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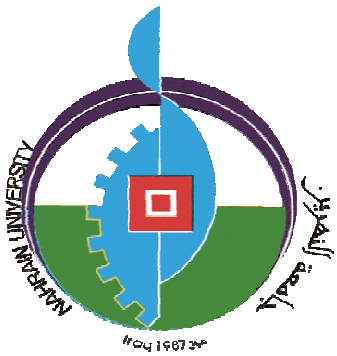

Production, Characterization and Immobilization of Laccase Enzyme from *Bacillus cereus* Local Isolate

A Thesis

Submitted to the Council of College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Master of Science in Biotechnology

By

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Dedication

To the candle that burned to enlighten my way in life

My Mother

To the man who was the best supporter to me in my life

My Father

To the man who was always beside me

My Husband

To the people who were always encouraging me in my life

My Brother& sister

Marwan and Mariam

Mina

Acknowledgment

At the beginning, thanks to great Allah, Lord of the whole creation who gave me the faith and strength to accomplish this work.

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Finally, I wish to thank my family and my husband for their patience and support asking God to bless them.

Mina

Supervisor Certification

I, certify that this thesis entitled **(Production, Characterization and Immobilization of Laccase Enzyme from *Bacillus cereus* Local Isolate)** was prepared by **(Mina Majid Allos)** under my supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of **Master of Science in Biotechnology.**

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Summary

In this study, fifty five soil samples were collected from different locations in Baghdad city. Many Gram positive and negative bacterial isolates were obtained; of which 39 were identified as *Bacillus* spp. upon subjecting to morphological and microscopic tests. Laccase enzyme activity was determined by quantitative methods using a syringaldazine as substrate for growth of these isolates. Results indicated that 17 of these isolates were laccase producer with different specific activity ranged between 98-600U/mg protein. Results of the biochemical test and Vitek 2 system showed that isolate B5 of *Bacillus cereus* was the most efficient in production of laccase when its specific activity reached 600 U/mg protein.

Therefore, *Bacillus cereus* B5 was chosen to determine the optimum conditions for laccase production. Maximum laccase production was achieved after supplementation of the minimal salt medium(pH7) with 0.5% dextrose, 0.5% yeast extract with on inoculums size 10^5 after incubation at 35^{o}C in a shaker incubator (200 rpm) for 24h. Under these conditions, the specific activity of laccase produced in culture supernatant was increased to 7000 U/mg protein.

Laccase produced under the optimum conditions was purified by precipitation with 70% saturation of ammonium sulfate, ion exchange chromatography by DEAE-Cellulose and gel filtration chromatography throughout Sephacryl S-200 column. After the last purification step, specific activity of the purified enzyme was jumped to 230.000 U/mg with 32.8 purification fold and 49.2% overall yield.

when biochemical characteristics of the purified enzyme were studied, results showed that the molecular weight of laccase produced by *Bacillus cereus* B5 was about 66000 Dalton, and an optimum pH of 7 for enzyme activity with a wide

range of pH stability (7-9). The optimum temperature for enzyme activity was 35^{o}C, while the enzyme was stable with its full activity at a range of between 30-40^{o}C.

The effect of chelating, reducing agents and heavy-metal ions on the purified laccase activity was investigated. Results indicated that the enzyme activity was inhibited by Zn^{+2} and Cu^{+2}, EDTA and Sodium azide.

Two immobilization supports (polyacrylamide and agarose) gel were used for entrapment of purified laccase enzyme. Result showed there were two fold increase in the laccase activity with agarose gel as compared to the original enzyme activity.

When storage stability of the free and immobilized laccase was studied, the immobilized enzyme showed good stability by retaining 66% of activity compared to only 5% for the free laccase after 61 days storage.

List of contents

List of Tables

List of Figure

List of Abbreviation

Abbreviation	Full name
µg	**Microgram**
µm	**Micrometer**
BSA	**Bovine serum albumin**
Cm	**Centimeter**
DEAE	**Dietheyl aminoethyl**
EDTA	**Ethylenediaminetetraacetic acid**
KDa	**Kelodalton**
Rpm	**Rotation per minute**
SDS- PAGE	**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis**
TEMED	**N,N,N',N'-tetramethylethylenediamine**
U	**Units**

Chapter One

Introduction

and

Literatures Review

1. Introduction and Literatures Review

1.1 Introduction

Biocatalysis involves the utilization of natural catalysts, i.e. enzymes, to perform chemical transformations of organic compounds. Living, whole-cells producing enzyme, or isolated enzymes, are used in biocatalysis. The key advantage of biocatalysis is the specificity of the biocatalyst, which potentially results in high yields of a particular product (Burton *et al*., 2002). New biocatalytic processes are based on the availability of interesting, useful new enzymes, usually obtained by screening for microbes that carry out the desired target reaction. Exploration of extreme environments can provide unique microbial culture collections that can be used in screening for suitable enzymes to perform a desired biocatalytic reaction. These enzymes may then be used as biocatalysts in industrially relevant bioprocesses(Schmid *et al*., 2001).

Laccases are belonged to the group of polyphenoloxidases and are multi-copper-containing enzymes which reduce molecular oxygen to water (Elshafei *et al*., 2012). This enzyme is classified as blue copper protein that catalyze the oxidation of a wide variety of organic and inorganic compounds by using molecular oxgen as the electron acceptor (Imran *et al*., 2012).

Laccase exhibit broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups including diphenols, polyphenols, diamines and aromatic amines(Sivakumar *et al* ., 2010). The syringaldazine [4-hydroxy-3,5-dimethoxy benzaldehyde azine] is considered as the substrate oxidized only by laccase enzyme (Thurston, 1994).

Laccases are produced by four types of living organisms including bacteria, insects, higher plants and fungi. A very few bacterial origin laccase

enzymes have been purified and characterized. The first study on this subject is the prokaryotic laccase which was derived from the a rizospheric bacterium *Azospirillum lipoferum* (Verma and Shirkot, 2014). In recent years, bacterial laccases have gained increasing attention due to overcoming the disadvantages of instability when compared to fungal laccases. They are highly active and much more stable at high temperatures and high pH values. Bacterial laccases become an industrially important enzyme that are applied in various processes like detoxification of industrial effluents, mostly from paper and pulp, textile and petrochemical industries, important tool for medical diagnosed, cleaning agent for certain water purification system and catalyst for manufacturing anti-cancer drugs. The important obstacles to commercialize the bacterial laccases was lack of sufficient enzyme stocks and cost order to achieve cheep over production of this biocatalyst and also alteration of enzyme by chemical means to obtain more robust and active enzyme (Muthukumarasamy and Murugan, 2014).

The purification of a laccase is an essential step for the determination of accurate parameters due to the possible presence of compounds from the host that may act as natural mediators, or the presence of similar enzymes that may exhibit significantly different reactions. The most commonly used method for laccase purification is salt elution from an anion-exchange resin, probably due to the higher stability of laccase at neutral to slightly alkaline pH, as well as the isoelectric point (pI) of laccases (Patel *et al.*, 2013).

Immobilized enzymes are generally preferred over free enzymes for many of the application purposes due to its potential advantages like increased stability and reusability. Also, the newer technological developments in the field of immobilized biocatalysts can offer the possibility of a wider and more economical exploitation in industry, waste treatment, medicine, and in the development of bioprocess monitoring devices like biosensors(Cheng *et al.*,

2006). The method of immobilization is the most important because equipped steadiness of enzymes depend on it. Entrapment is preferred over other methods as this method is easier and cheaper, stable derivatives are formed and the structure of the enzyme remains secure (Almeid *et al*., 2012).

According to those mentioned above, this study was aimed to isolate a locally higher laccase producer *Bacillus* sp. and to increase the activity and stability of the enzyme produced, and these were achieved by:

1. Isolation of *Bacillus* sp. from different soil samples.
2. Screening the ability of local isolates for laccase production and selecting the efficient one.
3. Optimization, purification and characterization of laccase enzyme produced by the efficient *Bacillus cereus* isolate.
4. Immobilization of purified laccase enzyme and studying its storage stability.

1.2 Literatures review

1.2.1The genus Bacillus

In 1872, Ferdinand Cohn, a student of Robert Koch, recognized a rod like bacteria in the soil and named it *Bacillus subtilis*. This organism is part of large and diverse genus of bacteria, the genus Bacillus, and was placed in the family Bacillaceae. Members of the genus Bacillus are characterized as Gram-positive, rod-shaped, aerobic or facultative anaerobic, endosporeforming bacteria (Bergquist, 1987; Yang Yang, 2007). The genus includes thermophilic and psychrophilic, acidophilic and alkalophilic, fresh- water and halophilic bacteria that utilize a wide range of carbon sources for heterotrophic growth or grow autotrophically. Bacillus include both free living and pathogenic species .Under stressful environmental conditions, the cells oval endospore can stay dormant for extend periods (Turnbull, 1996). Born *et al.* (1999) mentioned that the nonpathogenity of most species and their ability to secrete proteins make these bacteria interesting for the use in the pharmaceutical, food, and cosmetics industry. Bacilli produce many different industrially important enzymes, including: proteases, amylases, glucanases, lipases, nucleases and phosphatases.

On the other hand Bacilli currently account for 60% of the commercially available proteins synthesized on an economical scale. As microbiologists in the 1990’s explored earth’s extreme environments, novel endospore-forming halophiles, acidophiles, alkaliphiles and thermophiles turned up in large numbers. One might expect the list of approved Bacillus-like genera to expand rapidly during the coming decade (Nazina *et al*., 2001).

1.2.2 *Bacillus cereus*

Bacillus cereus group contains *Bacillus cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus weihenstephanensis*, *Bacillus mycoides* and *Bacillus pseudomycoides* (Drobniewski, 1993; Lechner *et al*., 1998; Nakamura, 1998).

Bacillus cereus is a motile, endospore forming, aerobe or facultative anaerobe gram positive bacterium that occurs ubiquitously in soil and in many raw processed foods such as rice, milk and dairy products, vegetables and spices (Choma *et al*., 2000; Guinebretiere *et al*., 2003). The spores of *Bacillus cereus* can tolerate harsh physical and chemical conditions, and will also survive pasteurization. Consequently they will germinate in processed foods when temperature and other conditions are desirable for vegetative growth, and competitive flora is absent. Aslo, many researchers stated that spores coat contains certain enzymes, such as laccases which may be active even when the spore core is devoid of metabolic activity(Hullo *et al*., 2001; Martins *et al*., 2002). In the study reported by Sowmya *et al.* (2014) degradation of polyethylene was carried out with *Bacillus cereus* which was isolated from dumpsite soil. This organism was able to degrade polyethylene and the enzymes responsible for this degradation was identified as laccase .

1.3 Enzymes

Enzymes are biomolecules able to catalyze a variety of chemical reactions such as hydrolysis, polymerization (bond formation), functional group transfer, oxidation, reduction, isomerization and dehydration. Enzymes and chemical catalysts increase the rate of reaction and lowering the activation energy. The enhancements of enzyme-catalyzed reactions are typically several orders of magnitude over background, and take place under mild condition. Unlike most chemical catalysts, enzymes are able to catalyze specific and often different chemical transformation in aqueous solutions at room temperature and

atmospheric pressure (Tao and Cornish, 2002). Furthermore, enzymes are generally environmentally friendly, economical and clean catalysts and they currently have commercial applications ranging from food or paper processing to fine chemical synthesis and diagnostic/research reagents(Wahler and Reymond, 2001).

Commercial sources of enzymes are obtained from three primary sources: animal tissue, plants and microbes. Enzyme manufacturers produce enzymes in accordance with all applicable governmental regulations. Most of the industrial enzymes are produced by a large numbers of microorganisms like *Aspergillus* and *Trichoderma* fungi, *Streptomyces imperfecti* and *Bacillus* bacteria. Yeasts are not good produces of extracellular enzymes and are rarely used for this purpose (Mojsov, 2014).

1.4 Multicopperoxidases (MCOs) family

Multicopperoxidases (MCOs) are a family of enzymes comprising laccases (EC1.10.3.2), ferroxidases (EC1.16.3.1), ascorbateoxidase (EC1.10.3.3) and ceruloplasmin. This family in turn belongs to the highly diverse group of blue copper proteins which contain from one to six cupper atoms per molecule and about 100 to1000 amino acid residues in the single peptide chain (Ryden and Hunt, 1993). MCOs have the ability to couple the oxidation of a substrate with a four-electron reduction of molecular oxygen to water. The electron transfer steps in these redox reactions are coordinated in two copper centres that usually contain four copper atoms. In a redox reaction catalyzed by an MCO, electrons from the substrate are accepted in the mononuclear centre (type1copper atom) and then transferred to the trinuclear cluster (one type 2 and two type 3 copper atoms), which serves as the dioxygen binding site and reduces the molecular oxygen upon receipt of four electrons (Ducros *et al.*, 1998; Hakulinen *et al*., 2002 ; Piontek *et al*., 2002). Yoshida

(1883) explained that laccases in the broader sense by far make up the largest subgroup of MCOs, originating from bacteria, fungi, plants and insects.

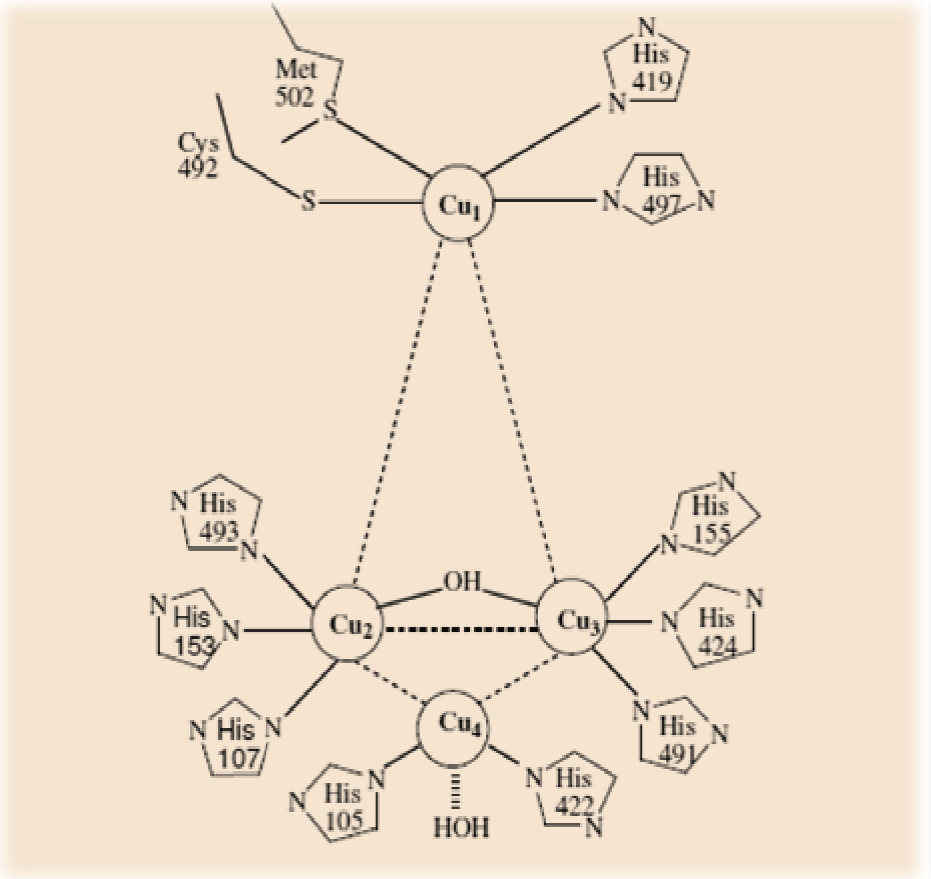
1.4.1 Laccase enzyme

Laccase nomenclature is defined in the Enzyme Commission (EC 1.10.3.2) indicates oxidoreductases EC 1.10 acting on diphenols and related substances as donors E.C.1.10.3 with oxygen as acceptor (Thurston,1994). Also laccase is a multicopper blue oxidase that couples the four electron reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, polyphenols, anilines, and even certain inorganic compounds by a one-electron transfer mechanism (Sakurai *et al.*, 1992 ; Xu *et al.*, 1996 ; Piontek *et al.*, 2002). Laccase was first discovered in the sap of the Japanese lacquer tree *Rhus vernicifera*, and its characteristic as a metal containing oxidase was discovered by Bertrand in 1985. Since then, laccases have also been found in various basidiomycetous and ascomycetous fungi and thus far fungal laccases have accounted for the most important group of multicopper oxidases with respect to number and extent of characterization (Giardina *et al*., 2010). Mayer and Staples (2002) stated that, the large quantity of laccases have been widely reported inside white-rot fungi. While, Claus (2003) mentioned that laccase in nature can be found in eukaryotes as fungi (principally in Basidiomycetes), plants, *Oscillatoria*, and insects. However, there has been increasing evidence for its existence in prokaryotes. Many researchers published the existence of corresponding laccase genes in the gram-negative and gram-positive bacteria (Hullo *et al.*, 2001; Kim *et al*., 2004). Pratheebaa *et al*. (2013) reported that laccase are gaining increasing attention due to their possible use in food and textile industries, pulp and paper manufacturing, wastewater treatment, bioremediation and nano-biotechnology. Recently, increasing interests have been put on the application of laccases as a new biocatalyst in organic synthesis because the environmentally friendly oxidation has become a very

important field in green chemistry (Aktas *et al*., 2000 ; Ciecholewski *et al*., 2005).

1.4.2 Structure of laccase

The laccase molecule, as an active holoenzyme form, often occurs as isoenzymes that oligomerize to form multimeric complexes (Flurkery, 2003). The molecular mass of the laccase monomer ranges in size from 50 to 110 kDa. The linked carbohydrates moiety can reach mass amounts between 10 and 45% of that of the entire molecule (Mayer, 1987). Claus (2004) and Baldrian (2006) explained laccase active site which consists of three copper centers that are classified according to their spectroscopic properties into Type I (T1), Type II (T2) and Type III (T3) (Figure 1-1).



Figure(1.1):Copper centres of *Bacillus subtilis* laccase (Enguita *et al*., 2003).

The T1 absorbs light at wavelength 610 nm and responsible for the blue cooler of the enzyme. The T2 copper is undetectable spectrophotometrically; however, it generates a characteristic electron-para-magnetic resonance (EPR) signal. The bi-nuclear T3 copper is diamagnetic; it exhibits a characteristic absorbance at 330 nm and displays a characteristic fluorescence spectrum (Shin and Lee, 2000). All these copper sites are indispensable for the four-electron reduction of dioxide with the release of an activated phenolic radical as intermediate (Quintanar *et al*., 2005).

1.4.3 Mechanism of laccase oxidation

The reaction mechanism of the phenolic oxidation via laccase is based on the activity of the four copper centres in the enzyme (Claus, 2003). The enzyme drives electrons from a reducing substrate to molecular oxygen. The catalysis of laccase occurs with reduction of one molecule of oxygen to water with the adjunct oxidation of one electron of a wide range of phenolic or non phenolic substrates (Gianfreda *et al*., 1999). The catalyzed free radical can be consequently oxidized to generate quinone species. Laccase catalysis occurs in three steps: (1) T1 Cu reduction by substrate; (2) electron transfer from T1 Cu to the trinuclear center composed of the T2 Cu and T3 Cu; (3) reduction of oxygen to water at the trinuclear cluster (Duran and Esposito, 2000 ; Claus, 2003). The reactivity of laccases has been directly related to their standard redox potential of the T1 site which is thought to play a major role in the performance of the enzyme and its substrate-specificity and the redox potential of T1 centers can vary widely between laccases from different sources (Shleev *et al*., 2004; Alcalde, 2007). Laccases are also able to oxidize substrates of lower or higher redox potential, when coupled with mediators resulting in a higher rate of substrate oxidation and broader substrate specificity (Alcalde, 2007). Gianfreda *et al*. (1999) mentioned that laccase coupled with mediators can thus be used to extend its substrate range to non phenolic compounds. So, laccase-catalyzed

reaction becomes a two-step process, where the mediator first is oxidized by the enzyme yielding an oxidized mediator and this later then reacts with the substrate (Figure 1-2) . The most common synthetic mediators are 1-hydro-xybenzotriazole (HOBT), N-hydro-xyphthalimide (NHPI), and 2,2 -azinobis-3-ethyl- thiazoline-6-sulfonat (ABTS) (Gochev and Krastanov, 2007).

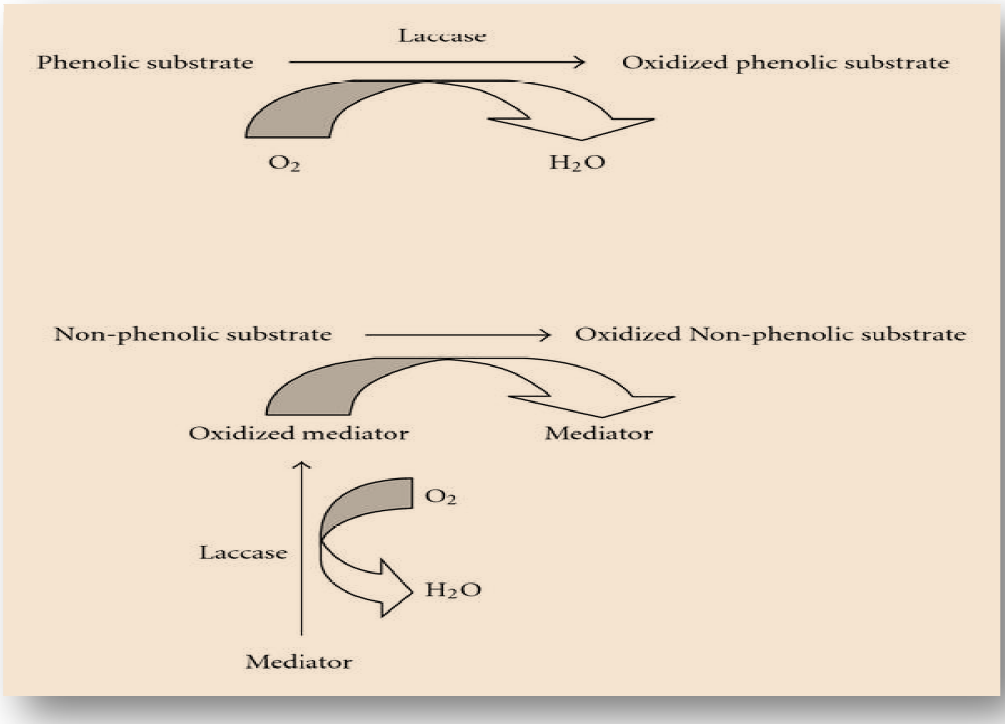


Figure (1.2):Mechanism of laccase action for both phenolic and non – phenolic substrates (Gochev and Krastanov, 2007).

1.4.4 Assay methods for laccase activity

The laccase activity shows a lack of strict substrate specificity, which allows the use of a wide range of substrates and detection techniques. Several techniques were reported in the literatures, such as measurement of oxygen consumption (Gigi *et al*., 1981), spectrofluorimetric method (Zuyun *et al*., 1998) and spectrophotometric estimation with various chromogenic substrates or products (Cantarella *et al*., 2003). The colorimetric techniques offer a reliable

sensitivity, with rapid and specific quantifiers of laccase activity, but it can be affected by organic solvent in the reaction, the pH and the stability of the product (Felby, 1998; Flurkey, 2003). It should be known that it is hard to specify the definition of laccase activity for two reasons including, the range of substrates oxidized by laccases which varies from one laccase to another and the presence of wide range of oxidative enzymes that can oxidize the same substrates (Alcalde, 2007). A suitable pH, substrate and buffer must be determined experimentally for each laccase and substrate used (Flurkey, 2003).

1.4.5 Occurrence of laccase enzyme

Laccase is most widely distributed in a wide range of higher plants and fungi (Benfield *et al*., 1964) as well as in bacteria (Diamantidis *et al*. 2000). Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine, 1965). Also, it has been detected in various plant species, including lacquer, mango, mung bean, peach, pine, prune, and sycamore, etc. Laccase is also present in a dozen of insects genera that include *Bombyx, Calliphora, Diploptera, Drosophilia, Lucilia, Manduca, Musca, Oryctes, Papilio, Phormia, Rhodnius, Sarcophaga, Schistocerca,* and *Tenebrio* (Xu, 1999).

Although laccase enzymes are distributed in plants and insects. Laccase activity has been reported in few bacteria, including *Azospirillum lipoferum, Marinomonas mediterranea, Streptomyces griseus,* and *Bacillus subtilis* (Octavio *et al*., 2006) and the first indication that laccases may be present in bacteria was based on the phenol-oxidase activity observed in *Azospirillum lipoferum* about 20 years ago (Givaudan *et al*., 1993). On the other hand, Assavanig *et al*. (1992) mentioned that most of the laccase was isolated from higher fungi. However, laccase from *Monocillium indicum* was the first laccase to be characterized from an ascomycete showing peroxidative activity (Thakker

et al., 1992). Most common laccase producers are the wood rotting fungi *Trametes versicolor, T. hirsuta, T. ochracea, T. villosa, T. gallica, Cerena maxima, Coriolposis polyzona, Lentinus tigrinus, Pleurotus eryngii,* and *P.ostreatus* etc. (Morozova *et al*., 2007).

1.5 Optimum conditions for laccase production

Laccase production under different fermentation conditions is influenced by physical and chemical environment of cultural conditions ,such as quality and quantity of carbon and nitrogen source, pH, temperature, aeration, presence of inducers, vitamins, amino acids, phosphorus and metal ion. For effective laccase production, it is essential to optimize simultaneously the culture conditions and composition of media. Therefore, the optimum conditions for laccase production should be investigated for a cost effective commercial production (Dhawan and Kuhad, 2002; Gnanamani *et al* ., 2006 ; Liang *et al.,* 2012).

1.5.1 Carbon and nitrogen source

Major factors that can affect laccase production include the concentrations and the types of carbon and nitrogen sources in the culture media (Xiao *et al*., 2004). Glucose, mannose, maltose, fructose and lactose are the commonly used carbon sources, whereas yeast extract, peptone, urea, ammonium sulfate and sodium nitrate are the commonly used nitrogen sources (Shraddha *et al*., 2011).The concentration of these nutrients is also determinant for the level of enzyme production, The carbon sources in the medium play an important role in ligninolytic enzyme production and the diversity of carbon sources is as large as the number of reported laccase producers. Among them, glucose is the easiest carbon source for laccase production. Although it has been found to enhance the production in *Galerina* sp., but it repressed the production of laccase in *Trametes pubescens* and *Phlebia* sp.(Galhaup *et al*., 2002, Arora and Rampal,

2000). Monteiro and De-Carvalho (1998) reported high laccase activity with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7:8 g/g). Revankar and Lele (2006) were studied the effect of different nitrogen sources, (yeast extract, peptone, asparagines and ammonium sulfate) on laccase activity. Among the nitrogen sources selected, ammonium sulfate resulted in the minimum laccase activity, whereas the maximum activity was obtained using yeast extract.

1.5.2 Initial pH for laccase production

The optimum pH for enzyme activity is an important criteria for any enzyme because it depends on many factors such as temperature, buffer nature, concentration of activators, inhibitors, the substrate and the presence of cofactors (Segel, 1976). Many researches showed that the optimal pH for laccase production depends on the substrate that is used to assay the enzyme activity. Usually, bacterial laccases are produced in range of pH 5-6, when the substrate is a hydrogen atom donor compound 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). When the substrate is a phenolic compound (e.g. syringaldazine), the optimal pH for production is shifted to 6-7. Researchers noted the shift in pH is a result of the balance of redox potentials between the substrate and the inhibition of the T2/T3 copper site by binding of an OH- ion. In general, laccase activity has a bell-shaped profile with an optimal pH that varies considerably. This variation may be due to changes in the reaction caused by the substrate, oxygen or the enzyme itself (Xu, 1997; Heinzkill *et al*., 1998).

Nyanhongo *et al*.(1998) was found that an initial pH of 7.0 was the best for optimal growth and laccase production by a newly isolated strain of *Tramete modesta*. The optimal pH for laccase production by *Geobacillus*

thermocatenulatus MS5 was ranged between 6.0-8.0, this result were recorded by Verma and Shirkot (2014).

1.5.3 Optimum temperature for laccase production

Incubation temperature plays an important role in the metabolic activities of microorganisms. However, laccase was active over a wide range of temperatures, typical optimum temperature range for laccase production was 35-45°C. The optimal temperature of laccase differs greatly from one strain to other. Increase and decrease in temperature lead to the gradual decrease in protein products (Peter and Vandana 2014). Adejoye and Fasidi (2009) were reported that maximum laccase production (51.5 ± 2.21 U/ml) in *Schizophyllum commune* was achieved at 28°C, while 60°C was reported as optimum temperature for production of laccase by *Ganoderma lucidum* (Ding *et al.* 2012).

1.6 Laccases purification

Protein isolation is endeavoring to purify a particular protein, from some biological (cellular) material, or from a bioproduct, since proteins are only synthesized by living systems. The objective is to separate the protein of interest from all non-protein material and all other proteins which occur in same material. Removing the other proteins is the difficult part because all proteins are similar in their properties. In an ideal case, where one was able to remove the contaminating proteins, without any loss of the protein of interest, clearly the total amount of protein would decrease while the activity (which defines the particular protein of interest) would remain the same (Clive, 2002).

Ammonium sulphate fractionation is being commonly employed from years to partially purify laccase from the cruds filtrate of their source. However, researches reported thus far have also been experimented with much efficient

methodologies such as membrane filtration techniques,various chromatographic techniques and have resulted in many commercially available purified froms of enzyme. A single-step purification procedure for *Neurospra crassa* laccase is using celite chromatograghy and obtained a specific activity of 338U mg^{-1} protein with 54% fold purification (Judewicz *et al*., 1998). In the others study, noted the laccase enzyme was purified from the *Geobacillus thermocatenulatus* bacteria extracellularly in three steps ; the ammonium sulphate (60-80)% precipitation, DEAE-cellulose and Sephacryl S200 gel filtration chromatography (Verma and Shirkot, 2014). Han *et al* . (2005) purified laccase from *Trametes versicolar* using ethanol precipitation, DEAE-sepharose, Phenyl-Sepharose and Sephadex G-100 chromatography and *Trametes versicolar* 951022 excretes a single monomeric lacaase showing a high specific activity of 91,443 U mg^{-1} for ABTS(2,20-azino-bis[3-ethylbenzothiazoline-6-sulphonicacid] as a substrate. In other study submitted by Khammuang and Sarnthima (2009) laccase was purified from fruiting bodies using ammonium sulphate precipitation with 40–70% saturation and DEAE cellulose chromatography then 1.34 and 3.07 fold purification is obtained, respectively.

1.7 Laccase characterization

1.7.1 Molecular weight

Many experimental reported the molecular weight of laccase is in the range of 50-97 kDa and an important feature is that a covalently-linked carbohydrate moiety (10-45% of total molecular mass) may contribute to the high stability of the enzyme (Desai and Nityanand, 2011).

Ruijssenaars and Hartmans (2004) determined a molecular weight for purified laccase by SDS-PAGE from *B .subtilis* to be 55kDa. A molecular weight of 56kDa for a laccase from *Bacillus halodurans* and 50 kDa from a *Bacillus sp*. HR03 has been reported by Bains *et al*. (2003). From few years

ago, Mohammadian *et al.* (2010) showed a molecular weight of 65 kDa for a laccase from *Bacillus sp*. Also, Reiss *et al.* (2011) estimated a molecular weight of 58 kDa for a purified Cot A -type laccase from *Bacillus pumilus* . On the other hand, the molecular weight of purified laccases from *Trametes* sp. was found to be approximately 66 kDa through calibrated gel filtration and SDS-PAGE (Cordi *et al*., 2007) .

1.7.2 PH effects on laccase activity and stability

Since enzymes are proteins, they are very sensitive to change in pH. Each enzyme has its own optimum range for pH where it will be most active. The result of pH effect on a combination of factors: (1) the binding of enzyme to substrate, (2) the catalytic activity of the enzyme, (3) the ionization of the substrate, and (4) the variation of protein structure (Clive, 2002). Hydrogen ion concentration affects enzyme by two ways: The first is that activation energy of enzyme combine with ionic state for active site and the change in pH can affect ionization of active site groups, moreover it affect substrate if it contains ionizing groups and their ability of binding with the active site, while the second way is that the change in ionizing groups can cause a change in the tertiary structure of the enzyme and the change in pH may cause denaturation. Although some enzymes tolerate big changes in pH, but most enzymes are active in a limited range of pH, and the best pH for enzyme activity is called optimum pH (Mckee and Mckee, 1996). Nevertheless, acidic and alkaline optimum pH values have also been observed for polyphenol oxidases from bacterial species such as *Bacillus thuringiensis* (pH 9.0) (Liu *et al*., 2004), *Bacillus licheniformis* pH 4.2 (Koschorreck *et al*., 2008) and *Thermomicrobium roseum* (pH 9.5) (Kong *et al*., 2000). Laccases from *Streptomyces psammoticus* and *S. ipomoea* showed unusually high activity at the slightly alkaline pH values 7–8 (Niladevi *et al*., 2008; Molina-Guijarro *et al*., 2009). Hublik and Schinner (2000) founded that laccase from *Pleurotus ostreatus* have an optimum pH for activity 5.8 with

syringaldazin as substrate ,while the range of pH stability was between 5-9 . In another study, Stoilova *et al*. (2011) were proved that optimum pH for laccase activity produced by *Trametes versicolor* was 4.5 , while the pH stability range was 4-5.

pH stability studies have been carried out with polyphenol oxidases from other bacteria. such as thermophilic *Bacillus* sp. in which the laccase enzyme retained more than 80% of its activity in the pH range of 5-8, however lost 40% of its activity at pH 9. This enzyme was found to be stable as it retained most of its activity through a broad range of pH after 1.5 hour incubation period (Güray, 2009) . *Thermomicrobium roseum* polyphenol oxidase retained more than 70% activity in the pH range of 8.5-10.0 but lost approximately 75% of activity below pH 6.0 and above 11.0 upon incubation in various buffers at 4 ºC for 20 hours(Kong *et al.* 2000).

1.7.3 Temperature effects on laccase activity and stability

Functional studies of temperature and enzyme linked properties have defined as " enzyme temperature optimum" which is being derived from a complex mixture of both activity and thermal stability effects, and dependent on assay duration. Consequently, it is of limited value for measuring enzyme temperature adaptation (Lee *et al*., 2007). In other word all enzymatic reactions are affected by temperature where the rise in temperature accelerate the reactions which comes from the increase in molecules number that contain enough energy to enter transition state, the reactions rate of the enzymatically activated reactions also increases by temperature increase, and because they are protein molecules they denatured in high temperatures (Mckee and Mckee, 1996). Enzyme stability depends on many factors such as the nature of the enzyme protein, concentration of the substrate, affinity of the enzyme interaction with substrate, pH of the buffer, the presence of activators and

inhibitors, and incubation period (Fullbrook, 1983). The maximum temperature was recorded to be 92ºC for *Thermus thermophilus* laccase (Miyazaki, 2005), 85ºC with *Bacillus licheniformis* laccase (Koschorreck *et al*., 2008), 75ºC with *Bacillus thuringiensis* and CotA protein of *Bacillus subtilis* (Liu *et al*., 2004; Martins *et al*., 2002), 70ºC with *Thermomicrobium roseum* (Kong *et al.*, 2000), and 55ºC with both *Bacillus* sp. HR03 (Dalfard, *et al*. 2006) and γ-*proteobacterium* JB (Bains *et al*., 2003). Temperature stability of hyperthermophilic laccase from *Thermus thermophilus* was found to be resistant to incubation at 85ºC for 10 minutes, also the enzyme retained two-thirds of its activity at 100ºC for 10 minutes (Miyazaki, 2005). Laccase from *Bacillus thuringiensis* was most stable at 75ºC (Liu *et al.*, 2004). Also, *Thermomicrobium roseum* laccase was very stable between 30-70ºC with 10 minutes incubation period (Kong *et al.*, 2000). While, laccase isolated from *Ganoderma lucidum* showed optimum temperature of 20-25°C and was found to be stable between 10- 50°C for 4 hours (Ko *et al*., 2001).

1.7.4 Heavy metals and some chemicals agents effect on laccase activity

Given the natural occurrence of laccases in soil, the inhibition by heavy metals and humic substances must be taken into account (Zavarzina *et al*., 2004). Heavy metals normally found in the environment, might affect the stability or activity of enzymes (Couto *et al*., 2005). The role of copper in the enhancement of laccase activity has been well demonstrated in both fungi and bacteria (Givaudan *et al.*, 1993). Niladevi (2008) noticed that the purified laccase enzyme from *Streptomyces psammoticus* activity was enhanced by metal ions such as Fe^{+2}, Cu^{+2}, Zn^{+2}, Na^{+2} and Mg^{+2} (each at 2 mM) while the heavy metals like Hg^{+2}, Cd^{+2}, Co^{+2} and Ni^{+2} reduced the activity. Other study submitted by Sadhasivam *et al* .(2008) showed that the metals such as Co^{+2} , Hg^{+2} ,Fe^{+2}, K^{+2}, Mg^{+2}, Mn^{+2}, Na^{+2}, Ba^{+2} and Ca^{+2} at a concentration of 1mM had no significant effect over *Trichoderma harzianum* WL1 laccase activity except

Hg ,which caused 17.2% inhibition .The purified laccase from the edible mushroom *Lentinula edodes* was inhibited in the presence of 1mM Sn^{2+}(99%), Ca^{2+}(70%), Zn^{2+}(64%), Hg^{2+}(55%), K^{+}(54%) and Cd^{2+}(45%) (Nagai *et al*., 2002).

Sodium azide is a common inhibitor of metalloproteins and laccase inhibition by this compound has been well established. Sodium azide has been reported to prevent the substrate oxidation by laccase (Johannes and Majcherczyk, 2000). Ryan *et al.* (2003) founded that laccase activity was completely inhibited by sodium azide (NaN3)at the concentration of 9mM. It was reported that the binding of sodium azide to the types 2 and 3 copper sites and disrupts electron transfer ,thus inhibiting the activity of *Sclerotium rolfsii* laccase .On the other hand, EDTA is another well known metal chelating agent fully inhibited the purified laccase at 2 and 5 mM concentrations. Similar results have also been reported from *Streptomyces cyaneus* (Arias *et al.*, 2003). Interestingly, the presence of 1mM EDTA barely effected the action of polyphenol oxidase and the enzyme showed 95% of its activity. However laccase from *Streptomyces griseus* and *Bacillus thuringiensis* showed 67% and 72% of their activity in the presence of EDTA with same concentration, respectively (Endo *et al.*, 2003, Liu *et al.*, 2004). In contrast to inhibitory effect, the activator effect of EDTA on *Bacillus thuringiensis* laccase, in the concentration range of 200-400mM, have been reported (Liu *et al*., 2004).

1.8 Applications and uses of laccases

Currently, the catalytic properties of laccases are being exploited for a range of biotechnological applications, thus studies on laccase-producing organisms have been intensified (Niladevi and Prema, 2007). Laccases have attracted scientific attention due to their application in diverse industrial sectors such as the paper and pulp industry, removal of xenobiotics from waste streams,

stabilization of fruit and vegetable juices, oxidation of phenolics in wine, denim washing, textile dye decolourisation, dye bleaching, lignin bleaching, and bleaching of cork for bottled wine (Mayer and Staples, 2002; Niladevi and Prema, 2007; Ergül, *et al*., 2009). Most of the varied uses of laccase can be attributed to the ability of the enzyme to produce a free radical from a suitable substrate. The consequent secondary reactions are responsible for the versatility of laccases in producing many different products (Mayer and Staples, 2002). Researchers have focused on the biochemical properties of laccases, on their applications in biotechnological and bioremediation processes, and their use in chemical reactions (Riva, 2006). The main limitation to the use of these 'green' enzymes (laccases use air and produce water as the only by-product) has been their limited availability (Nicotra *et al*., 2004). Increased knowledge of these enzymes will encourage laccase-based industrial bioprocesses in the future. Since, oxidative biocatalytic systems can provide a means to controlled and predictable formation of polymers, a feat that remains challenging in organic synthetic chemistry, and thus laccases are receiving attention as an alternative to conventional synthetic chemical approaches (Burton, 2003; Riva, 2006). In addition to a wide substrate specificity: laccase does not require cofactors; its cosubstrate is oxygen, which is usually present in its environment; they are often produced extracellularly, making purification procedures easy; they are stable in the extracellular environment; and their inducible expression allows for their application in bioprocesses (Baldrian, 2006). Kunamneni *et al*. (2007) studied that laccases can be use in biosensors to detect various phenolic compounds, detection of morphine and codeine, catechol amines, plant flavonoids and also for electro immunoassay have been developed. Abdulah (2011) found that crude laccase which extracted from the fungal culture of Pleurotus *sapidus* was able to degrade aflatoxin B1(AFB1) and its ability of degradation increase with increasing the incubation period, and the most effective degradation of AFB1 was obtained after 72hr of incubation.

1.8.1 Enzyme Immobilization

Enzyme immobilization is a technique designed to restrict the freedom of enzyme movement by it fixation in or into a support (Cao, 2006).

Immobilization provides a physical support for enzymes. There are five principle immobilization methods including adsorption, covalent binding, entrapment, encapsulation and cross-linking. The key properties of immobilization techniques that should be considered for the selection of a support and method of immobilization of a specific enzyme, includes the physical and chemical properties of the support, its stability, resistance, safety, cost and reactivity (Bickerstaff, 1997). Straathof *et al*. (2002) estimated that only 20% of biocatalytic processes involve immobilized enzymes. Initially, the main challenge was to find suitable immobilization methods to allow multiple uses of enzymes for the same reaction. With the advancement in immobilization techniques, the focus has shifted to the development of modulated enzymes with the desired properties for certain specific applications. Immobilization has its associated advantages of allows for multiple, repetitive, or continuous use and has minimum reaction time, high stability, improved process control, multi enzyme system, easy product separation, while it is less labor intensive and more cost effective, safe to use, and environmentally friendly (Polizzi *et al* ., 2007).

1.8.2 Immobilization method and supports

a. Adsorption

Imobilization by adsorption is the simplest immobilization method. It involves reversible surface interaction between enzyme and support material (Bickerstaff, 1997). Enzyme immobilization via non-covalent interactions can be either nonspecific or specific. The nonspecific adsorption is a physical

immobilization that involves hydrogen bonding, van der Waals forces, or hydrophobic interactions, whereas the specific one involves ionic binding, hydrophobic adsorption and affinity binding (Guisan, 2006). Immobilization by adsorption is a mild, easy to perform and reversible process that preserves the catalytic activity of the enzyme; in fact, the nature of the forces involved in non-covalent immobilization can be reversed by changing the conditions that influence the forces of interaction such as the pH, ionic strength, temperature or polarity of the solvent (Cao, 2006; Guisan, 2006).

b. Covalent Bonds

Immobilizations of proteins by methods based on the formation of covalent bonds are among the most widely used. These methods form stable and irreversible bonds between the enzyme and the support material. Multipoint attachment can result in a more rigidly immobilized enzyme and thus in a more stable biocatalyst. The bonds involve specific functional groups that are suitable for the participation in covalent bond formation including, the amino (NH2), carboxyl (COOH), hydroxyl (OH) and sulfydryl group (SH) (Cao, 2006). Bickerstaff (1997) and Guisan (2006) have been stated that a low catalytic activity can be observed after covalent immobilization due to the involvement of the active site in the covalent linkage to the support. The use of analogue substrates might be a simple procedure that could improve the activity yield. A variety of support materials had been used for covalent binding and the extensive range of supports available reflects that no ideal support exists. Also, additional matrix modification or activation had been also employed for enzyme immobilization.

c. Cross-Linking

Enzyme immobilization via cross-linking is a support-free immobilization that involves joining the enzymes to each other to form a large three-

dimensional complex structure, this can be achieved by chemical or physical methods (Bickerstaff, 1997). The bond formation between the enzymes is accomplished by the addition of a bi- or multifunctional reagent, such as glutaraldehyde and toluene diisocyanate. The immobilization via cross-linking of enzyme molecules with a bifunctional agent limit the dilution of the catalytic activity resulting from the introduction of large proportion of noncatalytic mass that play the role of the support in the other immobilization techniques (Guisan, 2006). However, this method had several drawbacks, such as low activity retention, poor reproducibility, low mechanical stability and difficulties in handling the gelatinous cross linked enzymes (Bickerstaff, 1997; Sheldon, 2007).

d. Entrapment

Enzyme entrapment refers to the process by which the enzyme is confined in a matrix formed by chemical or physical means such as cross-linking or gelation. The entrapment matrix is usually formed during the immobilization process. The enzyme molecules can be physically embedded or covalently linked to the matrix. Thus, entrapment can be also classified as covalent entrapment and physical entrapment (Cao, 2006). The matrix can be prepared by polymerization using unsaturated monomers and co-monomers as cross-linker; the polymerization of the matrix can be irradiation-initiated, photo or chemically initiated. The enzyme entrapment will allow the transfer of substrate and product, while the enzyme is retained into the matrix. Although the enzyme molecules are free within the polymer, the entrapment can lead to serious substrate and product diffusion limitation; thus reducing the apparent activity of the enzyme. On the other hand, the mass transfer limitation can have a useful advantage by limiting the mass transfer of desactivators and inhibitors. Depending on the application and the method of entrapment, the geometric

properties of the entrapped enzymes can be easily adapted in various forms such as beads, films, and fiber (Bickerstaff, 1997; Sheldon, 2007).

e. Encapsulation

Enzyme encapsulation is an entrapment-like immobilization but within spherical membrane acting like a semi permeable barrier that permits the diffusion of the substrate and product, yet it limit the movement of the enzyme (Cao, 2006). The pores of the membrane must be smaller than the size of the enzyme molecules and the diffusion limitation is seriously affected with the enzyme encapsulation as compared to other enzymes immobilization techniques. On the other hand, several materials have been used to prepare capsules of variable diameters ranging from a few to hundreds of micrometers (Smidsrød and Skjåk-Bræk, 1990). Gibbs *et al.* (1999) stated that several methods can be used for the preparation of the enzyme encapsulation including the interfacial processes, phase inversion methods, template leaching, and post-loading encapsulation. The encapsulated enzyme can be used in numerous applications such as in food industry, medicine and agriculture.

1.8.2 Immobilization of Laccase

Efforts have been made to immobilize laccase on solid supports, in order to enhance its industrial utility by increasing its activity, stability and half-life time. An extensive review on laccase immobilization was presented by Duran *et al*. (2002) summarizing all the immobilization methods employed, the various supports used and their potential applications. Various supports types were used for the laccase immobilization, including Sepharose, alumina oxide, alginate beads, Silica gel, DEAE cellulose and glass beads. Laccases have been immobilized using different immobilization methods including adsorption, covalent binding, entrapment, encapsulation and cross-linking (Bryjak *et al*., 2007).

Laccase enzyme produced from *Sterptomyces pasmmoticus* was partially purified by ammonium sulphate precipitation and immobilized in alginate beads by entrapment method using calcium and copper. The copper alginate beads proved a better support for laccase immobilization by retaining 61% of activity when compared to calcium alginate beads which retained 42.5% of laccase activity (Niladevi and Prema, 2008).

Chapter Two

Materials

and

Methods

2.Materials and Methods

2.1 Materials

2.1.1 Apparatus and equipments

The following apparatus and equipments were used in this study:

Apparatus	Company/Orgin
Autoclave	Express / Germany
Compound light microscope	Olympus / Germany
Cooling centrifuge	Harrier / Uk
Distillator	GFL / Germany
Fraction collector	Bio-Rad / Italy
Hot plate with magnetic stirrer	Gallenkamp / UK
Incubator	Gallenkamp
Laminar air flow	Memmert / Germany
Mircopipette	Brand / Germany
pH-meter	Metter-Tolledo/ UK
Refrigerator	Beko / Turkey
Sensitive balance	Delta Range / Switzerland
Shaker incubator	GFL
U.V/ visible spectrophotometer	Shemadzu / Japan
Vacuum pump	Value / China
Vortex	Buchi / Switzerland
Water bath	Gallenkamp

2.1.2 Chemicals

The following biological and chemical materials were used in this study:

Material	Company\ Origin
Acetic acid	BDH/England
Agar	Himedia/ India
Ammonium acetate	BDH
Ammonium chloride	BDH
Ammonium presulfate	BDH
Ammonium sulfate	BDH
Bovin serum albumine	BDH
Bromophenol blue reagent	Himedia
Comassie birlliant blue-G250	BDH
Copper sulphate	BDH
Cystine	Sigma/USA
DEAE-Cellulose	Whattman/England
EDTA	Himedia
Ethanol 96%	BDH
Gelatin powder	Himedia
Glucose	BDH
Glycerol	BDH
Gram stain set	BDH
Hydrogen peroxide	BDH
Iso butanol	Fluka /Switzerland
KH_2po_4	Sigma
Lactose	Himedia
Magnesium chloride	BDH
Mercaptoethanol	BDH
Mercury chloride	Himedia
Methanol 95%	Fluka
Na_2Hpo4	Sigma
Peptone	Himedia
Sephacryl-S-300	Pharmacia Fine Chemical/Sweedan
Sodium acetate	BDH
Sodium azide	Himedia
Sodium chloride	BDH
Sodium nitrate	BDH
Starch	Himedia

Sucrose	Himedia
Syringaldazine	Sigma
TEMED	Sigma
Tris-Hcl	Himedia
Yeast extract	Himedia

2.1.3 Media

2.1.3.1 Readymade media

The following media were used in this study and prepared according to the instructions on their containers by the manufacturing companies and sterilized by autoclaving at 121°C for 15 min. :-

Medium	Company/ Origin
MR-VP	Himedia/India
Nitrate agar	Himedia
Nutrient agar	Himedia
Nutrient broth	Himedia
Simmon citrate	Himedia
Urea agar base	Biolife/Italy

2.1.3.2 Laboratory prepared media

All media were prepared according to the related references after they were brought to boiling to dissolve the constituents completely, sterilized by autoclaving at 121℃ for 15 min., then poured in sterile petri dishes and incubated at 37 ℃ for 24 hours to ensure the sterilization .

a. Skim milk agar (Sneath *et al*., 1986)

This medium was prepared by dissolving 5 g of skim milk in 50 ml distilled water (D.W.) and sterilized by autoclaving, then 2 g of agar were dissolved in 50 ml D.W. and sterilized by autocalving, cooled to 45°C, mixed together then distributed into sterilized plates.

b. Gelatin (Cruickshank *et al*., 1975)

This medium was prepared by dissolving 12g of gelatin in 100 ml of nutrient broth medium, then distributed in tubes and sterilized by autoclaving.

c. Peptone Water (Atlas *et al*., 1995)

This medium was prepared by dissolving peptone (20g) and sodium chloride (5g) in 950 ml D.W.; pH was adjusted to 7.0, then volume was completed up to 1L with D.W., mixed thoroughly, distributed into tubes and sterilized by autoclaving.

d. Urea agar (Collee *et al*., 1996)

It was prepared by adjusting pH of the urea agar base (Christensens media) to 7.0 before autoclaved, then cooled to 50°C, and 50 ml of 40% urea (sterilized by filtration) was mixed together and distributed into sterilized tubes and let solidfied as slant.

e. Luria- Bertani (LB) (Nazina *et al*., 2001)

This medium was prepared by dissolving tryptone (10g), yeast extract (5g), and NaCl (5g) in 950 ml D.W., pH was adjusted to 7.0, then the volume was completed to 1L with D.W. and sterilized by autoclaving.

f. Starch agar (Zimbro *et al*., 2009)

This medium was prepared by dissolving 10g of soluble starch in 1000 ml of nutrient broth media,. sterilized by autoclaving.

h. Minimal salt medium (Sambrook *et al.*,1989)

This medium was composed of the following basal salts

Components	Concentration(g/L)
KH_2Po_4	4
Na_2Po_4	4
NaCl	0.5
NH_4Cl	2

All components were dissolved in 950 ml of distilled water, pH was adjusted to 7.0, the volume was completed to 1 L with D.W and sterilized by autoclaving. Then aseptically, 2ml of sterile 1.0M $MgSo_4$ solution was add.

2.1.4 Reagents and Dyes

2.1.4.1 Catalase reagent (Atlas *et al*., 1995)

Catalase reagent was prepared to be consist of 3% hydrogen peroxide.

2.1.4.2 Oxidase test reagent (Atlas *et al*., 1995)

This reagent was freshly prepared by dissolving 1 g of tetramethyl-p-phenylenediamine dihydrochloride in 100 ml D.W. and stored in dark bottle until use.

2.1.4.3 Methyl red indicator (Collee *et al*., 1996)

This indicator was prepared by mixing the following components

Methyl red	0.05 g
Ethanol (95% v/v)	150 ml
D. W.	100 ml

2.1.4.4 Barritt's reagent (Collee *et al*., 1996)

This reagent was consisting of two solutions:

- **Solution A**: Potassium hydroxide (40%)
- **Solution B**: Prepared by dissolving 5 g of α-naphthol in absolute ethanol to make a final volume of 100 ml.

Equal volumes of solution A and solution B were immediately mixed before use.

2.1.4.5 Nitrate test reagent (Atlas *et al*., 1995)

This reagent was consisting of two solutions:

- **Solution A:** It was prepared by dissolving 0.8 g of sulfanilic acid in 5N acetic acid to make a final volume of 100 ml.

- **Solution B:** It was prepared by dissolving 0.5 g of Dimethyl-α-naphthylamine in 100 ml of 5N acetic acid.

  Equal volumes of solution A and B were immediately mixed before use.
- Zinc Dust

2.1.4.6 Kovac's reagent (Atlas *et al*., 1995)

This reagent was prepared by dissolving 0.5g of ρ-Dimethyl-aminobenzaldehyde in 75 ml isoamyl alcohol with heating in a water bath at 50°C and 25 ml 37% HCl was added slowly. The reagent was prepared in small quantities and stored in dark botle at 4℃.

2.1.4.7 Gram stain compounds

Stains and reagents were prepared according to Atlas *et al*.,1995

2.1.4.8 Bromophenol blue(0.25%) (Blackshear,1984)

It was prepared by dissolving 0.25 g of bromophenol blue in D.W. up to 100ml, mixed until dissolved completely.

2.1.5 Buffers and Solutions

2.1.5.1 Laccase assay solution (Rid, 1980)

a. Potassium phosphate buffers(100mM) pH 6.8

It was prepared by dissolving 1.3 g of monobasic potassium phosphate in 100 ml of D.W.

b. Syringaldazine solution (0.216mM)

It was prerpared by dissolving 0.007 g of syringaldazine in 100 ml of 95% methanol.

2.1.5.2 Protein assay solution (Bradford, 1976)

a. Coomassie Brilliant Blue G-250 solution

It was prepared by dissolving 0.1 g of coomassie brilliant blue in 50 ml of ethanol 95%, then 100 ml of 85% phosphoric acid was added under cold condition, and the volume was completed to 1litter with D.W.

b. Tris-HCl buffer (0.1M) and pH 8.0

c. Bovine Serum Albumin (BSA)

It was prepared by dissolving 10 mg of BSA in 10 ml of Tris-HCl buffer pH 8.

2.1.5.3 Laccase purification solution

a. Sodium hydroxide (0.25 M)

This solution was prepared by dissolving 4 g of sodium hydroxide in suitable amount of D.W, then the volume was completed to 500 ml with D.W.

b. Hydrochloric acid (0.25 M)

It was prepared by adding 20.8 ml of concentrated hydrochloric acid to suitable volume of D.W., then the volume was completed to 1 liter with D.W.

c. Sodium chloride (0.25 M)

It was prepared by adding 14.25 g of sodium chloride to suitable volume of D.W., then the volume was completed to 1 liter with D.W.

2.1.5.4 Polyacrylamide gel electrophoresis solutions (Laemmli, 1970)

- **Stacking gel solution (0.626 M)**

This solution was prepared by dissolving 7.56 g Tris base in 40 ml of D.W., pH was adjusted to 6.8, then the volume was completed to 100 ml with D.W.

- **Resolving gel solution (1.5 M)**

It was prepared by dissolving 22.7g of Tris base in 80 ml of D.W., pH was adjusted to 8.8, then the volume was completed to 100 ml with D.w.

- **Sodium dodecyl sulphate (SDS) Solution (10%)**

It was prepared by dissolving 10 g of SDS in 90 ml of D.W., then the volume was completed to 100 ml with D.W.

- **Resolver buffer (Tris-Glycin)**

It was prepared by dissolving 3.0 g Tris base, 14.4 g Glycin in 900 ml of D.W. , then 10 ml of SDS (10%) was added, and the volume was completed to one liter with D.W.

- **Acrylamide-bisacrylamide solution**

It was prepared by dissolving 30 g of acrylamide and 0.8 g bisacryamide in 60 ml of D.W., then the volume was completed to 100 ml with D.W. and kept in a dark bottle in refrigerator until use.

- **Ammonium presulfate solution (10%)**

This solution was freshly prepared by dissolving 1 g of ammonium presulfate in 10 ml of D.W.

- **N, N, N, N- tetramethylene diamine (TEMED)**

TEMED was used as mentioned by the manufacturer.

- **Fixing solution**

It was prepared from 40% methanol and 10% trichloracetic acid (TCA).

- **Destaining solution**

It was prepared from 40% methanol and 10% acetic acid.

- **Sample buffer solution (2X)**

It was prepared by mixing 2 ml of staking gel buffer, 3.2 ml of 10% SDS, 1.6 ml glycerol, 0.8 ml of 2-mercaptoethanol and 0.4 ml of 0.25% bromophenol blue, mixed well and stored at -20°C.

2.1.5.5 Laccase characterization solutions

- Sodium acetate solution (50mM, pH 5-6)
- Monobasic potassium phosphate (100mM, pH 7-8)
- Tris- HCl (50mM, pH 9-10)

2.1.5.6 Chemicals agents and metal ion solutions

Each of inhibitors(EDTA, sodium azide and L-cystine) and metal ions ($CaCl_2$, $ZnSO_4$, $MgCl_2$, $CuSo_4$ and $HgCl_2$) was prepared alone by dissolving in D.W. as stock solution to give a final concentration of 10 mM, then 2 mM was prepared from each stock with D.W.

2.1.5.7 Agarose solution (3%)

It was prepared by dissolving 1.5g of agarose in 50ml of distilled water and dissolved by microwaves for 30 seconds .

2.2 Methods

The main steps of the method plan were summarized in scheme (2-1).

2.2.1 Sterilization Methods (Colline and Lyne, 1987)

The following sterilization methods were used in this study

- **Moist- Heat Sterilization (Autoclaving)**

  Media and solutions were sterilized by autoclaving at 121°C (15Ib/in2) for 15 min.

- **Dry-Heat Sterilization**

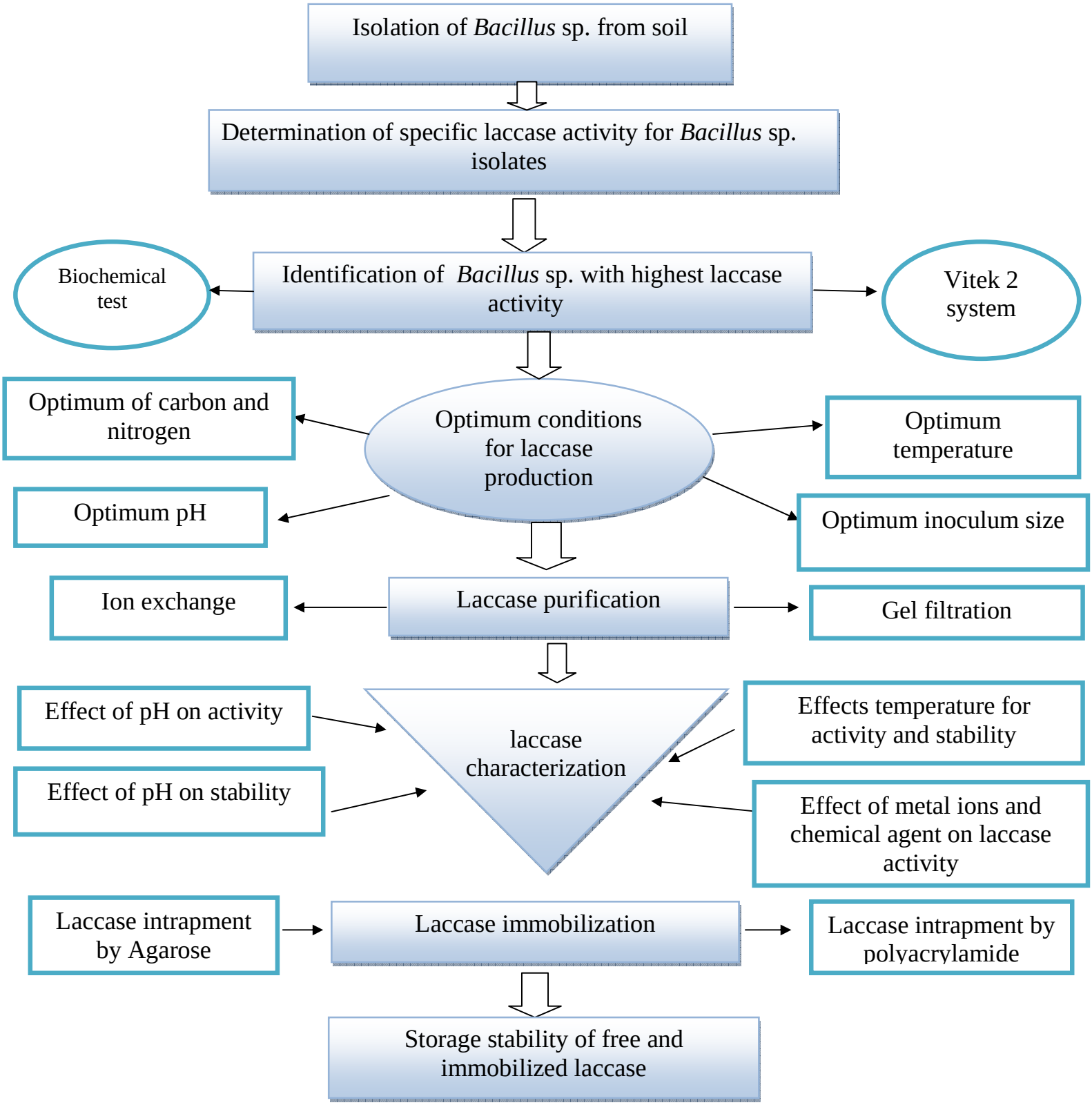
  Electric oven was used to sterilize glasswares at 160-180°C for 3-2 h.

- **Membrane Sterilization (Filtration)**

  Membrane filtration was used to sterilize heat sensitive solutions using millipore filters (0.22) µm.

2.2.2 Samples collection

For the isolation of *Bacillus* species, fifty five soil samples were collected from different locations in Baghdad city. A portion of 1g of each sample was suspended in 9 ml sterile D.W., agitated vigorously in shaker water bath at 37°C .Then serial dilutions for each sample using sterilized D.W. then 0.1 ml aliquot from appropriate dilution was spread on LB ager plates, incubated at37°C for 24h.



Scheme (2-1): Main steps of the methods used.

2.2.3 Identification of the isolates

In order to identify the selected isolates, the following examination were performed:

2.2.3.1 cultural and microscopic characteristics (Atlas *et al*., 1995)

colonies (size, shape, edge, color, and margin) of the bacterial isolates were studied. Then, a loopfull of each bacterial suspension was fixed on a slide, and stained by Gram staining method to examine Gram reaction, shape grouping, and spore forming of the isolated bacteria.

2.2.3.2 Biochemical tests

a. Catalase test (Atlas *et al*., 1995)

A single colony from each isolate was placed onto a clean glass of microscope slide with a sterile toothpick, then a drop of hydrogen peroxide (3%) was added onto the colony. Production of gaseous bubbles indicates a positive result.

b. Oxidase test (Atlas *et al*., 1995)

This test was done by using moistened filter paper with few drops of a freshly prepared solution of tetramethyl-ρ-phenylene diamine dihydrochloride. Aseptically, a clump of cells was picked up from the slant growth with a sterile wooden stick and smeared on the moistened paper. Development of a violet or purple color within 10 seconds indicates a positive result.

c. Methyl red test (Collee *et al*., 1996)

MR-VP broth were inoculated with a single colony of bacterial isolate and incubated at 37°C for 24 hours, then five drops of methyl red reagent was added and mixed gently. Presence of bright red color indicates a positive result.

d. Voges-proskauer test (Collee *et al*., 1996)

MR-VP broth were inoculated with a single colony of bacterial isolates and incubated at 37°C for 24-48 hours, then 1 ml of Barritt's reagent (2.1.4.4 A) and 3 ml of Barritt's reagent (2.1.4.4 B) were added to 5 ml of cultured broth and shacked for 30 seconds. Formation of pink to red color indicates a positive result.

e. Nitrate reduction test (Atlas *et al*., 1995)

Single colony of each bacterial isolate was used to inoculate nitrate ager media, then test tubes were incubated at 37°C for 24 hours, After incubation, 0.1 ml of the test reagent (2.1.4.5 A, B) was added to each tube. The immediate formation of red color indicates a positive result.

f. Indole test (Collee *et al*., 1996)

Peptone water broth was inoculated with a single colony of each bacterial isolates and incubated at 37˚C for 24 hours. Fifty micro litter of Kovac's reagent (2.1.4.6) was added and mixed gently. Appearance of a red ring on the surface of liquid media indicates a positive result.

g. Gelatin liquefaction test (Cruickshank *et al*., 1975)

Tubes of gelatin media were inoculated with a single colony of bacterial isolates and incubated overnight at 37℃ then transferred to refrigerator for an hour. Liquefaction of tubes indicates positive result.

h. Citrate utilization test (Atlas *et al*., 1995)

Simmon's citrate agar slants were inoculated with a single colony of bacterial isolates, and incubated for 24 hours at 37℃. Changing of color to royal blue indicates a positive result.

i. Urease test (Atlas *et al*., 1995)

Urease activity was detected by streaking Christensen urea agar slants with the bacterial growth and incubated at $37°C$ for 24 hours. Appearance of a red-violet color indicates a positive result.

2.2.3.3 Identification of bacteria by VITEK 2 system (Logan and Turnbull, 2003)

The VITEK2 is an automated microbiology system utilizing growth-based technology. The system accommodate colorimetric reagent cards that are incubated and interpreted automatically.

Suspension preparation:

A sterile swab was used to transfer a sufficient number of colonies of a pure culture and to suspend them into 3.0 ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to pH 7.0) in a 12 x 75 mm clear plastic (polystyrene) test tube. The turbidity is adjusted to (BCL 1.80-2.20) and measured using a turbidity meter called the DensiChekTM.

2.2.4 Maintenance of Bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*. (1982) as follows:

2.2.4.1 Short-Term Storage

Each of bacterial isolates was first prepared in Lb broth medium at 37℃ for 24h. then maintained for month on LB agar slants. They were tightly wrapped with parafilm, and then stored at 4°C.

2.2.4.2 Medium-Term Storage

Bacterial isolates were maintained as stab cultures for few months. Such cultures were prepared in small screw-capped bottles containing 2-3 ml of LB agar medium and stored at 4°C.

2.2.4.3 Long-Term Storage

Single colonies were cultured in LB broth and incubated for 24 h. then 8.5 ml of bacterial culture mixed with 1.5 ml of glycerol, and stored at -18℃ for a long time.

2.2.5. Determination of laccase activity (Rid, 1980)

A volume of 100 µl of fresh culture of each bacterial isolate was used to inoculate the 100 ml LB broth in a conical flask and incubated in a shaker incubator (150 rpm) at 37ºC for 24h. After incubation, the culture was centrifuged, pellets were discarded, and supernatants were taken and assayed for laccase activity. Activity of laccase was assayed by measuring oxidation of syringaldazine at 530nm for 10min.The assay mixture contained: 0.3ml of 0.216mM syringaldazine , 2.20ml of 100mM monobasic potassium phosphate buffer pH(6.5) and 0.5ml of laccase.

Laccase activity was calculated from the following formula:

Enzyme activity(U/ml) = $\frac{Sample\Delta A530nm = (Test - Blank(df))}{(0.001)(0.5)}$

df=dilution factor

0.001= the change in A530 nm/min. per unit of laccase at at 30℃ in pH 6.5 a 3 ml reaction mix.

0.5 = volume (in milliliters) of enzyme used .

Enzyme activity was expressed in units; 1U being defined as the amount of enzyme causing the formation of 1µmol of product per minute under the assay conditions used (Saito *et al*., 2003).

2.2.6 Determination of protein concentration

Protein concentration was determined according to the method described by Bradford, (1976) and as follow:

- Standerd curve of bovine serum albumin (BSA) was plotted by using different concentrations from the BSA stock solution (as prepared in item 2.1.5.2) according to the following volumes.

Table(2.2): Bovine serum albumin standard curve composition

BSA (µl)	Tris-HCL Buffer (µl)	Protein Amount (mg)	Final Volume (ml)
0	100	0	0.1
20	80	20	0.1
40	60	40	0.1
60	40	60	0.1

- Then 2.5 ml of Coomassi brilliant blue G-250 dye was added, mixed and left to stand for 2 min at room temperature.

- The absorbance at 595 nm was measured; the blank was prepared from 0.5 ml of Tris-HCl buffer and 2.5 ml of the dye reagent.
- A standard curve was plotted between the BSA concentrations against the corresponding absorbance of bovine serum albumin.
- Protein concentration was estimated by mixing 0.1ml of the tast sample, 0.4 ml of Tris-HCl and 2.5 ml of Coomasi brilliant blue G-250, left to stand for 2 min at room temperature then measuring the absorbance at 595 nm.

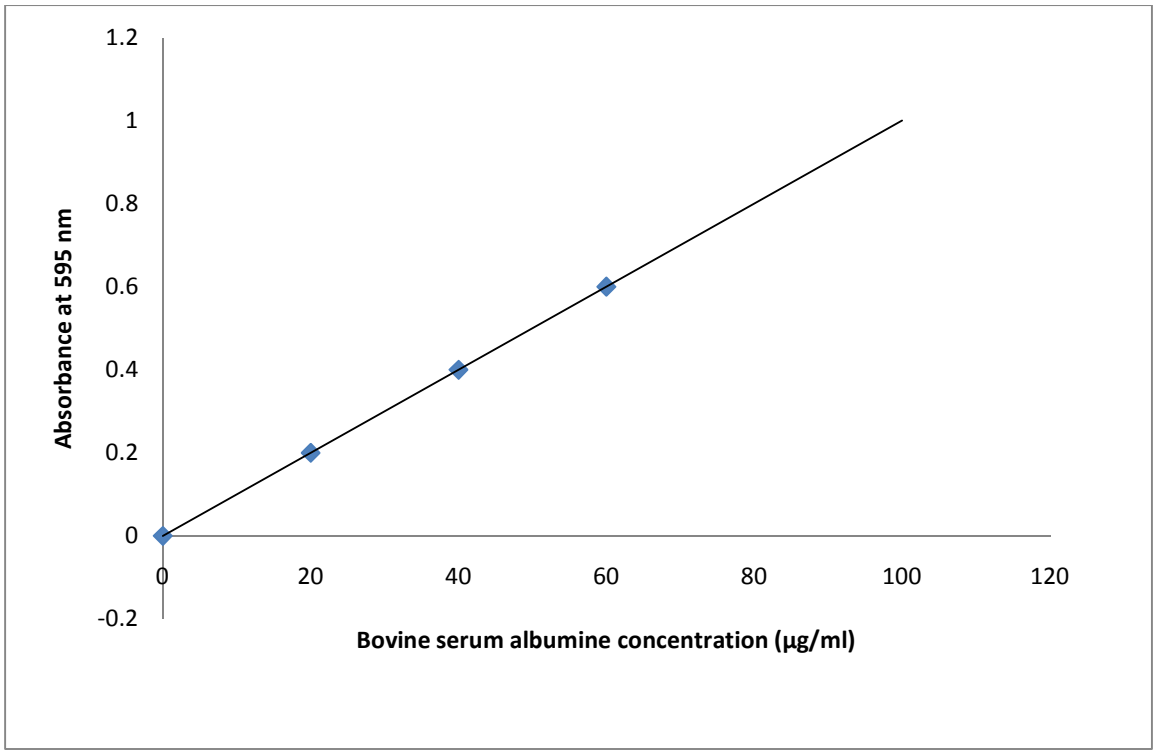


Figure (2.1): Bovine serum albumin standard curve

2.2.7 Optimum conditions for laccase production

The main optimal conditions for laccase production were performed in order to achieve higher production and as following;

2.2.7.1 Optimum carbon and nitrogen sources for laccase production

Various source of carbon (glucose, dextrose, sodium citrate and sucrose) and nitrogen (yeast extract, sodium nitrate, peptone and tryptone) were supplemented individually to the minimal medium. All these sources were added in the medium at a final concentration of 0.5% (w/v). The initial pH of the medium was set at 7.0. Laccase activity was determined in the supernatants after inoculation of the medium with 1% of the isolate *Bacillus sp.* B5log phase culture, and incubation at 37℃ for 24h.

2.2.7.2 Optimum pH

Optimal pH for production of laccase was determined by preparing the minimal salts medium with different pH values (6, 6.5, 7, 7.5, 8, 8.5, 9). Laccase activity was measured in the supernatant after inoculation of the production medium with the isolate *Bacillus sp.* B5log phase culture, and incubation at 37°C under shaking (200 rpm) for 24h.

2.2.7.3 Optimum incubation temperature

The isolate *Bacillus sp.* B5 was grown in the production medium and incubated at different temperatures (30, 35, 40, 45) °C under shaking (200 rpm) for 24h. Laccase activity was determined in supernatants after centrifugation at 6000 rpm for 10min.

2.2.7.4 Optimum inoculum size

Effect of different inoculum size of the selected the isolate *Bacillus sp.* B5 on laccase production was studied. This was achieved by inoculating the production medium, individually with inoculum sizes ranging between 10^2 ,10^3, 10^4, 10^5, 10^6, 10^7, $10^8$and 10^9cell/ml.

2.2.8 Laccase Purification

The laccase purification steps were carried out according to Bertus *et al.* (1989) and as follows:

2.2.8.1 Enzyme production and preparation of cell free filtrate

The isolate *Bacillus sp.* B5 was grown in the production medium under optimum conditions. Crude laccase filtrate was collected by centrifugation at 6000 rpm for 15 min. at 4℃, then 100 ml of the cell free filtrates containing laccase were collected and laccase activities and protein concentration were determined.

2.2.8.2 Ammonium sulfate precipitation

Ammonium sulfate precipitation was achieved by adding ammonium sulfate to the crude enzyme gradually with continuous mixing on ice a saturation ratio of 70%, the mixture was centrifuged at 6000 rpm for 20 min at 4°C. The resulted pellets was dissolved in 2 ml of potassium phoshpate buffer pH(6.5). Laccase activity and protein concentration were determined.

2.2.8.3 Dialysis of crude enzyme

The obtained ammonium sulfate precipitate (in solution) was introduced into dialysis tube (special plastic bag) with 10000 MWcutoff against 100M KH_2Po_4 buffer pH6.5, for 3 h., followed by dialysis against the same buffer over night. The obtained laccase enzyme preparation was kept at 4°C for further purification steps.

2.2.8.4 Purification by ion exchange chromatography

The dialyzed laccase was further purified by ion-exchange chromatography technique using DEAE-Cellulose column (2.5× 15) which prepared according to

Whitaker and Bernard (1972) by dissolving 20 g of resin in 1L of distilled water. Then beads were left to settle down and washed several times with D.W. until getting clear appearance. The suspension was filtered throughout Whattman No.1 using Buchner funnel under discharging. The resin was resuspended in 0.25 M sodium chloride and sodium hydroxide solution. The suspension was filtered again as mentioned above and washed several times with 0.25 M hydrochloric acid solution and next by distilled water before it was equilibrated with 100mM potassium phsophate pH6.5 buffer. After preparation, column was equilibrated with 100mM potassium phsophate buffer pH 6.5. The column was washed with an equal volume of the same buffer, while attached proteins were eluted with gradual concentrations of sodium chloride (0.1-0.9 M). Laccase enzyme was eluted at flow rate of 30ml/h (3 ml/fraction). Absorbance of each fraction was measured at 280 nm using UV-VIS spectrophotometer, then laccase activity was determined in each fraction as described in(2.2.5). Fractions presents laccase activity were pooled and kept for further steps of purification.

2.2.8.5 Purification by gel filtration chromatography `

The next step in the purification of laccase was achaived by gel filtration chromatography technique through Sephacryl S-200. It was prepared as recommended by Pharmacia Fine Chemicals Company. Aquantity of Sephacryl S-200 was suspended in100mM potassium phoshpate buffer pH6.5, degassed, and packed in a glass column (1.5×35 cm), and equilibrated with 100M KH_2Po_4 buffer pH6.5.

Elution was achieved at a flow rate of 3 ml/fraction using the same buffer. Absorbance of each fraction was measured at 280 nm. Laccase activity was also determined in each fraction.

2.2.9 Molecular weight determination (Bollag *et al*., 1996)

Gel electrophoresis was performed in the presence of SDS (SDS-PAGE) according to Laemmli (1970) using 5.0 % stacking and 10 % polyacrylamide resolving gels (0.7 mm thickness). Gels were run using a Mini-Protein II Cell apparatus (Bio-Rad). Low molecular weight proteins (phosphprylase b, Albumin, Ovalbumin, Carbonic anhydrase, Trypsin inhibitor, α Lactalbumin) were used as a standards (Amersham, Germany) to estimate the molecular weight of separated proteins. For 4x gel preparations, the Separating (resolving) gel was prepared by adding 2.5 ml of (acrylamide/bisacrylamide) solution, 2.5 ml of resolving gel buffer pH 8.8, 0.1ml of 10% SDS solution and 4.85 ml of distilled water, the solution was degassed for 10 min. using a vacuum pump, then 50 µl of 1.5 % ammonium persulphate and 5 µl of TEMED were mixed gently. Using pasteur pipet, the separating gel was transferred to the gel cassette, using another pipet, the top of the gel was covered slowly with isopropanol and allow the gel to polymerize about 1 hr at room temperature, then the layer of isopropanol poured off.

Stacking gel was prepared by adding 1.3 ml of (acrylamide/ bisacrylamide) solution, 2.5 ml of stacking gel buffer pH 6.8, 0.1ml of 10% SDS solution and 6.1 ml of distilled water, the solution was degassed for 10 min, using a vacuum pump, then 50 µl of 10 % ammonium persulphate and 5 µl of TEMED were added and mixed gently. Using pasteur pipette the stacking gel was transferred slowly over the separating gel at a level which allow the comb to be inserted with 5mm between the bottom and the well, the gel was allowed to polymerize about 1 h. at room temperature.

Samples for SDS-PAGE were prepared by adding an equal volume of sample buffer solution (1X) to the sample and mixed well. The samples were incubated at 90ºC for 5 min. and cooled to 25ºC.

The PAGE casette were submerged in Resorvier buffer, and 20 µl of the prepared sample was loaded on the gel. The power supply was connected to the cell and run at 20 mA. The total run time for a gel was about 4hr. with cooling at 4ºC.

2.2.10 Characterization of purified laccase

2.2.10.1 Determination of pH effects on laccase activity

Using buffer solutions described in (2.1.5.5) which had been distributed evenly into clean tubes, the pH was adjusted from (5-10). 0.5 ml of purified laccase, was added to 2.25 ml buffered . The activity of laccase was assayed and enzyme activity was plotted against the pH values to determine the optimal pH for laccase activity.

2.2.10.2 Determination of pH effects on laccase stability

Equal volumes of purified enzyme and buffer solutions (2.1.5.5) with pH range (5-10) were incubated at a 37℃ for 30 min. The enzymatic activity for each one was measured.The remaining activity (%) for laccase was plotted against the pH value of solutions .

2.2.10.3 Determination of temperature effect on activity and thermal stability of laccase

Laccase activity was measured at different temperatures (30, 35, 40, 45, 50, and 55) $°C$. The enzyme activity was plotted against the temperatures.

for thermal stability, equal volumes of purified laccase was incubated in water bath at (25, 30, 35, 40, 45, 50 and 55) °C for 30 min. enzyme activity was measured and the remaining activity (%) was plotted against the temperature.

2.2.10.4 Determination of metal ions and chemicals effects on laccase activity

The purified laccase was pretreated with 2mM(1:1 v/v) of each inhibitors and meta ions for 30 min at room temperature. The remaining activity was measured under standard assay conditions. The control was prepared for each one of the reagents tested under the same assay conditions without the presence of purified laccase.

2.2.11 Laccase immobilization

2.2.11.1 Laccase entrapment in polyacrylamide gel

Entrapment of enzyme by polyacrylamide gel was done according to the method of Kierstan and Bucke (1977) by dissolving 9% acrylamide and 1% bisacrylamide in 0.02M phosphate buffer (pH7.0), then 20ml of purified enzyme were mixed together. Gasses were removed by using vacuum pump . To this, 10ml of 0.5% ammonium pre sulphate (freshly prepared) and 0.6 ml of 50% TEMED were added for polymerization, the contents were stirred gently and 20 ml of this solution poured in to each petri plates (10 cm diameter) and kept for 10 min. at 20^0C for setting of the gel. The thickness of gel was 2.5mm^2, which it was cut into square blocks of 2.0×2.0 cm^2 and stored in 100mM potassium phosphate buffer pH 6.5. Enzyme activity was measured under standared assay conditions .

2.2.11.2 Entrapment of laccase by 3% agarose/agar gel (Om and Nivedita, 2011)

The purified enzyme was mixed with 3%(w/v) agarose .The mixture was quickly poured into Petri plates (10cm)and allowed to gel at 16℃. after 1 h, the plates were brought to room temperature and the stiff agarose-enzyme conjugte obtained (thickness of gel 1mm) was cut into square blocks (2.0× 2.0 cm2) and stored in 100mM potassium phosphate buffer, pH(6.5). Enzyme activity was measured under standard assay conditions.

2.2.11.3 Storage stability of immobilized laccase

The storage stability of free laccase (control) and laccase entrapped to agarose was investigated. The free and immobilized laccase were stored at 4°C in 100 Mm KH_2Po_4 buffer. The enzyme activity was determined after specified period; 2 months.

Chapter Three

Results

and

Discussion

3 . Results and Discussion

3.1 Isolation of *Bacillus* spp.

Fifty five soil samples were collected from different places in Bagdad city . Many Gram positive and negative isolates were obtained; of which 39 isolates were identified as *Bacillus* spp. depending on cultural and microscopic examinations. Cultural examination of *Bacillus* sp. on the LB agar gave colonies with rough surface with an unacceptable odor. Microscopic examination showed that bacterial cell was Gram positive *Bacillus* sp.

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

To screen the ability of local Bacillus sp. isolates for laccase production. Enzyme activity was determined by quantitative methods using Syringaldazine as substrate. Results indicated that 17 out of 39 isolates are laccase producing with different specific activities (Table 3-1). Depending on these results, the isolate symbol B5 was the most efficient in the production of laccase when its specific activity reached 600 U/mg protein, therefore it was chosen in next experiments .

Harkin and Obst (1973) were the first researchers to demonstrate the use of Syringaldazine as a substrate for laccase assay and later several other workers have also used syringaldazine as an assay substrate for laccase. Laccase catalyses the oxidation of syringaldazine to tetramethoxy-azo-bis (methylene quinone) that is measured spectrophotometrically at 530 nm. It was found that laccase activity correlated closely to the spore formation, which was the same as the laccases from *Bacillus sphaericus* (Claus and Filip, 1997) and *Bacillus* SF (Held *et al*., 2005). However, focusing on the quantitatively production of laccase, differences were showed comparing some bacterial species. *Pseudomonas putida* was able to

produce 11 U mg^{-1} (McMahon *et al.*, 2007), *Bacillus sp*. HR03 exhibited a DMPO-specific activity equal to 50 U mg^{-1} (Dalfard *et al* ., 2006) .and *Sinorhizobium meliloti* CE46 purified laccase- like enzyme was characterized by 266 U mg^{-1} of ABTSO activity (Castro-Swinski *et al* ., 2002).

Table(3.1): Specific activity of laccase produced by *Bacillus* sp. isolates from local soils.

Isolates symbol	Laccase specific activity(U/mg) protein	Isolates symbol	Laccase specific activity(U/mg) protein	Isolates symbol	Laccase specific activity(U/mg) protein
B1	470	B7	120	B13	188
B2	400	B8	140	B14	399
B3	460	B9	123	B15	250
B4	110	B10	89	B16	207
B5	**600**	B11	300	B17	336
B6	200	B12	114		

3.3 Identification of *Bacillus* B5 species

3.3.1 Biochemical characterization

Bacillus sp. B5 was subjected to some biochemical tests. Results illustrated show that the isolate was able to hydrolyse gelatine, casein, starch and able to reduce nitrate, producing catalase and unable to produce oxidase and urease enzyme , as well as postive for methyl red, citrate, Voges-Proskauer and Indole tests. Bergey and Holt (2000) in Bergey's Manual of Determinative Bacteriology declared that such characteristics usually are coming in accordance with those belonging to *Bacillus cereus*

3.3.2 Identification of *Bacillus* B5 isolate by VITEK system:

Identification of bacterial isolates was confirmed by the VITEK 2 system, recently installed at the Central Health Laboratory/Ministry of Health, by using the BCL (*Bacillus*) card..

Table (3-2) shows that this isolate gave the same results of identification given by the standard *Bacillus* card. The performance of VITEK 2 system for identification of the bacterial isolate was *B. cereus*

Table(3.2) : Vitek2 system result for identification of *Bacillus cereus* B5

Test	Result	Test	Result	Test	Result	Test	Result	Test	Result
BXYL	-	IRHA	-	PyrA	+	GLYG	+	AMAN	-
BGAL	-	dTAG	-	CDEX	+	MTE	+	NAG	-
APPA	+	NACL 6.5%	+	MdX	-	PLE	-	PVATE	+
ELLM	+	ProA	-	dMLZ	-	AGLU	+	dRIB	+
DMNE	-	BNAG	+	PHC	-	PSCNa	_	TTZ	-
BMAN	-	MdG	-	dGLU	+	POLYB-R	+	Phe	+
INU	-	DMAN	-	ESC	+	AspA	+	TyrA	+
OLD	-	BGLU	+	LeuA	-	AGAL	-	INO	-
LYSA	-	dTRE	+	ALaA	+	dGAL	-	Glya	-
KAN	+								

In general, the VITEK 2 system is an easy-to-handle system that provides a rapid (4 to 15 h) and reasonably accurate for the identification of significant aerobic endospore-forming species of the family *Bacillaceae*. Also, one of the most important advantages of the VITEK 2 system is the significant reduction in handling time, which will have a positive impact on the work flow of the clinical microbiology laboratory.

3.4 Optimum conditions for laccase production

3.4.1 Optimum carbon and nitrogen sources

Optimum nitrogen source was investigated using yeast extract, tryptone, peptone and sodium nitrate. *Bacillus cereus* B5 was cultivated in a minimal media containing 0.5% w/v from each of these nitrogen sources. Results (Table3.4) show that, the type of nitrogen source affected enzyme production, among the various nitrogen sources, maximum laccase production was obtained when yeast extract was supplemented to the minimal salt medium . other investeigated nitrogen sources were showed varying degress of laccase productivity.

Table(3.3): Effect of nitrogen source on laccase production by *Bacillus cereus* B5 isolate

Nitrogen source	Specific activity (U/mg protein)
Yeast extract	2084
Tryptone	1800
Peptone	971
Sodium nitrate	900

Also, Optimum carbon source was investigated using sucrose, dextrose, glucose and sodium citrate. *Bacillus cereus* B5 was cultivated in a minimal media containing 0.5% w/v from each of these carbon sources. Results (Table3.4) show that, *Bacillus cereus* B5 was capable of utilizing different carbon sources, and production of laccase was varied according to each carbon source. Dextrose was the best carbon source for laccase production, while glucose and sodium citrate were the less effectives ones.

Table(3.4): Effect of carbon source on laccase production by *Bacillus cereus* B5 isolate

Carbon source	Specific activity (U/mg protein)
Dextrose	3200
Glucose	2261
Sucrose	1500
Sodium citrate	1200

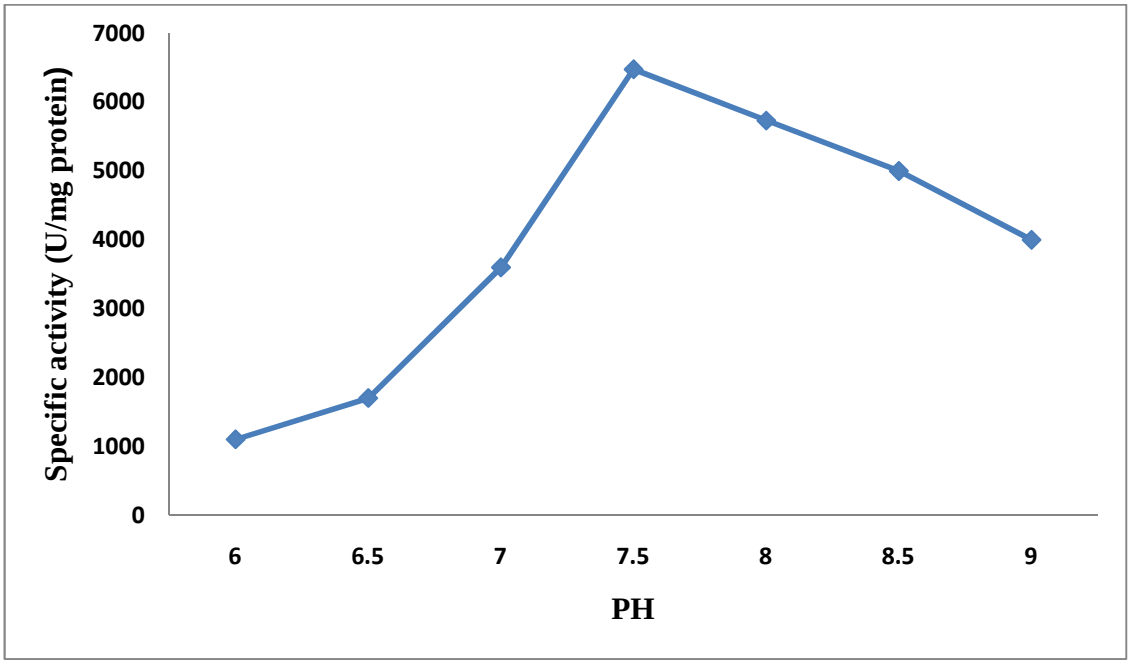
Nature and type of carbon and nitrogen sources are among the most important factors for any fermentation process (Pandey and Radhakrishnan, 1992). The enzymatic activities of any organism are highly regulated by cell metabolism, which in turn is regulated by medium composition such as carbon and nitrogen sources and their ratio in addition to other culture parameters. Thus, it was essential to provide optimum growth conditions to the microbe to obtain the desired results. Moreover, for maximum enzyme production by the microorganism, the basic need is to have prior information on growth and fermentation conditions (Pandey *et al*., 2000 ; Singh *et al.*, 2011).

Kaushik and Thakur (2014) observed that dextrose was the best carbon source producing 16.19(U/ml) laccase enzyme activity in the culture supernatant of *Bacillus* sp. , while Chhaya and Modi (2013) have been reported that dextrose was comparatively less repressive for laccase production from *Sterptomyces chartreusis*, which yielded 4.6 U/ml wheres all the other carbon sources reduced the enzyme yield considerably, and this was probably due to the reason that dextrose was a readily utilizable substrate which would promote the biomass production. In the same study, replacement of yeast extract with peptone failed to

elicit laccase production. This confirmed the suitability of yeast extract as the nitrogen source .

3.4.2 Optimum pH for laccase production

In order to investigate the effect of initial medium pH on laccase production by *B. cereus* B5 isolate, the production medium was adjusted to different pH values ranged between pH (6, 6.5, 7, 7.5, 8, 8.5, 9). Results in figure (3.1) shows that maximum laccase production was obtained when the pH value of the production medium was adjusted to 7.5, at this value the enzyme specific activity in culture filtrate was 6478 U/mg protein.



Figure(3.1): Effect of pH on laccase production by *Bacillus cereus* B5 isolate

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*

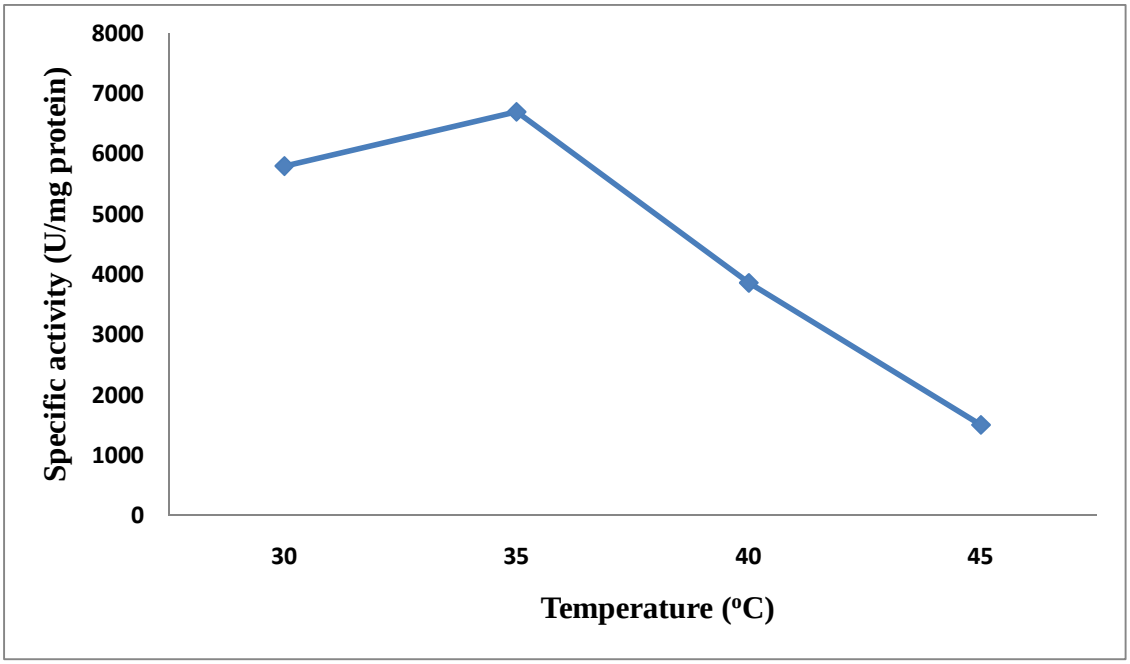
al., 2004). On the other hand, the effect of pH on enzyme production resulted from its role in the solubility of the nutritional substances and its effect on the ionization of the substrate and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme (Bull and Bushnel, 1976). Kaushik and Thakur (2014) observed that highly acidic and alkaline conditions were not suitable for *Bacillus* sp. to produce the laccase enzyme, whereas neutral pH was the best condition for laccase production.

Peter and Vandana (2014) showed that the highest laccase activity from *Pseudomonas aeruginosa* was at pH 7 (0.0341 U/ml), while lowest laccase activity was observed at pH 12 (0.0287 U/ml). The optimum pH of laccase production as reported in many *Streptomycetes* falls between 7-7.5 (Niladevi and Prema, 2008). However, the optimum pH of laccase was 6.5 from melanogenic marine bacterium MMB-1 (Solano *et al* ., 1997).

3.4.3 Optimum incubation temperatures

In this study, different incubation temperatures (30, 35, 40, and 45) ^{o}C were used to determine the optimum temperature for laccase production by the *B.cereus* B5 isolate. Figure (3.2) shows that laccase specific activity increased with increasing temperatures from 30 to 35℃, when the specific activity was 5800 U/mg protein at 30℃ has increased to 6700U/mg protein at 35℃. Generally, for any enzymatic reaction, temperature below or above the optimum 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). Forst (2007) stated that the depressed enzyme activity in low and high temperatures due to the indaequacy of these degrees for the growth of bacterial

cell, leading to slow the growth, and the temperature may also have a negative effect on dynamic energy of molecules, speed of reaction and metablic processes in the cell. 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).

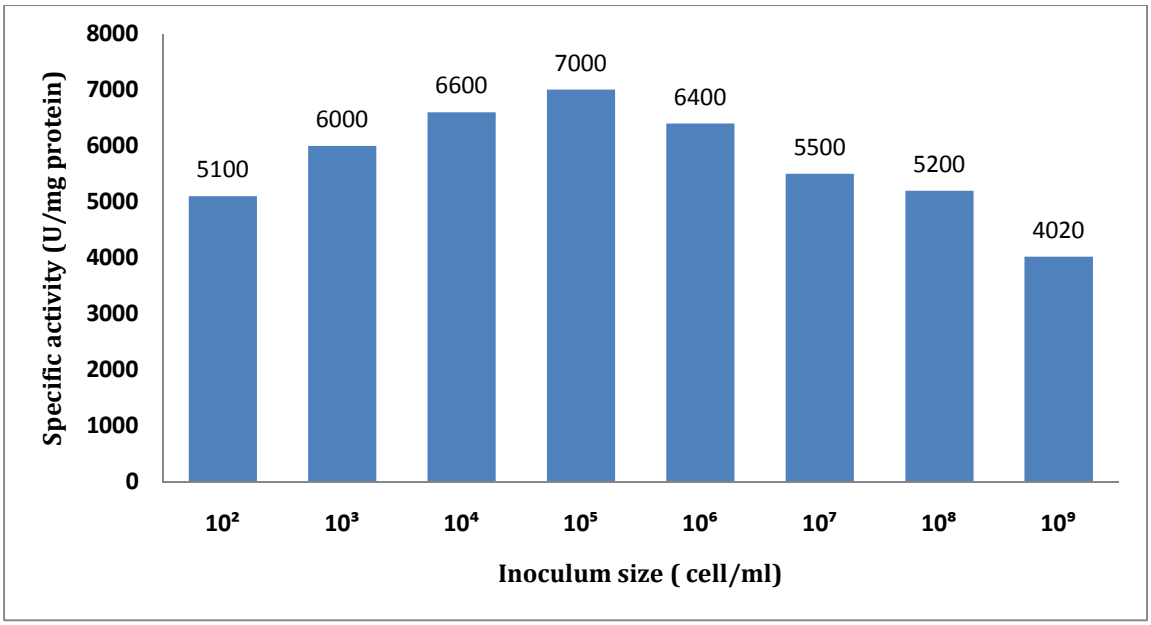


Figure(3.2): Effect of temperature on laccase production by *Bacillus cereus* B5 isolate

The optimum conditions of laccase activity are necessary for the efficiency of enzymes and their applications, in general, laccase of the bacteria had optimum temperatures in mild conditions (32 to 45°C), unlike fungal laccases which have high activities at high temperatures (40 to 70°C) (Baldrian, 2006). Dalfard *et al.* (2006) reported the *Bacillus sp*. HR03 laccase supernatant was produced at stationary phase of growth and the best temperature for production was 37˚C.

3.4.4 Optimum inoculum size for laccase production

Different inoculum sizes were used to determine the optimum for laccase production by *Bacillus cereus* B5. These inoculum sizes were ranged between (10^2-10^9) cell/ml. Results illustrated in figure(3.3) shows that production of laccase by B5 isolate was affected by the inoculum size and its production was increased slightly with increasing inoculum size to 10^5 cell/ml, then it was decreased above this size. However, maximum specific activity (7000 U/mg) was obtained using 10^5 cell/ml.



Figure(3.3): Effect of inoculum size on laccase production by *Bacillus cereus* B5

Production of enzyme in sufficient amount required optimum inoculums size of cells; lowering inoculum size required longer time for cells to multiply for sufficient number and produce enzyme. On the other hand, an increase in the number of the inoculum would ensure a rapid proliferation and biomass synthesis, after a certain time, enzyme production could be decreased because of the

depletion in the nutrients which may result in decreased in metabolic activity (Kashyap *et al.*, 2002). Niladevi *et al.* (2007) recorded that a very low inoculum size was found to be inadequate for enzyme production, while the inoculum level above the optimum reduced the yield probably due to the competition for nutrients. In the same reference, the optimization of inoculum size revealed that 1.5 x 10^7 CFU yielded maximum (33.4 U/mg) laccase production by *Streptomyces pasmmoticus* and the enzyme yield was reduced at lower and higher inoculum levels.

3.5 Laccase purification

Optimum culture conditions were used for the production of laccase from *B.cereus* B5isolate as described earlier. The crude was then subjected to the purification process (ammonium sulfate precipitation, dialysis, ion exchange and gel filtration chromatography).

3.5.1 Ammonium sulfate precipitation

Ammonium sulfate was widely used for the fractionation of protein by salting out, it is rather used as an inexpensive way of concentrating a protein extract (Clive, 2002). Cell free extract was subjected to ammonium sulfate precipitation (70% saturation) and the specific activity was increased to 21.250 U/mg. So a purification fold of 3 was achieved by this step (Table 3.5). On the other hand, dialysis of laccase enzyme after precipitation step showed no effect in the specific activity of the enzyme which may be due to the fact that laccase enzyme precipitated onto the walls of the dialysis tubing thereby impairing the passage of particles across the membrane. Which would be particularly problematic in the dialysis of a crude cell extract (Doonan, 2004). The saturation ratio of laccase enzyme was different from one organism to another, Laccase

produced by *Bacillus tequilensis* SN4 was partially purified by acetone precipitation at a concentration of 60 %(Sondhi *et al.*,2014). Peter and Vandana (2014) reported that 70% of ammonium sulfate saturation was contained high laccase activity of 0.059 U/ml and 0.82 mg/ml of protein as an initial step for purification of laccase from *Pseudomonas aeruginosa*. Also Niladevi (2008) mentioned that, laccase produced by *Streptomyces psammoticus* was purified by ammonium sulphate precipitation (30-60 % saturation) with an increased in the specific activity from 1.78 to 7.6 U/mg and a purification fold of 4.3 was achieved by this step. *Pleurotus ostreatus* ARC280 laccase was purified using ammonium sulfate precipitation 40-80% (Othman *et al* ., 2014).

3.5.2 Ion exchange chromatography

Ion exchange chromatography technique was used to purify negatively charged laccase produced by *B. cereus* B5 after ammonium sulfate precipitation and dialysis step. In this technique, the dialyzed laccase was applied to DEAE-cellulose column, then the column was washed with an equal volume of 100 mM potassium phosphate buffer to wash uncharged and positively charged proteins in laccase sample. The bounded proteins (negatively charged) were then eluted using gradient concentrations of sodium chloride ranging between 0.1and 0.9 M. Four peaks appeared in elution step, the first one with fractions from(13 to 18)showed most enzyme activity (Figure 3.4). The purification fold of this step was 14.2 and yield 53.5%and gave specific activity of 100.000 U/mg (Table 3-5).

DEAE-Cellulose chromatography has many advantages including: high resolution power, high capacity, easy handling, good separation, and ability of reactivation for using many times besides the simplicity of separation principle which depending on charge differences (Karlesson *et al*., 1998).

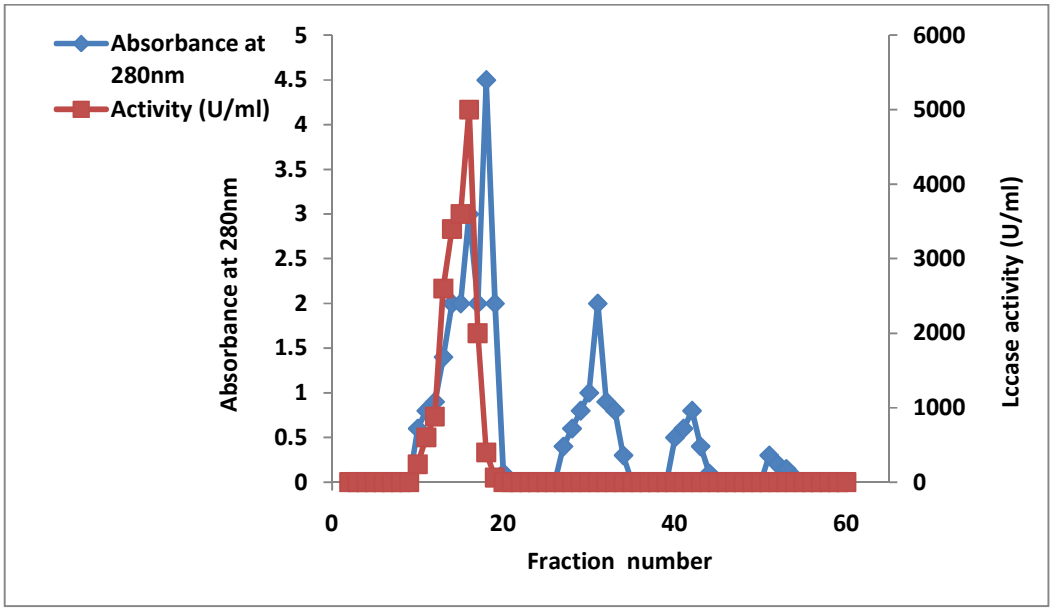


Figure (3-4):Elution profile: Ion exchange chromatography for purification of laccase produced by the *Bacillus cereus* B5 isolate using DEAE-Cellulose column (2x20cm) with a flow rate of 30ml/hour

Sondhi *et al*. (2014) have been reported the use the DEAE-cellulose column to purify the laccase enzyme from *Bacillus tequilensis* SN4, with fold of purification reached 28.46 and a yield of 13.34%. On the other hand, DEAE cellulose column was used to purify laccase enzyme from *Streptomyces psammoticus* and the specific activity has increased to 20.3 U/mg with 11 fold of purification (Niladevi, 2008). Purification of an extracellular laccase produced by *Bacillus* sp. ADR was done by two steps (Acetone precipitation and DEAE-anion exchanger) to get purified laccase with specific activity of 3.33 U/mg, purification folds of 56 and 33 yield (Telke *et al* ., 2011).

3.5.3 Gel filtration chromatographgy

Gel filtration chromatography technique was the last step in the purification of laccase produced by *B. cereus* B5. After purification by the ion exchange,

fractions representing laccase activity were collected and pooled for applying to sephacryl S-200 (1.5× 35) cm previously equilibrated with 100mM potassium phosphate buffer (pH6.8). Results in Figure (3.5) shows that only one peak representing laccase activity appeared after elution step. Activity and specific activity of laccase were measured, and results in (Table 3-5) shows that there is high final specific activity of purified enzyme reached 230.000 U/mg with purification fold 32.8 and 49.2% over all yield.

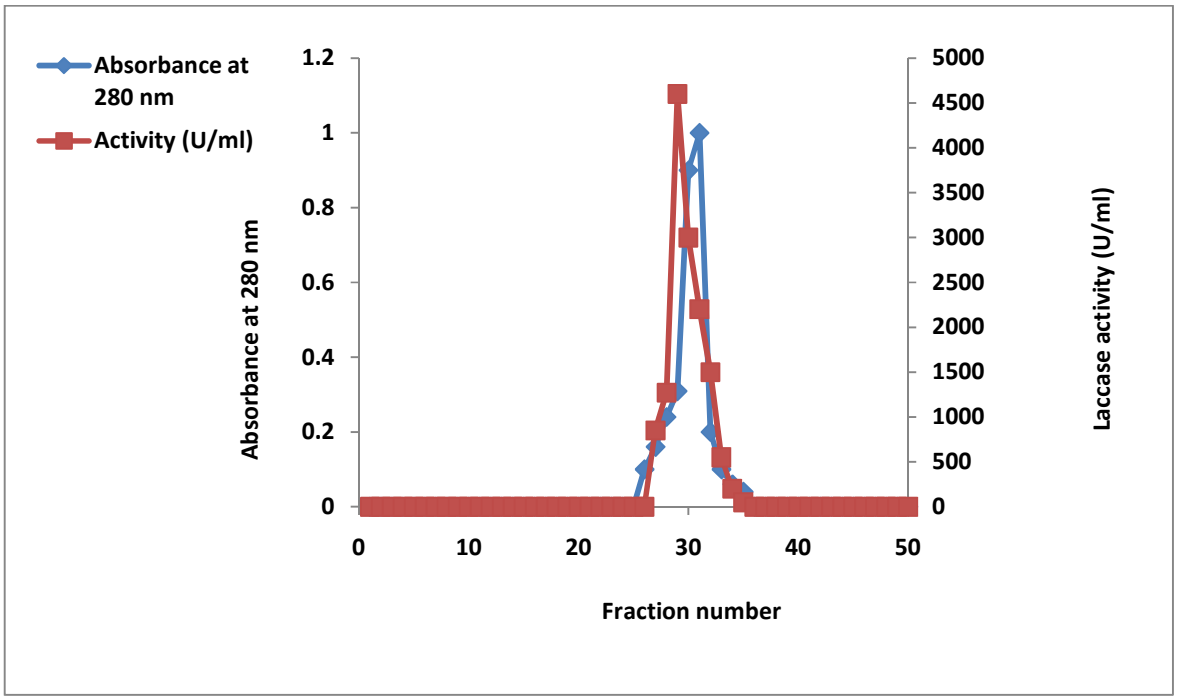


Figure (3-5): Elution profile: Gel filtration chromatography of laccase produced by *Bacillus cereus* B5 isolate using sephacryl-S200 column (1.5cmx35cm) equilibrated with 100Mm potassium phosphate buffer pH 6.8, fraction volume was 3ml at flow rate of 30ml/hour.

Sephacryl S-200 has separation limits ranging between (5000-300,000) dalton which allowed more efficient ability of separation with high degree of purification. Furthermore, it is characterized by simple preparation, fast runing, high recovery and stability for a long time which permit the re-use of the gel for several times in

protein separations (Pharmacia, 1985; Stellwagen, 1990). In the other study, McMahon *et al*. (2007) were successed in purifying *Pseudomonas putida* F6 laccase by using Superose 12 gel filtration as last steps of purification after (DEAE-sepharose) to get purified enzyme with specific activity of 4570 U/mg, with 418-fold and 30% yield.

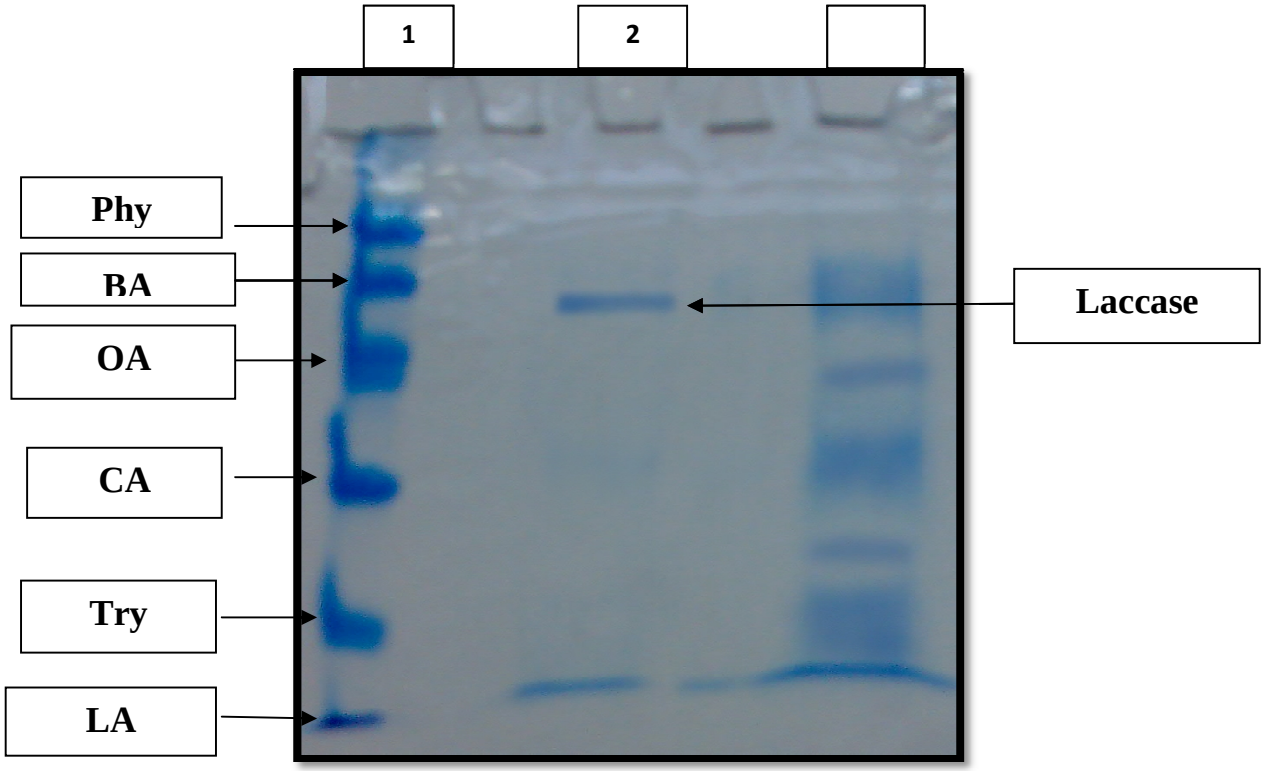
Table (3-5): Purification steps of laccase produced by *Bacillus cereus* B5 isolate

Step	Volume (ml)	Enzyme activity (U/ml)	Protein con.(mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield(%)
Crude enzyme	100	1400	0.2	70.00	140000	1	100
Ammonium sulphate precipitation	30	3400	0.16	21.250	102000	3	72.8
DEAE-cellulose	15	5000	0.05	100.000	75000	14.2	53.5
Sephacryl S-200	15	4600	0.02	230.000	69000	32.8	49.2

3.6 Molecular weight determination of laccase by SDS- PAG

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS- PAGE) is an anionic detergent, meaning that a net negative charge obtained when its molecules were dissolved. A polypeptide chain binds amounts of SDS in

proportion to its relative molecular mass and the negative charges on SDS destroy most of the complex structure of proteins, and are strongly attracted toward an anode (positively-charged electrode) in an electric field (Hassan, 2009). Results of protein profile by SDS-PAGE revealed that one band was obtained with molecular weight of about 66 kDa when compared with standard proteins as in Figure (3-6).



Figure(3-6): Polyacrlamide gel electrophoresis of purified and crude laccase produced by B. cereus B5 isolate where:(1) Protein markers: Phosphorylas Phy(97,000), Bovine Albumin BA(66,000), Ova Albumin OA(45,000), Carbonic Anhydrase CA(30,000), Trypsin Inhibitor Try(20,000) and α-Lactalalbumin LA(14,000),(2) Purified enzyme after gel filtration step and (3) Laccase protein band in crude filtrate

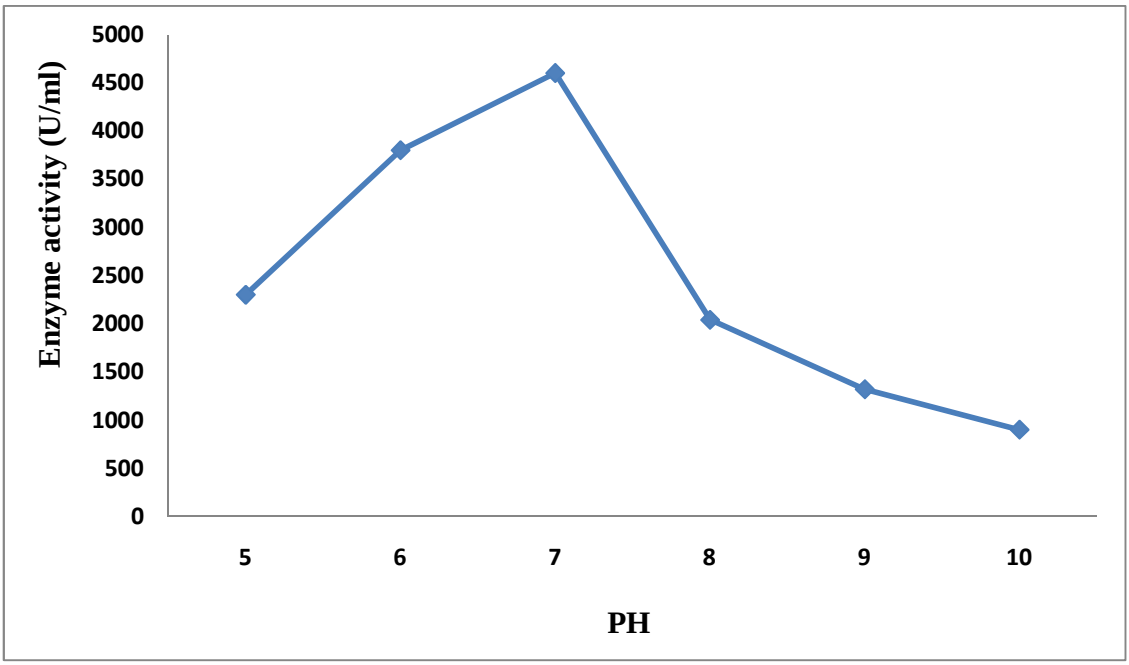
Molecular weight of laccase from other bacterial species vary from 14 to 120 KDa. Since, the molecular weight of laccase purified from *γ-proteobacterium* JB is 120kDa (Singh *et al*., 2007), *Streptomyces griseus* is 114 kDa (Endo *et al*., 2003), *Streptomeyces lavendulae* is 73 kDa (Suzuki *et al.*, 2003), *Bacillus subtilis* and

Bacillus licheniformis are 65 kDa (Martins *et al*., 2002, Koschorreck, *et al*., 2008), also, *Thermus thermophilus* has molecular of 53 kDa (Miyazaki, 2005) .

3.7 Characterization of purified laccase

3.7.1 Effect of pH on laccase activity

The effect of pH on purified *B. cereus* B5 isolate laccase was studied in a pH range of 5-10. Results in Figure (3-7) show that pH7 was an optimum pH. From these result, it can be concluded that the enzyme activity at neutral or nearly neutral values was higher than the activity at highly acidic or basic values.



Figure(3.7):Effect of pH on the activity of purified laccase produced by *Bacillus cereus* B5 isolate

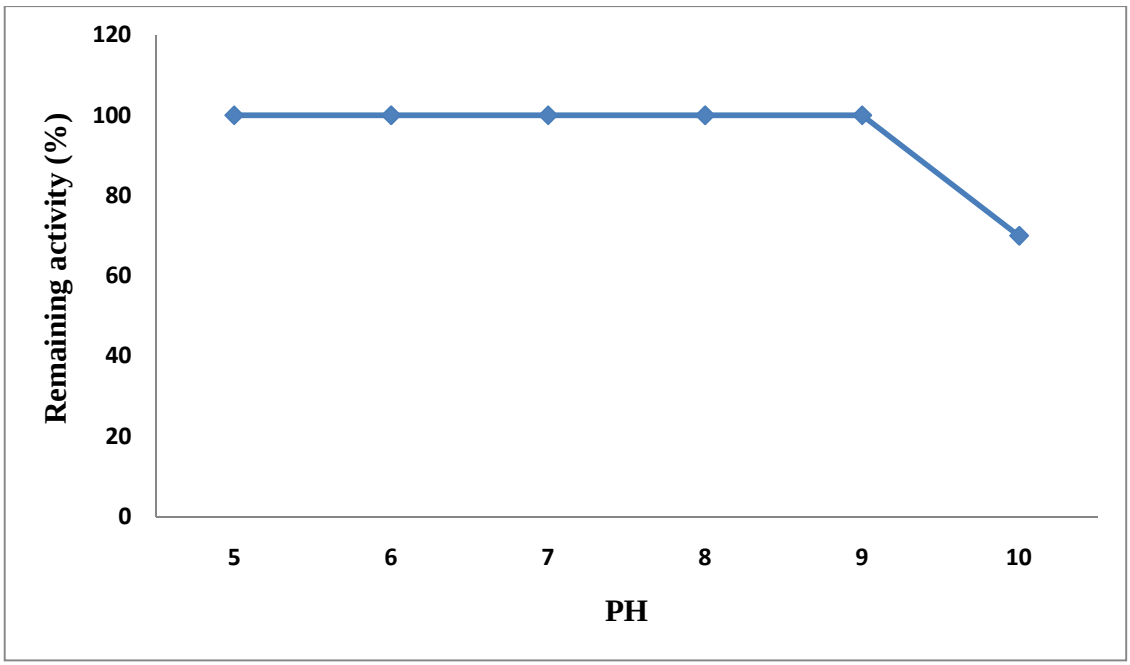
This result could be returned to the effects of pH on the state of ionization of acidic or basic amino acids. Since, acidic amino acids have carboxyl functional groups in their side chains. While, basic amino acids have amine functional groups in their side chains. So if the state of ionization of amino acids in a protein is

altered then the ionic bonds that help to determine the 3-D shape of the protein can be altered. This lead to altered protein recognition or an enzyme might become inactive. In other words, changes in pH may not only affect the shape of an enzyme but it may also change the shape or the properties of the substrate so that either the substrate cannot bind to the active site or it cannot undergo catalysis (Chesworth *et al*., 1998). The maximum activity of laccase isolated from *Bacillus subtilis* WD23 spores was at pH 6.8 against syringaldazine (Wang *et al* ., 2011). PH maxima near to neutrality was also observed for other bacterial laccases, i.e. *Martellea mediterranea* (Jimenez-Juarez *et al.,* 2005). While Margot *et al.* (2013) has founded that laccase from *Streptomyces cyaneus* activity was strongly dependent on the pH with for instance, an order of magnitude increase between pH 5.5 and 4.5 with ABTS(from 84 to 87 U $l^{-}1$) and with syringaldazine (from 8 to 9 U l^{-1}), and a rapid decrease of activity below pH 3.5 with all substrates.

3.7.2 Effect of pH on enzyme stability

The pH of enzyme stability was studied because it is an important criteria to determine the optimum conditions for purification and storage of the enzyme. Figure (3-8) indicated that purified laccase was stable over wide range of pH (7-9), because the enzyme gain maximum remaining activity 100%, while the activity was decreased slightly at acidic and alkaline pH. The lowering in enzymatic activity in extreme acidic and extreme basic conditions may be due to change in secondary and tertiary structure of the ionic state of active site for the enzyme and substrate (Lemacher and Bisswanger, 1990).On The other hand, Moat *et al*. (2002) have been discussed several factors for the effect of pH of the environment on an enzyme ; First of each, enzyme has its pH optimum, at which the enzyme is most active, but the enzyme is stable within certain limits on each side of the optimum. Secondly, the environmental pH of the enzyme may influence its stability, and at

extremes of acidity or alkalinity the enzyme may be denatured. Thirdly, the pH of the reaction mixture may cause dissociation of the substrate and so by its action on the substrate influence the character of the pH activity curve.

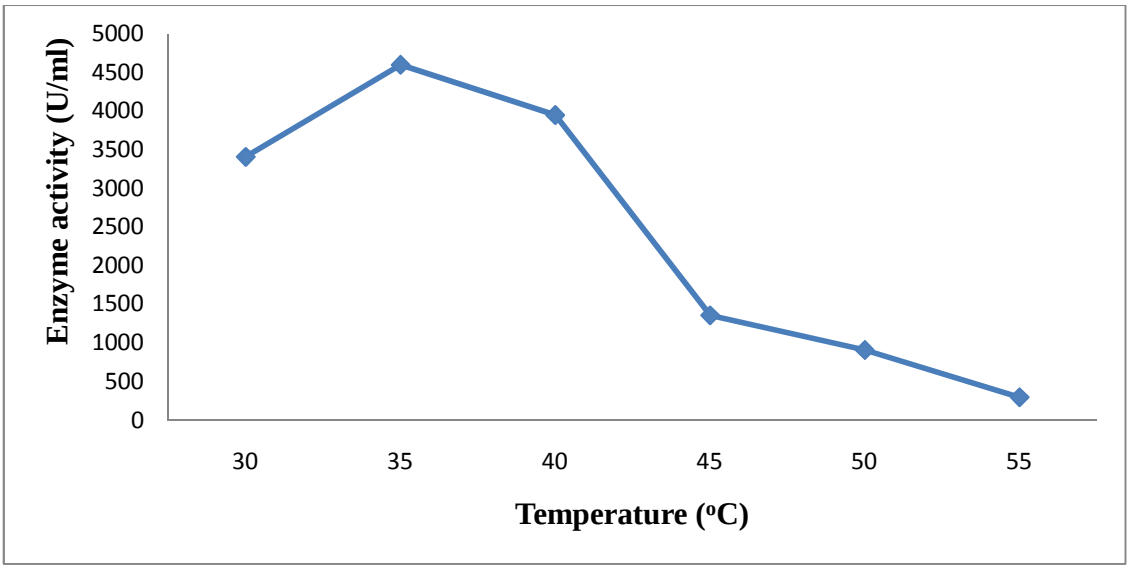


Figure(3.8): Effect of pH on stability of purified laccase produced by *Bacillus cereus* B5 isolate

Ruijssenaars and Hartmans (2004) stated that the pH optima of the phenolic substrates towards alkaline range can be explained by the redox potential difference between the phenol and the T1 copper of laccase, (the driving force for electron transfer) which increases with increase in pH. Also, laccase enzyme purified from *Bacillus tequilensis* SN4 was found to be highly stable in the alkaline pH range and the enzyme could retain 50% activity even after 24 h incubation at pH 9.0 (Sondhi *et al* ., 2014).

3.7.3 Effect of temperature on laccase activity

Temperature is an important factor which affects enzyme activity. The favorable temperature for laccase activity may differ with different laccase sources. In order to determine the optimum temperature for laccase activity, enzyme activity was assayed at different temperatures values, ranged between 30 and 55^{o}C. Results in figure (3-9) shows that 35^{o}C was the optimum temperature for laccase activity, enzyme activity was decreased at less or a higher temperatures of incubation.



Figure(3.9):Effect of temperature on activity of purified laccase produced by *Bacillus cereus* B5

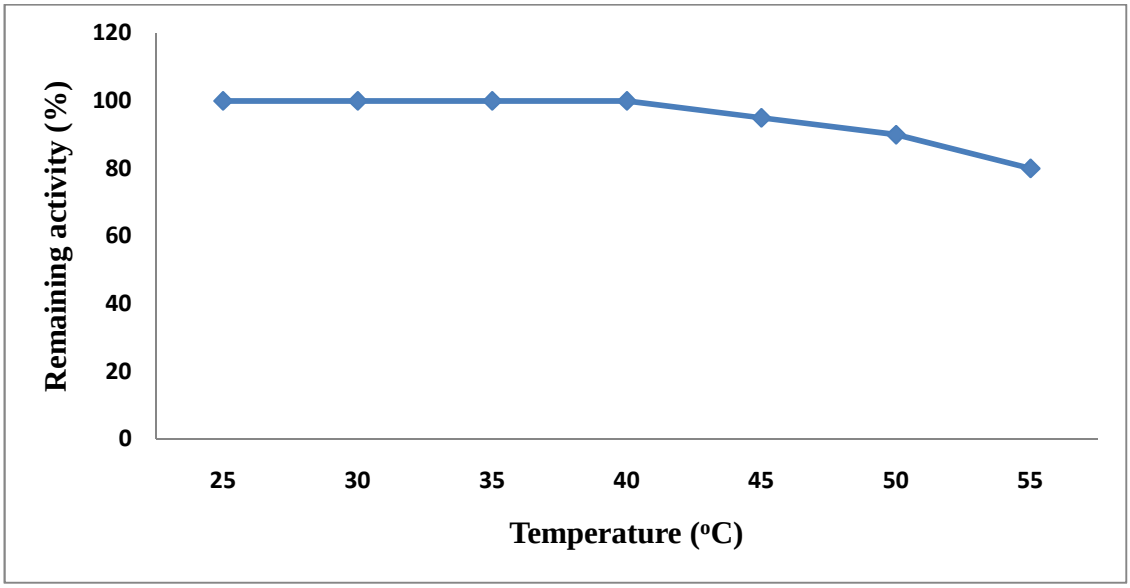
This is due to the fact that speed of enzyme interaction increases with increasing temperature within a certain range due to increased energy kinetics and collisions between enzyme molecules and substrate, except that high temperatures within certain limits lead to denature the enzyme and loss of three dimensional structure and then decline enzyme activity (Muro *et al*., 1984). The optimum temperature for laccase usually depend upon the source of organism. In general,

laccases have optimum temperatures at 30-50°*C* and rapidly lose activity at temperatures above 60°*C* (Galhaup *et al*., 2002, Palonen *et al*., 2003). While, *Bacillus tequilensis* SN4 laccase was found to be highly thermostable, having temperature optimum at 85℃ and could retain more than 80% activity at 70℃ for 24 h (Sondhi *et al* ., 2014). Also, McMahon *et al.* (2007) founded that laccase enzyme purified from bacterial *Pseudomonas putida* F6 displayed maximal activity at 30 ˚C and pH 7.0. However, at temperatures above 30℃ laccase activity was significantly reduced, with 50% and 30% of the activity observed at 50 and 70 ℃ respectively.

3.7.4 Thermal stability of purified laccase

Laccase enzyme incubated at different temperature (25, 30, 40,45, 50 and 55)℃ for 30 min to determine its stability. Figure (3-10) shows that laccase had wide range of thermo stablility ranged between 25-55 ℃, However 15% of laccase activity was reduced at 55 ℃.

High temperature more than 45℃, lead to break various bonds in the enzyme , which are responsible for building stability of the enzyme and may lead to the denaturation of enzyme by destructing the three dimensional structure of protein and formation of random polypeptide chains, or by causing a change in the active site leading to inactivation of enzyme (Rodwell, 1996). Segal (1976) stated that crude and partially purified enzymes are more stable than purified enzymes due to the existence of carbohydrates and other proteins protecting them. It has been reported that the stability of laccase at a higher temperature increased under neutral pH conditions (Min *et al*., 2001; Jung *et al*., 2002).



Figure(3.10): Effect of temperature on stability of purified laccase by *Bacillus cereus* B5 isolate

Weang *et al*. (2011) was reported that laccase purified from *Bacillus subtilis* WD23 spores showed temperature half-life stability for 2.5 h at 80℃ and the laccase activity disappeared after 18 h. In addition, the laccase had a high stability at the optimum temperature at 60 ℃. In other research, laccase enzyme purified form *Trametes versicolor* showed to be very stable at temperatures ranging from 0 to 50℃ for a long time, but the activity decreased to about 40% after 1 h at 60℃ , (Han *et al* ., 2005).

3.7.5 Effect of Metal ions and some chemicals agents on laccase activity

Heavy metals normally found in the environment, might affect the stability or activity of enzymes. The effect of some heavy metals ($MgCl_2$, $CaCl_2$, $ZnSo_4$, $CuSo_4$ and $HgCl_2$) and chemicals agent (Sodium azid, EDTA and L-cystein) on the activity of the purified laccase was examined . Result (Table3.6) shows that $ZnSo_4$ and $CuSo_4$ ions were characterized by their strong inhibitory effect on the enzyme. While, only $HgCl_2$ has increased laccase activity about 114%. Bollag and

Leonowicz (1998) stated that laccases are inhibited by metal ion chelators, also, it was mentioned that the requirement for specific metal ions depends on the source of enzyme (Kumar and Takagi, 1999). The same results was reported by Tamayo-Ramos *et al.* (2012) in which different concentrations of $CuSO_4$ (0.1, 0.5, 1.0 and 2.5 mM) highly inhibited purified laccase from *Aspergillus niger* . Also, Rosconi *et al* . (2005) founded that *Sinorhizobium meliloti* CE52G laccase inhibited to different extents by Cu^{+2} , Fe^{+2} and Mn^{+2} suggesting that these metal ions interfere with oxidation of the classical organic laccase substrates and laccases can be inhibited by metals such as Fe^{+2}, Mn^{+2}, Zn^{+2} and Ag^{+2} (Zavarzina *et al* ., 2004 ; Couto *et al* ., 2005). Other studies submitted by Scheel *et al.* (2000) and Soden and Dobson (2001) showed that Hg^{+2} can stimulate lacccase expression.

On the other hand, three potential chemicals agents (Sodium azide, EDTA and cysteine) were tested to check the properties of laccase in this study. The result (Table3.6) show that 2mM of sodium azide barely effected the action of laccase, 14% and 25% of enzyme activity was reduced with EDTA and L-cystein respectively. This result could be due to the fact that, anions such as the halides, azide, cyanide and hydroxide bind to the types 2 and 3 copper atoms of laccases, which disrupts the electron transfer system, resulting in enzyme inhibition (Gianfreda *et al.*, 1999). This result was closed to Güray and Şanlı-Mohamed (2013) since only 5% of thermophilic Bacillus laccase activity was decreased when laccase enzyme was treated with 1mM EDTA. However, laccase from *Streptomyces griseus and Bacillus thuringiensis* showed 67% and 72% of their activity in the presence of EDTA with same concentration, respectively (Endo *et al*., 2003; Liu *et al*., 2004) .Saito *et al.* (2003) have reported that sodium azide has exerted little inhibition on laccase from a fungal strain at a concentration of l0 mM

while EDTA had no effect on the enzyme even at a very high concentration of 25 mM.

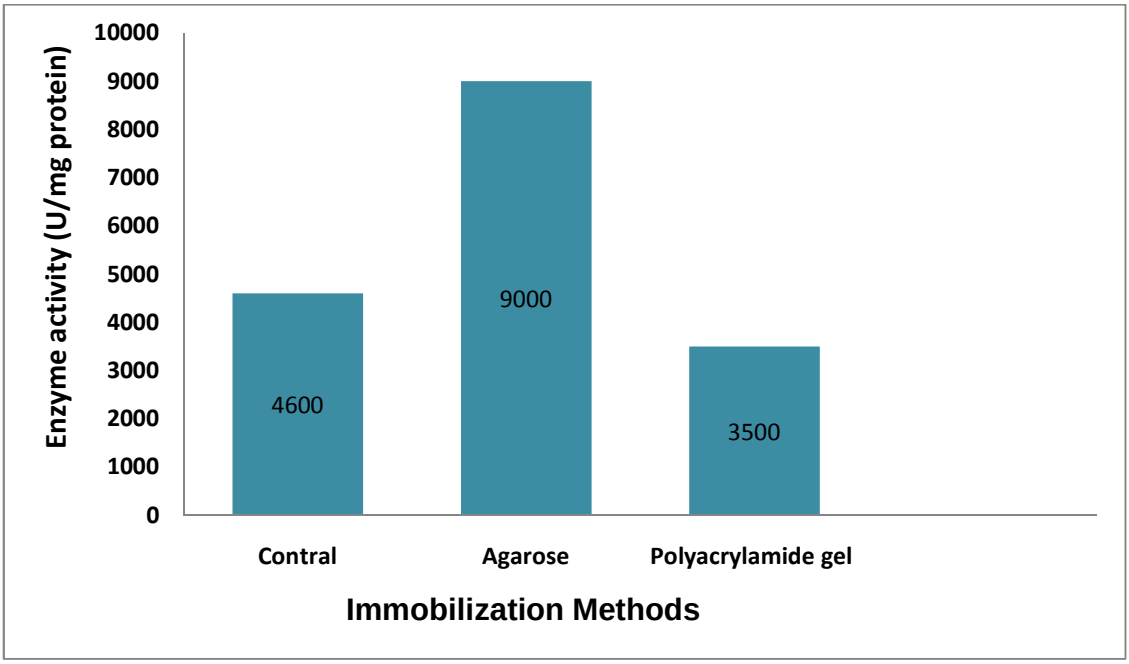
Table (3-6): Metal ions and inhibitors effect on laccase activity produced by *Bacillus cereus* B5 isolate

Reagent	Concentration (mM)	Remaining activity (%)
Control	_	100%
$HgCl_2$	2mM	114%
$CaCl_2$	2mM	86%
$MgCl_2$	2mM	74%
$ZnSo_4$	2mM	28%
$CuSo_4$	2mM	23%
Sodium azide	2mM	93%
EDTA	2mM	86%
L-Cystein	2mM	75%

3.8 Immobilization of laccase enzyme

Although the best methods of immobilization might differ from enzyme to enzyme. From application to application and from carrier to carrier, depending on the peculiarities of each specific application, criteria for assessing the robustness of the immobilized enzyme participate remaining the same-industrial immobilization enzyme must be active (high active in a unit of volume, U $g^{-}1$ or $ml^{-}1$) , highly selective (to reduce side reaction) , high stable (to reduce cost by effective reuse), cost-effective(low cost contribution thus economically attractive), safe to use (to meet safety regulations) and innovative (for recognition as intellectual property) (Cao, 2005).

The result in Figure(3.11) shows the efficiency of two used entrapment media : agarose and polyacrlymide gels in immobilization of laccase purified from *Bacillus cereus* B5. It was found that agarose gel retained the highest activity with about two folds increase in specific activity compared to the original activity. However, slightly decrease in laccase activity when entrapped in polyacrylamide gel compared to the original activity. This result could be due to the fact that gels (agarose and polyacrylimide) prepared from natural and synthetic biopolymers have been quite commonly and successfully employed for the entrapment of whole cell and proteins (Kierstan and Bucke, 1977; Tanaka *et al*., 1984). Also, They offer simplest methods because relatively milder conditions are required for immobilization of enzyme (Palmieri *et al* ., 1994). Gupta and Mattiasson (1992) were stated that agarose is an excellent matrix that has been extensively used. In addition to its high porosity, which leads to a high capacity for proteins, also agarose is used as a matrix for its hydrophilic character, ease of derivatization, absence of charged groups (which prevents nonspecific adsorption of substrate and products), and commercial availability. In a similar study, Poonkuzhali and Palvannan (2012) purified laccase from *Pleurotus florida* and immobilized it on agarose/ager/polyacrylamide gel, glass beads and alginate beads. The laccase entrapped in agarose gel exhibited 88% of immobilization yield as compared to laccase immobilized on glass beads (72%) and Ba-alginate (50%). In other study, Partially purified laccase (eluted from DEAE-Sepharose column) immobilized on various supports (Nitrocellulose membrane, Polyvinyl alcohol membrane, agarose membrane and nylon membrane) showed the different activity order . Since, laccase showed 100% activity after immobilization on nitrocellulose membrane (Singh *et al*., 2010).



Figure(3.11): Laccase enzyme immobilization in agarose and polyacryamied gel

3.8.1 Storage stability of immobilized laccase

To investigate the effect of immobilization step on the storage stability of laccase, free enzyme and laccase immobilized by agarose gel were stored in 100 mM potassium phosphate buffer pH 6.8 at 4^{o}C and the laccase activity of free and immobilized enzyme was determined at every 7 days up to 60 days(Table 3-7). Results showed that laccase entrapped in agarose gel remain stable up to 61 days since it was retained highest residual activity of about 66% when compared to the free enzyme which lost most activity during the same period. Neeru and Sawhney (2002) showed the loss of enzyme activity during storage could be du to lattice structure of the gel at lower storage temperature, which facilitated faster diffusion of the reactants and products of the reaction. Several studies reported that immobilization of laccase in a suitable support with appropriate buffer result in an increase of enzyme stability (Leonowicz *et al*. 1988 ; Kunameni *et al*. 2008). Also,

Poonkuzhali and Palvannan (2012) invistigated that storage stability of immobilized laccase entrapped by agarose gel retained the residual activites of 65% after 61 days .

Table (3-7) Storage stability of free and laccase immobilized on agarose gel.

Storage stability (d)	Free enzyme (%)	Agarose gel (%)
0	100	100
7	84	95
14	77	90
21	69	88
28	55	80
35	46	77
42	30	74
49	24	73
56	15	70
61	5	66

Chapter four

Conclusions

and

Recommendations

4. Conclusions and Recommendations

4.1 Conclusions:

1. The local soil isolate *B. cereus* B5 was the most efficient laccase producers among the other 39 isolates.
2. Optimum conditions for laccase enzyme produced by *B. cereus* B5 isolate was achieved after the minimal salt medium(pH7) was supplemented with 0.5% dextrose , 0.5% yeast extract, inoculum size 10^5 and incubated at 35^{o}C in shaker incubator (200 rpm) for 24h.
3. Purification protocol of isolate B5 of *Bacillus cereus* gave higher laccase specific activity with higher fold of purification.
4. Purified laccase enzyme was characterized on stable at neutral to alkealine pH and at a wide range of temperatures .
5. Agarose gel was good support for laccase immobilization, moreover the immobilized laccase retained more than half of its activity after two months storage.

4.2 Recommendations :

1. Increasing the stability of laccase using others immobilization techniques.
2. Studying the antitumor activity of the purified laccase against different tumor cell lines in *vivo*.
3. Studying more applications and uses of the enzyme.

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Appendix

Appendix (1): Biochemical tests in the VITEK2 system.

Test substrates on BCL card.

Well	Test	Mnemonic	Amount/Well mg
1	BETA-XYLOSIDASE	BXYL	0.0324
3	L-Lysine-ARYLAMIDASE	LysA	0.0228
4	L-Aspartate ARYLAMIDASE	AspA	0.024
5	Leucine-ARYLAMIDASE	LeuA	0.0234
7	Phenylalanine ARYLAMIDASE	PheA	0.0264
8	L-Porline ARYLAMIDASE	ProA	0.0234
9	BETA-GALACTOSIDASE	BGAL	0.036
10	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	0.018
11	ALPHA-GALACTOSIDASE	AGAL	0.036
12	Alanine ARYLAMIDASE	AlaA	0.0222
13	Tyrosine ARYLAMIDASE	TyrA	0.0282
14	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	0.0408
15	Ala-Phe-Pro ARYLAMIDASE	APPA	0.0384
18	CYCLODEXTRIN	CDEX	0.3
19	D-GALACTOSE	dGAL	0.3
21	GLYCOGEN	GLYG	0.1875
22	Myo-INOSITOL	INO	0.3
24	METHYL-A-D-GLUCOPYRANOSIDE acidification	MdG	0.3
25	ELLMAN	ELLM	0.3
26	METHYL-D-XYLOSIDE	Mdx	0.3
27	ALPHA-MANNOSIDASE	AMAN	0.036
29	MALTOTRIOSE	MTE	0.3
30	Glycine ARYLAMIDASE	GlyA	0.012
31	D-MANNITOL	DMAN	0.3
32	D-MANNOSE	DMNE	0.3
34	D-MELEZITOSE	DMLZ	0.3
36	N-ACETYL-D-GLUCOSAMINE	NAG	0.3
37	PALATINOSE	PLE	0.3

39	L-RHAMNOSE	IRHA	0.3
41	BETA-GLUCOSIDASE	BGLU	0.036
43	BETA-MANNOSIDASE	BMAN	0.036
44	PHOSPHORYL CHOLINE	PHC	0.0366
45	PYRUVATE	PYVATE	0.15
46	ALPHA-GLUCOSIDASE	AGLU	0.036
47	D-TAGATOSE	dTAG	0.3
48	D-TREHALOSE	DTRE	0.3
50	INULIN	INU	0.12
53	D-GLUCOSE	DGLU	0.3
54	D-RIBOSE	dRIB	0.3
56	PUTRESCINE assimilation	PSCNa	0.201
58	GROWTH 6.5% NACL	NACL 6.5%	1.95
59	KANAMYCIN RESISTANCE	KAN	0.006
60	OLEANDOMYCIN RESISTANCE	OLD	0.003
61	ESCULIN hydrolysis	ESC	0.0225
62	TETRAZOLIUM RED	TTZ	0.0189
63	POLYMIXIN_B RESISTANCE	POLYB_R	0.00093

الخلاصة

جمعت في هذه الدراسة خمس وخمسون عينه تربه من أماكن مختلفة في محافظة بغداد. تم الحصول منها على عزلات بكترية موجبه وسالبه لصبغه غرام، شخصت تسع وثلاثون عزله منها على إنها تعود للجنس*Bacillus* وذلك اعتمادا على صفاتها المظهرية والمجهريه. بعدها تم البحث عند قدره هذه العزلات على إنتاج إنزيم اللاكيز بواسطة الطريقة الكميه وباستخدام syringaldazine كماده أساس، أشارت النتائج إن سبعة عشر عزله منها كانت منتجه لإنزيم اللايكيز وبفعالية نوعيه مختلفة تراوحت بين 98-600 وحد/ ملغم بروتين وكانت العزلة *Bacillus cereus* B5 التي تم الكشف عنها بواسطة الاختبارات الكيموحيوية واختبارات الـ2 Vitek هي الأكفأ في إنتاج اللاكيز إذا بلغت الفعالية النوعية لها 600 وحده/ ملغم بروتين. لذلك تم اختيار العزلة *Bacillus cereus* B5 لدراسة الظروف المثلى لإنتاج إنزيم اللايكيز.

أظهرت نتائج دراسة المثلى لإنتاج إنزيم الايكيز كفاءة وسط الأملاح المعدنية ذو الرقم الهيدروجيني7 ب 0.5% دكستروز، 0.5% خلاصه الخميرة و حجم اللقاح 10^5 و الحضن بحرارة 35°م في الحاضنة الهزاز بسرعة 200 دوره/ دقيقه لمدة 24 ساعة, حيث ازدادت الفعالية النوعية لإنزيم اللايكيز المنتج تحت هذه الظروف لتبلغ 7000 وحده/ملغم بروتين.

لدى تنقيه الإنزيم المنتج تحت الظروف المثلى للإنتاج بواسطة الترسيب بكبريتات الامونيوم وبنسبه إشباع 70%ثم التبادل ألايوني بواسطة المبادل ألايوني الموجب DEAE-Celluloseوالتنقية بالترشيح الهلامي باستخدام سيفاكريل200-S. إن الفعالية النوعية للإنزيم المنقى قد ارتفعت بعد أخر خطوه تنقية الى230000وحده/ملغم بروتين وبعدد مرات تنقيه 32 وحصيلة أنزيمية مقدراها 49.2%.

أظهرت نتائج التحري عن بعض الخصائص الكيموحيوية للإنزيم المنقى، أن الوزن الجزيئي لإنزيم اللاكيز المنتج من بكتريا *Bacillus cereus* B5كان 66000دالتون تقريبا، و الرقم الهيدروجيني الأمثل لفعالية الإنزيم هو 7 فضلا عن ثباته الإنزيم ثابتا عند الأرقام الهيدروجينية التي تراوحت 7-9،كما و كانت الدرجة الحرارية المثلى لفعالية الإنزيم 35°م واظهر الانزيم ثباتا حرارية كاملا بمدى حرارة تراوحت بين 30-40 °م.

عند دراسة تأثير بعض المواد الكلابية ،المختزل وأيونات المعادن على فعالية إنزيم اللايكيز، أظهرت النتائج إن فعالية الإنزيم قد ثبطت بوجود Cuو Zn ، ال EDTA وال Sodium azideولكن بنسب مختلفة.

أدى استخدام نوعين من دعائم التقيد (متعدد الاكرامايد والاكاروز) لحجز إنزيم اللايكيز المنقى، والى زيادة الفعالية الإنزيمية إلى الضعف عند التقيد مع الاكاروز مقارنه بفعالية الإنزيم الاصليه.أظهرت نتائج من جهة أخرى درسه ثباته الإنزيم الحر والمقيد أن الإنزيم المقيد أظهر ثباتيه خزينة جيده وذلك عندما احتفظ ب 66% من فعاليته مقارنه ببقاء 5% فقط من فعالية الإنزيم الحر بعد61 يوم من الخزن على التوالي.

†بسم الأب والابن والروح القدس الإله الواحد آمين†

14أَنْتُمْ نُورُ الْعَالَمِ. لاَ يُمْكِنُ أَنْ تُخْفَى مَدِينَةٌ مَوْضُوعَةٌ عَلَى جَبَل،15 وَلاَ يُوقِدُونَ سِرَاجًا وَيَضَعُونَهُ تَحْتَ الْمِكْيَالِ، بَلْ عَلَى الْمَنَارَةِ فَيُضِيءُ لِجَمِيعِ الَّذِينَ فِي الْبَيْتِ. 16 فَلْيُضِئْ نُورُكُمْ هكَذَا قُدَّامَ النَّاسِ، لِكَيْ يَرَوْا أَعْمَالَكُمُ الْحَسَنَةَ، وَيُمَجِّدُوا أَبَاكُمُ الَّذِي فِي السَّمَاوَاتِ.

متى5: 14-16

جمهورية العراق
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قسم التقانة الإحيائية

أنتاج و توصيف وتقييد أنزيم اللاكيز من بكتريا *Bacillus cereus* المعزولة محليا

رسالة

مقدمه إلى مجلس كلية العلوم / جامعة النهرين

كجزء من متطلبات نيل درجة الماجستير علوم في التقانة الإحيائية

من قبل

مينا ماجد اللوس

بكالوريوس علوم / تقانة احيائية/ كليه العلوم/ جامعه النهرين/ 2012

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