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Overexpression of fungal laccase enzymes and their application in waste treatment

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Abstract: Phenolic compounds like phenol, 2,4-dichlorophenol, bisphenol A, 2,4-dinitrophenol, 4-chlorophenol, besides 4-nitrophenol are well-known to be extremely harmful to humans and living organisms. Therefore, it is important to develop appropriate remediation techniques to remove these phenolic compounds effectively from industrial effluents. Biodegradation using enzymatic technology is investigated in detail as it is a promising biotechnological solution to sustainable combat of water pollution caused by phenolic compounds as part of a defined environmentally optimized strategy need to do it.

Objective: The current paper's objective is to study production of laccases enzyme from white rots fungi and use of immobilized laccases to degrade phenolic compounds in dyes.

Results: Among tested fungal isolates, only onefungus., *Pleurotus sp*. was showed a highly laccase enzyme productivity,wherein the optimization process for laccases productivity showed to be necessiate .It is showed that pH 7.0 and temperature 30c were the bestphysical conditions for optimization of enzyme.As well as,, carbon source such as glucose and sucrose , , nitrogen source(i.e., urea and ammonium chloride mixtures) were crucial nutritional sources affected on the productivity of laccase enzymes. . At molecular biology level, gene coding for laccase was partially estimated by 831bps, and its sequence analysis showed 98.44% with Pleurotus ostreatus,s oxidoreductase (MIA40). Further, laccase protein was estimated by 55-57KDa.Laccase enzyme and it exhibits activity against phenolic compounds when(revised these sentences) (Azo Dye from Egyptian German For Pigments & Resins) used as a substrate for the enzyme, total phenolic compounds concentration in untreated substrate was 476.4 µg/ml. there was a remarkable reduction in this concentration when laccase enzyme applied to Azo dye as substrate, it showed 127.2 (26.7%) µg/ml

keywords: *Pleurotus ostreatus* ,Laccase Enzyme,Phenolic Compounds

Introduction

Nowadays, the bioremediation process is an economical and ecologically beneficial way to remediate persistent and harmful substances, frequently xenobiotics. It has been established that microbial activity converts variety of contaminants found in soil, compost, or wastewater. Bacterial species have properties that make them appropriate for a range of bioremediation applications, including the capacity to thrive in a variety of settings and utilise a wide range of chemicals as food sources. As eukaryotes, fungi, on the other hand, prefer more temperate surroundings, such as those with a smaller temperature range, and

frequently need aerobic conditions, but some can survive in hostile settings.Nonetheless, a large number of saprophytic fungi that break down garbage and wood generate nonspecific extracellular oxidising enzymes with a high redox potential that are intended to break down the most challenging natural polymer, aromatic lignin [1]. As a result, aromatic compounds—which are usually big molecules that bacteria cannot break down intracellularly—have drawn the most attention in fungal bioremediation [2]. These substances stay in the water system because they are either fully or partially destroyed, as in activated sludge wastewater treatment facilities, for instance.

Xenobiotics are typically utilised in soil in an established form and are frequently linked to humic compounds [3]. White rot fungus have been the focus of bioremediation investigations since it is thought that fungi break down these pollutants mostly through their lignin-modifying enzymes, such as laccase in addition to lignin-modifying peroxidase. Lignin is one of three main polymers present in the cell walls of wood-grown plants, along with cellulose and hemicellulose.

As the only creatures that can efficiently break down and even mineralize lignin, wood-decomposing white-rot fungus and soil litter-decomposing fungi are crucial to the Earth's carbon cycle [1]. For many years, lignin degradation by white rot fungus has been extensively researched, mostly for biotechnological uses in the pulp and paper sector, wastewater treatment in pulp mills or industrial textiles, and soil biological treatment.

Textile dyes, personal care products and pharmaceuticals (PCPP), and endocrine disruptors (also known as endocrine disrupting chemicals, or EDCs), which are substances that mimic natural hormones, have been the focus of wastewater biological treatment [4,5,6,7]. Polychlorinated aromatic hydrocarbons (PAHs), halogenated compounds (polychlorinated biphenyls (PCBs), chloro- or bromophenols, polychlorinated dibenzo-p-dioxins, and polychlorinated dibenzofurans (PCDD/F)), a variety of products, agricultural chemicals used as pesticides or herbicides, and nitroaromatic substances like trinitrotoluene (TNT) used in explosives are among the contaminants found in soil systems [8]. These substances are frequently produced by gas plants, wood processing facilities, and military operations. White rot fungi have been shown to degrade all of these types of pollutants, and some in vitro research using fungal oxidative enzymes has also been conducted. The biological treatment process can benefit greatly from the use of enzymes, an environmentally benign industrial biocatalyst. Fungal laccases are appealing enzymes for the restoration of water and soil systems contaminated with different organic contaminants due to their capacity to oxidise a broad variety of substrate molecules by molecular oxygen reduction to water [9]. Furthermore, testing for the breakdown of these enzymes has been made feasible by the availability of multiple commercially manufactured fungal laccases and the general ability of fungi from various eco-physiological groups to create a range of laccase isoenzymes. In various biological treatment systems, Xenobiotic chemicals are destroyed [10,11,12,13,14]. Numerous laccase candidates have been produced as a result of the growing availability of whole fungal genome sequences [15,16,17,18]. Additionally, new developments in genome editing and genetic engineering will make it possible to produce recombinant fungal laccases more cheaply and enhance their biochemical characteristics for use in bioprocessing [19].

Our study aims to remove dyes pollution from the environment mainly from water through microbial laccase enzyme which has a fundamental role in phenolic compounds decomposition.

Materials and Methods

2.1. Materials

2.1.1. Fungal isolation

Twenty one fungal isolates were isolated from seven soil samples from different regions of Egypt, ,for isolation of laccase producing fungi.

2.1.2. Reagents

Hydrochloric acid HCl solution with a concentration of 0.1N: This solution was prepared by adding 8.73 ml of concentrated hydrochloric acid to a volume of distilled water to make a final volume of 1 liter of distilled

water. The resulting solution was used to adjust the pH of the media used in the study.

Sodium hydroxide solution NaOH with a concentration of 0.1 N: This solution was prepared by dissolving 4 g of sodium hydroxide in distilled water to a volume of 1 liter of distilled water. The resulting solution was used to adjust the pH of the media used in the study [20].

2.1.3. Media

A- Potato dextrose agar media

Potato dextrose agar (PDA) medium was prepared by dissolving 200 gm peeled and sliced potato,20 gm dextrose, 20 gm agar in 1 liter distilled water. The medium was sterilized in an autoclave at a temperature of 121 °C and a pressure of 1.5 atm for 20 min. This medium was used for activation and preservation of isolates and for comparison purposes [20].

B- Potato dextrose broth

This medium was used to cultivate fungal isolates and assay of laccase enzyme, it prepared by dissolving 200 gm peeled and sliced potato,20 gm dextrose in 1 liter distilled water., this medium sterilized at 121^{O}C at 1.5 atm for 20 min. medium was cooled then inoculated with fungal spores and cultivated for 5 days at 30^{O}C [20].

2.2. Methods

2.2.1. Sampling

Soil samples were collected in May 2022 from different regions within Dakahlia governorate, Egypt. Samples were separated and labeled according to their location. Soil samples were collected from the plow layer (0–15 cm depth) of the soil. Approximately 100 g of soil was collected and packed into labeled sterile bottles [21].

2.2.2. Sterilization Technique

All glass ware was sterilized in a hot air oven at 180 ^{0}C for 2 h. All the prepared media were sterilized in an autoclave at 121^{0}C for 20 min. The isolation and inoculation were carried out in a laminar air flow chamber.

2.2.3. Isolation and purification of laccase-producing fungi

Fungi were isolated and purified from different soil samples diluted in 0.85 g/LNaCl saline using the serial dilution method. 200 µL in triplicate for each sample. Lastly, they were cultured in an incubator at 28 °C for 3–5 days of culture, mycelium grew on his PDA medium. Colonies with the most similar morphology when cultured in the dark at 28 °C for 3–7 days were classified into the same type according to colony color and morphology and uniformly numbered. To avoid bacterial contamination, they were collected with a pipette and spread on PDA medium plates supplemented with appropriate antibiotics (gentamicin 8 µg/ml) [22].

2.2.4. Inoculum Preparation

Fungal isolates were obtained from different polluted soil samples within Dakahlia governorate in Egypt to obtain laccase-producing fungi which could be applied in bioremediation of synthetic dyes that are used in many industrial fields particularly in textile field, these dyes depend mainly in their color on phenolic compounds which the target of our laccase enzyme. Seven soil samples which carried out, from each dilution about 200µl transferred into PDA agar plates supplemented with Gentamycin (8 µg/ml) to eliminate yeast and mold growth, beside bacteria incubated at 30^{O}C for 6 days. Grown colonies were transferred to freshly prepared PD broth medium for enzyme assay which incubated in a shaking incubator at 30 °C / 120 rpm for 5 days to allow mycelial growth through spore germination [23].

2.2.5. Laccase Activity Assay

Extracellular laccase enzyme activity was estimated through enzymatic extracellular crude extract by spectrophotometry, monitoring the change in absorbance due to the oxidation of 2,6-dimethoxyphenol (2,6-DMP) through UV/Vis Shimadzu spectrophotometer. The standard reaction mixture (1 mL) contained 2,6-DMP (2 mM) in K2HPO4 (0.1 M, pH 6.5) and 40 µL of enzymatic extracellular crude extract. The reaction was monitored at 468 nm for 4 min at 30 °C The laccase activity in U/mL was calculated using this formula:

E. A=A V/t x e x v

where:

E. A=Enzymeactivity , A=Absorbance ,V=Total mixture volume (mL), v =Enzyme volume(mL)

[23].

2.2.6. Optimization of cultural and nutritional growth conditions for laccase production.

Through Minitab software, conditions for maximum enzyme productivity were optimized,and twenty-one trials were obtained as experimental prediction. These trials were for the most producer laccase isolate [24,25].

2.2.6.1. Effect of initial pH

For each culture, fermentation fungal tubes were incubated at different pH values (5.0, 6.0 and 7.0) to confirm the maximum production of laccase activity. These culture bottles were incubated at 100 rpm on a rotary shaker for detection which give maximum productivity.

2.2.6.2. Effect of incubation time

The effect of incubation time on laccase activity was calculated at different incubation periods being 4 to 6 days. Fermentation flasks were incubated at 100 rpm for determination which give maximum laccase productivity.

2.2.6.3. Effect of temperature on laccase activity

The effect of cultivation temperature on laccase activity was investigated at 25 ^{0}C, 27.5 ^{0}C and 30 ^{0}C through 6 days of cultivation.

2.2.6.4. Effect of carbon source

The effect of carbon source on laccase activity was investigated by adding each of glucose and sucrose with the concentrations of 1.5 to 2.5 g/L considering the previous optimized conditions.

2.2.6.5. Effect of nitrogen source

The effect of nitrogen source on laccase production was studied using NH4Cl, and urea, as inorganic and organic nitrogen sources with different concentrations (1.5, 1.75, 2, and 2.5 g/L).

2.2.7. Identification of laccase- producing Fungi

2.2.7.1. Macroscopic and microscopic examination of isolated fungi

The morphology of the fungi was examined macroscopically by describing colony characteristics (color, size, shape, hyphae), and microscopically by observing lactophenol cotton blue-stained slides on which mycelium was attached these slides were finally examined and identified using standard taxonomic references [26].

2.2.7.2. Molecular Identification

2.2.7.2.1. PCR amplification of Pleurotus Laccase coding gene sequencing

PCR was performed on a programmable DNA thermal cycler PCR system. The PCR reaction mixture was optimized to 25 µl containing 40 ng (6 µl) template (fungal) DNA solution, and 8.5 µl master mix containing dNTP mix, MgCl2, Taq polymerase, and PCR buffer. The primers were listed in **[Table 1]** added separately (1 µmol/L of each primer) after preparation from lyophilized stock solutions. The amplified DNA product was separated into TAE along with the DNA marker [Gene Ruler 100 bp DNA Ladder (SM0241)] by electrophoresis using a 1% agarose gel (Hamed et al., 2019). Gel was stained with ethidium bromide and band profiles were recorded through UV Gel documentation system, then Amplified DNA products were purified and sequenced [27].

Table (1): specific PCR primers for amplification of laccase coding gene from the isolated strains

Primer	5-3 sequence	GC%	Extinction coefficient l/(mol˙cm)	Tm ^{0}C
FW-Kh20	ATGTTCAGACGCG CCGCGAA	60%	194200.0	69
RW-Kh20	CACAGCGAACCTG TAGATGC	55%		64

2.2.7.2.2. SDS-PAGE fingerprinting of bacterial strains:

A- Extraction of total proteins content:

Approximately 1o ml of Fungal fermentation was inoculated into 50 ml of PD broth and incubated at 37°C for 6 days. 50 ml of culture was centrifuged for 10 min at 10,000 rpm in a tube with a green cap. The pellet was then resuspended in 1 mL of distilled water,

transferred to a 1.5 mL Eppendorf tube, and centrifuged at 13,000 rpm for 2 min. After discarding the water, the pellet was washed twice with 1000 µL of TNT I buffer and centrifuged. After discarding the upper layer, the pellet was again washed twice with 1000 µL of TNT II buffer and centrifuged. Laemmli buffer (350–400 µL) was added to the pellet and mixed well.

B- SDS-PAGE Gel Preparation

A 100 ml gel was prepared by diluting 40.9 ml of acrylamide stock solution with 25 ml of stock separation gel buffer according to [28]. 100 µl of N, N, N, N`-tetramethylethylenediamine (TEMED) was added, and the volume was adjusted to 99 ml with double distilled water. After degassing and immediately pouring the gel, 1 ml of freshly prepared 10% ammonium persulfate solution was added. To prepare 50 mL of stacking gel, 6.5 mL of acrylamide stock solution was diluted with 12.5 mL of stacking gel stock buffer, 50 µL of His TEMED was added, and the volume was brought to 49.5 mL with double-distilled water. The mixture was degassed and 0.5 ml of freshly prepared 10% ammonium persulfate was added.

C- SDS-PAGE electrophoresis:

Total proteins were subjected to SDS-PAGE in 1 mm thick gel plates (3.5 cm, 4% stacking gel and 16.5 cm, 12.5% resolution gel) as described in (Laemmli, 1970). Electrophoresis was performed at 100 volts, 30 mA, and 4°C for 12 h. The gels were then stained with Coomassie Brilliant Blue R-250 and left on a low-speed shaker overnight. After removing the staining solution, 100 ml of the staining solution was added. The gel was gently stirred for 1 hour. Stirring was repeated three times with different target solutions until the gel background became clear. A photograph of the gel was then taken.

D- Data analysis

Gels were examined by naked eyes directly and the protein profiles were recorded through Gel analyzer software to determine protein bands against marker lane and estimate laccase enzyme protein molecular weight.

2.2.8. Biodegradation of phenol by laccase enzyme [29].

To execute the oxidation of standard phenol by crud enzyme, 1 milliliter of enzyme filtrate was mixed with 2 milliliters of 0.1M citrate-phosphate buffer (pH 6) at several concentrations of phenol (476 µg/l). The mixture was kept in a shaking water bath at 25 0C for incubation. Following zero, two, four, six, and twenty-four hours, the percentage(%) of phenol degradation was computed using the following

equation:

Phenol degradation (%) = (Phenol concentration after incubation at different times x 100)/(Phenol concentration at zero time)

Result and Discussion

3.1 .Collection and Isolation of Specimens

Fungal bioremediation is essential to environmental restoration, since fungi have the extraordinary capacity to breakdown and eliminate a wide range of contaminants. These adaptable microbes could degrade a wide range of toxins, such as organic pollutants, heavy metals, hydrocarbons, and dyes into less dangerous materials[2]. Within 6 days any single colony grown was picked up, transferred into a new plate of PDA, and coded for the next step. Twenty-one fungal isolates belonged to five fungal genera isolates belonged to *Fusarium sp*, eleven isolates to *Aspergillus sp*, three isolates to *Mucor sp*, and two isolates to *Alternaria sp*, and one belongs to *Pleurotus sp*., in **[Figure 1].**

3.2 .Production and Optimization of Laccase Enzyme Assay

Laccase enzyme assay from twenty-one isolates indicates that enzyme productivity was confirmed into one isolate only (M21),that determined laccase enzyme productivity e with activity 0.47 U/ml **[Figure 2,3].**This producing isolate -M21- was optimized through seven factors, i.e., pH,, temp, incubation time, carbon source, and nitrogen source, for the laccase productivity.

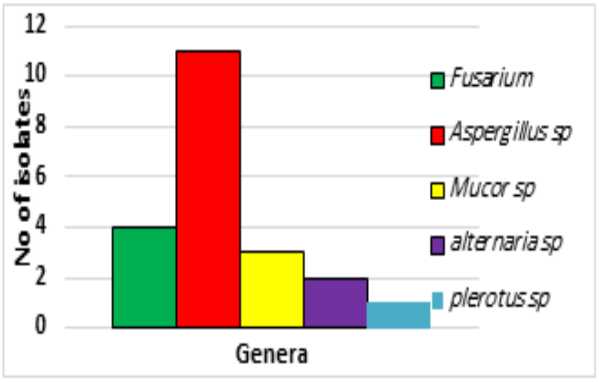


Figure (1): Five fungal genera which twenty-one isolates belonged to them.

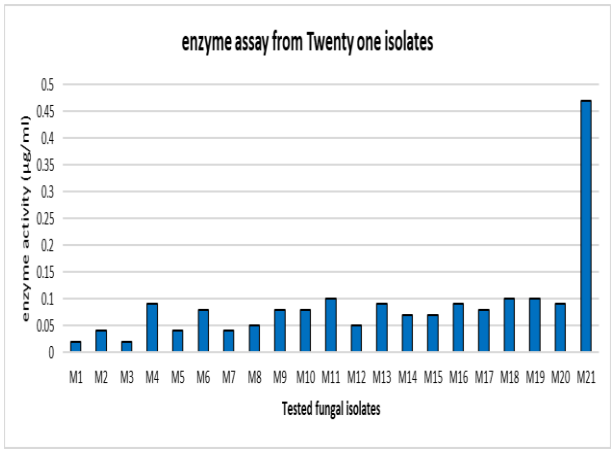


Figure (2): Laccase enzyme activity of twenty-one fungal isolates.

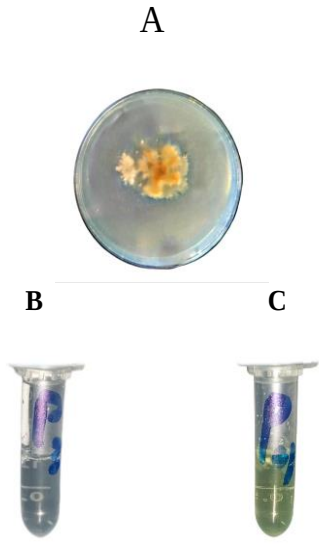


Figure (3): (A) *Pleurotus sp.* on PDA Media with DMP as substrate, (B) *Pleurotus sp.* on PD broth media without DMP, (C) *Pleurotus sp.* on PD broth media with DMP.

Isolate M21 was optimized for its enzyme activity through many factors, firstly, different three pH (5, 6 and 7) were tested for enzyme activity. Enzyme activity was with the least value at pH 5, while activity improved at pH 6 and 7 where the maximum activity was at pH7. Also, regarding fermentation time effects on the enzyme activity, different fermentation periods were studied which were 4, 5 and 6 days of fermentation, maximum enzyme productivity was achieved after 6days of fermentation. Different temperature degrees of fermentation were also evaluated for enhancing enzyme productivity 25, 27.5 and 30OC, and our study noted that 30℃ was the best for enzyme production. The best carbon source concentration was (1.5 g/L) from glucose and (2 g/L) sucrose also glucose and sucrose concentrations 2 and 1.75 g/L were recommended for improves enzyme activity but slightly lower than 2.5 g/L for both sugars. To determine the best nitrogen source, concentrations of urea and NH4Cl were tested. Urea improves the enzyme activity with concentration 2 g/L, while ammonium chloride was enhancer for enzyme productivity in concentration 2.5 g/L. based on optimized conditions as previously mentioned, five subcultures of *Pleurotus sp.* were the best enzyme producers among of twenty-one, these subcultures coded as 2, 4, 11, 16 and 20. The most frequent fermentation time for all subcultures was 140h, followed by 100h, then 120h as the least frequent fermentation time **[Figure 4].**

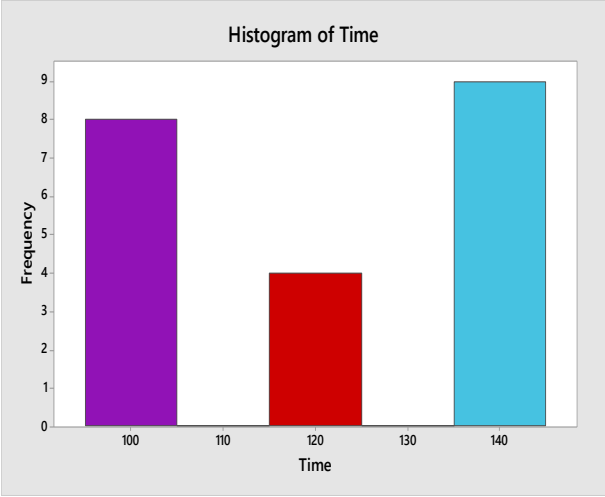


Figure (4): Histogram for fermentation time freuqency for 21 subcultures of isolate M21.

Based on pH, the most freuqent pH was 7 followed by 5, then 6 as described in histogram in **[Figure 5] .**

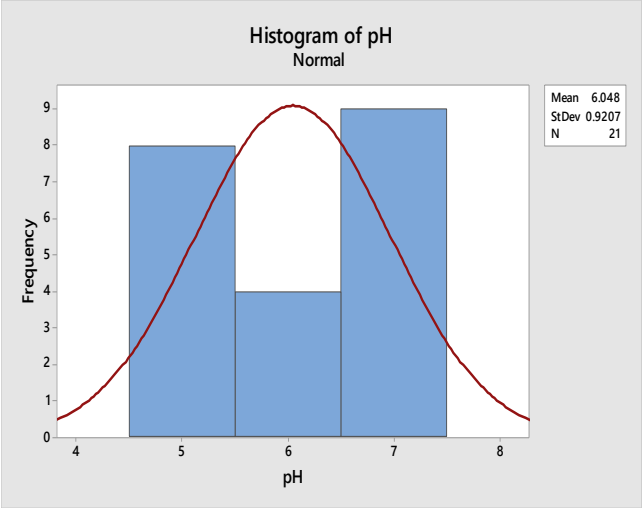


Figure (5): Histogram for fermentation pH freuqency for 21 subcultures of isolate M21 based on optimized conditions for each one separately.

Optimization was also applied to temperature, different degrees were tested, 25, 27.5 and 30^{O}C, the most frerquent temperature was 30^{O}C with 11, followed by 25^{O}C with 7, then 27.5^{O}C with 3 as in **[Figure 6].**

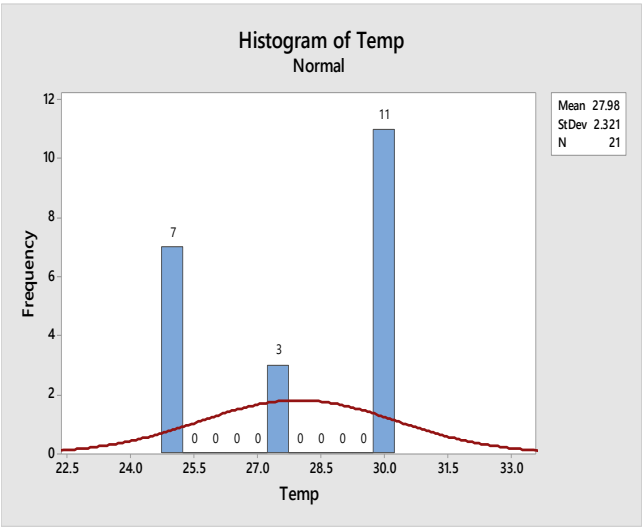


Figure (6): Histogram for fermentation pH freuqency for 21 subcultures of isolate M21.

[30] Li et al. (2011) proposed a potential explanation, demonstrating that the overproduction of G. lucidum laccase was caused by the carbon source succession brought on by Candida sp. converting glucose into glycerol during the co-culture process. The best carbon source concentration according to [31] *Li* et al. 2011 was (2 g/L) from glucose and sucrose together with 1:1 ratio among different ratios of these two sugars, also glucose and sucrose concentrations 1.5 and 1.75 g/L were recommended for improving enzyme activity but slightly lower than 2 g/L for both sugars.

In our study, The best producing fungus coding number(11) with productivity reaching 0.67 U/ml followed by (2) with 0.65 U/mL, then number (16) with 0.63 U/mL, then number (20) with 0.60 U/mL , while number (4) was the least producer among the best-chosen fungi with 0.46 U/mL . All these fungal cultures optimized at temp (30^{O}C), also pH values 6 and 7 were the best for productivity in these cultures. All subcultures reached maximal productivity after 6 days of fermentation while only one (4) reached its maximal productivity after 4 days of fermentation. Two subcultures (2 and 4) were optimized for carbon source (glucose and sucrose) in concentrations of 2g/L for both, while one subculture (11) was optimized at 1.5 and 2 g/L for glucose and sucrose respectively. Subculture (16) requires 1.75 g/L glucose and 1.5 g/L sucrose. Subculture (20) was optimized at 1.5 g/L glucose and 1.75 g/L sucrose. In the study of [31] Durán- Sequeda *et al.*, 2021, two nitrogen sources (i.e., ammonium sulfate- and Yeast Extract) were selected to determine which best enhances fungal biomass production and laccase- activity in Submerged fermentation. This approach enables evaluation of laccase activity in the medium in the absence or presence of laccase-inducing copper sulfate, comparing the performance of nutrient-rich, carbon-only, and nitrogen-rich conditions, YE was a more effective nitrogen source than ammonium sulfate that stimulated biomass and laccase activity under the media conditions evaluated. In current study, Regarding nitrogen source, only subculture (16) gave maximum production 2 g/L for both urea and ammonium chloride, subcultures (2 and 11) optimized at 2 and 2.5 g/L for urea and ammonium chloride respectively, subcultures (4 and 20) shared the same urea concentration 1.5 g/L while (4) optimized at 2.25 g/L while (20) optimized at 2.5 g/L for ammonium chloride **[Table 2].**

3.3 .Molecular Identification of Laccase Enzyme

Oxidoreductase coding gene within *Pleurotus sp.* (M21) was detected, amplified, and sequenced, DNA amplicon was estimated through agarose gel electrophoresis which

exhibits a size of 831bps when compared with known DNA ladder **[Figure 8],** followed by alignment the resulted sequence against the most similar sequences on NCBI through NCBI BLASTn, our result evaluate sequencing of oxidoreductase gene with 831bps of DNA **[Figure 9],** which codes for 332 amino acids including start codon for methionine and stop codon **[Figure 10].**

Table (2): Optimized production conditions for laccase enzyme from the most potent subcultures.

subcultures	PH	Time	gluose	sucrose	urea	NH4Cl	Average of Laccase Activity U/mL	Temp
2	6	144	2	2	2	2.5	0.651	30
4	7	96	2	2	1.5	2.25	0.469	30
11	7	144	1.5	2	2	2.5	0.670	30
16	7	144	1.75	1.5	2	2	0.631	30
20	6	144	1.5	1.75	1.5	2.5	0.601	30
M21	7	144	1.5	2	1.5	2	0.47	30

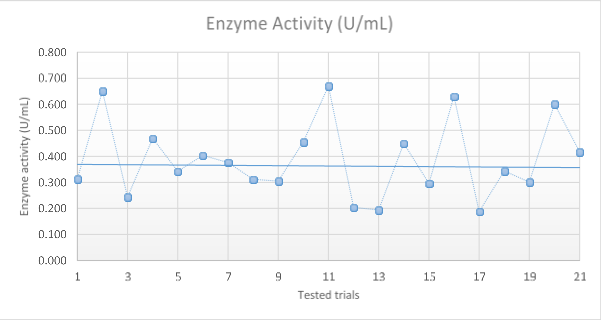


Figure (7): Optimized production conditions for laccase enzyme .

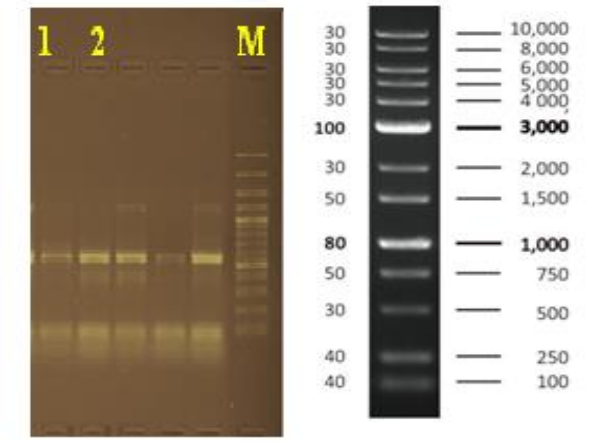


Figure (8): gel documentation of 1% Agarose gel electrophoresis of two subcultures of Pleurotus ostreatus where, Band 1 represents strain without laccase substrate, and Band 2 represents strain with laccase with its substrate at optimal culture conditions against band M which represents DNA ladder (1Kb PLUS DNA ladder, 100-10Kb).

>MK_312024. *Pleurotus ostreatus* Oxidoreductase (Mk10), partial mRNA 831bps

ATGTTCAGACGCGCCGCGAACTCGTCGCTACGAAG
AAGTCTGGATACCTCGACGCCGCGTTGGGGTCCCG
CACCGCGTTTGAACCCGCTTGGCTTCGCGCTTGGTG
CCTCGACCGTGGTCGCCACTTACCTGACGTTGCGGT
TGAGTCTGGATAGCCAAAAAGTCGCGTTAGATGGT
CCTGCCAGTGGTTCAGCATCTTCCAAGCCTCCGCAA
TCCGACTTCAGGTATCCCAGCTTTTCGCCTGCGCAT
GTCCCCCAGCCTGTGCGCGCCGCCGAGCCAGAGGT
ATCGTCCTTTGACGACGCAGAATCATCTTCGGCACC
TTCTGAAACCACGGCCGAGCCCTCCTAAGCATCCG
AGGACACCACAGATGCTTCTTCCACCACACCGGAG
TCAGAAACTACTGGCGTCGCAGGAGAAGAGGACTC
TGCTGAAGCTGGCGCAGGGGGAGGTGGTGCATACG
ACCCCGTTACCGGCGAGATCAATTGGGACTGCCCG
TGCTTAGGTGGTATGGCTCATGGACCGTGTGGCCCA
CAGTTCCGCGAAGCTTTCTCGTGCTTTATCCACTCG
GAGGACGAACCGAAGGGTATCAACTGCGTCGAAAA
GTTCAAGGACATGCAGAATTGCTTCAGAGAGCATC
CGGAGATCTATGCCGAAGAAATTATGGATGATGAT
GACGACGAGCCAGAGGGTGTAGCCATCGCGGAGTC
CGCTGCTGCTGACGAAGTTCGACCTCCCCCAGAGTC
GGAGAAGTATGAGCCTCCAGTAGAGCACATCAACT
CCTCTTCAGCAGACTCGCCGCCCTGGGACACGGCA
TCTACAGGTTCGCTGTGA

Figure (9): FASTA format of Oxidoreductase gene coding region of *Pleurotus ostreatus.*

>sequence 1 *Pleurotus ostreatus* [Oxidoreductase_partial] mRNA 831bps

MFRRAANSSLRRSLDTSTPRWGPAPRLNPLGFALGAST
VVATYLTLRLSLDSQKVALDGPASGSASSKPPQSDFRY
PSFSPAHVPQPVRAAEPEVSSFDDAESSSAPSETTAEPS
MVLPVVQHLPSLRNPTSGIPAFRLRMSPSLCAPPSQRYR
PLTTQNHLRHLLKPRPSPPKHPRTPQMLLPPHRSQKLLA
SQEKRTLLKLAQGEVVHTTPLPARSIGTARAMAHGPCG
PQFREAFSCFIHSEDEPKGINCVEKFKDMQNCFREHPEI
YAEEIMDDDDDEPEGVAIAESAAADEVRPPPESEKYEP
PVEHINSSSADSPPWDTASTGSL*

Figure (10): FASTA format of amino acids sequence of oxidoreductase gene of Pleurotus ostreatus generated through snapgene software.

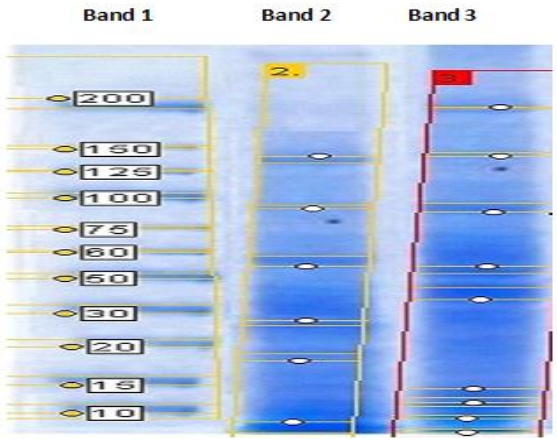
Sequence analysis of gene indicates that our gene sequence highly similar with 98.44% with *Pleurotus ostreatus* Oxidoreductase (MIA40), partial mRNA, followed by 82.9% similarity with Pleurotus ostreatus genome assembly, chromosome 3, the top ten hint of similar sequences with gene of interest was summarized in Table (3) and [figure 11].

Table (3): Top ten hint of the most similar sequences with oxidoreductase of Pleurotus ostreatus in NCBI BLASTn

Description	Scientific Name	Per. Ident	Accession
Pleurotus ostreatus Oxidoreductase (MEA-40), partial mRNA	*Pleurotus ostreatus*	98.44%	XM_037773451
Pleurotus ostreatus genome assembly, chromosome: 5	*Pleurotus ostreatus*	92.89%	OX344737.1
Epithelial cryptase uncharacterized protein (BXZ75DRAFT_980358), partial mRNA	*Epithelial cryptase*	80.00%	XM_046802781.1
Psilocybe cubensis uncharacterized protein (JR316_0002565), partial mRNA	*Psilocybe cubensis*	76.78%	XM_047888157.1
Fomitopsis rosea uncharacterized protein (C8Q71DRAFT_680166), partial mRNA	*Rhodofomes roseus*	79.49%	XM_047819745.1
Coprinopsis cinerea okayama7#130 intermembrane space import and assembly protein 40 (CC1G_01882), mRNA	*Coprinopsis cinerea okayama7#130*	77.87%	XM_001830194.2
Postia placenta MAD-698-R-SB12 hypothetical protein (POSPLADRAFT_1053243), partial mRNA	*Postia placenta MAD-698-R-SB12*	78.11%	XM_024805681
Gloeophyllum trabeum ATCC 11539 uncharacterized protein (GLOTRDRAFT_74525), mRNA	*Gloeophyllum trabeum ATCC 11539*	78.11%	XM_007866588.1
Auricularia subglabra uncharacterized protein (BODA9DRAFT_300922), mRNA	*Auricularia subglabra*	76.89%	XM_046857452.1
Pseudozyma flocculosa PF-1 uncharacterized protein (PFL1_02524), partial mRNA	*Pseudozyma flocculosa PF-1*	80.95%	XM_007880041.1

[32] Contato *et al.,* 2020 state Although laccases from basidiomycetes are typically monomeric proteins with molecular masses ranging from 50 to 80 kDa, the culture circumstances may have a major impact on the formation of isoforms. However, using denaturing and non-denaturing SDS-PAGE, Souza and Peralta (2003) discovered an isoform of isolated laccase of *P. pulmonarius* CCB-19 with a molecular mass of 22.4 kDa. The zymogram confirmed that the crude laccase had a major band with a molecular weight of 23 kDa and 46.5 kDa. Syringaldazine, dimethoxyphenol (DMP), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) were used as substrates for the kinetic investigations [33]. In 2018, Brugnari *et al.,*

In our study, According to previous result, *Pleurotus ostreatus* strain (M21) as the most potent laccase enzyme producing strain, so laccase enzyme from this strain was evaluated through SDS-PAGE to estimate its molecular weight, firstly the strain was fermented in its optimal conditions, gel analysis of SDS-PAGE showed laccase enzyme protein band in range 55-57 KDaltons, the enzyme as showed in **[Figure 11]** was characterized with and without phenolic compounds (DMP) which indicates the presence of band in either DMP presence or absence that reflect that gene coding laccase enzyme is constitutive in its expression



57KDaltons **55KDaltons**

Figure (11): SDS-PAGE analysis through gel analyzer software for total cellular protein of standard strain, where from left to right, Band 1: Bovin serum albumin ladder, band 2: strain without DMP, and band 3: strain with DMP.

3.4. Bio degradation of Phenolic Compounds using Laccase Enzyme produced by *Pleurotus ostreatus*

In bioremediation, the laccases degrade phenolic compounds that are important environmental pollutants. These polyphenol oxidases have been obtained from the fermentation of agricultural waste according to [34] Menezes, Silva and Durrant, 2009.

In current study, Oxidoreductase enzyme also was exhibits activity against phenolic compounds when (Azo Dye from Egyptian German For Pigments & Resins) used as a substrate for the enzyme, total phenolic compounds concentration in untreated substrate was 476.4 µg/ml. there was a remarkable reduction in this concentration when laccase enzyme applied to Azo dye as substrate, it showed 127.2 (26.7%) µg/ml **[Figure 12].**

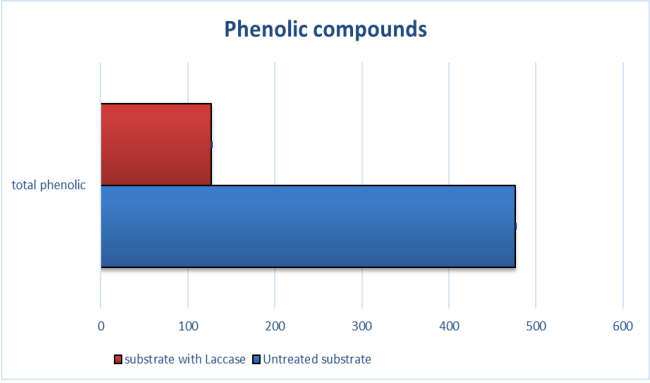


Figure (12): Total phenolic compounds reduction by laccase enzyme on azodye as a substrate.

Conclusion

Laccase is used in the synthesis of organic substances, where typical substrates are amines and phenols, the reaction products are dimers and oligomers derived from the coupling of reactive radical intermediates. *Pleurotus ostreatus* was the best fungal strain among other basidiomycetes in enzyme productivity. *Pleurotus ostreatus* Laccase productivity was reached the maximum value at both pH 7, within four and six days of incubation with shaking at 150 r.p.m, 30^{O}C, 1.5 g/l from glucose and 2g/l sucrose, Also, urea and ammonium chloride mixtures were effective in ratios 2 g/l and 2.5 g/l respectively. Gene coding for laccase was partially estimated by 831bps, and its sequence analysis showed 98.44% with *Pleurotus ostreatus* Oxidoreductase (MIA40). Also enzyme protein was estimated by 55- 57KDa. Laccase enzyme also was exhibits activity against phenolic compounds which found in wastes and dyes. Laccase enzyme productivity may be increased through genetic improvement through Directed and random DNA mutations that predict a higher production of enzyme than environmental optimization.

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