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Biodegradation of some hydrocarbon pollutants by laccase produced from local isolate of *Bacillus* sp.

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بِسْمِ اللهِ الرحْمن الرحيم

((قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا ۖ إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ))

صدق الله العظيم

سورة البقرة

آية (32)

Dedication

To whom who sent to us as a merciful prophet ((**Mohammed**)) peace be upon him…

To who planted the seed of patience in myself and walked in my long way ((My darling **father**))…

To a candle that burned to lighten the way ((My beloved **mother**))…

To the best supporters in the world ((My **brothers** and my **sister**))…

To my life love ((My **wife**)) Thank you very much for your support whenever I need you…

To the best gift in the world ((my **sons**))…

To all my friends, especially my close friends ((**Mohammed, Qusay, Haidar**))…

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Finally, I apologize from all those who helped me and are not mentioned in this acknowledgment truly I thank them all.

Ahmed

Certification

We certify that this thesis was prepared under our supervision at the University of Baghdad, College of science, and Department of Biotechnology as a partial requirement for the Degree of Master of Science in Biotechnology

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Summary

Fifty seven isolates of *Bacillus* spp. were isolated from soil samples of different sites in Al-Diwaniyah and Baghdad governorates. The bacterial isolates were initially identified as *Bacillus* spp. according to the cultural and microscopic characteristics

Primary screening of *Bacillus* spp. isolates for laccase production indicate that, only 10 isolates were developed a pink color within 15 seconds. These isolates were selected for secondary screening, *Bacillus* sp. 54 was appeared the highest spore-bound laccase activity (12.8) U/ml.

The selected isolate with highest level of spore-bound laccase activity was identified as *Bacillus subtilis* according to the biochemical tests.

The optimization process for laccase production proved that, the optimum conditions for *B .subtilis* laccase production were: 3 days of incubation, 37 °C, pH 7.0, 0.2 mM $CuSO_4$, 3% glucose as carbons source, 0.2% tryprone as nitrogen source, supplemented with 1 mM of KCl and pyrogallol as inducer. laccase activity was increased up to (439.23) U/ml.

The optimum pH and temperature of laccase activity were (6.8) and (40 °C) respectively, and the spore-bound laccase was retained its initial activity after 4 days of incubation at 10 °C.

Dyes decolorization by *B. subtilis* was studied in solid medium containing dyes with concentration of 150 ppm after 3 days of incubation at 37^{o} C, the bacterial growth in solid medium caused clear halo around the growth area to the following dyes: crystal violet, eriochrome black T, azur B and methyl violet dyes.

For textile (blue) dye, a halo was formed, but it was not clear. Finally, no halo appeared around the growth area for methyl orange.

Dyes decolorization by *B. subtilis* laccase was studied with different concentrations (25, 50, 75,100 and 125 ppm). The results showed that the dyes: eriochrome black T, crystal violet and azure B were completely decolorized in all concentrations within 10 minutes. Same result was observed with textile (blue) dye but at concentrations of 25, 50 and 75 ppm, while in concentrations: 100 and 125 ppm was completely decolorized after 20 minutes. methyl violet and methyl orange dyes were decolorized in different percentages.

Anthracene and phenanthrene degradation by *B. subtilis* laccase was studied with different concentrations (25, 50, 75, 100 and 125 ppm). The results revealed that, the degradation of these hydrocarbons was occurred in different percentages, but phenanthrene degradation percentage was more than anthracene with all concentrations, and the degradation of both hydrocarbons was decreased with increasing their concentrations.

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List of Abbreviations

ABTS	**2, 2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate)**
BOD	**Biological oxygen demand**
COD	**Chemical oxygen demands**
EC	**Enzyme Commission**
EPR	**Electron paramagnetic resonance**
HMW	**High Molecular Weight**
K_{ow}	**Octanol-Water Partition Coefficient**
LMW	**Low Molecular Weight**
MCD	**Magnetic circular dichroism**
PAHs	**Polycyclic Aromatic Hydrocarbons**
PCBs	**Polychlorinated biphenyl**
ppm	**part per million**
rpm	**round per minute**
spp.	**Species**
US-EPA	**United State Environmental Protection Agency**
UV/VIS spectrophotometer	**Ultra Violet/visible spectrophotometer**

Introduction

Introduction

Hydrocarbons are organic compounds consisting entirely of hydrogen and carbon (Barroso *et al.*, 2008).

Environmental pollution with hydrocarbons and Dyes was a very serious problem whether it comes from petroleum, pesticides or other toxic organic matter. Environmental pollution caused by petroleum was of great concern because petroleum hydrocarbons are toxic to all forms of life and the pollution by crude oil was relatively common because of its widespread use and its associated disposal operations and accidental spills (Abha and Singh, 2012).

Bacterial enzymes play a major role in degradation of toxic dyes and hydrocarbons. Low molecular weight alkanes were degraded most rapidly whereas mixed cultures carry out more extensive biodegradation of petroleum through pure cultures (Levin *et al.*, 2003; Ghazali *et al.*, 2004).

Laccase (benzenediol:oxygen oxidoreductase; EC 1.10.3.2.) is a multi-copper blue oxidase capable of oxidizing ortho- and para-diphenols an aromatic amines by removing an electron and a proton from a hydroxyl group to form a free radical (Coll *et al.*, 1993). In most cases laccases are monomeric glycoproteins contain around 500 amino acids with molecular weights in the range 60–85 kDa, depending on the carbohydrate content (Lyashenko *et al.*, 2006). Laccases were widely distributed among plants, fungi (Morozova *et al*., 2007), insects (Hoegger *et al.*, 2006) and bacteria (Claus, 2003). More than a hundred laccases have been isolated and characterized. Most of them were derived from fungi, including especially white rot basidiomycetes, plant and bacteria (Mayer and Staples, 2002; Morozova *et al*., 2007).

In contrast to fungal laccases, only a few bacterial laccases have been so far studied, although recent rapid progress in whole genome analysis suggests that these enzymes were widespread in bacteria (Alexandre and Zhulin, 2000; Claus, 2003; Sharma *et al*., 2007). Bacterial laccases have advantageous properties compared to fungal laccases with respect to industrial applications; they were highly active and much more stable at high temperature and high pH value than fungal laccases (Sharma *et al*., 2007).

The aim of the present study is an attempt to biodegradation of some hydrocarbons and toxic dyes by *Bacillus* spp. laccase. To achieve this aim, the following steps were carried out:

1- Isolation and identification of *Bacillus* spp. isolates from different soil samples.
2- Primary and secondary screening of laccase producing *Bacillus* spp. using solid and liquid medium respectively.
3- Determining the optimal conditions for laccase production from selected isolate.
4- Degradation of some hydrocarbons and toxic dyes by spore-bound laccase.

Chapter one
Literature review

1. Literature review

1.1 Sources of laccase

The first laccase was reported in 1883 from *Rhus vernicifera*, the Japanese lacquer tree, from which the designation of laccase was derived and subsequently, laccases were discovered largely in plants and fungi (Mayer and Staples, 2002). However, bacterial laccase was first reported in 1993 (Givaudan *et al*., 1993). Since that time, more and more studies have shown that laccases were widespread among bacteria, based on homology searches in protein databases and bacterial genomes (Alexandre and Zhulin, 2000).

Actual laccase-like activity has been found in *Bacillus sphaericus* (Claus and Filip, 1997), *Azospirillum lipoferum* (Diamantidis *et al*., 2000), *Escherichia coli* (Grass and Rensing, 2001; Kim *et al*., 2001), *Marinomonas mediterranea* (Sanchez-Amat *et al*., 2001), *Bacillus subtilis* (Hullo *et al*., 2001) and *Streptomycetes* (Endo *et al*., 2002; Suzuki *et al*., 2003).

Bacterial laccases contain four copper-binding domains, but show little overall sequence similarities with fungal laccases. In addition, the kinetics and reaction conditions may differ from those of classical laccases. The oxidation rate may be slow, Cu^{+2} supplementation may be required, and the enzyme may be inactivated during turnover (Solano *et al*., 2001).

1.1.1 Fungal laccase

Fungi from the deuteromycetes, ascomycetes (Aisemberg *et al*., 1989) as well as basidiomycetes are good producers of laccase (Sadhasivam *et al*., 2008). White-rot fungi produces efficient amount of laccase among basidiomycetes

(Revankar and Lele, 2006). *Trametes versicolor*, *Chaetomium thermophilum* and *Pleurotus eryngii* were well known producers of laccase (Kiiskinen. *et al*., 2004).

1.1.2 Plant's laccase

The plants in which the laccase enzyme has been detected includes lacquer, mango, mung bean, peach, pine (Arora and Sharma, 2010). Laccase has been expressed in the embryo of maize seeds (Bailey *et al*., 2004). Laccases were produced by both plants and fungi but when they are produced by plant, it takes part in radical based polymerization of lignin whereas fungal laccase contributes to lignin biodegradation (Ranocha *et al*., 2002).

1.1.3 Insect's laccase

Different insects (Bombyx, Calliphora and Diploptera) have also been reported for the production of laccase (Arora and Sharma, 2010).

1.1.4 Bacterial laccase

First laccase was found in plant root associated bacterium *Azospirillum lipoferm* (Givaudan *et al*., 1993). Nowadays laccase was found in many bacteria including *B. subtilis*, *Bordetella compestris*, *E. coli* and *Pseudomonas syringae* (Alexandre and Zhulin, 2000).

The *Bacillus* genus weas known to be one of the most important sources of enzymes and other biomolecules of industrial interest, being responsible for the supply of about 50% of the market for enzymes (Schallmey *et al*., 2004).

CotA, which is the endospore coat component of *B. subtilis*, was the most-studied bacterial laccase (Hullo *et al*., 2001). Since spores allow microorganisms to

survive under drastic conditions, spore coat enzymes might also withstand high temperatures or extreme pH values. Since most fungal laccases were unstable at pH values higher than 7.0, their detoxification efficiencies for pollutants often decrease under alkaline conditions. This limits the industrial potential of fungal laccase as many processes were performed in alkaline conditions. Alternatively, spore laccases which are active in the alkaline pH range could be used for bioremediation or application in membrane reactors (Held *et al*., 2005).

1.1.4.1 The Genus *Bacillus*

Members of the genus *Bacillus* are rod-shaped spore-forming bacteria belonging to the Firmicutes, the low G+C gram-positive bacteria. The genus is large, including more than 60 species with a great genetic diversity, most of which were non-pathogenic. Bacillus species divided into six groups (groups I–VI), based on 16S rRNA phylogeny or phenotypic features. The *Bacillus* genus encompass a range of species of human interest. This is mostly due to the use of the bacteria in industrial applications, such as for example in the making of biotechnological products (insect toxins, peptide antibiotics, enzymes for detergents, etc.) and the employment of the spore as a model system for studying bacterial cellular differentiation, and its resistance to decontaminating agents or treatments (Priest, 1993). *B. subtilis* was defined as the type species (Soule, 1932).

1.2 History and Distribution of Laccases

Laccase (EC 1.10.3.2) is an extra cellular, multicopper enzyme that uses molecular oxygen to oxidize various aromatic and nonaromatic compounds by a radical-catalyzed reaction mechanism. Laccases are |N-glycosylated multi copper oxidases belonging to the group of the blue copper proteins (Thurston, 1994). Laccases were widely distributed in fungi, higher plants, bcteria (Messerschmidt and Huber, 1990) and in a lower proportion in insects and bacteria. Laccase was first described by Yoshida in 1883 when he extracted it from the exudates of the Japanese lacquer tree *Rhus vernicifera* , from which the name laccase was derived (Thurston, 1994). Even though the detection and purification of laccases from plants were restricted due to the large amount of oxidative enzymes contained in the crude plant extracts, laccase from *R. vernicifera* was studied extensively, especially regarding its spectroscopic properties (Woolery *et al*., 1984).

In 1896, Bertrand and Laborde demonstrated the presence of laccases in fungi (Revankar and Lele, 2006). Since then, the presence of laccase was shown in ascomycetes, deuteromycetes and basidiomycetes. Moreover, laccases have been particularly found in many white-rot fungi involved in the lignin metabolism (Thurston, 1994). Laccases are thought to be nearly ubiquitous among fungi; actually the presence of laccases has been documented in virtually every fungus examined for it. Laccases are produced in multiple isoforms depending on the fungal species and environmental conditions. Although the structure of the active site seems to be conserved in all the fungal laccases, there is great diversity in the rest of the protein structure and in the sugar moiety of the enzyme (Mayer and Staples, 2002).

The first bacterial laccase was detected in the plant root-associated bacterium *Azospirillum lipoferum* (Givaudan *et al*., 1993), where it was shown to

be involved in melanin formation (Faure *et al*., 1994). An atypical laccase containing six putative copper-binding sites was discovered from *Marinomonas mediterranea*, but no functional role has been assigned to this enzyme (Sanchez-Amat *et al*., 2001). *Bacillus subtilis* produces a thermostable CotA laccase which participates in pigment production in the endospore coat (Martins *et al*., 2002). Laccases have recently also been found in *Streptomyces cyaneus* (Arias *et al*., 2003) and *Streptomyces lavendulae* (Suzuki *et al*., 2003).

1.3 Laccase properties

In most cases laccases are monomeric glycoproteins contain around 500 amino acids. These enzymes contain 15– 30% carbohydrate and have a molecular mass of 60–90 kDa. These are copper containing 1,4-benzenediol: oxygen oxidoreductases (EC 1.10.3.2) found in higher plants and microorganisms. These are glycosylated polyphenol oxidases that contain four copper ions per molecule that carry out 1 electron oxidation of phenolic and its related compound and reduce oxygen to water (Couto and Herrera, 2006). When substrate is oxidized by a laccase, it loses a single electron and usually forms a free radical which may undergo further oxidation or nonenzymatic reactions including hydration, disproportionation, and polymerization (Faccelo and Cruz, 2008). These enzymes are polymeric and generally contain 1 each of type 1, type 2, and type 3 copper centre/subunit where the type 2 and type 3 are close together forming a trinuclear copper cluster (Gianfreda and Bollag, 1999).

1.4 Substrate specificity

Laccases are remarkably non-specific as to their reducing substrates, and the range of substrates oxidised varies from one laccase to another. These enzymes catalyse the oxidation of a wide variety of organic and inorganic substrates, including polyphenols, methoxy-substituted phenols, aromatic amines and

ascorbate with the concomitant four-electron reduction of oxygen to water (Thurston, 1994).

Laccase is a blue copper protein, but also falls within the broader description of polyphenol oxidases. Polyphenol oxidases are copper proteins with the common feature that they are able to oxidise aromatic compounds with molecular oxygen as the terminal electron acceptor. Polyphenol oxidases are associated with three types of activities:

Catechol oxidase or *o*-diphenol: oxygen oxidoreductase (EC 1.10.3.1)
Laccase or *p*-diphenol: oxygen oxidoreductase (EC 1.10.3.2)
Tyrosinase or monophenol monooxygenase (EC 1.14.18.1) (Bar, 2001).
There are several compounds that have been used as substrates by spectrophotometry methods such as 2, 2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), syringaldazine, o-dianisidine and guaiacol (Li *et al*., 2008).

Syringaldazine [4-hydroxy-3, 5-dimethoxy benzaldehyde azine] is considered the substrate oxidized only by laccase enzyme. Thus, several organic compounds which contain hydroxyl, acid, or amino groups can act like substrates (Gardiol *et al*, 1998).

In most cases, the oxidation of the substrates by laccase finally leads to polymerization of the products through oxidative coupling. Products of oxidative coupling reactions result from C-O and C-C coupling of phenolic reactants and from N-N and C-N coupling of aromatic amines (Hublik and Schinner**,** 2000).

When oxidization occurs by laccase, the reducing substrate loses a single electron and usually forms a free radical. The unstable radical may undergo further laccase-catalysed oxidation or non-enzymatic reactions including hydration, disproportionation and polymerization (Jhadav *et al.*, 2009).

1.5 Laccase active site

Laccase contains four copper atoms that have been classified according to their electron paramagnetic resonance (EPR) features: Type 1 or blue, Type 2 or normal and Type 3 or coupled binuclear copper site where the coppers are antiferromagnetically coupled through a bridging ligand [EPR undetectable] (Durán *et al.*, 2002). In their oxidized states Type 1 and Type 2 copper ions each exhibit specific EPR signals. Type 1 is associated with an intense optical absorption band near to 610 nm. In addition, coppers Type 3 nonparamagnetic are supposed to be associated with a cooperative two-electron acceptor, which shows a strong optical absorption at about 340 nm in the oxidized state (Andréasson *et al*., 1976).

Spectroscopy combined with crystallography has provided a detailed description of the active site in laccase. Magnetic circular dichroism (MCD) and X-rays absorption spectroscopy of laccase have shown that the Type 2 and 3 centers combine to function as a trinuclear copper cluster with respect to exogenous ligand interaction including reaction with dioxygen (Cole *et al*., 1990). The Type 2 center is 3-coordinate with two histidine ligands and water as ligands. The Type 3 coppers are each 4-coordinate, having three histidines ligands and bridging hydroxide (fig. 1-1). The structural model of bridging between the Type 2 and 3 has provided insight into the catalytic reduction of oxygen to water (Magnus *et al*., 1993). It has been elucidated that the Type 2 copper is required for the reduction of oxygen since bridging to this center is involved in the stabilization of the peroxide intermediate. Reduction of oxygen by laccase appears to occur in two 2e− steps. The first is rate determining.

In this Type 2/3 bridging mode for the first 2e− reduced, the peroxide-level intermediate would facilitate the second 2e− reduction (from the Type 2 and 1

centers) in that the peroxide is directly coordinated to reduced Type 2 copper, and the reduced Type 1 is coupled to the Type 3 by the covalent Cys–His linkages (Clark and Solomon, 1992). It is clear that the Type 2 Cu is required for dioxygen reactivity in laccase and that dioxygen reduction occurs in the absence of the Type 1 Cu. This demonstrates that the Type 2/3 trinuclear Cu site represents the active site for the binding and multielectron reduction of dioxygen. The Type 1 Cu is clearly not necessary for reactivity with dioxygen (Cole *et al*., 1990).

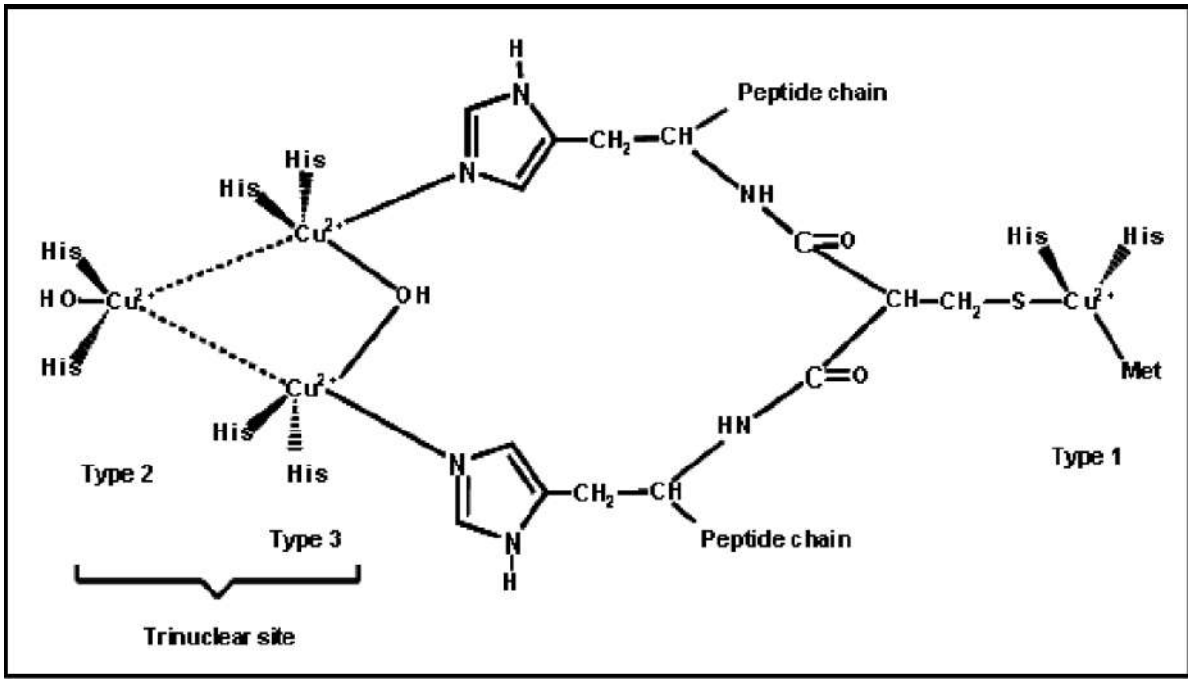

Figure (1-1): The laccase active site. The Type 1 copper coordinate with two histidine ligands and two sulphurs one of methionine and the other of cysteine. The Type 2 center is 3 coppers coordinate with two histidine ligands and water as ligands. The Type 3 coppers are each 4-coordinate, having three histidines ligands and bridging hydroxide, adapted from Durán *et al*., (2002).

1.6 Factors effecting laccase production

1.6.1 Temperature

The temperature is a critical parameter that must be controlled and varied from microorganism to other. The effect of temperature on enzyme production was not well understood (Chaloupka, 1985). However, some studies showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake (Frankena *et al*., 1986). Temperature influencing the rates of biochemical reactions which effect on enzyme production (Strnadova *et al*., 1991).

1.6.2 Hydrogen ion concentration (pH)

Many enzymatic process and transport of the compounds across the cell membrane were effected by pH of the medium (Shanmugam *et al*., 2008). A decrease or increase in hydrogen ions (H^+) concentration causes pH changes in the culture medium which may lead to drastic changes in the three-dimensional structure of proteins because H^+ and/or OH^- compete with hydrogen bonds and ionic bonds in an enzyme, resulting in enzymes denaturation (Tortora *et al*., 2004). Kaushik and Thakur, (2014) observed that highly acidic and alkaline conditions were not suitable for the *Bacillus* spp. to produce the laccase enzyme, whereas neutral pH was the best condition for laccase production.

1.6.3 Carbon and Nitrogen sources

The organism grown in the defined medium contains 0.1%w/v yeast extract and 1% (w/v) different carbon sources as well as nitrogen sources. Glucose, mannose, maltose, fructose, and lactose were the commonly used carbon sources. The excess glucose and sucrose reduce the production of laccase by obstructing the initiation. This problem of production of enzyme can be improved by using polymeric substrates like cellulose (Lee *et al*., 2004). Yeast extract, peptone, urea,

$(NH_4)_2SO_4$, and $NaNO_3$ were the commonly used nitrogen sources. Laccase production was triggered by nitrogen depletion but some nitrogen sources do not affect the enzyme activity (Leatham and Kent Kirk, 1983). Some studies show that the elevated laccase activity was achieved by using low carbon-to-nitrogen ratio (Monteiro and De Carvalho, 1998) while others show that it was achieved at high carbon-to-nitrogen ratio (Buswell *et al*., 1995).

1.6.4 Inducer

The use of an appropriate inducer can greatly enhance laccase production, and can be a prerequisite for effective production on a large/industrial scale. Inducers are usually natural substrates or substrate analogues for the enzyme.

Aromatic compounds have been widely used to elicit enhanced laccase production by different organisms and the nature of the compound that induces laccase activity differs greatly with the species. The most effective and commonly used inducer of laccase in fungi was 2, 5-xylidine and copper sulphate (CuSO4). Inducers reported to be used for laccase production, were copper sulphate (Revankar and Lele, 2006), guaiacol, ferulic acid and veratryl alcohol, ethanol, 4-hydroxy-benzoic acid, 2, 5-xylidine (Arora and Gill, 2001). Copper atoms serve as cofactors in the catalytic core of laccase; thus, a minimum concentration (millimolar range) of copper ions was necessary for the production of the active enzyme.

Copper ions were also an inducer of laccase in several fungi. In *Tinea versicolor*, copper regulates laccase at the level of gene transcription. Excess copper may have a toxic effect on fungal biomass, and thus decrease laccase production. The addition of copper during the exponential phase of fungal growth gives optimal laccase activity, while minimizing the inhibitory effect of copper on fungal growth (Revankar and Lele, 2006).

1.7 Laccase activity assay

Usually, there are several compounds that have been used as substrates by spectrophotometry methods such as 2, 2'-azinobis-(3 ethylbenzthiazoline-6-sulfonate) (ABTS). The assay mixture contained 2mM ABTS and 0.1M sodium acetate buffer (pH 4.5). Oxidation of ABTS was followed by absorbance increase at 420 nm (Couto and Herrera, 2006).

Minussi1*et al.*, (2007) used (syringaldazine), enzymatic activity was assayed by measuring oxidation of syringaldazine at 525 nm with assay mixture contained 0.1 ml of 1.0 mM syringaldazine, 0.3 ml of 50 mM citrate-phosphate buffer (pH 5.0) and 0.6 ml of culture filtrate.

Unal and Kolankaya, (2001) used guaiacol for measuring laccase activity. Crude enzyme was added to 50 mM Na-acetate buffer (pH 4.5) containing 1mM guaiacol as substrate, to make a final volume of 5 ml. The tubes were incubated at 37^{o} C for 15 minutes. The blank contained substrate and the source enzyme that was inactivated by boiling. The optical density of the reaction tubes was measured against reagent blank in spectrophotometer at 465 nm wavelengths.

1.8 Factors effecting laccase activity and stability

1.8.1 Hydrogen ion concentration (pH)

Enzymes are said to be ampholytes that are both their acidic and alkaline groups dissociate. In most cases the change in enzymatic activity at different pH levels was caused by changes in the ionization of the enzyme, substrate or the enzyme – substrate complex (Cato, 2005).

Effect of pH on an enzyme reaction is crucial for most enzyme activities, since substrate binding and catalysis are dependent on the charge distribution of substrate and enzyme molecules. Some enzymes were active over a broad range of pH values, although most are active over a narrow range. The pH optimum varies for different enzymes. In addition, enzyme stability was affected by pH and

typically the stability range tends to be much greater than the activity range. Both pH impacts have to be considered during processing (Tucker, 1995).

In general, enzyme activity has a bell shaped profile with an optimal pH that varies considerably. This variation may be due to changes to the reaction caused by the substrate, oxygen or the enzyme itself (Xu, 1997).

The optimum pH for laccase activity was around 4.6, when measured with phenolic substrates. The decrease in laccase activity in neutral or alkaline pH values was affected by increasing hydroxide anion inhibition, because as a small anion, hydroxide ion was also a laccase inhibitor, on the other hand, the increasing in pH decreases the redox potential of the phenolic substrate, which makes the substrate more susceptible to oxidation by laccase (Kiiskinen, 2004).

Usually, bacterial laccases exhibit pH optima in the range of 5- 6, when the substrate was a hydrogen atom donor compound (i.e., ABTS). When the substrate is a phenolic compound (e.g., syringaldazine), on the other hand, the optimal pH was shifted to 6-7. This shift in pH was a result of the balance of redox potentials between the substrate and the inhibition of the T2 /T3 copper site by the binding of an OH- ion.

1.8.2 Temperature

The structure of a native, catalytically active enzyme, and thus of a protein, is stabilized by a sensitive balance of disulfide bonds and various non-covalent forces such as hydrogen bonds and hydrophobic, electrostatic and van der Waals interactions. Upon heating, all these forces were weakened particularly due to molecular fluctuations and the protein molecule denatures or unfolds. The aminoacid residues that form the active center of an enzyme are brought together only in the native structure of the molecule and unfolding results in disassembling of this domain and thus, in enzyme inactivation (Ing, 2006).Temperature stabilities

of laccases vary considerably, depending on the source of organisms (Kiiskinen, 2004).

1.9 Laccase applications

1.9.1 Laccase in Degradation of Polyaromatic Hydrocarbon

Polycyclic aromatic hydrocarbons (PAHs) are organic compounds comprising benzene rings arranged linearly, angularly, or in clusters. These are ubiquitous environmental contaminants found in air, soil, and aquatic environments (Han *et al*., 2004).

Most PAHs are toxic to living organisms, and some of them and their metabolites are mutagenic and carcinogenic to humans. PAHs are resistant to biodegradation and therefore tend to accumulate to substantial levels in the environment and have been detected in a wide range of soils and sediments. There have been many studies on the biodegradation of various aromatic hydrocarbons, but their low water solubility and subsequent low degradation rates hamper the bioremediation of PAH-polluted environments (Levin *et al*., 2003).

Therefore, knowledge of microorganisms having a high PAHs degrading capability is essential for efficient remediation of PAHs contamination. Most biodegradation studies have been focused on degrading bacteria. However, white rot fungi that can degrade lignin and various recalcitrant polyaromatic hydrocarbon compounds have several potential advantages over other degrading microorganisms. Compared to most degrading enzymes of bacteria which have narrow substrate specificity, the ligninolytic enzymes of these fungi are very nonspecific and extracellular. Therefore, white rot fungi can degrade various insoluble organic pollutants. The lignin degrading system was induced more in response to nutrient exhaustion than by the presence of pollutants. This allows the fungi to degrade pollutants to essentially nondetectable levels. Under such conditions, white rot fungi produce certain extracellular enzymes lignin

peroxidases, manganese peroxidases, and laccases. These ligninolytic enzymes of white rot fungi oxidize PAHs to corresponding PAH quinones and subsequently degrade the material further to CO2. Laccase of *T. versicolor* oxidizes most of the 16 PAHs listed by the US-EPA as priority pollutant chemicals. Benzo[α]pyrene and perylene are partially converted to polymeric products. Small amounts of quinones and ketones are the main oxidation products from anthracene, benzo[α]pyrene, and fluorene. Laccase in combination with 1-hydroxybenzotriazole oxidizes acenaphthene and acenaphthylene to 1,2-acenaphthenedione and 1,8-naphthalic acid. Purified Lac from *T. versicolor* has been shown to oxidize a range of 3-5 ring PAH in the presence of the chemical mediators HBT and ABTS (Collins *et al*., 1996).

1.9.1.1 Properties and environmental fate of Polyaromatic Hydrocarbons (PAHs)

Generally, PAHs are lipophilic compounds that show high affinity for organic matter. However, individual PAHs differ substantially in their physico-chemical properties. As shown in table (1-1), properties such as aqueous solubility and vapor pressure range in five and twelve orders of magnitude, respectively, moving from two to seven benzene rings in the PAH-molecule. Thus, low molecular weight (LMW) PAHs are much more water soluble and volatile than their high molecular weight (HMW) relatives, while the HMW PAHs show higher hydrophobicity than the LMW compounds (Mackay *et al*., 1992).

The difference in hydrophobicity was also reflected by the octanol-water partitioning coefficient (Kow) (Table 1-1). These physico-chemical properties largely determine the environmental behavior of PAHs, and indicate that transfer and turnover will be more rapid for LMW PAHs than for the heavier PAHs (Wild and Jones, 1995).

The semi-volatile nature of the LMW PAHs means that they exist in the atmosphere partly as vapors and are therefore highly susceptible to atmospheric degradation processes. Similarly, in aqueous environments, the LMW PAHs were partly dissolved, making them highly available for various degradation processes. The HMW PAHs, on the other hand, were primarily associated with particles in the atmosphere and water, and were therefore less available for degradation. Furthermore, PAHs adsorbed to particles may be transported over long distances in the atmosphere and are therefore ubiquitous in the environment (Wilson and jones, 1993).

Table (1-1): Properties of the 16 US-EPA PAHs (Mackay *et al*., 1992).

Hydrocarbons	**Number of rings**	**Molecular weight**	**Aqueous solubility mg/l**	**Vapor Press. (Pa)**	**Log K_{ow}**
Naphthalene	2	128	31	1.0x102	3.37
Acenaphthylene	3	152	16	9.0x10-1	4.00
Acenaphthene	3	154	3.8	3.0x10-1	3.92
Fluorene	3	166	1.9	9.0x10-2	4.18
Phenanthrene	3	178	1.1	2.0x10-2	4.57
Anthracene	3	178	0.045	1.0x10-3	4.54
Pyrene	4	202	0.13	6.0x10-4	5.18
Fluoranthene	4	202	0.26	1.2x10-3	5.22
Benzo[*a*] anthracene	4	228	0.011	2.8x10-5	5.91
Chrysene	4	228	0.006	5.7x10-7	5.91
Benzo[*b*]fluoranthene	5	252	0.0015	-	5.80
Benzo[*k*]fluoranthene	5	252	0.0008	5.2x10-8	6.00
Benzo[*a*]pyrene	5	252	0.0038	7.0x10-7	5.91
Dibenzo[*a,h*]anthracene	6	278	0.0006	3.7x10-10	6.75
Indeno[1,2,3-*cd*]pyrene	6	276	0.00019	-	6.50
Benzo[*ghi*]perylene	6	276	0.00026	1.4x10-8	6.50

1.9.1.2 PAHs in soil

In soil, most PAHs are strongly sorbed to the organic matter, making them relatively unavailable for degradation processes (Wild and Jones, 1995). HMW PAHs can therefore remain in the soil for many centuries, posing a long-term threat to the environment (Howsam and Jones, 1998), although LMW PAHs are partly lost through degradation processes, volatilization and leaching (Wild and Jones, 1995).

The effect of sorption generally increases as the number of benzene rings in the PAH-molecule increases (Bossert and Bartha, 1986), since this implies higher lipophilicity. Furthermore, it has been shown that the degradability and extractability of organic compounds in soil decreases with the time they have been in contact with the soil: a phenomenon referred to as 'aging' or 'weathering' (Hatzinger and Alexander, 1995).

Aging is mainly a result of slow diffusion into the soil organic matter, but other mechanisms involved include the formation of bound residues and physical entrapment within soil micro pores. The processes of sorption and aging limit, on one hand, the degradability of the contaminants. On the other hand, these processes reduce the toxicity of the soil contaminants, by lowering the fraction available for uptake by living organisms (Alexander, 1995).

1.9.1.3 PAHs toxicity

A wide range of PAH-induced ecotoxicological effects in a diverse suite of biota, including microorganisms, terrestrial plants, aquatic biota, amphibians, reptiles, birds and terrestrial mammals have been reported (Delistraty, 1997).

Effects have been documented on survival, growth, metabolism and tumor formation, *i.e.* acute toxicity, developmental and reproductive toxicity, cytotoxicity, genotoxicity and carcinogenicity. However, the primary focus of the

toxicological research on PAHs has been on genotoxicity and carcinogenicity. In these studies, several PAHs have been shown to damage DNA and cause mutations, which in some cases may result in cancer. However, for the unsubstituted PAHs it is not the original compound that reacts with DNA. The PAHs require metabolic activation and conversion to display their genotoxic and carcinogenic properties (Pickering**,** 1999). This happens as the PAHs are metabolized in higher organisms. PAHs do not accumulate in the same manner as some other lipophilic organic compounds, *e.g.* PCBs. Instead, they are converted to more water-soluble forms, which facilitates their subsequent excretion from the organism. Unfortunately, this may also lead to the formation of reactive intermediates that may react with DNA to form adducts, preventing the gene involved from functioning normally. The DNA-damage may be repaired, but if the repair fails a mutation, *i.e*. an irreparable genetic damage, will have occurred. Mutations may affect many different functions of a cell, but above all they may induce cancer (IARC, 1983).

1.9.1.4 Biological degradation of PAHs

Biological degradation appears to be the main process responsible for the removal of PAHs in soil (Wilson and jones, 1993).

Microorganisms, such as bacteria and fungi, may transform the PAHs to other organic compounds or to inorganic end products such as carbon dioxide and water. The latter process has been referred to as mineralization.

Some PAH-degrading microorganisms, primarily bacteria, were capable to use the PAHs as a carbon and energy source, and may thus transform the contaminants into molecules that can enter the organisms central metabolic pathways (Cerniglia, 1984).

Other microorganisms have the ability to degrade PAHs, while living on a widely available substrate. Such cometabolism does not always result in growth of

the microorganism, and sometime the cosubstrate, *i.e.* the PAH, was only transformed into another compound without any apparent benefit for the organism. This may lead to partial degradation, if no enzyme capable of transforming the metabolite was available (Gibson, 1993).

For PAHs, the contribution of the cometabolic degradation processes increases as the number of rings in the PAH-molecule increases, since far fewer microorganisms were capable of using the HMW PAHs as carbon and energy sources (Cerniglia, 1992).

PAH-degrading bacteria generally use the PAHs as a carbon and energy source, while fungi metabolize the PAHs to more water-soluble compounds, thereby facilitating their subsequent excretion. Bacteria and fungi therefore have different metabolic pathways (Fig. 1-2).

The general fungal pathway was quite similar to the transformation pathways found in humans and other mammals. Fungi oxidize PAHs via the cytochrome P-450 enzyme system to form phenols and *trans*-dihydrodiols, which can be conjugated and excreted from the organism. The bacterial degradation of PAHs generally begins with a dioxygenase attack on one of the aromatic rings to form a *cis*-dihydrodiol, which is subsequently dehydrated to catechol. Catechol was a key intermediate from which ring cleavage can occur. The aromatic ring was cleaved between the hydroxyl groups (*ortho* fission) or adjacent to one of the hydroxyl groups (*meta* fission). Successive ring degradation may then occur, so that the structure was ultimately degraded to molecules that can enter the central metabolic pathways of the bacteria (Cerniglia, 1992).

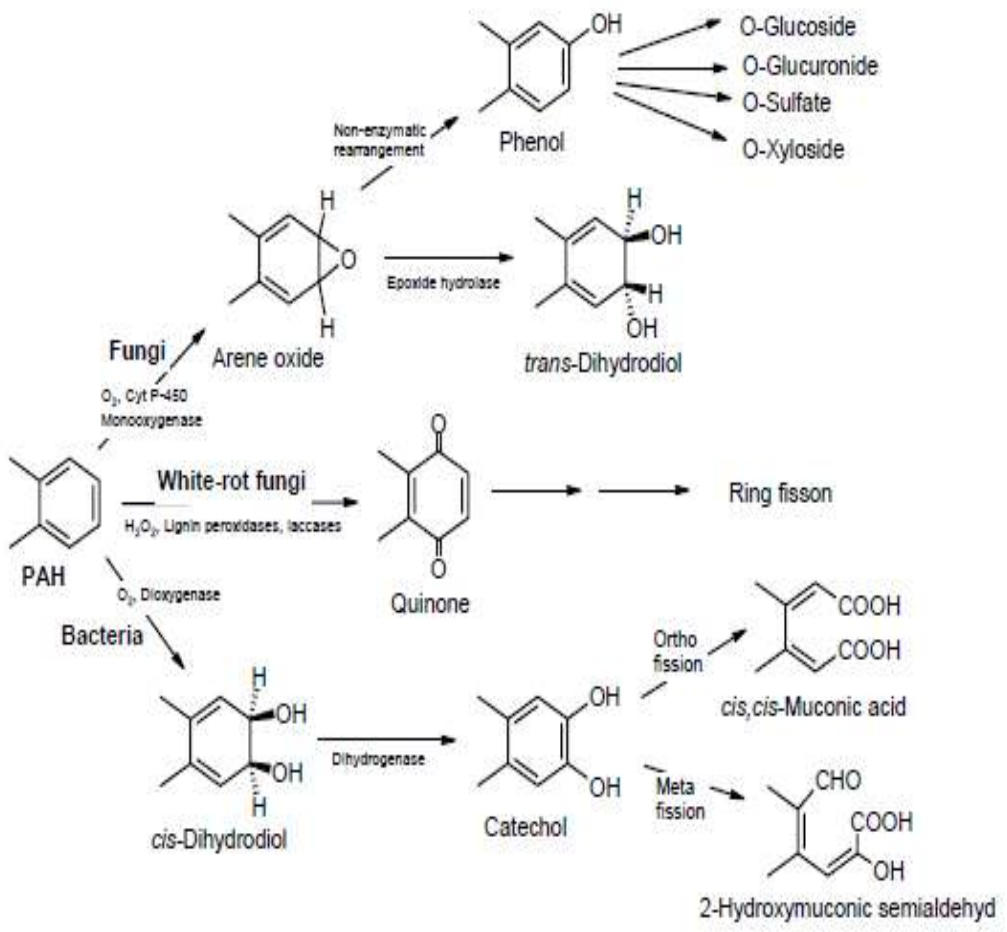

Figure (1-2): General pathways to degrade PAHs by bacteria and fungi, based on Cerniglia, (1992)

1.9.1.5 Chemical degradation of PAHs

The PAHs in soil were also degraded through abiotic processes. Oxidation reactions were the most important in this context, although photochemical reactions may contribute significantly to the degradation on the surface of soils (Kochany and Maguire, 1994). In addition, most of the oxidants that commonly initiate the oxidation reactions in the environment, *i.e.* singlet oxygen (O_2), organic peroxides, hydrogen peroxide, ozone and radicals such as alkoxy radicals (RO•),

peroxy radicals (RO2•) and hydroxyl radicals (HO•), were directly or indirectly generated through photochemical processes (Berg Van Den *et al.*, 1995*)*.

However, some can also be produced from inorganic salts and oxides, especially those of iron and manganese (Kochany and Maguire, 1994). Chemical oxidation reactions involving hydroxyl radicals, generated from hydrogen peroxide, and ozone, have been most widely studied. Hydroxyl radicals were strong, relatively unspecific oxidants that react with aromatic compounds, such as PAHs, at near diffusion-controlled rates (*i.e.,* KOH>10^9 M^{-1} S^{-1}) by abstracting hydrogen atoms or by addition to double bonds (Haag and Yao, 1992).

The ozone molecule may attack double bonds directly, but it can also form reactive hydroxyl radicals by decomposing water. The reaction pathways that follow are very complex, and numerous intermediates are formed. The final reaction products include, for both oxidants, a mixture of ketones, quinones, aldehydes, phenols and carboxylic acids. Photochemical degradation of PAHs often involves the same oxidative species that are produced during the pure chemical oxidation of PAHs, *i.e.* oxygen, hydroxyl radicals and other radicals. Consequently, the reaction products include similar complex mixtures of ketones, quinones, aldehydes, phenols and carboxylic acids (Kochany and Maguire, 1994).

1.9.2 Laccase in Decolorization of Dyes

Approximately 10,000 different dyes and pigments are produced annually worldwide and used extensively in the dyeing and printing industries. The total world colorant production is estimated to be 800,000 tons per year, and at least 10 % of the used dyestuff enters the environment through wastes (Palmieri *et al*., 2005).

Most of the dyes are very stable to light, temperature, and microbial attack, making them recalcitrant. These industrial effluents are toxic and are characterized by high chemical oxygen demands (COD)/ biological oxygen demand (BOD), suspended solids, and intense colour. Physical and chemical methods such as adsorption, coagulation-flocculation, oxidation, filtration, and electrochemical methods may be used for colour removal from wastewater. These methods are quite expensive and have operational problems. Hence there is a need to develop a practical biological method of dye waste treatment that can be used for a wide range of wastes (Kapdan *et al*., 2000).

Bacterial anerobic reduction of azo dyes (which comprise a large percentage of synthetic dyes) generates colorless, deadend aromatic amines, which are generally more toxic than the parent compounds. Bacterial aerobic dye degradation has been confined to chemostat-enriched cultures adapted to a single dye (Swamy and Ramsay, 1999).

Since effluent contains a range of dyes, successful decolorization of single dye does not indicate the suitability of an organism for a decolorization system. A biodecolorization system should sustain high activity upon repeated exposure to various dyes. Ligninolytic fungi have been shown to possess a remarkable potential for degrading various types of dyes. White rot fungi were the most efficient ligninolytic organisms capable of degrading various types of dyes such as azo, heterocyclic, reactive, and polymeric dyes (Novotny *et al*., 2000).

The decolorization of dyes by white rot fungi was first reported by Glenn and Gold (1983), who developed a method to measure the lignin lytic activity of *Phanerochaete chrysosporium* based upon the decolorization of sulphonated polymeric dyes. White rot fungi offers significant advantages for decomposition of

recalcitrant compounds. Ligninolytic enzymes produced by white rot fungi were substrate-nonspecific, and therefore they can degrade wide variety of recalcitrant compounds. Because the enzymes were extracellular, the substrate diffusion limitation into the cell, generally observed in bacteria, is not encountered. These organisms do not require preconditioning to particular pollutants, because enzyme secretion depends on nutrient limitation, nitrogen, or carbon rather than presence of pollutant. In addition, extracellular enzymes enable white rot fungi to tolerate high concentration of pollutants (Kapdan *et al*., 2000). This paved the way for a wealth of studies on the decolorization of dyes under conditions in which white rot fungi produce lignin-modifying enzymes. The capability of white rot fungi to degrade various dyes is due to extracellular nonspecific and non-stereoselective enzyme systems composed of laccases (EC 1.10.3.2), lignin peroxidases (EC 1.11.10.14), and manganese peroxidases (EC 1.11.1.13) (Heinzkill *et al*., 1998).

Laccases catalyze the oxidation of both phenolic and nonphenolic compounds (Bourbonnais *et al.,* 1995) and thus can mineralize a wide range of synthetic dyes (Swamy and Ramsay, 1999). This nonspecific mechanism of laccase makes it a versatile bio catalyst suitable for several applications such as biopulping, biobleaching, and industrial wastewater treatment. Due to the stringent environmental legislation, the textile industry is seeking to develop effective wastewater remediation technologies. The development of processes based on laccase enzyme seems an attractive solution due to the potential of these enzymes in degrading dyes of diverse chemical structure. Therefore considering the importance of laccases in removal of dyes from industrial effluents more studies to find the optimal production conditions are currently required.

Chapter two
Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Apparatus and Equipments

The following equipments and apparatus were used throughout this study table (2.1)

Table (2-1): Apparatus and Equipments used in this study.

Apparatus and equipments	Company	Origin
Autoclave	Hirayama	Japan
Balance	Sartorius	Germany
Centrifuge	CL008	Belgium
Distillator	K&K SCIENTIFIC SUPPLIER	Korea
Hood	Thermo Scientific	Korea
Incubator	UniEquip	Italy
Loop	K.D.SURGICAL	India
Magnetic stirrer with hot plate	Memmert	Germany
Micropipette	JOANLAB	China
pH-meter	Crison	USA
Refrigerator	DENKA	Korea
Shaking Water bath	Thermo Scientific	Korea
UV – Visible spectrophotometer	Biotech Engineering Management Co. Ltd	United Kingdom

2.1.2 Chemical and biological materials:

The Chemicals and Biological materials which were used in this study listed in table (2-2):

Table (2-2): Chemical and Biological materials used in this study

Chemical and biological materials	Company	Origin
Potassium phosphate KH_2PO_4, Potassium phosphate monobasic K_2HPO_4, Zinc sulphate$ZnSO_4$, Potassium chloride Kcl, Ferric sulfate $Feso_4$, Sodium Hydroxide (NaOH), Citric acid, Magnesium sulphate, Ammonium dihydrogen phosphate, pyrogallol, Anthracene, Hydrogen peroxide H_2O_2, Phenol red	BDH	England
Tetramethylparaphenylnene- diamine- dihydrochloride, Guaiacol ,Catechol, Copper sulfate $CuSO_4$, Hydrochloric acid HCl, Dibasic sodium phosphate $Na_2HPO_4.12H_2O$, Sodium chloride NaCl, Sodium citrate, Calcium chloride $CaCl_2$, Manganese (II) chloride $Mncl_2$,	CDH	England
Balachite green, Eriochrome black T, Azur B, Crystal violet, Methyl violet, Methyl orange, Phenanthrene, Safranine, , Fructose, Glucose, Sucrose, Maltose, Lactose, Galactose	Fluka	England
Peptone, Yeast extract, Starch, Agar, Meat extract, Tryptone, Urea, Gelatin, Tris base, Skim milk,	Hi-media	India
Syringaldazine,	Sigma	USA
Methanol, Ethanol	Solvochem	England

2.1.3 Buffers, solutions and reagents

2.1.3.1 Citrate phosphate buffer (0.1M): (Whitaker and Bernard, 1972).

This buffer consists of two solutions:

-Solution A:

0.1M of citric acid was prepared by dissolving 1.921 gm of citric acid in final volume of 100 ml distilled water.

-Solution B:

0.2M of dibasic sodium phosphate was prepared by dissolving 3.56 gm of $Na_2HPO_4.12H_20$ in final volume of 100 ml distilled water.

A (ml) + B (ml), diluted to a total of 200 ml using distilled water**.** This buffer was prepared to constitute different pH values as below:

A(ml)	B(ml)	pH
39.8	10.2	3
34	16	3.5
30.7	19.3	4
27.8	22.2	4.5
24.3	25.7	5
22.2	27.8	5.5
21.0	29.0	5.6

2.1.3.2 Potassium phosphate buffer (0.1 M)

This buffer consist of two solutions A and B

- Solution A:

1 M of K_2HPO_4 was prepared by dissolving 87.09 gm of K_2HPO_4 in 500 ml of distilled water

- Solution B:

1 M KH_2PO_4 was prepared by dissolving 68.045 gm of KH_2PO_4 in 500 ml of distilled water

Potassium phosphate buffer 0.1 M was prepared as shown below:

A(ml)	B(ml)	pH
8.5	91.5	5.8
13.2	86.8	6
19.2	80.8	6.2
27.8	72.2	6.4
38.1	61.9	6.6
49.7	50.3	6.8
61.5	38.5	7
71.7	28.3	7.2
80.2	19.8	7.4
86.6	13.4	7.6
90.8	9.2	7.8
94.0	6.0	8

The combined 0.1 M stock solutions were diluted to 1 liter with distilled water

2.1.3.3 Tris-HCl buffer (0.1 M)

Trsi – HCl buffer was prepared by dissolving 6.05 g of Tris base in 30 ml of distilled water. Then the pH was adjust to 9 with 1 M HCl and adjusted to final volume 50 mL with distilled water, then autoclaved at 121 for 15 min and stored at room temperature.

The combined solutions were diluted to 1 liter with sterilized distilled water

2.1.3.4 Sodium hydroxide (NaOH) solution (1 M)

It was prepared by dissolving 40 gm of sodium hydroxide NaOH in amount of distilled water and the volume was completed to 1 liter of distilled water.

2.1.3.5 Hydrochloric acid (HCl) solution (1 M)

It was prepared by the addition of 83 ml of 37% HCl to appropriate amount of distilled water to get the final volume of 1000 ml.

2.1.3.6 Sodium chloride (NaCl) solution (0.5 M)

It was prepared by dissolving 29.22 gm of NaCl in an amount of distilled water and the volume was completed to 1000 ml.

2.1.3.7 Gram stain solutions

Gram stain including Crystal Violet, Iodine, Ethanol and Safranine, stains were provided by Hi-media, India

2.1.3.8 Oxidase test reagent

This reagent was prepared instantly by dissolving 1gm of tetramethylparaphenylnene- diamine-dihydrochloride in 100 ml distilled water and stored in a dark bottle (Collee *et al.*, 1996).

2.1.3.9 Catalase reagent (3%)

It was prepared by adding 1ml of 6 % hydrogen peroxide solution to 1 ml of distilled water (Cruikshank *et al*., 1976).

2.1.3.10 Endospores stain (Schaeffer and Fulton, 1993)

Endospores stain composed of two solutions

A- Malachite green 5 g/100 ml in water.

B- Safranin 0.05 g/100 ml in 95% ethanol

2.1.4 Dyes preparation

Six dyes [eriochrome black T, azur B, crystal violet, methyl violet, methyl orange and textile (blue)] were used in this study. Stock solution (1000 ppm) was prepared by dissolving 1gm of each dye in one liter distilled water then different concentrations (25, 50, 75, 100, 125 and150 ppm) were prepared form each dye.

2.1.5 Polycyclic aromatic hydrocarbons preparation

Two compounds of PAHs (anthracene and phenanthrene) were used in this study. Stock solution (1000 ppm) was prepared by dissolving 1gm from each compound in one liter distilled water for enthracene and 10% of methanol for phenanthrene, then different concentrations (25, 50, 75, 100 and 125 ppm) were prepared from each compound.

2.1.6 Substrate: Syringaldazine (0.5 mM)

Prepared by dissolving 0.018 mg of syringaldazine in 100 ml of 95% methanol.

2.1.7 Culture media

2.1.7.1 Ready-made media

The ready-made media which were used in this study are illustrated in table (2-3).

Table (2-3): List of the ready-made media

Culture media	Company	Origin
Mannitol fermentation medium	Himedia	India
Nutrient agar		
Nutrient broth		
Nutrient Gelatin		
Simmons Citrate Agar		
Urea broth		

These media were prepared according to the manufacturer's instructions and sterilized by autoclaving at121˚C for15 min, and cooled in water bath at 55◦C..

2.1.7.2 Prepared media

2.1.7.2.1 Starch agar medium

This medium was prepared from the following components:

Soluble Starch	10gm
Beef extract	3gm
Agar	15 gm
Distilled water	1000 ml

All components (28 gm) were suspended in amount of distilled water and completed to final volume 1000 ml, the pH was adjusted to 7.5 and sterilized by autoclaving at121˚C for15 min, and cooled in water bath at 55◦C, then poured in Petri dishes. This medium was used for starch hydrolysis test (MacFaddin, 2000).

2.1.7.2.2 Tryptone broth

This medium was prepared from the following components:

Tryptone	10gm
NaCl	5gm
Distilled water	1000 ml

The ingredients were suspended in 1000 ml distilled water, the pH was adjusted to 7.5 and dispensed in tubes and then sterilized by autoclaving at 121˚C for 15 min), and cooled in water bath at 55◦C. This medium was used for indole test (Difco, 1998).

2.1.7.2.3 Semi-Solid medium

This medium was prepared from the following components:

Peptone	5gm
Meat extract	3gm
Agar	5 gm
Distilled water	1000 ml

The ingredients were suspended in 1000 ml distilled water, the pH was adjusted to 7 and dispensed in tubes and then sterilized by autoclaving at 121˚C for 15 min, and cooled in water bath at 55◦C. This medium was used for motility test (Tittsler and Sandholzer, 1936) .

2.1.7.2.4 Skim milk agar

This medium was prepared from the following components:

Peptone	5gm
Skim milk	28 gm
Yeast extract	2.5gm
Dextrose	1gm
Agar	15 gm
Distilled water	1000 ml

The ingredients were suspended in 1000 ml distilled water, the pH was adjusted to 7 and sterilized by autoclaving at 121 ˚C for 3 min, and cooled in water bath at 55◦C, then poured in Petri dishes and kept at 4 ˚C. This medium was used for casein hydrolysis test (Wehr and Frank, 2004).

2.1.7.2.5 Carbohydrate fermentation medium

This medium was prepared from the following components:

Peptone	2gm
NaCl	5gm
K_2HPO_4	0.3gm
Phenol red	0.03gm
Glucose or Lactose or Galactose	10gm

The ingredients were suspended in 1000 ml distilled water, the pH was adjusted to 7.1 and dispensed in tubes and sterilized by autoclaving at 121˚C for 15 min, then cooled in water bath at 55◦C, and kept at 4 ˚C. This medium was used for carbohydrate fermentation test (MacFaddin, 1985).

2.2 Methods

2.2.1 Samples collection and bacterial isolation

Soil samples were collected in containers from different soils from Iraq – Baghdad and Al Diwaniyah regions in September, 2014. The following details about the samples were recorded (source, date of collection).

One gram of soil samples was added to 9ml of sterilized distilled water, then the samples placed in a water bath at 80 ℃ for 10 minutes and cooled, dilutions were carried out 6 times; this was done by using 0.1 ml of the previous solution and 9 ml of distilled water, 1ml of the each dilution was spread in nutrient

agar plates, using a sterile spreader. These plates were incubated at 30 ºC for 24 hrs, then a single colony was picked up and inoculate on nutrient agar plate. The colonies underwent gram staining, spore stain to verify their *Bacillus* nature (Koneman, *et al*., 1992).

2.2.2 Microscopic examination of *Bacillus* spp.

This technique was used to stain a slide to observe the *Bacillus* spp. based on their gram stain reaction. The slide with the smear of bacterial culture was heated by passing it over a heat source, the slide was passed very quickly through the flame and not be heated excessively. Then the slide was placed on the staining tray and flood the fixed smear with crystal violet solution for 1 minute , the crystal violet was rinsed with distilled water, then the slide was folded with iodine solution and allow to remain for one minute and the iodine solution risen off with distilled water, the slide was flooded with decolorizer for 20 seconds and the decolorizer was rinsed with distilled water. Finally, the slide was flooded with safranin for 30 seconds and the safranin was rinsed off with distilled water.The slide was dried by absorbent paper and place in an upright position. The slide was examined microscopically under the oil immersion lens (100X).

For endospore staining, the bacterial smear were dried and heat fixed on a glass slide, the slide was flooded with malachite green stain solution and steamed for 5 minutes over boiling water, the slide was kept moist by the addition of more dye, washed with tap water, then the slide was flooded with safranin for 30 seconds and washed with tap water.

After the slide was dryed, it was examined under the oil immersion lens (100X) for the presence of endospores (Beck, 2000).

2.2.3 Screening of bacterial isolates for laccase production

2.2.3.1 Primary screening

Fifty seven isolates of *Bacillus* spp. were grown in Petri plates containing nutrient agar at pH 7 supplemented with 0.4 mM Cu^{+2}. The plates were incubated at 30℃ for 24 hours, then 0.5 mM syringaldazine as prepared in section (2.1.6) was dropped on bacterial colonies to check its capability to generate laccase activity (Shukur, 2015). The time required from each isolate to develop a pink color was measured.

2.2.3.2 Secondary screening

For inoculums preparation, a sterile loop was used to transfer 4 pure colonies to a tube containing 5 ml of nutrient broth as prepared in section (2.1.7) and incubated at 30℃ for 24 hours.

A volume of 1 ml from each bacterial isolate (which required less time to display pink color from primary screening) were inoculated in 500 ml Erlenmeyer flask containing: 100 ml of production medium (nutrient broth supplemented with 0.4 mM $CuSO_4$), the pH of the medium was adjusted to 7. The flasks were incubated at 30℃ for 24 hours (Shukur, 2015).

The spores were collected from the flasks by centrifugation for 20 min at 4000 rpm and then washed with 0.5 mol/L NaCl and suspended in 0.1 M potassium phosphate buffer (pH 6.8).

Finally, 1 ml of spores suspension contained 100 mg wet cell was used for measuring the laccase activity (Wang *et al.*, 2011).

2.2.4 Laccase activity

Laccase activity of the spore suspension was determined using syringaldazine as a substrate. The oxidation of syringaldazine was detected by measuring the absorbance increase at 525 nm (ε525 = 65,000 L/(mol ·cm)) using a spectrophotometer (Biotech Engineering Management Co. Ltd/ United Kingdom). The reaction mixture (3 ml) contained 100 µl of spore suspension sample, 2.4 ml of potassium phosphate buffer (0.1 M, pH 6.5) and 500 µl of 0.5 mM syringaldazine (Wang *et al.,* 2011).

Spore sedimentation was not observed during incubation. The enzyme activity was calculated by the calculation described by Annuar *et al*., (2009) as follows:

$$\text{Laccase activity (U/L)} = \frac{\Delta \text{Abs}}{\Delta t \, \varepsilon \, l} \times \frac{\text{Total assay volume}}{\text{Enzyme sample volume}}$$

Where ΔAbs is the change in absorbance, Δt is the time of incubation (3 min), ε is the extinction coefficient of substrates (ε 525 = 65,000 (SGZ) in units of M^{-1} cm^{-1}), and l is the cuvette diameter (1cm). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol of substrate per minute.

2.2.5 Identification of *Bacillus* sp.

To identify selected *Bacillus* sp. to species level, the following biochemical tests were achieved:

2.2.5.1 Catalase test

A small amount of bacterial colony was transferred to a surface of clean dry glass slide using a loop and a drop of 3% H_2O_2 as prepared in (2.1.3.9) was placed on the slide and mixed by sterilized pipette tips. This reaction was evident by the rapid formation of bubbles (Duke and Jarvis, 1972).

2.2.5.2 Oxidase test

A small piece of filter paper was soaked in 1% Kovács oxidase reagent as prepared in (2.1.3.8) and let dry, then by a sterile loop a well-isolated colony was picked up from a fresh 24-hour culture bacterial plate and rub onto the treated filter paper. A positive reaction is indicated by an intense deep-purple color, appearing within 5-10 seconds and a negative reaction by the absence of coloration (Kovács, 1956).

2.2.5.3 Starch hydrolysis

After the bacterial isolate was inoculated on starch agar plate as prepared in (2.1.7.2.1), the plates were incubated for 24 hours at 37°C and the culture medium flooded with iodine. The color of the starch in the plate change to blue-brown by the iodine reagent. Areas where starch has been digested by bacterial growth exhibit clear halos in the midst of the dark plate, indicating a positive alpha-amylase, or starch hydrolysis test (Bird and Hopkins, 1954).

2.2.5.4 Gelatin liquefaction test

The bacterial isolate inoculated on nutrient gelatin tube with heavy inoculums and incubated for 24 hours at 37°C. The tubes were immersed in an ice bath for 15 minutes. Afterwards, tubes were tilted to observe if gelatin had been hydrolyzed. Hydrolyzed gelatin will result in a liquid medium even after exposure to cold temperature (ice bath), while the uninoculated control medium will remain solid (Clarke and Cowan, 1952).

2.2.5.5 Indole test

The bacterial isolate was inoculated in a tube of tryptone broth as prepared in (2.1.7.2.2) and incubated at 37°C for 48 hours, then 5 drops of Kovács reagent

was added directly to the tube. A positive indole test was indicated by the formation of a pink to red color in the reagent layer on top of the medium within seconds of adding the reagent (Baron and Finegold 1990).

2.2.5.6 Growth in aerobic or anaerobic conditions

The bacterial isolate was inoculated deeply on nutrient agar tube and incubated for 24 hours at 37 °C. In an obligate aerobic conditions, only surface growth above the agar was observed. In a strict anaerobic conditions, growth only at the bottom of the agar was observed and no surface growth. In a facultative anaerobic conditions, both surface growth and growth at the bottom of the agar was observed.

2.2.5.7 Urea hydrolysis

A heavy inoculum from 24-hour pure culture was inoculated in the urea broth tube, then the tube was gently shaken to suspend the bacterial isolate and incubated for 24 hours at 37^{o}C. The medium changed to magenta or hot pink in the presence of alkali/bases indicating a positive test (Clarke and Cowan, 1952).

2.2.5.8 Motility test

The bacterial isolate was inoculated on semi-solid medium as prepared in (2.1.7.2.3) using a sterile needle, then incubated for 24 hours at 37°C. A positive motility test is indicated by a diffuse cloud of growth away from the line of inoculation (Tittsler and Sandholzer, 1936).

2.2.5.9 Casein hydrolysis test

The bacterial isolate was inoculated on the skim milk agar plate as prepared in (2.1.7.2.4) and incubated for 48 hours at 37°C. A positive result was indicated by clear zone around the growth area (Clarke and Cowan, 1952).

2.2.5.10 Growth at 50 °C

Bacterial isolates were streaked on Petri plates containing nutrient agar and incubated at 50 °C for 24 hours. A positive result was indicated by bacterial growth.

2.2.5.11 Citrate utilization test

A single bacterial isolate was streaked lightly on the surface of the slant containing Simmons Citrate Agar, then the cap was placed loosely because citrate utilization requires oxygen. Finally, the tubes were incubated at 37°C for 24 hour. Citrate utilization positive was indicatd by bromothymol blue changing from the original green color to blue (Baron and Finegold, 1990).

2.2.5.12 Carbohydrate fermentation test

An inoculum from a pure culture was transferred aseptically to a sterile tube of carbohydrates (glucose (dextrose), lactose or galactose) broth medium as prepared in (2.1.7.2.5) and Mnnitol salt agar slant. The inoculated tube was incubated at 37°C for 24 hours (MacFaddin, 1985; Finegold and Baron, 1986). The positive result is detected by the color change of a pH indicator when acid products are formed.

2.2.6 Maintenance of bacterial isolates

The maintenance of bacterial isolates were performed according to modified method by Collee and Marr, (1996) as follows:

2.2.6.1 Short-term storage

Bacterial colonies were maintained for a period of few weeks on the surface of nutrient agar medium. The plates were tightly wrapped with parafilm and stored at 4°C.

2.2.6.2 Long-term storage

A sterile tube containing 2ml of nutrient broth with 15% glycerol was inoculated with the isolates and incubated at 37°C for 24 hours. When visible bacterial growth was seen, the tube was sealed tightly with parafilm and stored at -20°C.

2.2.7 Optimum conditions for laccase production

The following cultural conditions were studied for maximum enzyme production using basal production medium as prepared in (2.2.3.2). For each experiment, 500 ml Erlenmeyer flask containing 100 ml of production medium was inoculated with a volume of 1 ml from the bacterial isolate.

2.2.7.1 Incubation periods

In order to determine the optimum incubation time for the maximum laccase production, the time course for enzyme production was followed up to 7 days. The production medium was prepared and inoculated with selected isolate and incubated at 30℃ for 7 days. The spore-bound laccase activity was assayed at every 24 hours interval as in (2.2.4). Finally, 1 ml spore suspension from each time interval contained 100 mg wet cell was used to measure its spore-bound laccase activity (Shukur, 2015).

2.2.7.2 Temperatures

Laccase production was achieved at different temperatures (20, 25, 30, 37, 40, 45 and 50)℃ (Wang *et al.*, 2010). The production medium was inoculated with selected isolate and incubated at different temperature for 3 days at pH 7 and the spore-bound laccase activity was measured.

2.2.7.3 pH values

The effect of pH on laccase production was determined within a pH range of (5.0 and 10.0) by preparing the production medium at different pHs, then the culture medium was inoculated with the selected isolate and incubated at optimum temperature for 3 days (Wang *et al.*, 2010) and the spore-bound laccase activity was measured.

2.2.7.4 Concentrations of copper sulphate

The suitable concentration of copper sulphate for the maximum laccase production was determined by using laccase production medium containing different concentrations of $CuSO_4$ (0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7) mM (Sivakumar *et al*., 2010).

The medium was inoculated with the selected isolate and incubated at optimum pH and temperature for 3 days. The medium without $CuSO_4$ was used as a control and the spore-bound laccase activity was measured.

2.2.7.5 Carbon sources

Carbon sources (sucrose, galactose, maltose, glucose and fructose) were used at the concentration of 3% (w/v) in the production medium containing 0.2 mM $CuSO_4$. After inoculating with the bacterial isolate, the medium was

incubated at optimum temperature and pH for 3 days (Sivakumar *et al*., 2010). Spore-bound Laccase activity was measured to compare the effect of presence of different carbon sources on the enzyme production.

2.2.7.6 Nitrogen sources

The laccase production medium containing optimum carbon source and optimum concentration of $CuSO_4$ was supplied with 0.2% (w/v) of different nitrogen sources (tryptone, calcium nitrate, corn, peptone and yeast extract) (Sivakumar *et al*., 2010). After inoculated with selected isolate, the medium was incubated at optimum temperature and pH for 3 days, the spore-bound laccase activity was measured to compare the effect of presence of different nitrogen sources on the production.

2.2.7.7 Metal ions

Various metals were used to find their effect on the production of laccase by *B. subtilis*, the production medium containing optimum carbon and nitrogen sources supplemented with optimum concentration of $CuSO_4$ was supplied with different metal source ($CaCl_2$, $MnCl_2$, $FeSO_4$, KCl and $ZnSO_4$) at concentration of 1 mM and inoculated with the selected isolate (Wang *et al.,* 2010). Finally the medium was incubated at optimum temperature and pH for 3 days and the spore-bound laccase activity was determined by using bacterial spores suspension obtained from each treatment.

2.2.7.8 Enzyme inducers

Various hydrocarbons inducers were used to find their effect on the production of laccase by *B. subtilis*, the production medium containing optimum concentration of carbon source, nitrogen source, metal ion and $CuSO_4$ was

supplied with different inducers (Guaiacol, Catechol, Pyrogallol, Phenanthrene and Anthracene) at concentration of 1 mM and inoculated with the selected isolate (Niladevi and Prema, 2008). Finally, the medium was incubated at optimum temperature and pH for 3 days and the spore-bound laccase activity was measured.

2.2.8 Characterization of *B. subtilis* laccase enzyme

2.2.8.1 Optimum pH for laccase activity and stability

The effect of pH on the activity of the laccase was determined at 37°C in 0.1 M citrate-phosphate buffer (pH 3.0−5.6) as prepared in (2.1.3.1), 0.1 M potassium phosphate buffer (pH 5.8 – 8) as prepared in (2.1.3.2) and 0.1 M Tris-HCl buffer (pH 9.0) as prepared in (2.1.3.3).

The laccase activity was determined by using bacterial spores suspension and the activity was measured with different buffers according to paragraph (2.2.4).

The effect of pH on laccase stability was examined by adding 100 µl of spore suspension to a test tube containing 2.4 ml of buffer with different ranges of pH (3-9). Then the tubes incubated in an incubator at 37^{o} C for 1 hr. Laccase activity was estimated and the relationship between pH and the remaining activity % of laccase was plotted (Abdulah, 2011).

2.2.8.2 Optimum temperature for laccase activity and stability

Laccase activity was determined by incubating the spores in different ranges of temperature (10-90^{o} C) with optimum pH for 1 hour. Laccase activity was estimated according to paragraph (2.2.4). The relationship between temperature and the laccase activity was plotted.

The effect of temperature on laccase stability was examined by adding 100 µl of spores suspension to a test tube and incubated in an incubator at different

temperatures (10-90 °C) with optimum pH for 3 hr. Laccase activity was estimated and the relationship between temperature and the remaining activity % of spore-bound laccase was plotted (Abdulah, 2011).

2.2.8.3 Effect of operational time in laccase stability

A volume of 100 µl from spores suspension was added to a test tubes, then incubated at 10 ° C with optimum pH using different time range (1 to 10) days. Laccase activity was estimated according to paragraph (2.2.4). The relationship between operational time and the remaining activity % of spore-bound laccase was plotted (Abdulah, 2011).

2.2.9 Dyes decolorization

2.2.9.1 Dyes decolorization by *B. subtilis* on solid media

The dyes eriochrome black T, azur B, crystal violet, methyl violet, methyl orange and textile (blue) dye were prepared at concentration of 150 ppm in nutrient agar plates, the pH was adjusted at 7. The medium was inoculated in the center of the plate with *B. subtilis* isolate and incubated at 37℃ for 3 days (Montira and Sukallaya, 2012).

2.2.9.2 Dyes decolorization by *B. subtilis* laccase

For decolorization experiments, the dyes eriochrome black T , azur B, crystal violet, methyl violet, methyl orange and textile (blue) dye were used. The dyes were prepared in 100 ml distilled water at different concentrations (25, 50, 75, 100, 125 ppm) containing 10 ml of spore suspension (100 mg /ml), dye samples without spore suspension were used as a control, the pH was adjusted to 6.8.

Dyes was shaken at 120 rpm in a water bath at 40 ℃, samples were withdrawn every 10 min and centrifuged at 4000 rpm for 20 min and analyzed by

UV/VIS spectroscopy using spectrophotometer (Biotech Engineering Management Co. Ltd/ United Kingdom) with different wavelengths were used: 410 nm for eriochrome Black T, 425 nm for azur B, 590 nm for crystal violet, 570 nm for methyl violet, 620 nm for methyl orange and 436 nm for textile (blue) dye (Wang *et. al.* 2011).

Percent of decolorization was calculated as follows (Telke *et al*., 2010).

$$\text{Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100\%$$

2.2.10 Hydrocarbons degradation by *B. subtilis* laccase

Anthracene and phenanthrene aromatic hydrocarbons were prepared at different concentrations (25, 50, 75, 100, 125) ppm in 100 ml distilled water for anthracene and in 100 ml 10 % methanol for phenanthrene containing 10 ml of spore suspension (100 mg/ml), the pH was adjusted to 6.8 and shaken at 120 rpm in a water bath at 40 ̊C. The same procedure was used for the control, but without spores suspension, this procedure was done according to modified methods described by Munusamy *et al.,* (2008).

Samples were withdrawn every 10 min, centrifuged at 4000 rpm for 20 min, and analyzed by UV/VIS spectroscopy using spectrophotometer (Biotech Engineering Management Co. Ltd / United Kingdom) using wavelengths 288 nm for anthracene and 251 nm for phenanthrene as described by Rivera-Figueroa *et al*., (2004). Percent of degradation was calculated as follows:

$$\text{Degradation (\%)} = \frac{\text{Initial absorbance} - \text{final absorbance}}{\text{Initial absorbance}} \times 100\%$$

Chapter three
Results and discussion

3. Results and discussion

3.1Cultural and microscopic examination of *Bacillus* spp.

A colony of *Bacillus* spp. was circular with ragged edges and creamy color or white. The bacteria spread out from the center, keeping the ragged circular shape of the colony.

The bacterial cells were gram positive rod shaped under microscope at 100 X objective (Fig. 3-1). Endospores staining proved that, the bacterial spores were subterminal with very little swelling of vegetative cells, endospores were stained green and vegetative cells were stained red.

The results of cultural and microscopic examination of *Bacillus* spp. were compared with the characteristics of *Bacillus* spp. as documented by Claus and Fritze, (1989).

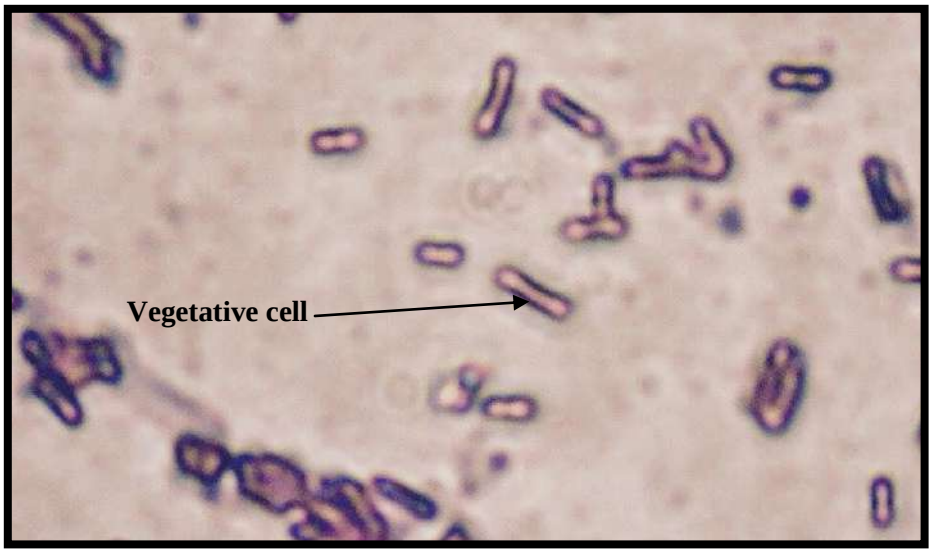

Figure (3-1): Bacterial isolate under microscope at 100X objective.

3.2 Screening of *Bacillus* spp. isolates for laccase production

3.2.1 Primary screening

Fifty seven isolates of *Bacillus spp.* were screened in nutrient agar medium supplement with 0.4 mM $CuSO_4$. It was found that, 41 isolates developed a pink color with different times, 10 isolates developed a pink color within (4-15) second after dropping 0.5 mM of syringaldazine (Fig. 3-2) and 16 isolates were negative (Table 3-1).

Shukur, (2015) observed that, developing of pink color in nutrient agar plates by *Bacillus* sp. was considered as an indicator for the laccase production.

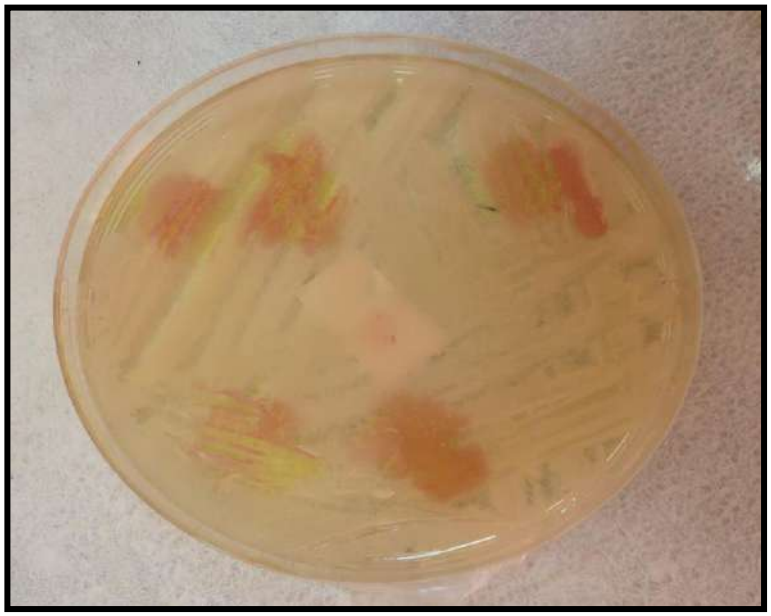

Figure (3-2): Primary screening for laccase production from *Bacillus* sp. after 24 hours of incubation at 30 ˚C.

Table (3-1): Primary screening for laccase production from *Bacillus* spp. isolates in solid media after 24 hour of incubation at 30 ˚C.

Source of isolates	Isolates number	Laccase production	time required (seconds)
Agricultural soils	1	+	134
	4	+	32
	5	+	589
	12	-	-
	13	+	33
	15	-	-
	16	+	45
	19	+	63
	21	-	-
	22	+	32
	23	+	7
	31	+	35
	33	-	-
	34	-	-
	35	-	-
	37	+	1457
	39	-	-
	42	+	29
	43	-	-
	46	-	-
	47	-	-
	2	+	36
	3	+	11
	7	+	76
	44	+	37
	45	+	31
	48	+	9

Soils contaminated with oils	49	+	40
	50	+	21
	51	+	29
	52	+	19
	53	+	22
	54	+	4
	55	+	18
	56	+	6
	57	+	20
Soils contaminated with sewage	6	-	-
	8	-	-
	9	+	28
	10	+	19
	11	+	42
	14	+	10
	17	+	20
	18	+	13
	20	-	-
	24	-	-
	25	-	-
	26	+	452
	27	+	22
	28	+	97
	29	+	81
	30	-	-
	32	+	9
	36	+	15
	38	+	50
	40	+	23
	41	+	5

3.2.2 Secondary screening

The isolates which exhibited less time to develop pink color from primary screening were examined in nutrient broth media containing 0.4 mM $CuSO_4$ for production of laccase quantitatively by measuring their spore-bound laccase activity.

It was found that, all *Bacillus* spp. isolates which selected from primary screening have the ability to produce laccase but with different levels (4.2 – 12.8) U/ml (Table 3-2). *Bacillus* sp. 54 was showed high level of laccase production, hence it was selected for optimization experiments. There are two factors which play an important role in enzyme production. The first is the source of the isolate and the second is the genetic factors (Adrio and Demain, 2014).

In present study *Bacillus* sp. 54 and 56 were exhibited the highest laccase producers (12.8 and 11.1) U/ml respectively. The higher laccase activity of these two isolates could be related to the source of isolates were isolated from isolated from contaminated soils with oils, play as enzyme inducers and the isolates have the genes, which were responsible for enzyme synthesis (Adrio and Demain, 2014).

Polyphenols, methoxy-substituted phenols, diamines and a considerable range of other compounds which can be used as substrates for laccase production, but syringaldazine (4-hydroxy-3,5-dimethoxybenzaldehyde azine) has been considered as the laccase-specific substrate (Wang *et al*., 2011).

Claus and Filip, (1997) indicated that, syringaldazine used as the substrate for laccase activity, and it was correlated closely with spore formation from *Bacillus sphaericus*.

Table (3-2): Secondary screening of *Bacillus* spp. for laccase production in broth medium supplemented with $CuSO_4$ after incubation for 24 hours at pH 7 and 30 ˚C

Sources of isolates	Isolates number	Laccase activity (U/ml)
Agricultural soils	23	10.7
Soils contaminated with oils	3	8.4
	48	6.2
	54	12.8
	56	11.1
Soils contaminated with sewage	14	9.3
	18	6.4
	32	9.5
	36	4.2
	41	10.3

3.3 Identification of *Bacillus* sp.

To identify the *Bacillus* sp. 54 to species level, the following biochemical tests were followed:

In catalase test, the selected isolate has the ability to produce catalase, which act as a catalyst in breakdown of hydrogen peroxide into water and oxygen, hence the appearance of bubbles (Fig 3-3a).

Bacillus sp. 54 was oxidase positive, the color of the indicator was changed to blue within 9 sec. (Fig. 3-3b). Oxidase test depends on the presence of cytochrome oxidase in bacteria that will catalyze the transport of electrons between electron donors and redox dye.

In starch Hydrolysis, after the culture medium was flooded with iodine, the bacterial isolate was produced a clear zone around the growth which means a positive reaction and indicates that the starch had been hydrolyzed in the area around the bacterial growth by amylase production (Fig. 3-3c).

In gelatin liquefaction test, after 24 hour of incubation, the tubes were immersed in to the ice bath for 15 minute the inoculated tube was liquefied and the uninoculated control tube was completely solidified, which means the *Bacillus* sp. 54 was gelatinase-positive, (Fig. 3-3d) and it was grown above the surface of agar in the tube, thus *Bacillus* sp. 54 are obligate aerobe**,** needing air in order to grow (Fig. 3-3e).

In indole test, the pink colored ring was not formed after addition of Kovács reagent. Thus, *Bacillus* sp. 54 has no tryptophanase enzyme hence it could not able to convert tryptophan to the indole (Fig. 3-3f).

In casein hydrolysis test, *Bacillus* sp. 54 growth in skim milk agar caused a clear zone around the growth area, which identified the presence of the enzyme protease (Fig. 3-3g).

In urea Hydrolysis, the urea broth medium remained light orange (no color change). Therefore, *Bacillus* sp. 54 have no urease enzyme which is not able to change the indicator phenol red to hot pink (Fig. 3-3h) and it was appeared motile by formation of a diffuse zone of growth spreading out from the stab inoculums line (Fig. 3-3i), and the bacterial isolate was grown at 50 ˚C.

In citrate Utilization test, *Bacillus* sp. 54 showed a positive result for this test, which was able to turn the color of the indicator from green to blue because using the citrate was led to rise in pH of the medium (Fig. 3-3j).

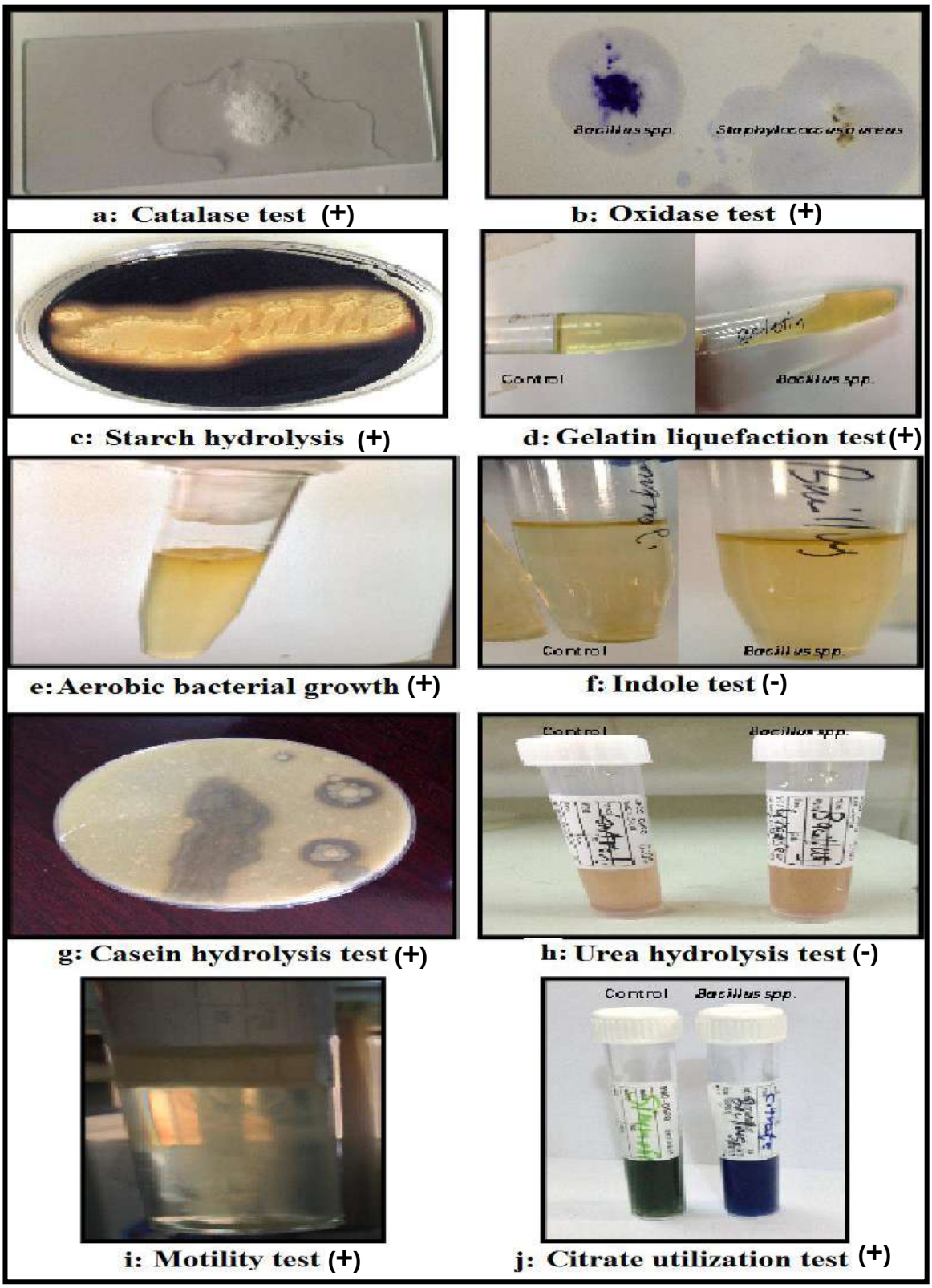

Figure (3-3): Biochemical test results of *Bacillus* sp. 54. [(+) positive test, (-) negative test].

In carbohydrate fermentation test, the fermentation reactions were detected by the color change of a pH indicator when acid products are formed. This was accomplished by adding a single sugar to carbohydrate medium containing phenol red as a pH indicator. Bacterial isolate can also utilize peptones in the medium resulting in alkaline by-products, thus the pH changed only when excess acid was produced because of carbohydrate fermentation (MacFaddin, 1985; Finegold and Baron, 1986).

Bacillus sp. 54 showed a positive glucose, galactose and mannitol fermentation tests while it was negative for lactose fermentation test (Fig. 3-4).

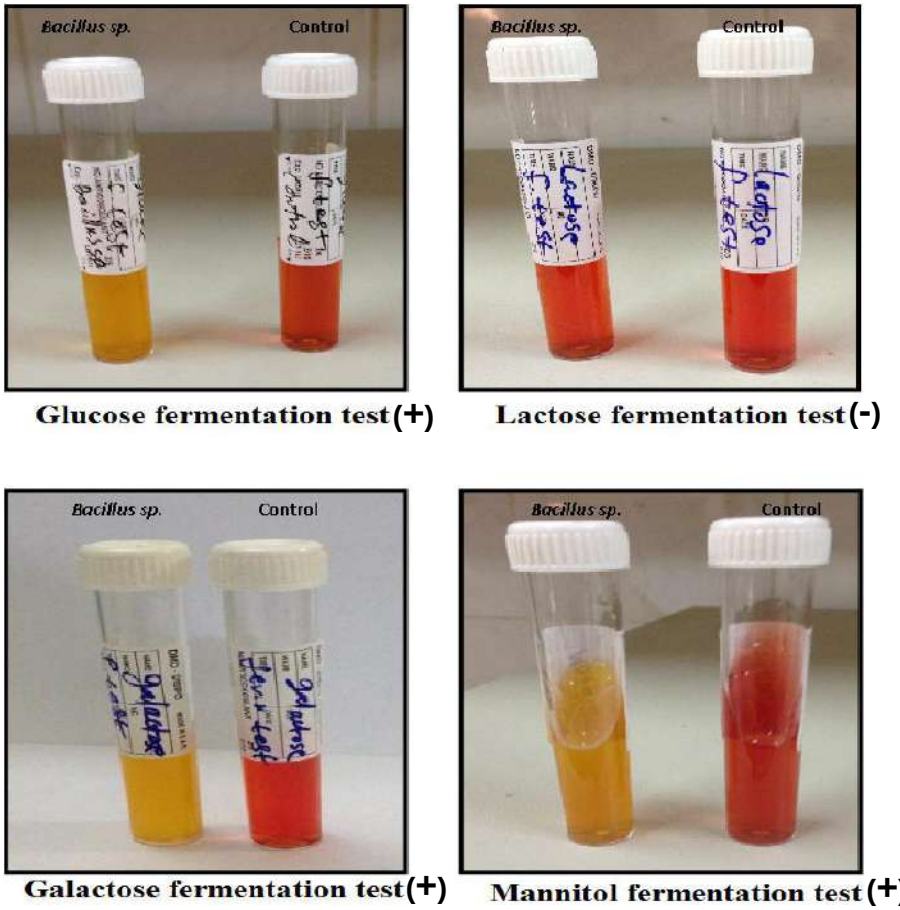

Figure (3-4): Carbohydrate fermentation test results of *Bacillus* sp. 54. [(+) positive test, (-) negative test].

The results of morphological, cultural and biochemical characteristics (Table 3-3) were compared with the characteristics of *Bacillus* spp. documented by others (Priest**,** 1989; Slepecky and Hemphill, 2006). Therefor, the selected isolate was identified as *Bacillus subtilis.*

Table (3-3): Morphological, cultural and biochemical characteristics of *Bacillus* sp 54.

Test	Result
Gram stain	+
Shape	Rod
Spores forming	+
Motility	+
Anaerobic growth	-
Growth at 50 ˚C	+
Catalase	+
Oxidase	+
Starch hydrolysis	+
Gelatin liquefaction	+
Indole	-
Urea hydrolysis	-
Casein hydrolysis	+
Citrate utilization	+
Carbohydrates fermentation	
Glucose	+
Lactose	-
Galactose	+
Mannitol	+

3.4 Optimum conditions for laccase production

3.4.1 Incubation periods

After *B. subtilis* isolate was grown in the production medium, the spore-bound laccase activity was measured at different time intervals of growth. The maximum laccase production was observed after 3 days of incubation at 30 ˚C with laccase activity (59.7) U/ml (Fig. 3-5).

The activity of the *Bacillus* SF spore bound laccase was dependent on the age of the spores and the maximum activity was achieved after 9 days of growth (Held *et al*., 2005).

The short incubation period for laccase production from *Bacillus* sp. than other bacteria and fungi offers unique potential for inexpensive enzyme production (Bernfeld, 1955; Saad, 2010).

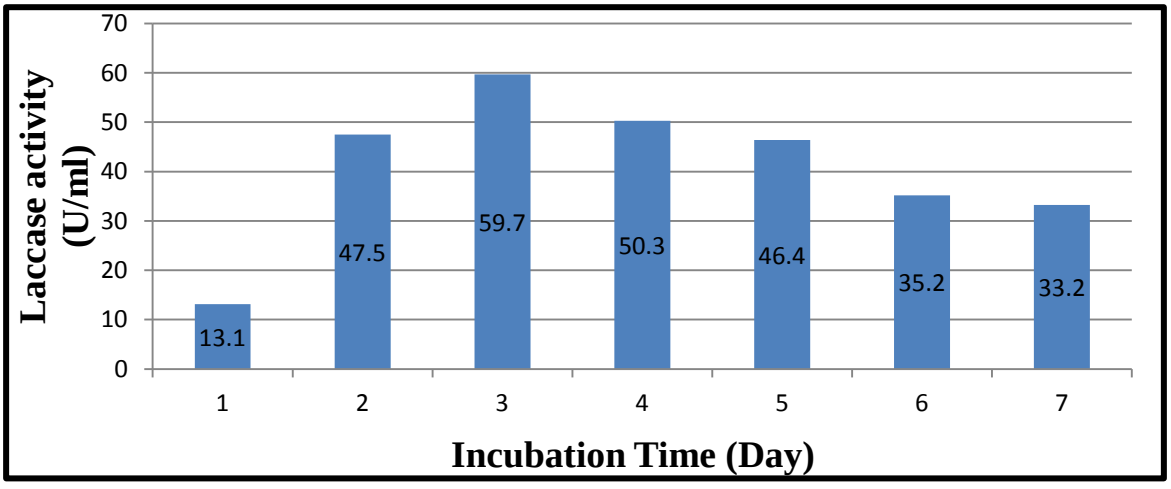

Figure (3-5): Effect of incubation peroids on laccase production from *B. subtilis* at 30 °C.

3.4.2 Temperatures

The selected isolate (*B. subtilis*) was able to grow and produce laccase at wide range of temperatures from 20 – 50 ˚C, Laccase production was found to be maximum at 37 ˚C with activity of (82.46) U/ml (Fig. 3-6).

Temperature influencing the rates of biochemical reactions either by inducing or repressing enzyme production (Strnadova *et al*., 1991). Wang *et al*., (2010) observed that the maximum laccase activity of *B. subtilis* WD23 was at 25 C˚.

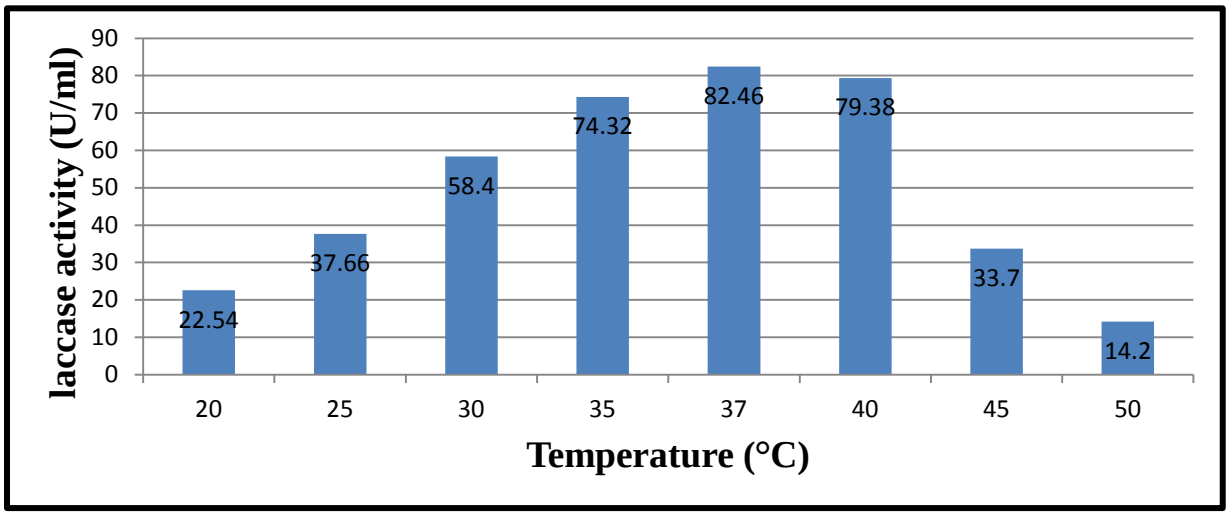

Fig. (3-6): Effect of temperatures on laccase production from *B. subtilis* after 3 days of incubation at pH 7.

3.4.3 pH values

As can be seen in figure (3-7) higher spore-bound laccase activity (82.73) U/ml was obtained at pH 7, increase or decrease pH value above or below 7, lead to reducing in enzyme activity.

The pH of the culture significantly influences many enzymatic process and transport of the compounds across the cell membrane (Shanmugam *et al*., 2008). This finding was agreed with Shukur, (2015) who reported that higher laccase activity of *Bacillus* sp. B16 was obtained at pH 7.

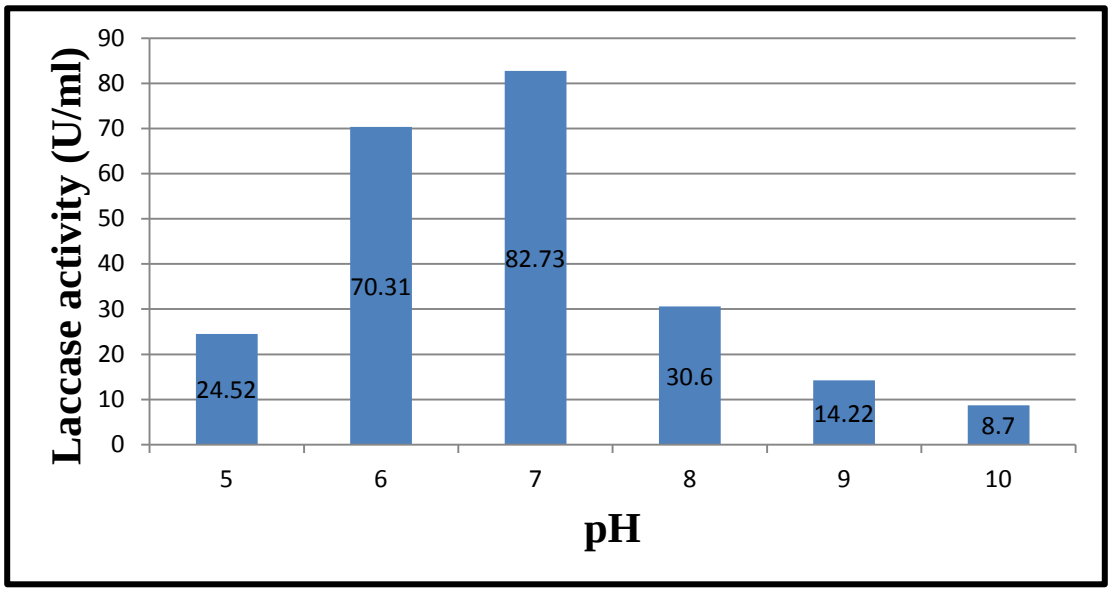

Figure (3-7): Effect of pH values on laccase production from *B. subtilis* in production medium after 3 days of incubation at 37 °C.

3.4.4 Concentrations of copper sulphate

The effect of different concentrations of $CuSO_4$on laccase production was studied. The result in figure (3-8) showed that 0.2 mM of copper sulphate induced laccase production with activity (227.23) U/ml. This suggested a possible role of Cu^{+2}, the laccase activity showed a distinct dependence on Cu^{+2} **(**Claus and Filip, 1997). Excess copper may have a toxic effect on microorganism, and thus decrease laccase production (Revankar and Lele, 2006).

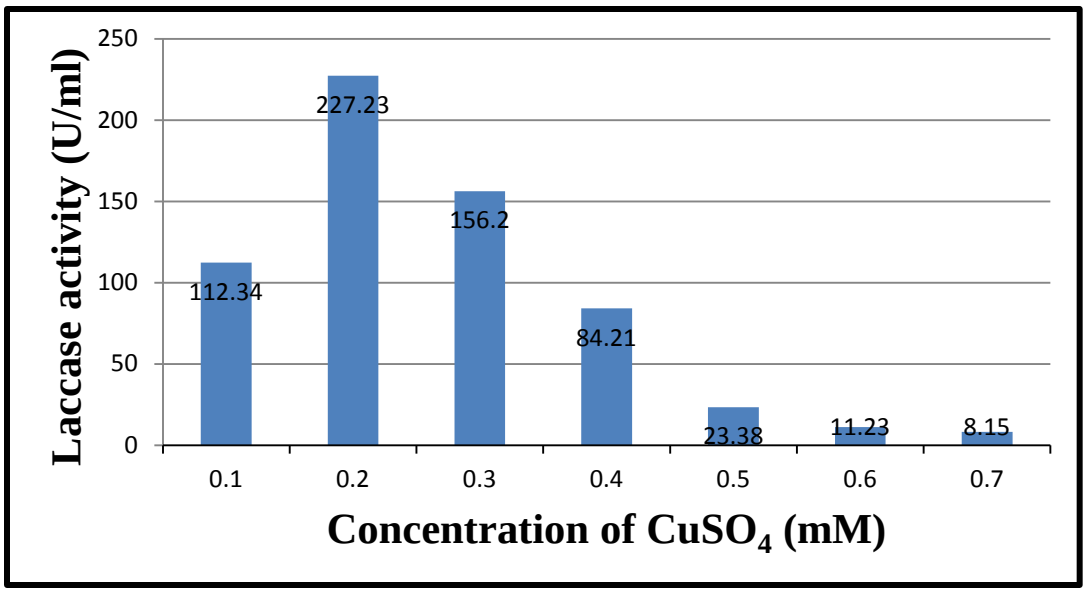

Figure (3-8): Effect of different concentrations of copper sulphate on laccase production from *B. subtilis* in production medium after 3 days of incubation at 37 °C and pH 7.

3.4.5 Carbon sources

Laccase production was detected in the presence of different carbon sources incorporated in the production medium with concentration of 3% (w/v). The laccase activity was increased in the culture supplemented with glucose (265.53) U/ml compared with maltose containing medium which was (114.61) U/ml. Among the five different carbon sources glucose supported good laccase production (Fig. 3-9).

The carbon source is the most important factor in laccase production, and that the addition of suitable amount of other sugar to the culture media has an influence on laccase synthesis (Sivakumar *et al*., 2010).

The effect of carbon source on enzyme production was reported in bacterial laccase production, glucose showed the highest potential for the production of laccase (Moldes *et al*., 2004).

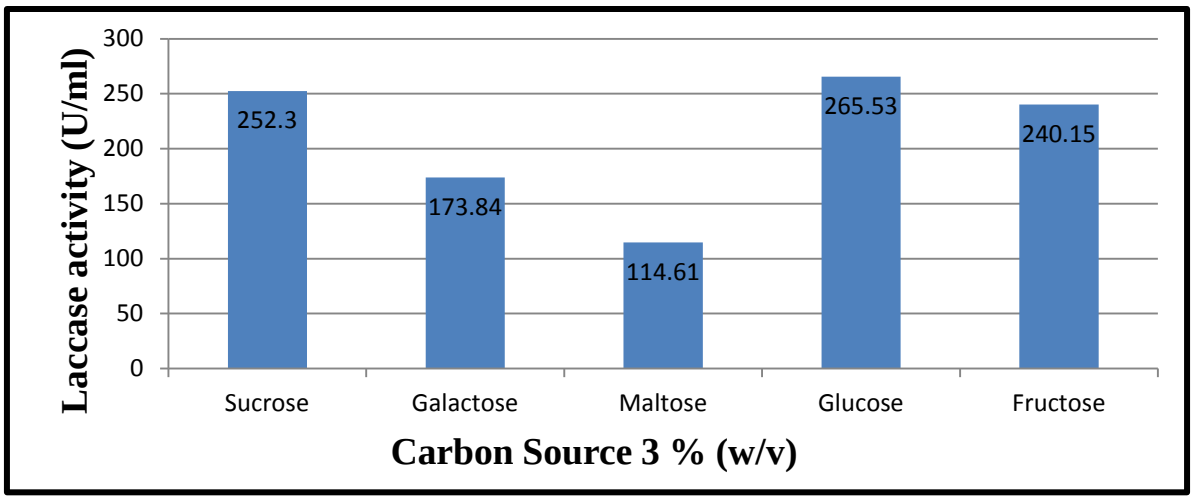

Figure (3-9): Effect of carbon sources on laccase production from *B. subtilis* in production medium after 3 days of incubation at 37 °C and pH 7 containing 0.2 mM of $CuSO_4$.

3.4.6 Nitrogen sources

The effect of different nitrogen sources was evaluated at optimum temperature, pH and carbon source. Based on the results, the laccase activity was increased to (345) U/ml in the culture contained tryptone compared with other nitrogen sources (Fig. 3-10). Tryptone was the best source of nitrogen that supported the growth and enzyme production from *Bacillus* sp. (Sharma *et al*., 2007).

Nitrogen source plays key role in laccase production, while the organic nitrogen source gave high laccase yields. The nature and the concentration of nitrogen sources in the culture medium for growth the organisms were essential for laccase production (Vandana and Peter, 2014).

Shanmugam *et al*., (2008) observed that addition of inorganic nitrogen source to the production medium resulted in low enzyme production.

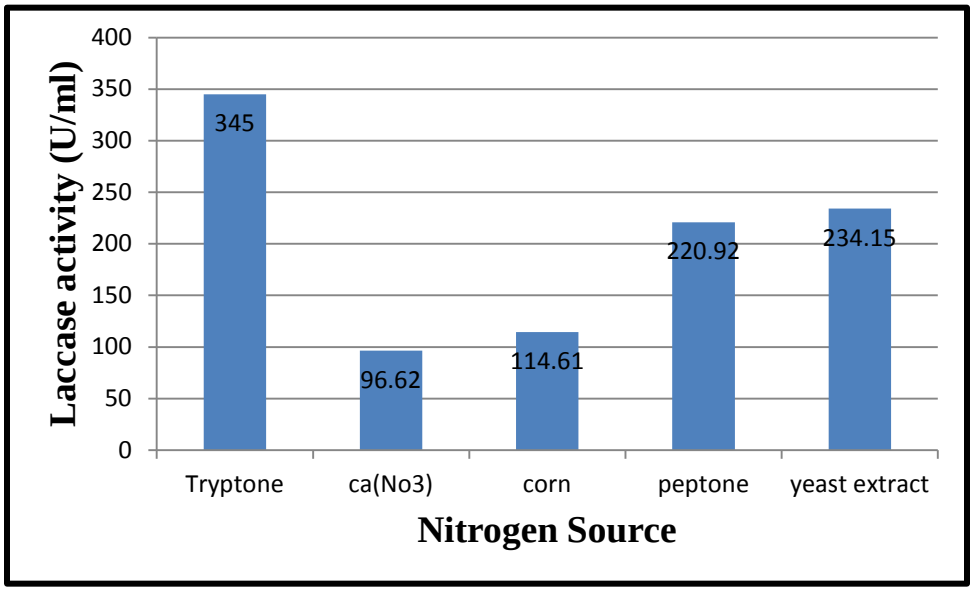

Figure (3-10): Effect of nitrogen sources on laccase production from *B. subtilis* in production medium after 3 days of incubation at 37 °C and pH 7 containing 0.2 mM of $CuSO_4$ and 3% glucose.

3.4.7 Metal ions

The effect of metals on the growth and production of laccase from *B. subtilis* was shown in figure (3-11). Based on the results obtained in this work, KCl showed the maximum induction on laccase production with highest activity of (397.69) U/ml.

Metals can be assimilated as part of enzymatic cofactors that lead to increase in enzyme activity and it may be adsorbed to surfaces of cells and be precipitated because of bacterial metabolism (Cliff *et al*., 2005).

Bacterial laccase production may be involved in the activation of gene transcription in presence of metal ions (Mongkolthanaruk *et al*., 2012).

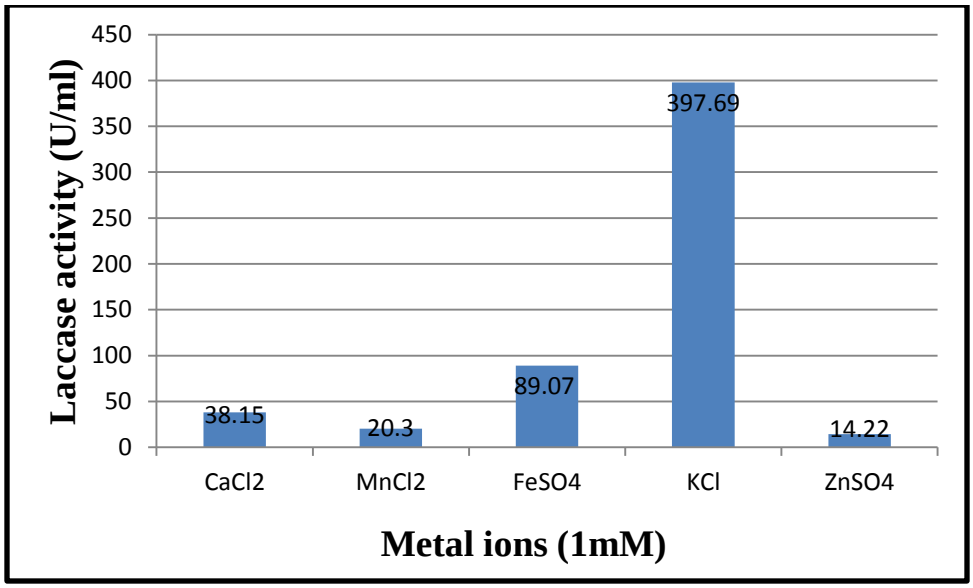

Figure (3-11): Effect of Metal ions on laccase production from *B. subtilis* in production medium after 3 days of incubation at 37 °C and pH 7 containing 0.2 mM of $CuSO_4$, 3% glucose and 0.2% tryptone.

3.4.8 Enzyme inducers

Laccase production was detected in the presence of different phenolic and hydrocarbons inducers added to the production medium. All inducers were enhanced laccase production, pyrogallol was supported the best laccase production with activity (439.23) U/ml (Fig. 3-12).

This finding was agreed with Niladevi and Prema, (2008) who reported that pyrogallol substantially enhanced laccase production. Aromatic compounds have been widely used to elicit enhanced laccase production by different organisms (Leonowicz *et al*., 2001).

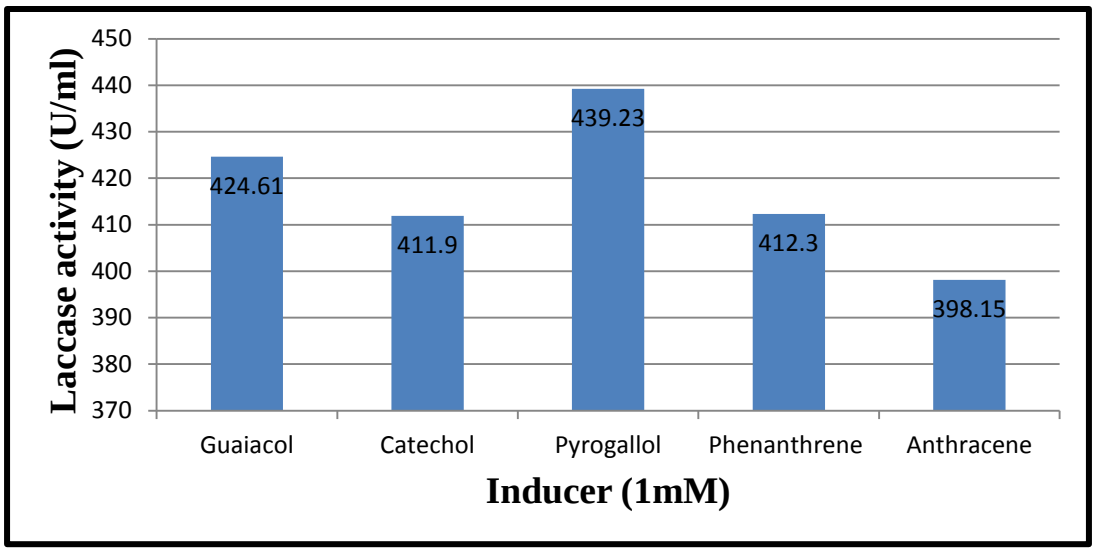

Figure (3-12): Effect of Inducers on laccase production from *B. subtilis* on production medium after 3 days of incubation at 37 °C and pH 7 containing 0.2 mM of $CuSO_4$, 3% glucose , 0.2% tryptone and 1 mM KCl.

3.5 Characterization of *B. subtilis* laccase enzyme

3.5.1 Optimum pH for laccase activity and stability

The effect of different ranges of pH values (3-9) in the activity of laccase were studied. The optimum pH for laccase activity was 6.8 with activity of (452.48) U/ml, with a decrease in laccase activity at the pH value directed towards alkaline range (8.0-90). It was also noticed a decrease in the activity of the enzyme at acidic values (3.0-5.0). However, the laccase activity was highest around neutral pH (6-7) (Fig. 3-13).

The result were in agreement with Claus and Filip, (1997) who reported that, the optimum pH for the laccase-like enzyme of *B. sphaericus* spores was about six.

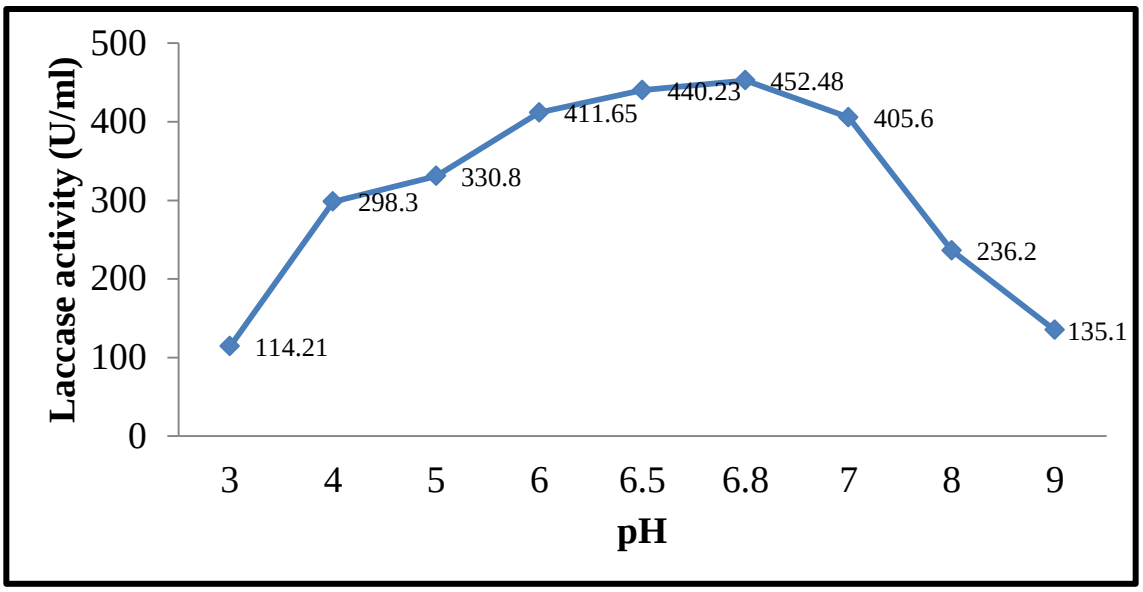

Figure (3-13): Effect of pH on laccase activity.

The spore-bound laccase was retained its initial activity (100%) at pH 6.8 after incubation for 1 hr at 37 °C, and the spore-bound laccase maintained 94-98 % of its initial activity at pH (6-6.5) respectively, while maintained about 76% of its activity at pH 9 (Fig. 3.14).

The difference in redox potential between the phenolic substrate and the T1 copper could increase oxidation of the substrate at high pH values, but the hydroxide anion (OH^-) binding to the T2/T3 coppers results in an inhibition of the laccase activity due to the disruption of the internal electron transfer between the T1 and T2/T3 centres. These two opposing effects can play an important role in determining the optimal pH of the biphasic laccase enzymes (Xu, 1997).

Held *et al*., (2005) observed that, the laccase activity of *Bacillus* SF spore showed higher stability over a broad pH ranges. Within a pH ranged from 5.0 to 7.0, the half-life was more than 120 h and showed a very high stability at alkaline pH values ($t_{1/2}$ = 48 h at pH 8.5).

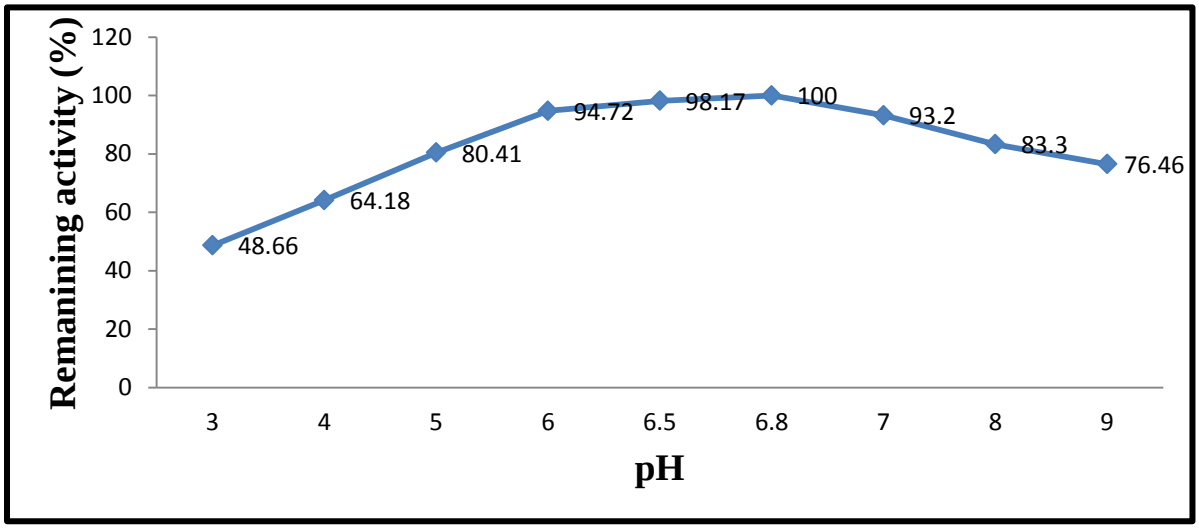

Figure (3-14): Effect of pH on laccase stability after 1 hour of incubation.

3.5.2 Optimum temperature for laccase activity and stability

The results of effect different temperatures (10-90) °C in laccase activity were shown in figure (3-15). The results indicated an increase in the activity at 40°C, the laccase activity reached to (468.2) U/ml, then the activity was declined with increasing temperature up to 40 °C with a minimum activity observed at 90 °C (146.75) U/ml. However, laccase activity was decreased below 40 °C too.

Wang *et al*., (2011) reported that, the optimum temperature of the spore-bound laccase was determined at pH 6.8, and the maximum activity was observed at 60°C. It showed higher activity within a temperature range from 40 to 70 °C.

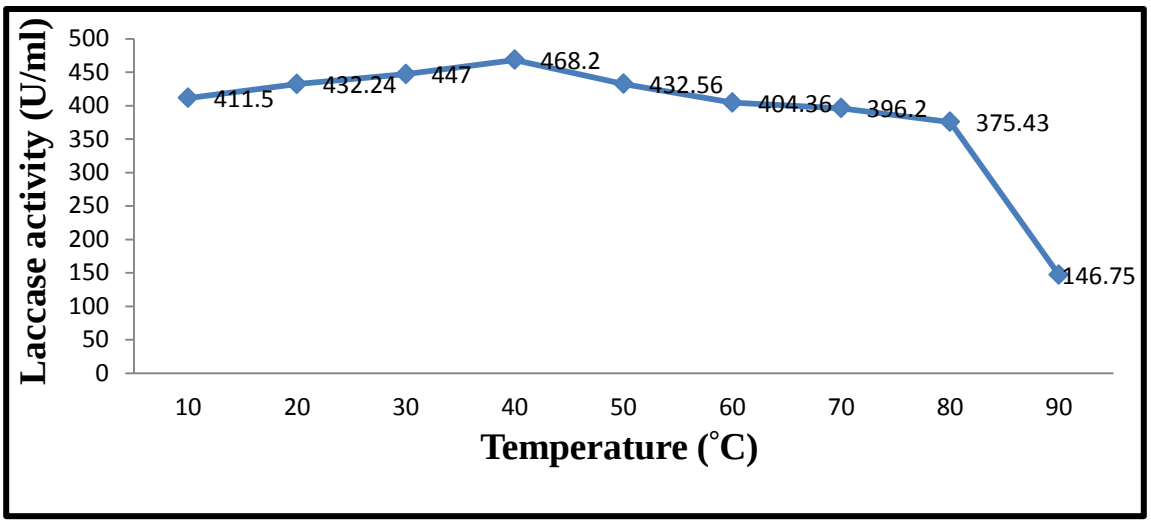

Figure (3-15): Effect of temperature on laccase activity.

B. subtilis spore-bound laccase retained its initial activity after 3 hr when incubated at temperature ranged (10-50) °C. Then the activity decreased with increasing temperature at 90 °C, the enzyme retained 22.67 % of the initial activity (Fig. 3-16).

Generally, for any enzymatic reaction, temperature below or above the optimal temperature will drastically reduce the rate of reaction. This may be due to the enzyme denaturation, or to losing its characteristics of three-dimensional structure. Denaturation of a protein involves the breakage of hydrogen bonds and other non-covalent bonds (Tortora *et al*., 2004).

Wang *et al*., (2011) reported that, the laccase had a high stability at the optimum temperature ($t_{1/2}$ = 68 h at 60 °C). The pH half-life of the spore-bound laccase was more than 6 months at pH 6.8.

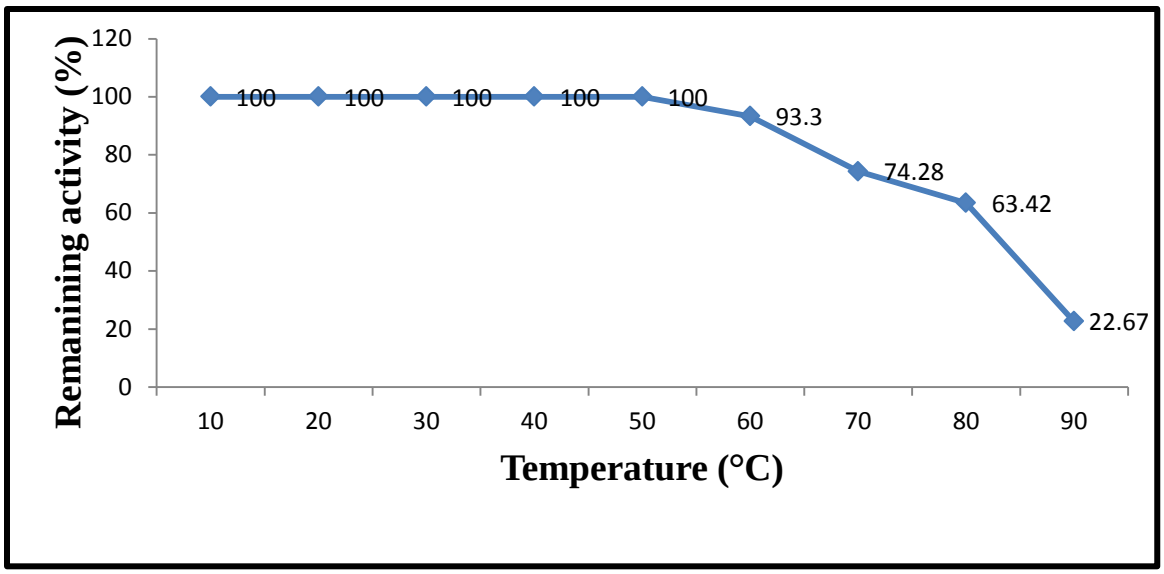

Figure (3-16): Effect of temperature on laccase stability after 3 hours of incubation.

3.5.3 Effect of operational time in laccase stability

To determine the operational time activity of *B. subtilis* spore-bound laccase, spores suspension was incubated for 10 days at 10 °C, pH 6.8. Enzyme activity started to decrease after 5 days of incubation period. The spore-bound laccase retained 100% of its activity after 4 days, 72.4% after 5 day and 23.78% after 10 days of incubation (Fig. 3-17). In contrast to fungal laccase, Kunamneni *et al*., (2008) found that, laccase isolated from *Myceliophthora thermophila* lost 36% of its activity after 24 hours of incubation at optimum conditions .

The high stability of laccase enzyme of *Bacillus* SF spores compared to the liberated enzyme result in a high potential for using of spores laccase in industrial processes like dye decolorization, detoxification, and transformation of phenolic and other compounds where high temperatures and pH values are common (Held *et al*., 2005).

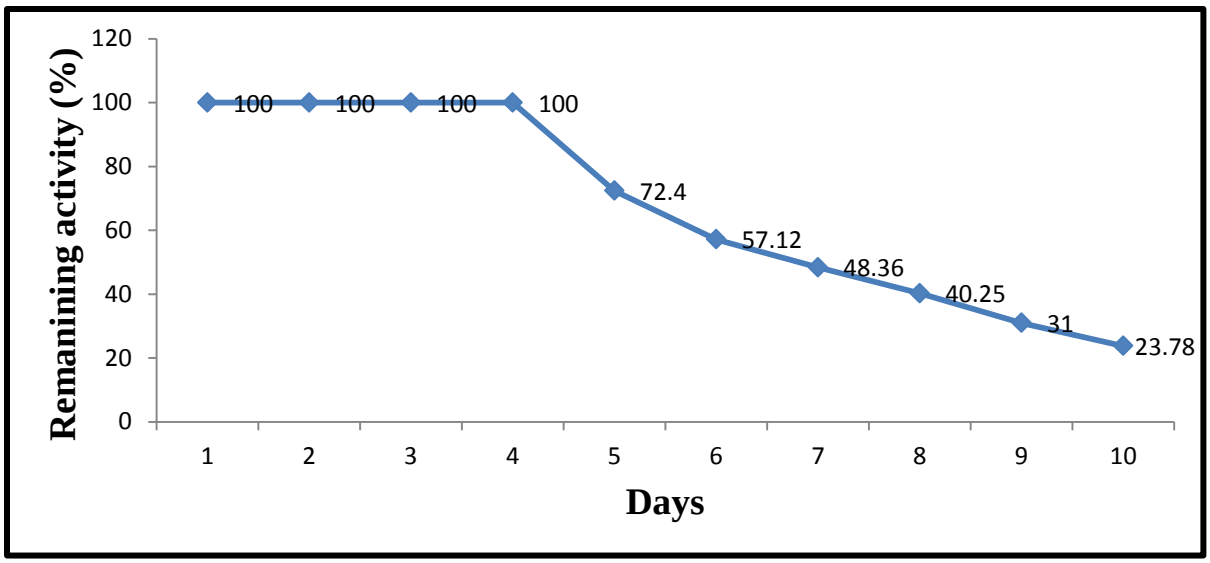

Figure (3-17): Effect of operational time on laccase stability.

3.6 Dyes decolorization

3.6.1 Dyes decolorization by *B. subtilis* on solid media

The *B. subtilis* growth after 3 days at pH 7 and 37 ℃ in nutrient agar plates containing dyes at concentration 150 ppm caused a clear halo around the growth area for: Crystal violet, Eriochrome Black T, Azur B and Methyl violet dyes. For textile (blue) dye, the halo was formed, but was not clear. Finally, no halo was appeared around the growth area for methyl orange, this may be due to the chemical structure of this dye (Fig. 3-18).

This finding is also in consistent with findings of Montira and Sukallaya, (2012) who reported bacterial decolorization ability was confirmed by the clear halo formed around each colony by plate assay within 48 h.

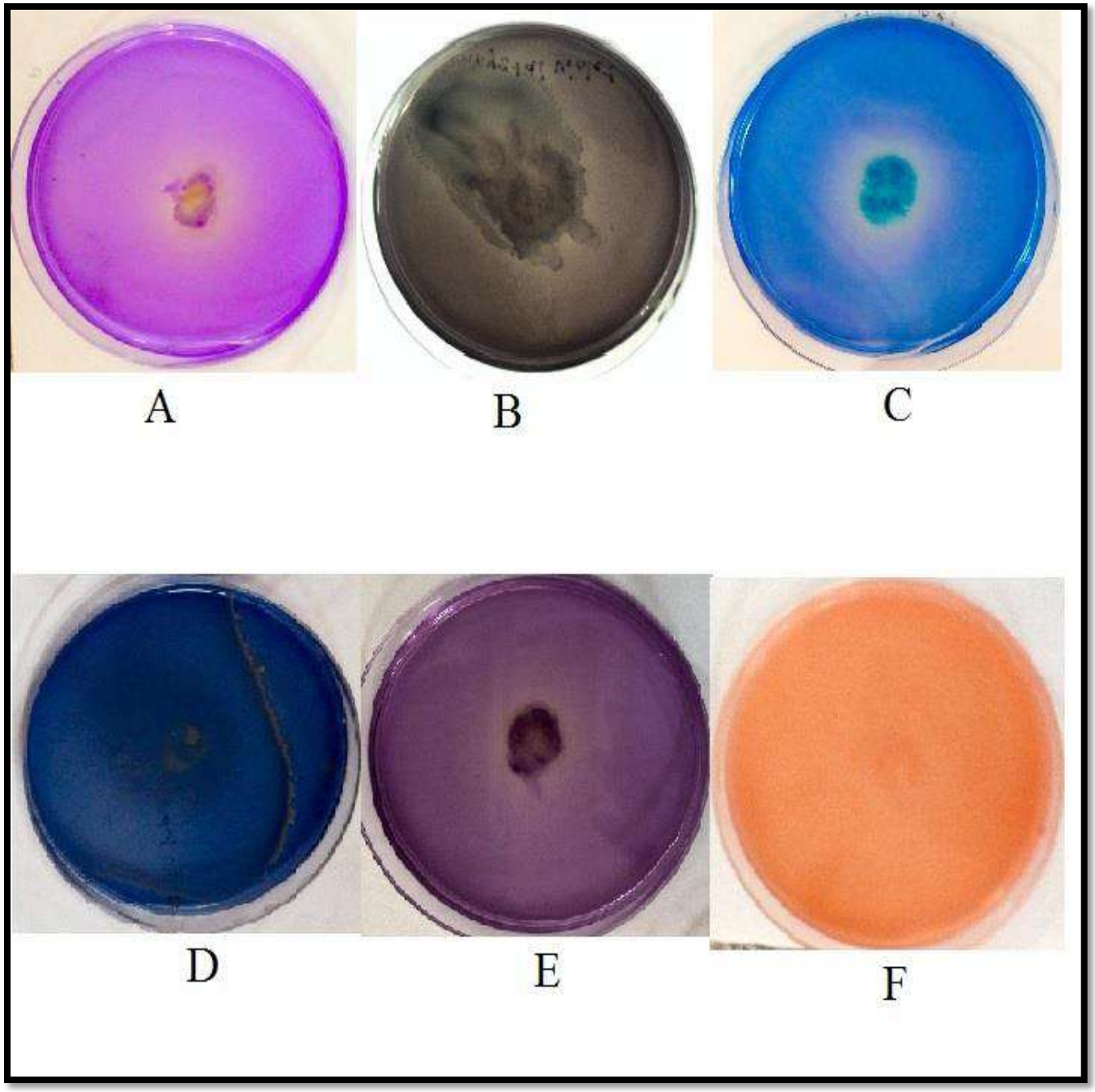

Figure (3-18): Decolorization of six dyes by *B. subtilis* isolate using solid medium supplement with each dye at concentration of 150 ppm after incubation for 3 days at pH 7 and 37 ˚C. (A)Crystal Violet , (B) Eriochrome Black T, (C) Azur B, (D) Textile (blue), (E) Methyl Violet, (F) Methyl orange.

3.6.2 Dyes decolorization by *B. subtilis* laccase

Dyes decolorization by *B. subtilis* laccase has been investigated using spores with initial laccase activity (468.2) U/ml in order to prove their potential application in the treatment of dyestuff wastewater. The reaction mixture composed of 100 ml of dyes at concentrations (25, 50, 75, 100, 125 ppm) in distilled water containing 10 ml of spore suspension, the dye decolorization was studied at optimum pH 6.8 and 40 ^{o}C stability. The results showed that, the dyes (eriochrome black T, crystal violet, azure B) were completely decolorized in all concentrations within 10 minutes, the same for textile (blue) dye in concentrations of (25, 50, 75 ppm) which was completely decolorized within 10 minutes, while in concentrations (100, 125 ppm) was completely decolorized after 20 minute. Methyl violet and Methyl orange dyes were decolorized in different percentage, (Table 3-4) and (Fig. 3-19).

Increasing concentration of methyl violet and the methyl orange dyes lead to decrease percentage of dye decolorization by spore-bound laccase. This may be interrelated to molar extinction coefficients and purity of each particular dye. Moreover, it was known that nature and position of the dye substituent group strongly affect the decolorization extent (Couto, 2007).

Wang *et al*., (2010) showed that the ability of spore laccase from *B. subtilis* to decolorize methyl orange and methyl violet dyes. The methyl orange was maximally decolorized (≈ 70 %), followed by methyl violet (≈ 50 %) at 5 days of treatment.

Table (3-4): Dyes decolorization (%) by *B. subtilis* laccase.

Dye concentration (ppm) / Incubation Times (min.)	Decolorization of Eroichrome Black T %			Decolorization of Crystal Violet %			Decolorization of Azure B %			Decolorization of Textile dye (blue) %			Decolorization of Methyl Violet %			Decolorization of Methyl Orange %		
	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30	10	20	30
25	100	–	–	100	–	–	100	–	–	100	–	–	77.4	83.4	98.7	24.2	27.6	29.5
50	100	–	–	100	–	–	100	–	–	100	–	–	64.6	69.3	73.5	13.2	16.3	20
75	100	–	–	100	–	–	100	–	–	100	–	–	52.1	56.8	60.5	10.8	12.2	13.8
100	100	–	–	100	–	–	100	–	–	90.3	100	–	37	39.3	41.3	2.2	3.4	4.1
125	100	–	–	100	–	–	100	–	–	87.6	100	–	14.3	15.2	17.4	1.7	2.3	2.6

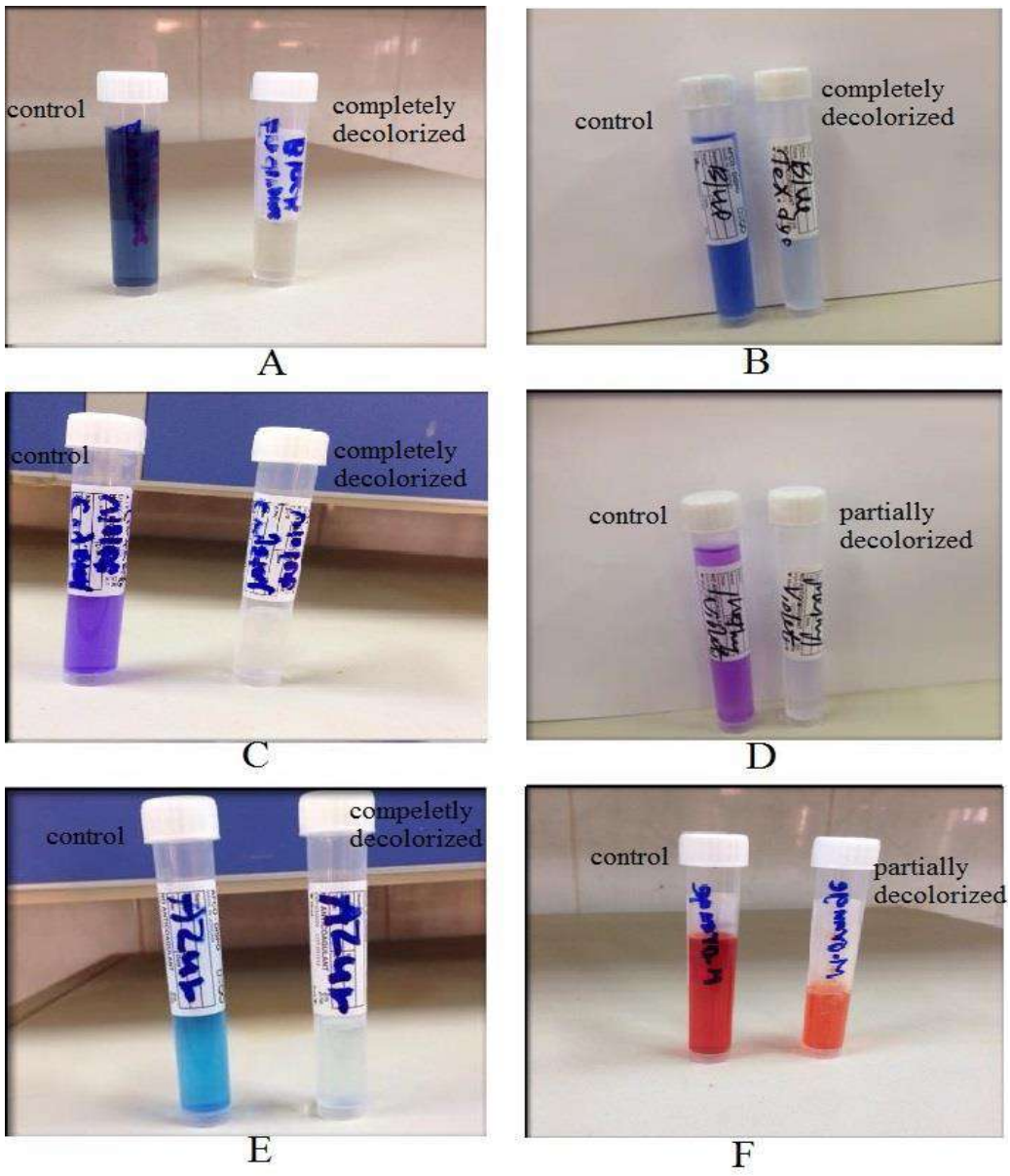

Figure (3-19): Dyes decolorization by *B. subtilis* laccase at pH 6.8 and 40 °C, all dyes with concentration of 25 ppm:

A: Eroichrome Black T was completely decolorized within 10 minute.

B: Textile dye (blue) was completely decolorized within 10 minute.

C: Crystal Violet was completely decolorized within 10 minute.

D: Methyl Violet was partially decolorized within 30 minute.

E Azure B was completely decolorized within 10 minute.

F: Methyl Orange was partially decolorized within 30 minute.

3.7 Hydrocarbons degradation by *B. subtilis* laccase

Anthracene and phenanthrene degradation by *B. subtilis* laccase was analyzed by spectrophotometer, this procedure was done to prove the potential application of *B. subtilis* spore-bound laccase in treatment of wastewater from hydrocarbons pollutants. *B. subtilis* spores were used with initial laccase activity (468.2) U/ml. Anthracene and phenanthrene were prepared at different concentrations (25, 50, 75, 100, 125 ppm) in 100 ml distilled water for anthracene and 100 ml 10 % of methanol for phenanthrene containing 10 ml of spore suspension, the hydrocarbons degradation was studied at optimum pH 6.8 and 40 ̊C.

The results showed that, the degradation of these hydrocarbons occurred in different percentages, but phenanthrene degradation percentage was more than anthracene at all concentrations. The degradation of both compounds was decreased with increasing the hydrocarbon concentration. Phenanthrene degradation in concentration of 25 ppm was 82.7% after 30 min, which considered the highest degradation percentage of both compounds, while anthracene degradation in concentration of 25 was 63.1% after 30 minutes of incubation with spore-bound laccase as shown in table (3-5) and figure (3-20).

Munusamy *et al.*, (2008) showed that the ability of laccase from *Pycnoporus sanguineus* (KUM 90953) to degrade phenanthrene and anthracene was 89% and 43% respectively after 24 hours. The degradation percentage depended on the solubility of phenanthrene and anthracene. Phenanthrene was more soluble than anthracene therefore, it was degraded faster.

Thus, microorganisms that can degrade various pollutants (e.g. nitroaromatics, chloroaromatics, polycyclic aromatics, biphe-nyls and components

of oil) have been isolated with the eventual goal of exploiting their metabolic potential for the bioremediation of contaminated sites (Spain et al., 2000; Dua et al., 2002).

PAHs were the real contaminants of petroleum matter, were detected in selected sites along Tigris river within Baghdad City in summer and winter time, analysis of samples from selected sites proved that the most abundant component of aromatic hydrocarbons were phenanthrene, naphthalene and acenaphthylene, followed by fluorene, acenaphthene, fluoranthene, benzo (a) pyrene, and anthracene (Ibraheem and Ibrahim, 2013).

A simple ultraviolet-visible (UV-VIS) spectrophotometric analysis can be a cost-effective method that can be used for biodegradation studies of PAHs. In this regard, PAHs generally absorb light in the 200–400 nm range as well as strongly fluoresce. UV-VIS absorption and fluorescence spectroscopic techniques are sensitive for PAHs detection (Das and Dash, 2015).

There are some PAHs with their reported absorption maximum under UV light. They include Benzo [α] anthracene (288 nm), Benzo [α] pyrene (297 nm), Benzo [κ] fluoranthracene (307 nm), Chrysene (268 nm) and phenanthrene (251 nm) (Rivera-Figueroa *et al*., 2004).

Table (3-5): PAHs degradation (%) by *B. subtilis* spore-bound laccase

PAHs concentrations (ppm) / Incubation Times (min.)	Degradation of Anthracene %			Degradation of Phenanthrene %		
	10	**20**	**30**	**10**	**20**	**30**
25	54.3	57.2	63.1	63.2	74	82.7
50	52.2	53.7	56	57.3	62.1	69.3
75	40.6	41.2	43.8	49.3	53.1	58.5
100	23.4	23.8	24.1	41.4	43.6	46.2
125	13.2	13.4	13.7	39.2	40.6	42.5

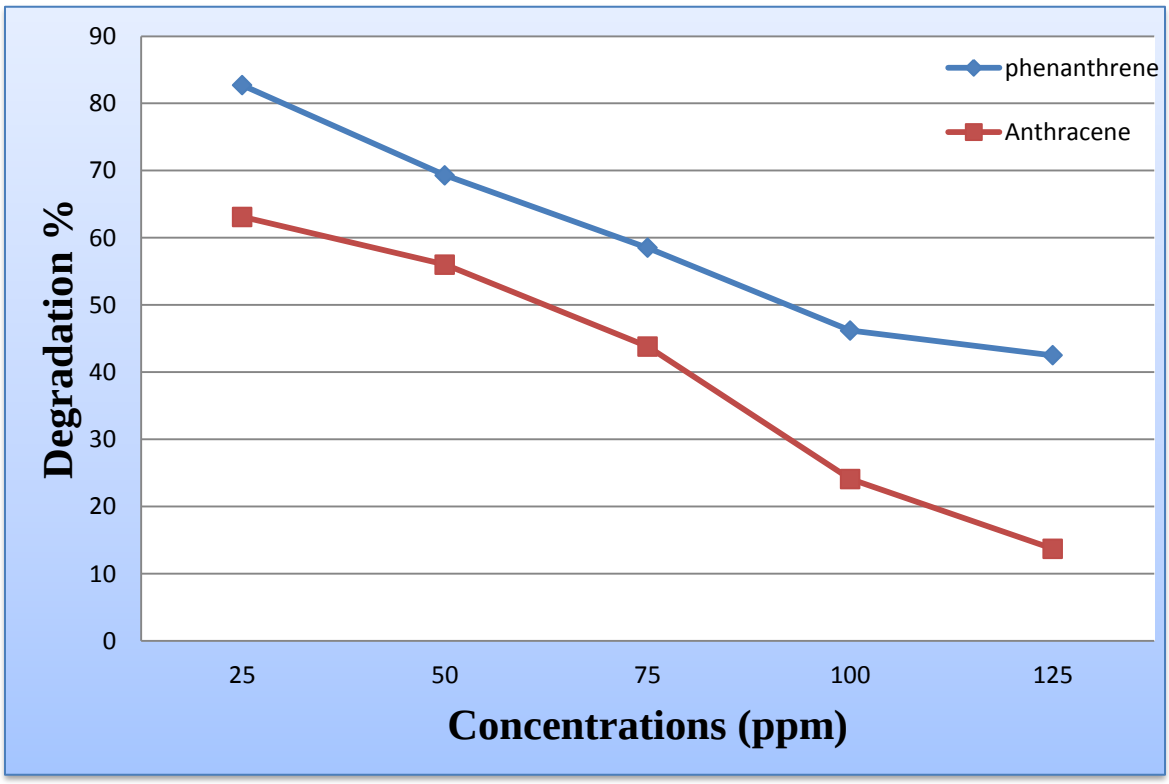

Figure (3-20): PAHs degradation by *B. subtilis* spore-bound laccase at pH 6.8 and 40 °C

Conclusions and Recommendations

Conclusions:

1- Primary and secondary screening for laccase production proved that the bacterial isolate that was isolated from soil contaminated with oils, was provided highest level of laccase production and identified as *B. subtilis* according to biochemical tests

2- The optimal conditions for *B .subtilis* laccase production were: 3 days of incubation, 37 °C, pH 7.0, 0.2 mM $CuSo_4$, 3% glucose as carbons source, 0.2% tryprone as nitrogen source, supplemented with 1 mM of Kcl and pyrogallol 1mM as inducer.

3- The optimum pH and temperature of spore-bound laccase activity are (6.8) and (40 °C) respectively, and the spore-bound laccase were retained its initial activity after 4 days of incubation at 10 °C.

4- Bacterial isolate and spore-bound laccase were able to decolorize dyes at different concentrations.

5- Spore-bound laccase was able to degrade hydrocarbons at different concentrations.

Recommendations:

1- Investigating other source of laccase producer.

2- Enhancement laccase production using cloning and expression of laccase gene from *B. subtilis* or by mutation

3- Studying different kinetics parameters of hydrocarbons degradation by *B. subtilis* spore-bound laccase in a bioreactor.

4- A comparative study among purified, crude laccase and spore-bound laccase to degradation and decolorization of hydrocarbons and dyes respectively.

5- Studying the degradation of other pollutants by bacterial spore-bound laccase.

6- A comparative study between free and immobilized laccase enzyme to degradation and decolorization of dyes and hydrocarbons.

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الخلاصة

تم عزل 57 عزلة من بكتيريا *Bacillus* spp. من نماذج ترب مختلفة (ترب زراعية وترب ملوثة بالزيوت وترب ملوثة بمياه المجاري) من مواقع مختلفة في الديوانية وبغداد. حيث تم تشخيصها اعتماداً على الصفات المظهرية والمجهرية.

اختبرت قابلية هذه العزلات على انتاج انزيم اللاكيز باستخدام الوسط الصلب المدعم بكبريتات النحاس بتركيز 0.4 ملي مول عن طريق اسقاط قطرات من syringaldazine بتركيز 0.5 ملي مول على مناطق النمو البكتري. وقد أظهرت نتائج الكشف ان 10 عزلات أعطت فعالية للانزيم خلال 15 ثانية.

استخدم الوسط السائل المدعم بكبريتات النحاس بتركيز 0.4 ملي مول لانتقاء العزلة الأكفأ من بين العزلات العشرة المنتجة خلال الغربلة الأولية. أظهرت النتائج ان العزلة 54 كانت هي الاكفأ وبفعالية قدرها U/ml (12.8).

تم تشخيص العزلة المنتخبة *Bacillus* sp. 54 على مستوى النوع اعتماداً على الفحوصات الكيموحيوية، وبينت النتائج انها تعود للنوع *Bacillus subtilis* .

حددت الظروف المثلى لانتاج الانزيم من العزلة المنتخبة *B. subtilis* حيث أظهرت النتائج ان الظروف المثلى هي: 3 أيام من الحضن ودرجة حرارة 37 مْ وبرقم هيدروجيني 7 و0.2 ملي مول من كبريتات النحاس و3 % كلوكوز كمصدر كاربوني و0.2 % من التريبتون كمصدر نيتروجيني وجهز الوسط بـ 1 ملي مول من كلوريد البوتاسيوم و1 ملي مول من pyrogallol كمحفز للانزيم. بعد هذه الظروف ازدادت فعالية اللاكيز الى U/ml (439.23).

تم تحديد الظروف المثلى الـ pH ودرجة الحرارة لفعالية اللاكيز المرتبط بالبوغ، حيث اظهرت النتائج ان اعلى فعالية للإنزيم كانت (6.8) و (40 مْ) على التوالي، والانزيم المرتبط بالبوغ استطاع استعادة فعاليته بعد اربعة ايام من الحضن وبدرجة حرارة 10 مْ.

اختبرت قابلية العزلة *B. subtilis* على إزالة لون الاصباغ على وسط صلب يحتوي اصباغ بتركيز 150 جزء بالمليون بعد الحضن لمدة 3 أيام وبدرجة حرارة 37 مْ، وقد سبب نمو البكتيريا على الوسط الصلب هالة حول منطقة النمو للاصباغ التالية (Crystal violet و Eriochrome Black T و Azur B

و Methyl violet). وقد تكونت هالة ايضاً بالنسبة لصبغة الانسجة الزرقاء لكن لم تكن واضحة. ولم تظهر هالة حول نمو البكتيريا لصبغة (methyl orange).

اختبرت قابلية انزيم اللاكيز المنتج من العزلة *B. subtilis* على إزالة لون الاصباغ وبتراكيز مختلفة من الاصباغ (25 و 50 و 75 و 100 و 125 جزء بالمليون)، وقد بينت النتائج ان الاصباغ (Crystal violet و Eriochrome Black T و Azur B) تم ازالة لونها بالكامل وبكل التراكيز خلال 10 دقائق، ونفس النتائج تم ملاحظتها لصبغة الانسجة الزرقاء بالنسبة للتراكيز (25 و 50 و 75 جزء بالمليون) ، اما بالنسبة للتراكيز (100 و 125 جزء بالمليون) تم إزالة لونها بالكامل بعد عشرين دقيقة. اما الصبغات (methyl violet و methyl orange) تم إزالة لونها بنسب مختلفة.

اختبر قابلية انزيم اللاكيز المنتج من العزلة *B. subtilis* على تحلل الـ anthracene و phenanthrene وبتراكيز مختلفة من الهيدروكربونات (25 و 50 و 75 و 100 و 125 جزء بالمليون)، وقد اظهرت النتائج ان تحلل هذه الهيدروكارونات تم بنسب مختلفة، لكن نسبة تحلل الـ phenanthrene كانت اكبر من نسبة تحلل الـ anthracene لكل التراكيز وتحلل كلا العينيتين قل بزيادة تركيزهما.

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قسم التقنيات الإحيائية

التحلل الحيوي لبعض الملوثات الهيدروكربونية بوساطة انزيم اللاكيز المنتج من العزلة المحلية *Bacillus* sp.

رسالة مقدمة الى
كلية العلوم – جامعة بغداد
وهي جزء من متطلبات نيل درجة الماجستير في
التقنيات الأحيائية

تقدم بها

احمد مجيد عبدزيد

بكالوريوس علوم حياة – جامعة القادسية 2007

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