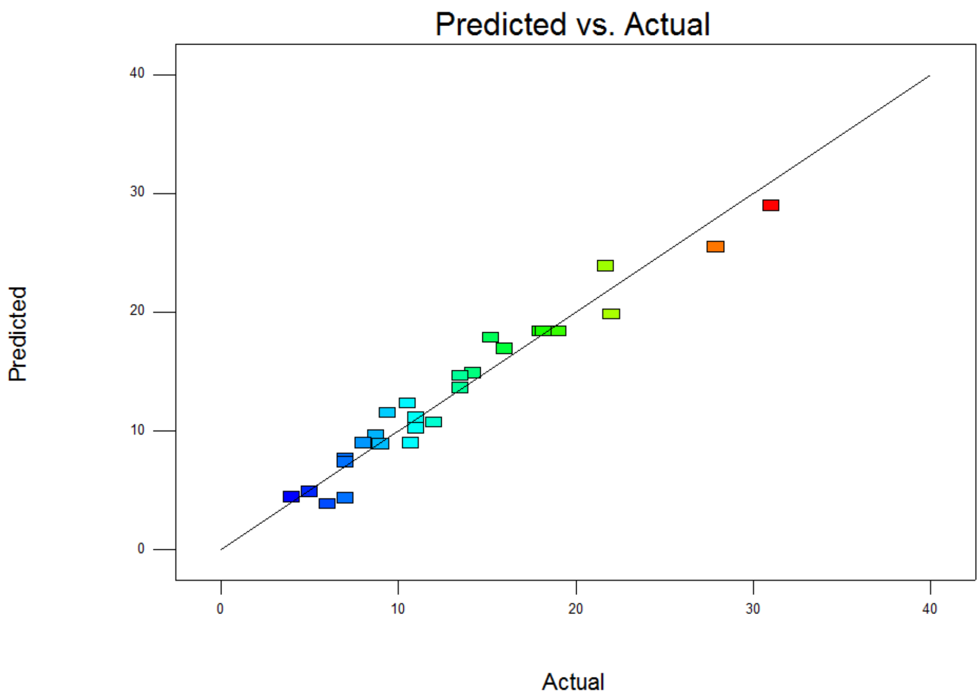
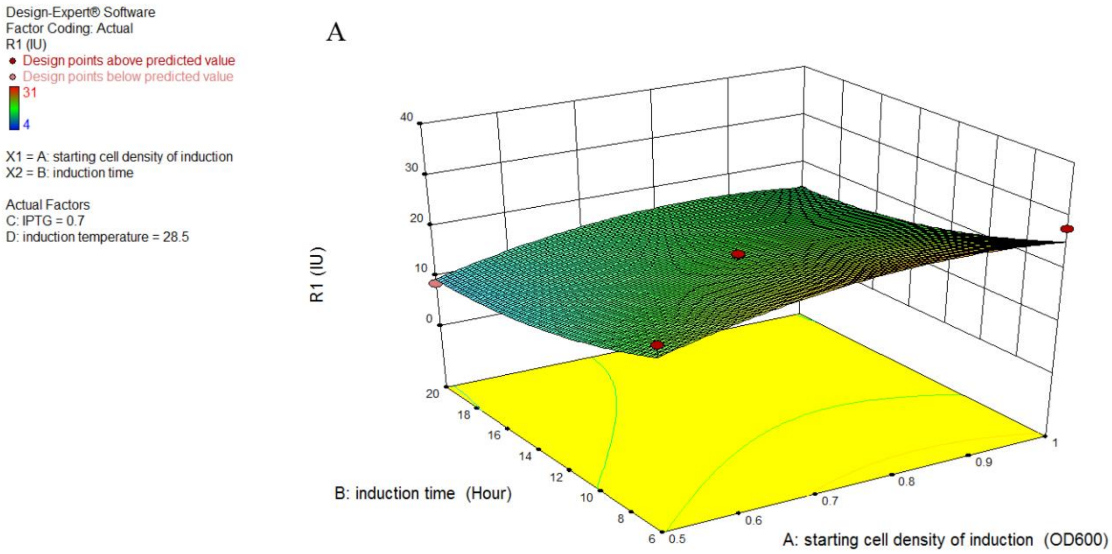
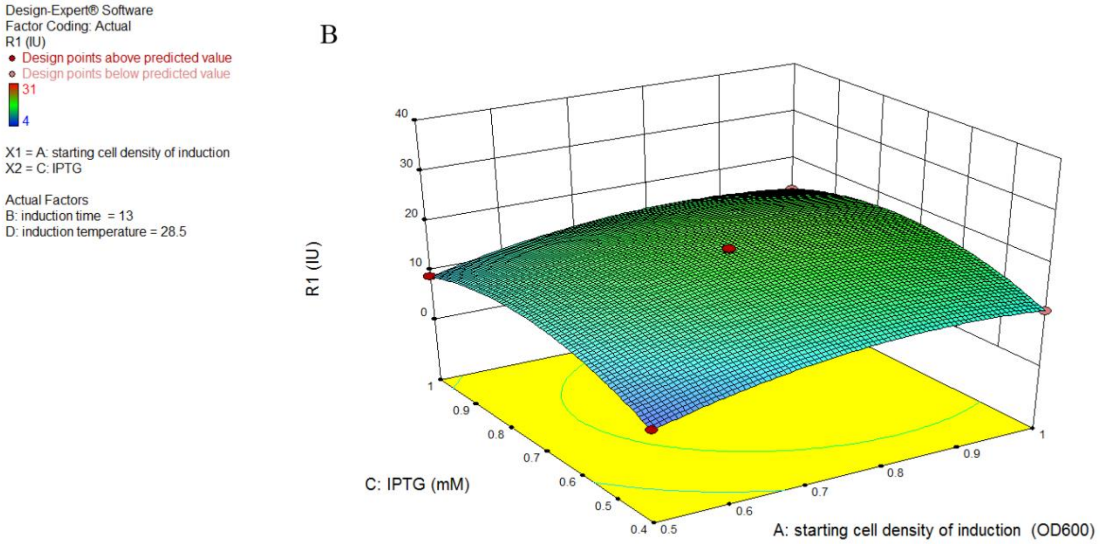
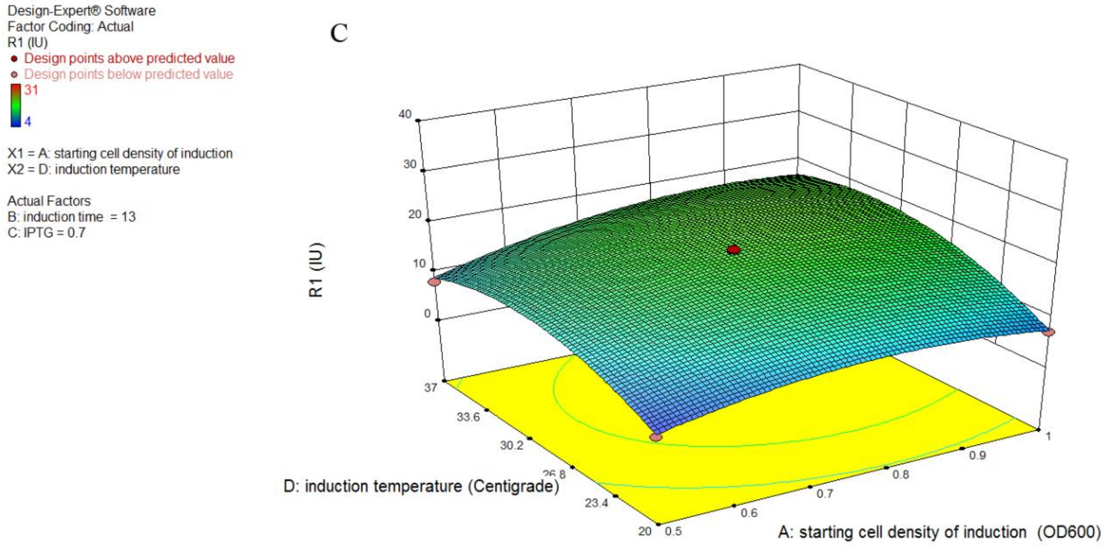
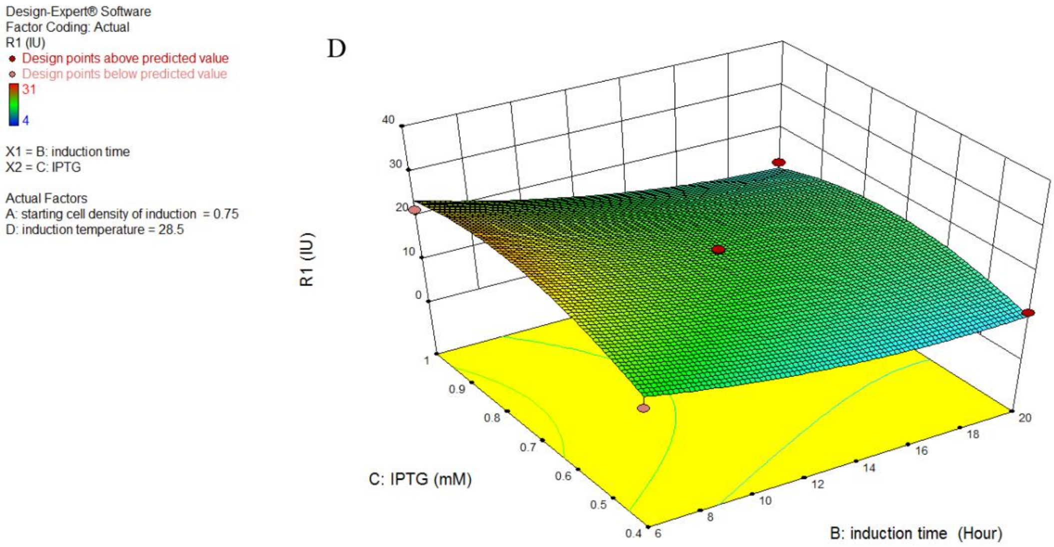


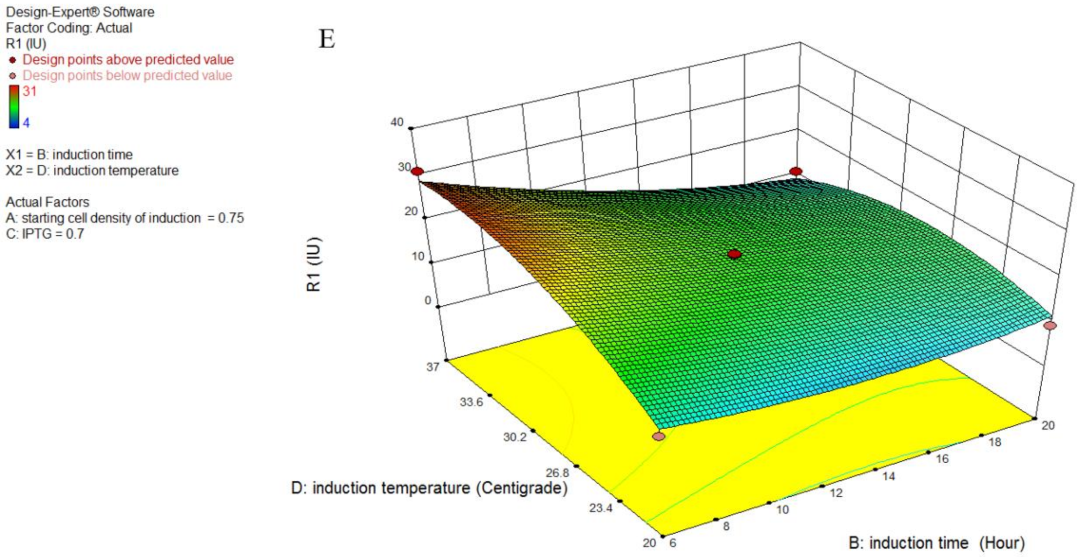
Figure 4.38. Plot of actual yield vs. predicted yield.


Design-Expert® Software
Factor Coding: Actual
R1 (IU)
Design points above predicted value
Design points below predicted value
31
4
X1 = A: starting cell density of induction
X2 = B: induction time
Actual Factors
C: IPTG = 0.7
D: induction temperature = 28.5
A
R1 (IU)
B: induction time (Hour)
A: starting cell density of induction (OD600)


Design-Expert® Software
Factor Coding: Actual
R1 (IU)
Design points above predicted value
Design points below predicted value
31
4
X1 = A: starting cell density of induction
X2 = C: IPTG
Actual Factors
B: induction time = 13
D: induction temperature = 28.5
B
R1 (IU)
C: IPTG (mM)
A: starting cell density of induction (OD600)


Design-Expert® Software
Factor Coding: Actual
R1 (IU)
Design points above predicted value
Design points below predicted value
31
4
X1 = A: starting cell density of induction
X2 = D: induction temperature
Actual Factors
B: induction time = 13
C: IPTG = 0.7
C
R1 (IU)
D: induction temperature (Centigrade)
A: starting cell density of induction (OD600)


Design-Expert® Software
Factor Coding: Actual
R1 (IU)
Design points above predicted value
Design points below predicted value
31
4
X1 = B: induction time
X2 = C: IPTG
Actual Factors
A: starting cell density of induction = 0.75
D: induction temperature = 28.5
D
R1 (IU)
C: IPTG (mM)
B: induction time (Hour)



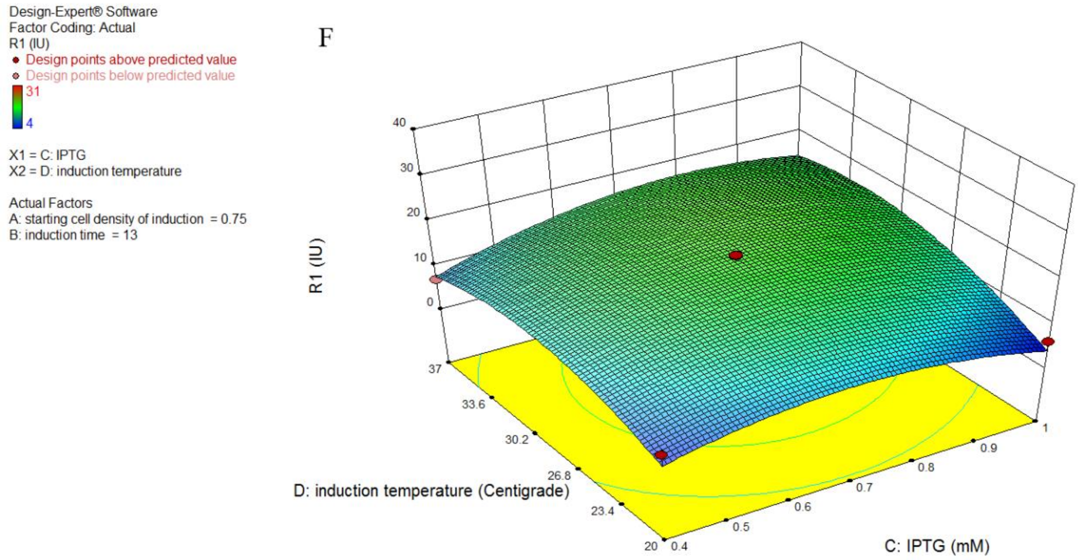


Figure 4.39. Response surface plot of the combined effects of starting cell density of induction, induction time, and IPTG and induction temperature on the tyrosinase enzyme production by *Bacillus megaterium* M36.

The response surface curves represented in Figure. 4. 39 A-F show the interaction between variants. By using the methods maximum recombinant enzyme production was obtained as 31 IU. And optimum condition for the production was determined as starting cell density of induction (OD 600=0.87), induction time (6.0 hours), IPTG concentration (0.82mM) and induction temperature (36.2 °C). In this condition predicted value for the enzyme production was 31.049 and actual value was 30.98. This result shows the the model to be appropriate for enzyme production.

4.2.10. Comparison of the Expression of *Bacillus megaterium* M36 Tyrosinase Enzyme in Two Different Systems

Two host cells (*E.coli* BL21 (DE3) pLysS and Rosetta-gami 2 (DE3) pLysS) having pET22b-tyr were subjected to protein expression as explained in material and methods under the optimized condition, the produced enzyme were evaluated. After purification by MagneHis protein purification system, 31 IU/ml of enzyme obtained from Rosetta-gami 2 (DE3) pLysS having pET22b-tyr. In all of the system the *Bacillus megaterium* M36 tyrosinase enzyme showed the bond of almost 35 kDa (Figure.4.40). This result was in agreement with the finding of the shuster et al. who has reported a 35 kDa tyrosinase enzyme from *Bacillus* megaterium.

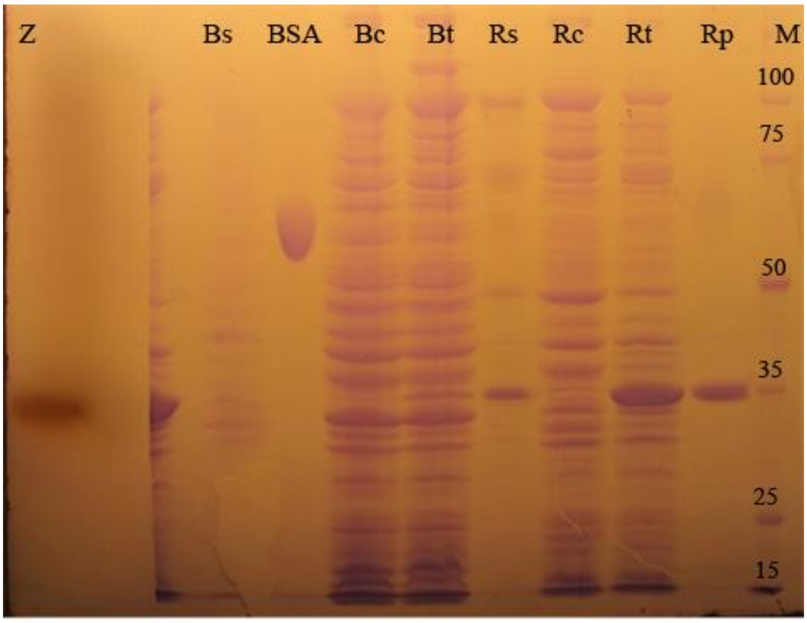


Figure. 4.40. Electrophoresis analysis for Comparison of the expression of *Bacillus megaterium* M36 tyrosinase enzyme in two different systems. Lane z represents the zymogram analysis of the recombinant *Bacillus megaterium* M36 tyrosinase enzyme, lane Bs is supernatant of the *E.coli* BL21 (DE3) pLysS, BSA is a bovine serum albumin protein. Bc shows the proteins obtained from non-induced *E.coli* BL21 (DE3) pLysS, Bt is relate to proteins of induced *E.coli* BL21 (DE3) pLysS, Rs is a supernatant of Rosetta-gami 2 (DE3) pLysS, Rc represents the proteins from non-induced Rosetta-gami 2 (DE3) pLysS, Rt is related to proteins from induced Rosetta-gami 2 (DE3) pLysS, Rp is a purified *Bacillus megaterium* M36 tyrosinase enzyme and M is protein marker from promega.

4.2.11. Effect of pH and Temperature on Activity and Stability of the Recombinant *Bacillus megaterium* M36 Tyrosinase Enzyme

The recombinant *Bacillus megaterium* M36 tyrosinase enzyme affected by different pH value and temperature as same as the native one with exception that the recombinant one was some more stable and active in high pH and temperature as compared to the native one (figure 4.41 and 4.42.).

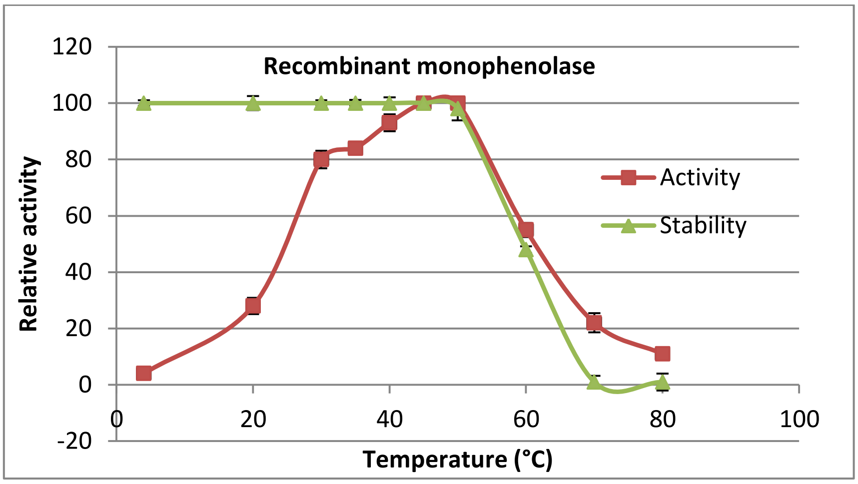


Figure 4.41. Effect of temperature on recombinant *Bacillus megaterium* M36 tyrosinase enzyme activity and stability. The enzyme showed maximum activity at 45-50°C and 93% of its maximum activity at 40°C. The activity of the enzyme was sharply decreased at temperature lower than 30°C and higher than 50°C. Also, the enzyme conserved its original activity at temperature ranging from 0°C to 45°C and 98% of that at 50°C.

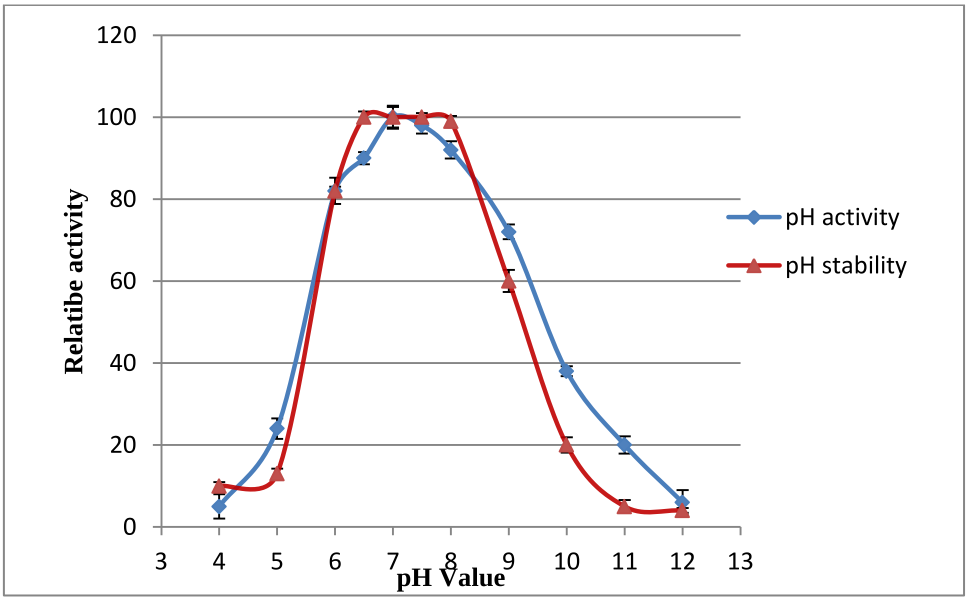


Figure 4.42. Effect of different pH values on recombinant tyrosinase enzyme activity and stability. The enzyme showed maximum activity at pH=7.0 and up to 90% of the maximum activity at pH ranging from 6.5 to8.0). Also, the enzyme conserved its original activity about 100% at pH 6.5-8.0.

4.2.12. Effect of Detergents on *Bacillus megaterium* M36 Enzyme Activity

The *Bacillus megaterium* M36 tyrosinase enzyme was studied in presence of various inhibitors (Fig.4.43). The enzyme was inhibited strongly by β-mercaptoethanol. β-mercaptoethanol is a reducing agent which inhibit dopachrom and melanin synthesis by chemical reducing of qinones (an intermediate) to L-DOPA. Similar results were obtained for *Bacillus megaterium* tyrosinase (Shuster and Fishman, 2009) and *Thermomicrobium roseum* tyrosinase (Kong et al. 2000) that was completely inhibited by β-mercaptoethanol (1 mmol).

The *Bacillus megaterium* M36 tyrosinase was inhibited about 90% by 5 mmol of EDTA (a chelating agent). The agent can inhibit the enzyme by chelating of Cu from its active site. Similarly *Bacills* megaterium tyrosinase was inhibited up to 27% by 1 mM EDTA (Shuster and Fishman, 2009) and *Bacillus* (HR03) tyrosinase enzyme was partially inhibited by 1mM EDTA (Dalfard et al. 2006). In contrast to the result of this research tyrosinase enzyme from *Bacillus* thuringiensis (El-Shora, and Metwally, 2008) was activated at high concentration EDTA from 200 to 400 mM.

Effect of different concentration of SDS (0.2- 30 mM) on the *Bacillus megaterium* M36 tyrosinase enzyme was studied. Although the enzyme was activated at the presence of 1 mM SDS, it was strongly inhibited at high concentration of (above 15 mM) SDS (Figure 4.44).

Previously, activating effect of SDS on tyrosinase enzyme from *Xenopus laevis* (Wittenberg and Triplett, 1985), *A. bisporus* (Espin and Wichers, 1999), *Bacillus* sp. (Dalfard et al., 2006) and *Bacillus megaterium* (Shuster and Fishman, 2009) has been reported which was in agreement with our result.

According to the paper published by Gandia-Herrero, although, active site of enzyme is not affected by SDS; a stepwise conformational change affected the enzyme activity by increasing accessibility of its active site to the substrate (Gandía-Herrero et al. 2005)

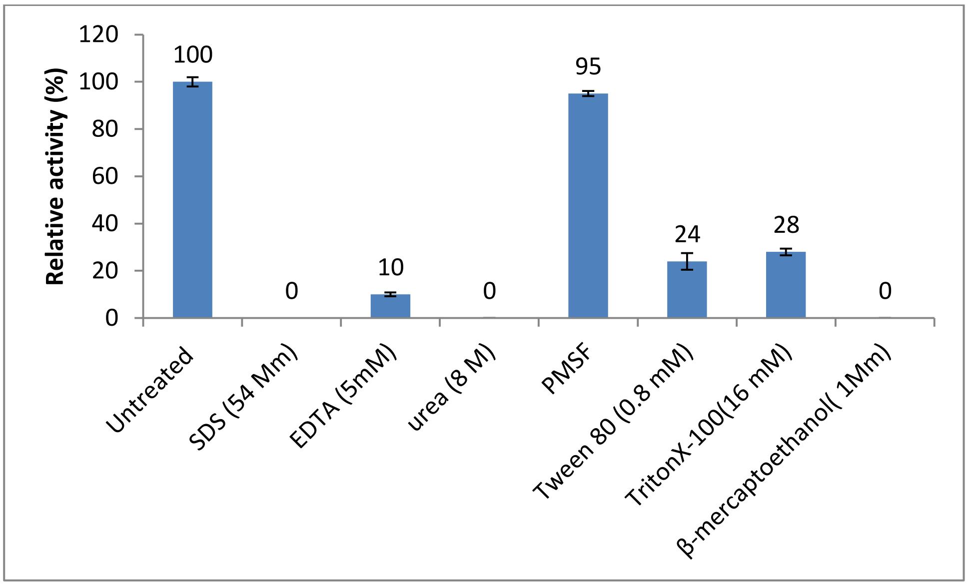


Figure 4.43. Effect of inhibitors on *Bacillus megaterium* M36 tyrosinase activity. The relative activity of the enzyme treated with SDS, EDTA, Urea, PMSF, Tween-80, TritonX-100, β-Mercaptoethanol was as 0, 10, 0, 95, 24, 28 and 0, respectively. The activity of the sample containing the enzyme without any of the additives was considered as control (100%).

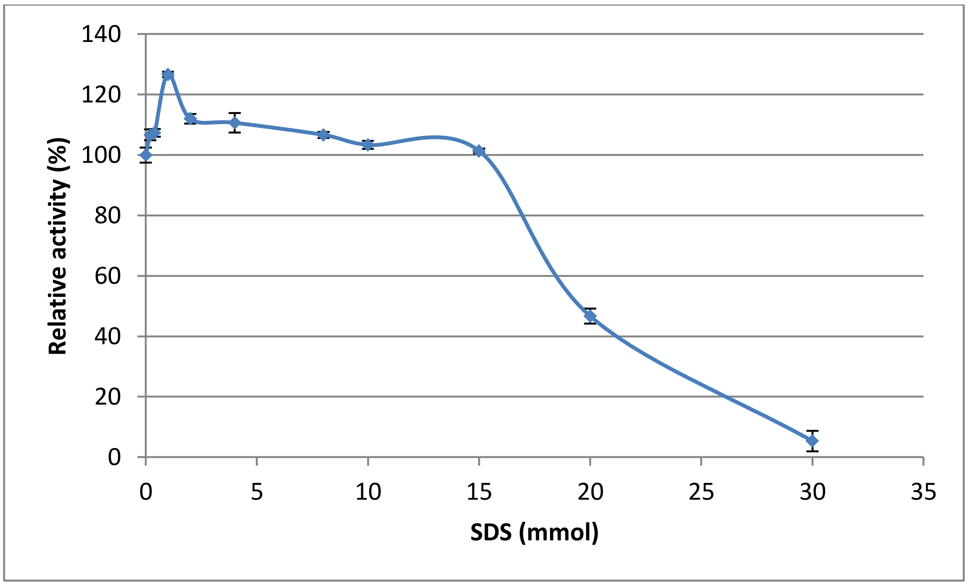
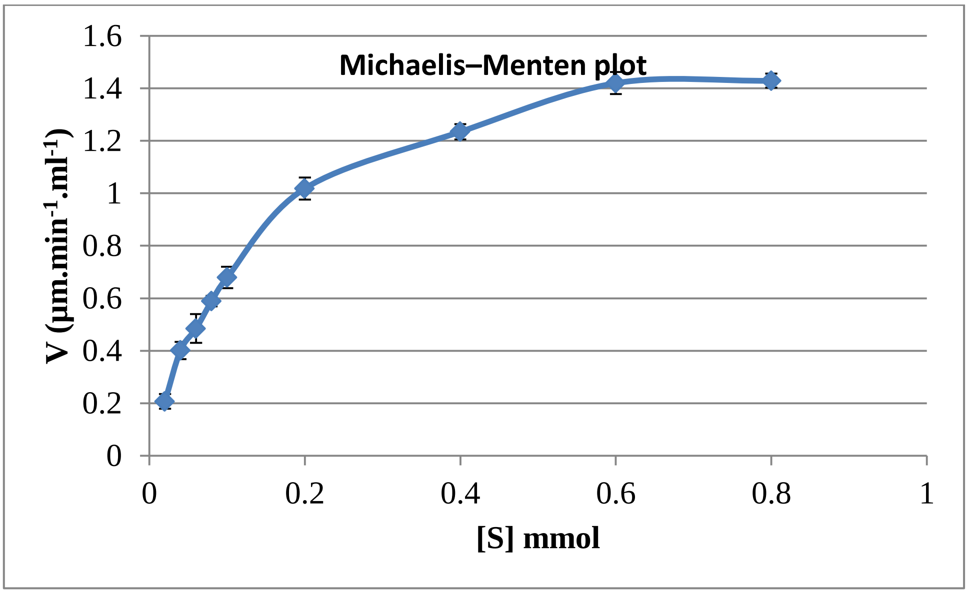


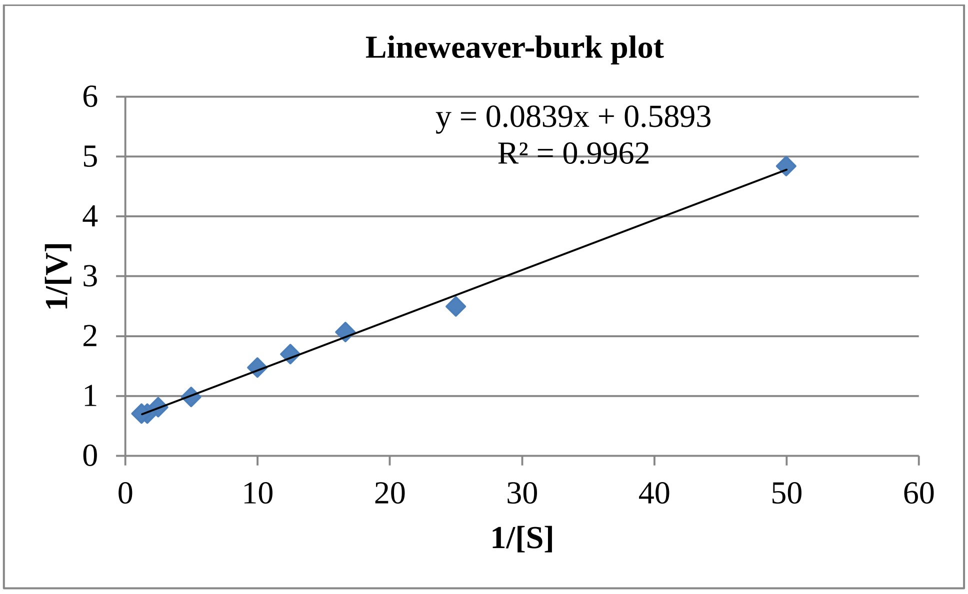
Figure 4.44. Effect of SDS concentration (w/v) on the *Bacillus megaterium* M36 tyrosinase activity. Sample having no SDS in reaction mixture was considered as a control (100%), the enzyme showed maximum activity (126.6%) at the presence of 1mM of SDS and its activity was gradually decreased at the SDS concentration more than 1mM, so that it reached to 5.3% at concentration of 30mM.

4.2.13. Kinetic Study of *Bacillus megaterium* M36 Tyrosinase Enzyme

The *Bacillus megaterium* M36 tyrosinase enzyme was shown to obey Michaelis-Menten kinetics when L-tyrosine and L-DOPA was used as a substrate. The Km value of *Bacillus megaterium* M36 tyrosinase for l-tyrosine (0.15mM) was lower than L-DOPA (0.58mM). The abtained V_{max} was 1.7µM.min^{-1}.ml $^{-1}$ for L-tyrosine and 6.2µM.min-1.ml -1 for L-DOPA (Figure.4.45).

Km value of *Bacillus megaterium* M36 tyrosinase enzyme for L-tyrosine was 0.15 mM. This is similar to the some previously reported Km values with the L-tyrosine, for example ; 0.2 mM for *Agaricus bisporus* (SelinheimoET AL. 2009) , 0.23mM for *P. putida* F6 (McMahon ET AL. 2007) and 0.19 mM for *Rhizobium etli* CFN42 (Cabrera-Valladares et al. 2006) , aslo it is higher than the value (0.075 mM) reported for *Bacillus megaterium* (Shuster and Fishman 2009) and it is less than the values 0.563 mM, 1 mM, 0.421mm reported for *Bacillus thuringiensis* (El-Shora and Metwally, 2008). , Streptomyces sp. REN-21(Ito, and Inouye , 2005) and *Verrucomicrobium spinosum* (Fairhead, and Thony-Meyer, 2010), respectively.





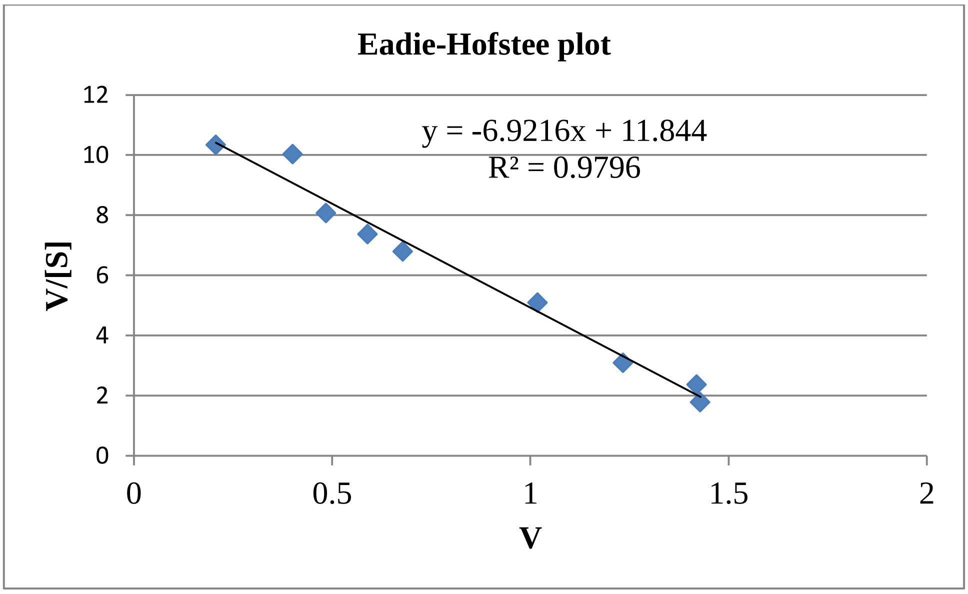


Figure 4.45. Michaelis-Menten plot, Lineweaver-Burk plot and Eadie-Hofstee plot of recombinant *Bacillus megaterium* M36 tyrosinase activity (velocity) versus tyrosine concentration (mM) to obtain values for the maximum velocity (V max) and Michaelis constant (Km).

4.3. Characterization of Melanin

The M36 melanin was produced using recombinant *Bacillus megaterium* M36 tyrosinase enzyme , extracted, dried and saved (figure 4.46.) for further analysis

Figure 4.46. Produced and dried *Bacillus megaterium* M36 melanin

4.3.1. FT-IR (Fourier- Transform Infrared) Spectroscopy Analyses of *Bacillus Megaterium* M36 Melanin

The infrared (IR) spectra of synthetic and water-soluble microbial melanin are presented in (Figure 4.47). The comparison of standard melanin and our microbial melanin showed a strong similarity at the main absorption peaks. In agreement for this some previous researchers approved their melanin products with FT-IR analysis (Aghajanyan et al. 2005).

A broad absorption at 3373 cm-1 indicates the presence of – OH and NH2 groups and small band at 2925 cm^{-1} can be assigned to stretching vibration of aliphatic C-H group. The characteristic strong band at 1623cm-1 (between 1650 - 1620 cm^{-1}) attributed to vibrations of aromatic ring C=C of amide I C=O and/or of COO- groups. Bands at ~1447 cm^{-1} can be due to aliphatic C-H groups and weak bands below 700 cm^{-1} ascribed to alkene C-H substitution in the melanin pigment. The observed IR patterns for the purified melanins were similar to the earlier reported DOPA-melanin study.

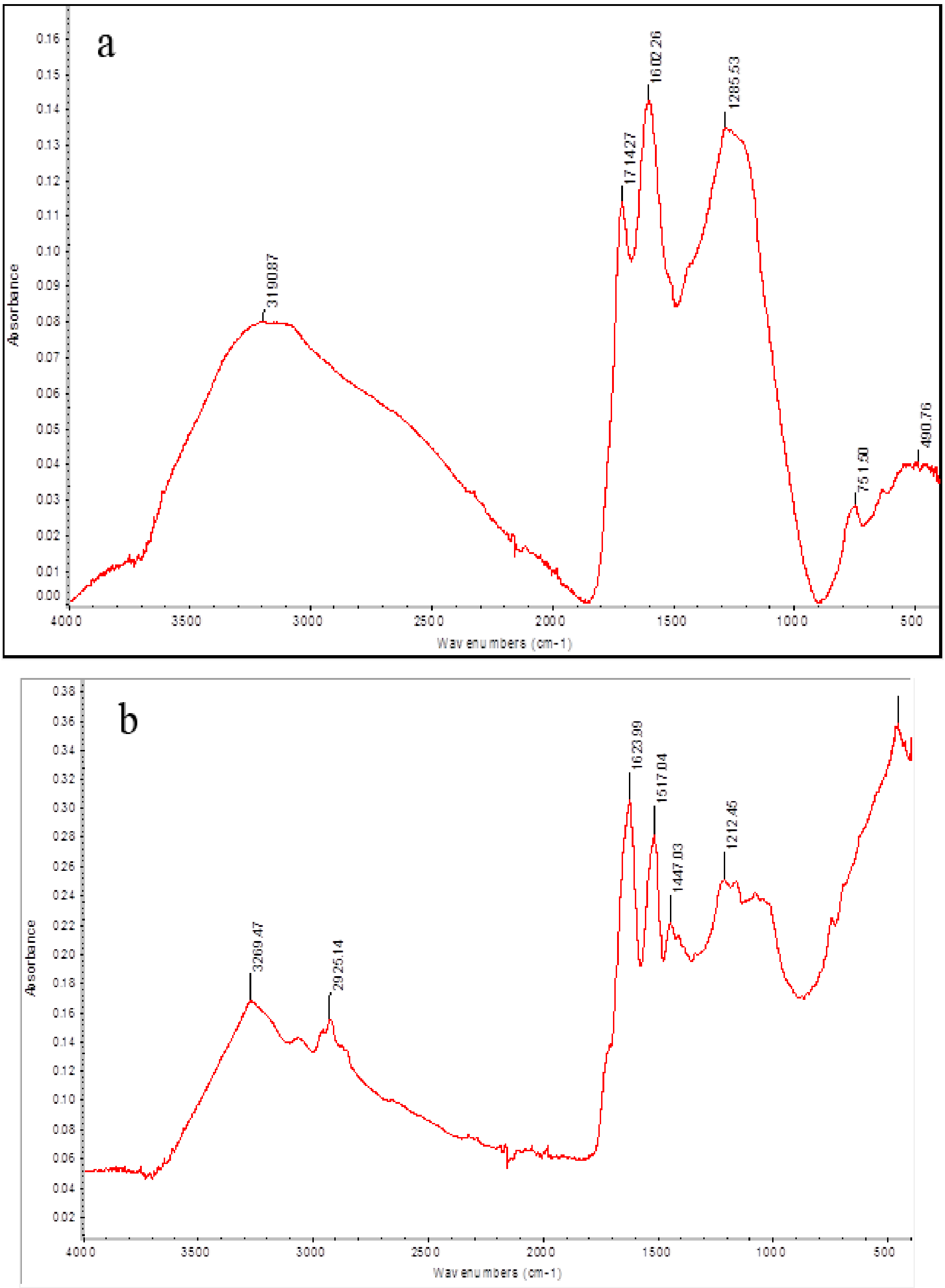


Figure 4. 47. FT-IR analysis of the *Bacillus megaterium* M36 melanin. a) represents the infrared (IR) spectra of standard synthetic melanin from sigma and b) shows the infrared (IR) spectra of melanin synthesized by recombinant *Bacillus megaterium* M36 tyrosinase enzyme.

4.3.2. EPR Spectroscopy Analyses of *Bacillus megaterium* M36 melanin

The major defining feature of all melanins is the presence of stable organic free radicals, which results in the characteristic electron paramagnetic resonance behavior. This property of melanin was exploited by studying EPR spectrum. In the present study, the EPR spectrum of the melanin pigment indicated the presence of free radicals (Figure.4.48). These results demonstrate the usefulness of spectroscopy in studying diverse aspects of melanin and melanization in microorganisms.

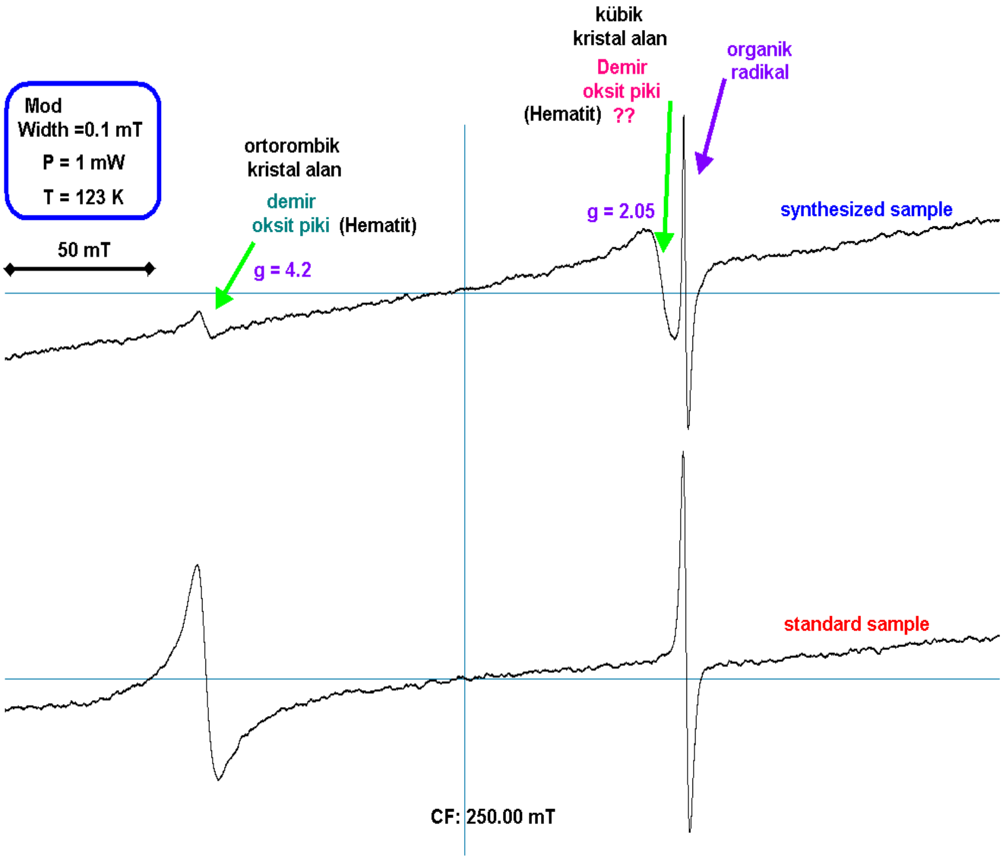


Figure. 4.48. EPR analysis of the *Bacillus megaterium* M36 melanin

4.3.3. Solubility in Various Solvents of *Bacillus megaterium* M36 melanin

Bacillus megaterium M36 melanin was soluble in NaOH (1N), HCL (1 N), H_2O, Ethanol, but insoluble in, Benzene, Chloroform, Acetone. In contrary of the finding of this research the melanin reported by Sajjan et al. was not soluble in ethanol (Sajjan et al. 2011). Melanin reported by Sajjan et al. was soluble NaOH (1N), HCL (1 N), H_2O.

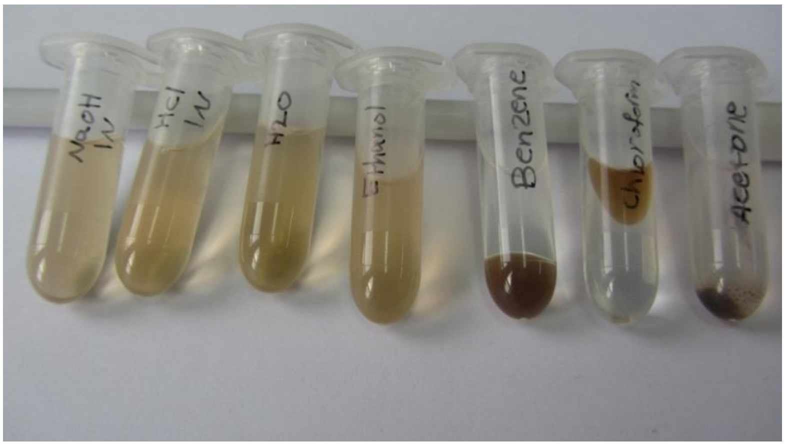


Figure. 4.49. Solubility of the *Bacillus megaterium* M36 melanin in various solvents. M36 melanin was soluble in NaOH (1N), HCL (1 N), H_2O, Ethanol, but insoluble in, Benzene, Chloroform, Acetone.

4.3.4. UV-Visible Spectroscopic Analysis of the Extracted *Bacillus megaterium* M36 Melanin

The UV-visible wavelength scan (180 to 900 nm) of the pigment is shown in (Figure.4.50).The absorption was highest in the UV region at 200-300 nm, but decreased towards the visible region, which is the characteristic property of melanin. This phenomenon is due to the presence of the very complex conjugated structure in melanin.

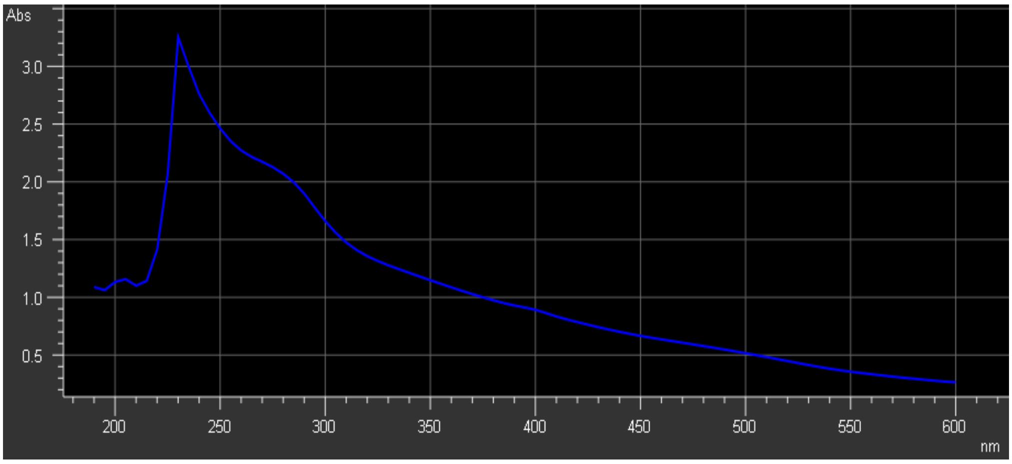


Figure 4.50. Absorption of the M36 melanin at various wavelength.

4.3.5. Antioxidant Effect of Melanin produced by recombinant *Bacillus megaterium* M36 tyrosinase enzyme

The phenolic hydroxyl group has a strong proton donating power. The more the hydroxyl group is the more hydrogen ions are provided to combine with more active free radicals. As shown in Figure. 4. 51, the M36 melanin like vitamin C showed strongly ·OH scavenging activity, this properties was similar to Plectania YM-421 melanin reported by Ye M. et al. (2011)

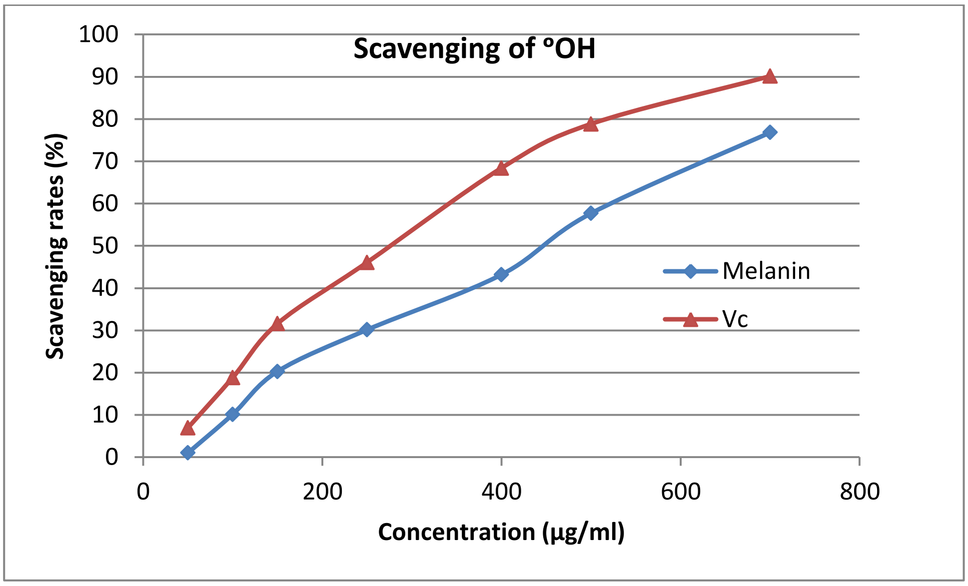


Figure. 4.51. The scavenging activity of LSM on ·OH. The *Bacillus megaterium* M36 melanin scavenged about 10% and 76.8% of radical ·OH at concentration of 10µg/ml and 700µg/ml, respectively. The ·OH scavenging ability of the M36 melanin was similar to Vc.

As shown in Figure. 4. 52. *Bacillus megaterium* M36 melanin has a strong O_2^{-}· scavenging activity. With the increase of the *Bacillus megaterium* M36 melanin concentration, the O2 -· scavenging activity of *Bacillus megaterium* M36 melanin increased gradually. This data is in agreement with of melanin from the muscles of Taihe Black-bone silky fowl (Tu et al. 2009).

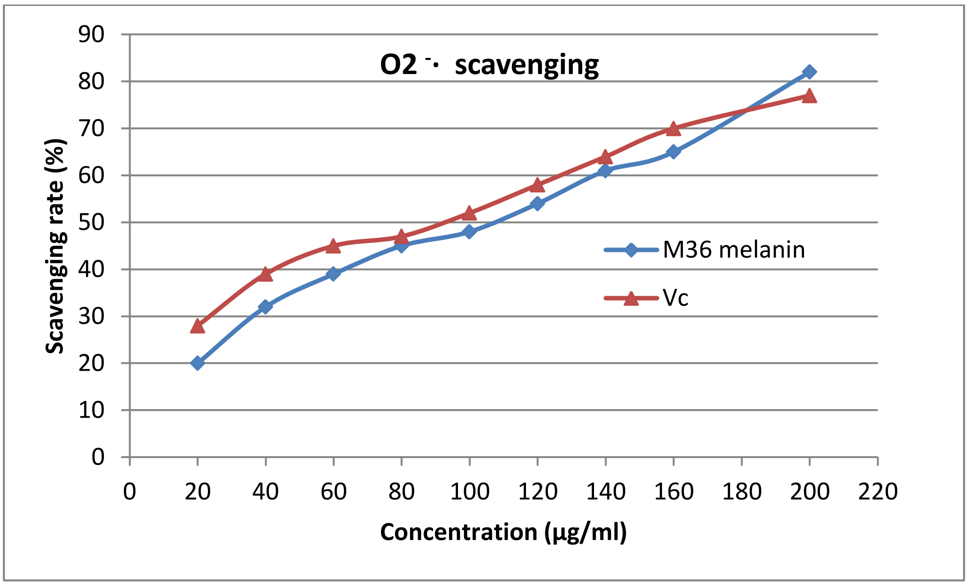


Figure. 4.52. The scavenging activity of *Bacillus megaterium* M36 melanin on O2-·. Concentration of 20µg/ml scavenged about 20% of O2-· and 200µg/ml of M36 melanin scavenged about 82% of O2-·. The scavenging ability of the M36 melanin was similar to Vc.

4.3.6. Antibacterial Effect of *Bacillus megaterium* M36 Melanin

Bacillus megaterium M36 melanin showed a good antibacterial effect. As shown at Figure.4.53. the minimal inhibitory concentration (MIC) of the *Bacillus megaterium* M36 melanin for some antibiotic resistance and hospital infectant bacterial strain such as *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Esherichia coli* and *Enterobacter aerogenes* was as 35, 35, 35, 40, 35 and 25 µg/ml respectively also, the Minimum Bactericidal Concentration (MBC) of the M36 melanin for the bacteria was as 70, 65, 65, 85, 55, 50 µg/ml respectively. Before the MIC of Melanin from Actinoalloteichus sp. MA-32 had been as 16, 19, 16 and 13µg/ml for *Esherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa, and Staphylococcus aureus* respectively (Manivasagan et al. 2013). The antibacterial effect of the melanin reported by Manivasagan was seemed more than that *Bacillus megaterium* M36 melanin. All of the bacteria tested by Manivasagan were sensitive to ampicillin, while according to the information tacked from the balcali hospital technicians and authorities, the strains used in this research were antibiotic resistance.

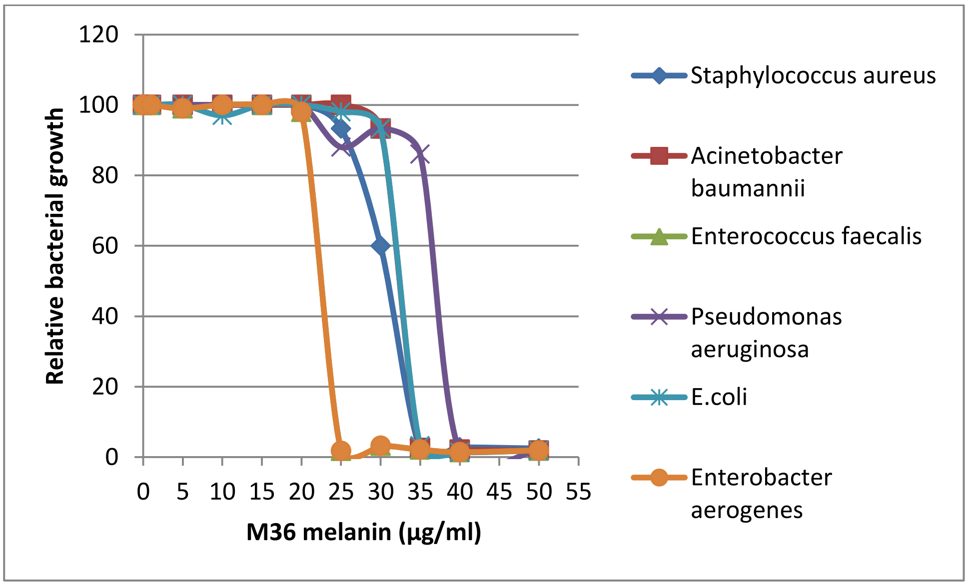


Figure. 4.53. Antibacterial effect of the *Bacillus megaterium* M36 melanin. The minimal inhibitory concentration (MIC) of the *Bacillus megaterium* M36 melanin for some antibiotic resistance and hospital infectant bacterial strain such as *Staphylococcus aureus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Esherichia coli* and *Enterobacter aerogenes* was as 35, 35, 35, 40, 35 and 25 µg/ml respectively.

4.3.7. Anticancer Effect of *Bacillus megaterium* M36 Melanin

To do MTT assay of the *Bacillus megaterium* M36 melanin effect on the mouse fibroblastoma cell line, the cells were treated with different concentrations of the melanin (0, 100, 200, 400, 600, 800, 1000 µg/ml) and incubated for 24 and 48 hours. After that cell concentration was assayed by spectrophotometer (OD_{560}) and subjected to calculation of relative cell concentration. The tests for each concentration was repeated 8 times and the result of the tests was statistically (T-test and R squared) analyzed and approved to be significant. According to the results in both of the 24h and 48h, the *Bacillus megaterium* M36 melanin showed concentration dependent inhibitory effect on cell growth. Before Sivaperumal et al.

(2014) had isolated and characterized a melanin from *actinobacterium Streptomyces* sp. and determined the half maximal inhibitory concentration (IC50) as 300µg/ml for the melanin. So in this research the needed concentration was selected as reported by Sivaperumal et al. (2014). According to our knowledge quantitatively study of the effect of melanin on cell line was done in this research for the first time.

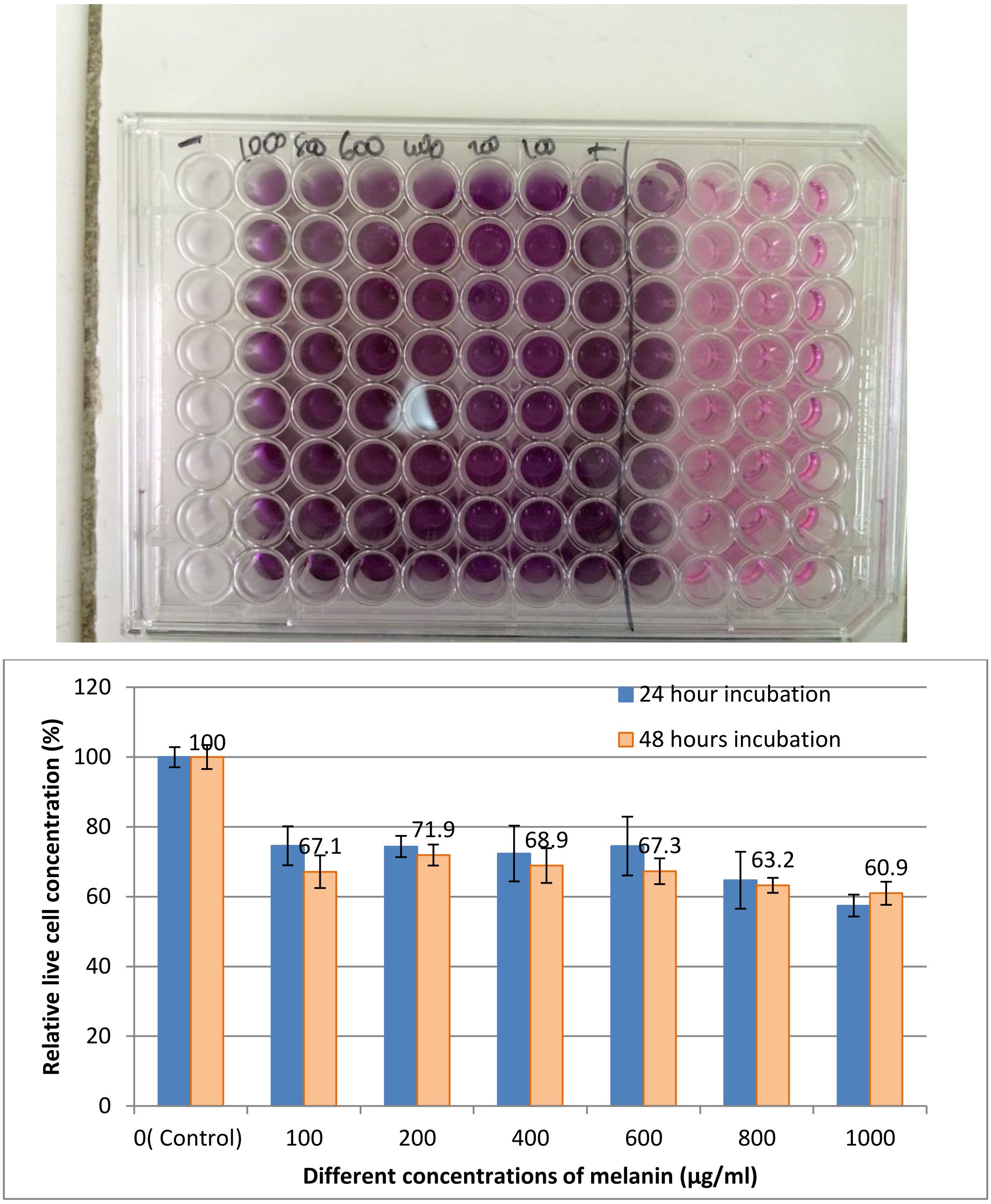


Figure 4.54. MTT assay of the *Bacillus megaterium* M36 melanin. The *Bacillus megaterium* M36 melanin was decreased growth of the cells about 33% at concentration of 100µg/ml and 40% at concentration of 1000µg/ml.

4.3.8. Dyeing experiment of *Bacillus megaterium* M36 Melanin

The cotton fabric was dyed by boiling of the fabric in melanin solution for 10 min at 100°C .After dying, the cotton fabrics could completely conserve its dye even after washing in tap water, liquid hand soap, concentrated dishwasher detergent (CIF) and boiling in water 100°C. After washing the fabric is fully protected melanin (Figure 4.55.). So this finding is better than the result reported by Ali et al. (2007) who reported that cotton fabrics dyed by *Streptomyces* melanin could conserve 50% of its dye after washing with normal detergent.

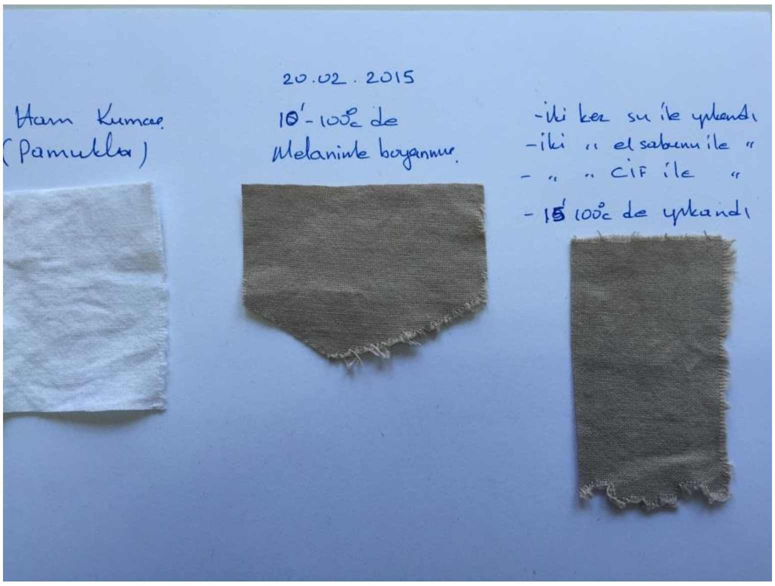


Figure 4.55. Dying of cotton fabrics by *Bacillus megaterium* M36 melanin. The cotton fabric was dyed by boiling of the fabric in melanin solution for 10 min at 100°C after that it was washed two times by tap water, two times by liquid hand soap, two times by dishwasher detergent (CİF) and finally it was boiled in water at 100°C for 15 min.

5. CONCLUSION

In this research bacilluse sp. (M36 *Bacilluse* sp.) having tyrosinase enzyme was isolated from native. The strain showed up to 99% homology with *Bacillus megaterium*. The tyrosinase enzyme was successfully produced by the M36 Bacillus sp., and characterized. By the native M36 tyrosinase enzyme, l-tyrosine was converted to both L-DOPA and melanin and this conversion was approved using TLC and HPLC.

The regarding gene fragment was cloned to *E.coli* BL21 (DE3) pLysS and Rosetta-gami 2 (DE3) pLysS using pET22b vector and its expression was accomplishedly took place. Comparison of the two systems showed that the expression of the enzyme in *Rosetta-gami* 2 (DE3) pLysS was streets a head of the expression in *E.coli* BL21 (DE3) pLysS.

Bioinformatics analysis of the sequenced M36 tyrosinase gene and protein showed that the enzyme was similar to the tyrosinase enzyme from *Bacillus megaterium* reported by shuster et al.

Production of the enzyme both by the native and recombinant cells was optimized using experimenta design by Design- Expert 9 program.

Production of the enzyme by the Native M36 *Bacillus* sp. was determined as 0.05IU/ml, after optimization of culture condition it was reached to 0.38 IU/ml, also this product reached to 31 IU/ml after optimization of recombinant M36 tyrosinase enzyme expression. M36 melanin produced in this research showed structural similarity with standard synthetic melanin from sigma according to the solubility, FT-IR and EPR analysis. Also it showed notable anti-bacterial, anti-UV and ant-oxidant effects. MTT assay showed the M36 melanin to have anticancer effect. Purified DNA treated by the M36 melanin was not been affected.

So this system is suitable for melanin production and the melanin has many biotechnological and industrial functions.

5.1. Suggestion

1- Using agricultural wastes as a substrate: By this way the cost of the melanin production will very decreased, at the other hand waste substance will be converted to a valuable product.

2- Cloning of the M36 tyrosinase gene in *Bacillus megaterium* and comparison with the E.coli system.

3- Study on the structural properties of the M36 tyrosinase enzyme using X-ray crystallography and circular dichroism and comparison with the data present in the litratures.

4- Further study on cytotoxic and genotoxic effect of the M36 melanin on the human cells.

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