fermentation

Article

Laccase Production from Local Biomass Using Solid State Fermentation

Shoaib Hasan [1], Zahid Anwar [1], Waseem Khalid [2], Fareed Afzal [2], Muddassar Zafar [1], Usman Ali [1], Mohammed Y. Refai [3], Mohamed Afifi [3,4,5], Ammar AL-Farga [3] and Moneera O. Aljobair [6,*]

[1] Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat 50700, Pakistan
[2] Department of Food Science, Faculty of Life Sciences, Government College University, Faisalabad 38000, Pakistan
[3] Department of Biochemistry, College of Sciences, University of Jeddah, Jeddah 21959, Saudi Arabia
[4] Department of Biochemistry, Faculty of Veterinary Medicine, Zagazig University, Zagazig P.O. Box 44519, Egypt
[5] Najla Bint Saud Al Saud Center for Distinguished Research in Biotechnology, Jeddah 21577, Saudi Arabia
[6] Department of Physical Sport Science, College of Education, Princess Nourah Bint Abdulrahman University, P.O. Box 84428, Riyadh 11671, Saudi Arabia
* Correspondence: moaljobair@pnu.edu.sa

Abstract: The large family of enzymes, known as polyphenols oxidases, includes laccase. Due to the inclusion of a copper atom in their catalytic core, laccases are frequently referred to as multi-copper oxidases. Laccases are versatile enzymes that can catalyze the oxidation of a wide range of phenolic and non-phenolic substances. In the current study, a local strain of *Aspergillus niger* was used for solid-state fermentation to produce fungal laccase, as well as purify and optimize laccase. The enzyme profile, which was acquired using guaiacol to measure enzyme activity, showed that after five days of incubation, wheat straw provided the highest level of laccase activity, or 2.551 U/mL. A technique called response surface methodology (RSM) was used to examine the effects of various conditions on the production of enzymes. The RSM results demonstrated that after five days of incubation, the enzyme activity was at its highest at 45 °C, pH 5.5, and 30% moisture level, inoculated with 2 mL mycelium. Through ammonium sulphate precipitation and dialysis, the enzyme was purified. Additionally, column chromatography was used to further purify laccase. The next step was enzyme characterization to evaluate how temperature and pH affected enzyme activity. At 45 °C and pH 5.5, the isolated enzyme produced its highest level of activity. The findings of the current study showed that *A. niger* is capable of producing laccase in an economical and environmentally friendly way. Due to its unique oxidative and catalytic features, this enzyme has received a lot of attention recently.

Keywords: laccase; *Aspergillus niger*; fermentation; enzyme

Citation: Hasan, S.; Anwar, Z.; Khalid, W.; Afzal, F.; Zafar, M.; Ali, U.; Refai, M.Y.; Afifi, M.; AL-Farga, A.; Aljobair, M.O. Laccase Production from Local Biomass Using Solid State Fermentation. *Fermentation* **2023**, *9*, 179. https://doi.org/10.3390/fermentation9020179

Academic Editor: Dharam Singh

Received: 30 December 2022
Revised: 1 February 2023
Accepted: 13 February 2023
Published: 16 February 2023

1. Introduction

The enzyme laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to a large class of enzymes, known as polyphenols oxidases, that are frequently referred to as multi-copper oxidases, because they have copper atoms in the catalytic center. Laccases have three different sorts of copper atoms, one of which gives them their distinctive blue color. White or yellow laccases are the names given to the enzymes that lack a blue copper atom. Typically, oxygen is reduced to water during laccase-mediated catalysis, and the substrate is then oxidized. Thus, laccases are oxidases that oxidize a variety of substances, including polyphenols, methoxy-substituted phenols, aromatic diamines and others [1]. Oxidation processes are important in many industries; however, many classical oxidation procedures have limitations regarding the usage of chemicals, due to environmental risks, nonspecific binding, and undesirable side effects. The ability to study novel oxidation approaches to biological systems, such as enzymatic oxidation has drawn a great deal of

attention from researchers. These systems are more stable and are carried out under mild conditions. In plants, the key components are lignocellulosic materials and they consist of cellulose, hemicellulose and lignin. A significant barrier protecting hemicellulose and cellulose from enzymatic attack is lignin [2].

Various microorganisms, such as fungi, have the capability to degrade the lignin of lignocellulosic waste. Numerous alternative methods are used by physiochemical and biological processes to separate the lignocellulosic waste. The microwave treatment is employed in physical procedures. In chemical methods, acids and alkalis are used, while in biological methods, enzymes are used that improve the pretreatment methods [3]. The biological method is more acceptable due to its high efficiency during the fermentation process, and this method decreases the drawbacks of physiochemical methods [4]. Laccases, unlike other lignin degrading enzymes, have a place with the super family of multi-copper oxidases [5].

In addition to being found in insects and bacteria, laccases are widely dispersed in higher plants and fungi [6,7]. Ascomycetes, deuteromycetes, and basidiomycetes all contain laccases, but several of the white-rot fungi that are involved in lignin metabolism are particularly rich in laccases [8,9]. Due to their higher redox potential (+800 mV) than bacterial or plant laccases, fungal laccases are used in a variety of biotechnological processes, most notably the breakdown of lignin [10]. For instance, the redox potentials of common laccase-producing fungus *Trametes villosa, Myceliophthora thermophila, Pycnoporuscinnab arinus,* and *Botrytis cinerea* were reported to be 790 mV, 450 mV, 750 mV, and 780 mV, respectively [11]. Therefore, laccases have a great deal of promise for application as food industry processing aids.

One area of biotechnology that is expanding is enzyme manufacturing. The majority of enzyme producers use submerged fermentation (SmF) processes to create their products. The utilization of the solid-state fermentation (SSF) method to create various enzymes has, however, become more popular during the past few decades. SSF is utilized, for instance, in the manufacturing of koji and sake, because it has a long history of use in Asian nations. Composting and ensiling are two examples of natural microbial processes that are replicated by this method. Studies on the production of fungal enzymes in SSF have demonstrated that SSF produces more temperature- and pH-stable enzymes than SmF, has greater volumetric productivities, and is less likely to experience substrate inhibition issues. Additionally, fermentation takes place faster and there is less chance of unfavorable proteases degrading the enzymes generated [12]. Castilho et al. [13] conducted a comparative economic analysis of the SSF and SmF methods for the *Ascomycete Penicillium restrictum* production of lipases. They discovered that the procedure based on SmF required a total capital investment 78% greater than the one based on SSF for a facility producing 100 m^3 lipase concentrate annually, and its product had a unitary cost 68% higher than the product market price. Additionally, Viniegra-González et al. [14] used SSF and SmF techniques to examine the productivity of three fungal enzymes: invertase, pectinase, and tannase. According to their findings, SSF cultivation operates as a fed-batch culture, with quick oxygenation, but a sluggish sugar supply. Additionally, SSF has the added benefit of being a static process without requiring mechanical energy. In addition, Roy et al. [15] recently revealed that SSF was a superior treatment approach to SmF for rubber biodegradation.

The first laccase was reported from the exudates of the *Rhus vernicifera* plant. The fungal origin of the laccase has been mostly studied because fungal laccase is implicated in both extra cellular and intra-physiological processes, including pigmentation and pathogenesis. It is reported that fungal laccase performs various functions, including degradation of lignin, interaction with pathogen host, as well as morphogenesis. Generally, laccases of fungi have monomeric globular-structure proteins, and their size is 60–70 kDa, with an isoelectric point at pH 4.0 [16]

Most of the fungal laccases secrete extracellular enzymes that are glycosylated, and the range of glycosylation is between 10 and 25%, but in some cases, it is also observed to be more than 30% [17]. Although laccases can be obtained from numerous plants and animals,

the industrial need is often met by microbial laccase. The most often utilized fungus for industrial laccase production is *A. niger*, because it has a high acid tolerance and is simple to keep free of bacterial contamination. *A. niger* is very useful due to its broad ranges of temperature and pH. It can easily grow at a pH between 1.4 and 9.8, and a temperature between 6 and 47 °C, while the optimum temperature is 35–37 °C [18]. Due to this secured ability, *A. niger* produces many useful enzymes, such as xylanase, lipase, protease, cellulase and laccase. Present research was conducted for the maximum production and isolation of laccase, and optimization of different physio-chemical parameters by *A. niger*.

A class of copper-containing oxidases, known as laccases, is used in the food and textile, pharmaceutical, biofuel, and dye decolorization sectors, among other industries [19,20]. Laccases have wide substrate ranges and utilize just oxygen as the final electron receptor. They catalyze the oxidation of a wide range of substrates as ortho- and para-diphenols, methoxy-substituted phenols and a few different compounds, to reduce the molecular oxygen to water with a one-electron oxidation system [21]. Almost all white-rot fungi contain laccases, which are believed to play a key role in the breakdown of lignin. It can follow up on a variety of substrates due to its low substrate specificity, which has attracted substantial interest from a number of industrial, ecological, and biotechnological domains [22]. Laccases have been viewed as a "Green Tool", since they require molecular oxygen as the main co-substrate for bio-catalysis, and not hydrogen peroxide (H_2O_2) [23].

Laccases can degrade different structures of synthetic and natural compounds, for example phenols, aromatics, and aliphatic amines. Hence laccases pull in a lot of research attention for their organic capacities in addition to their potential use in the industry and biotechnology. Juices made from natural products can also be stabilized using laccases. Many organic product juices typically contain phenolics and their oxidation products, which provide juice products color and flavor. After some time, the normal co-oxidation and polymerization response of phenolics and polyphenols causes irritating changes in the color and aroma. Juice made from natural products has a lot of polyphenols, which is why the color changes. When compared to other methods, laccase treatment results in the elimination of phenols with a high degree of efficiency. After laccase treatment, the juice phenolic content drops and the color stability improves. Additionally, laccase treatment has been found to be more effective for flavor stability than conventional treatments, such as adding ascorbic acid and sulfites. The high concentration of phenolics and polyphenols becomes the most important factor during the production of wine, especially during the pressing and crushing steps [24].

The commercial development of laccases is being pursued in a cost-effective manner and through the screening of low-cost substrates. The use of agro-industrial waste as substrates adds value to the sector and raises knowledge of recycling and energy usage. Pakistan is a largely agricultural country. All agro-based waste materials, including wheat straw, rice bran, stalk, leaves, sugarcane bagasse and corn cob seed husks, are easily accessible. The majority of these lignocellulosic wastes are disposed of throughout the world as a result of their inappropriate management, but these materials are now valued for their ability to serve as a substrate for the production of useful products. However, it is widely seen that residues from these crops are partially dried and burned for domestic purposes. In addition to the loss of valuable biomass resources that could be a source of carbon, bioactive compounds and energy, crop residues are burned [25]. Greenhouse gases can also be produced from the burning of these crops. Thus, the proper use of these tools for the production of energy and other useful purposes will lead to fruitful results.

The need for alternative procedures is now being realized with increasing scientific expertise and an environmentally friendly insight, and the demand for legislative authorities to contribute to the conservation of living systems and biological systems is of great importance in this regard. The current study focuses on producing, screening, optimizing, and purifying laccase from the local strain of *A. niger* that is a readily available substrate in our vicinity. The goal of this study is to offer a potential remedy for the large-scale solid waste management in our vicinity.

2. Materials and Methods

The methodology of the entire research for the production, purification and characterization of the laccase enzyme will be based on the following steps:

2.1. Isolation and Selection of Microorganism

The pure culture of *A. niger* IBP2013 [26] is available in the Biochemistry Lab, Department of Biochemistry and Biotechnology, University of Gujrat, Pakistan. *A. niger* is a filamentous fungus that is suitable for solid-state fermentation, because it can easily penetrate and colonize solid surfaces. More crucially, the US Food and Drug Administration has classified *A. niger* as "Generally Recognized as Safe", making it usable in the food industry [27]. Potato dextrose agar (PDA) media were used for the growth of the fungus. Potato dextrose (PD) media were used for the culturing of the fungus.

2.2. Media Maintenance

PDA media were prepared and transferred to the autoclaved test tubes to make the slants. Then the spores of the fungus were streaked to the test tubes, with the help of an iron loop, for growth. All of these procedures were performed in a sterilized environment, to make it free of all types of contaminations. After that, slants were incubated for the purpose of sporulation. Then these spores were transferred to the refrigerator and stored at 4 °C.

2.3. Preparation of the Fungal Spore

PDA media, which have all the components necessary for the formation of *A. niger,* were prepared for the growth of mycelium via spore germination.

The mixture of ingredients for the PDA media was taken in a conical flask and sealed with a cotton plug and aluminum foil. It was then sterilized in the autoclave, at a pressure of 15 pounds per square inch (psi) and 121 °C temperature for 15 min. The media needed to be sterilized immediately before the germs started depleting the nutrients, since it would have thousands of bacteria growing in it within a few hours. It was a 100% kill process. The thermal death time for the majority of the species was 15 min.

For the preparation of the media for inoculum, all given ingredients were added in distilled water. Then, spores of fungi were inoculated in these media and kept in the incubator for spore suspension.

2.4. Inoculum Preparation

With the aid of a sterilized iron loop, the *A. niger* spores were transferred to freshly prepared PD media, then kept in a shaking incubator at 30 °C at 120 rpm for seventy-two hours, for the growth of mycelium via spore germination. As the continuously increasing speed of the shaking incubator slowed down, the development of mycelia increased. Therefore, the fully developed suspension of fungal spores was kept at 4 °C for further use. After 15 days, the preserved culture was re-grown on new media, to preserve the strain's viability.

2.5. Lignocellulosic Substrates

Different types of lignocellulosic substrates, i.e., sugarcane bagasse, reed grass, rice bran, wheat bran, wheat straw, corn cobs, guava leaves and peanut husk, were collected locally. These agro-based substrates are cost-effective, and their ease of availability has made them suitable for research purposes. Firstly, all of the substrates were crushed, washed, dried with the help of an oven or under the sun, and then ground with the help of the lab grinding mill (manufactured by MRC Laboratory instruments) into small particles, with a mesh size of 0.2–2 mm. Then, these materials were stored in air-tight bottles to restore their moisture.

2.6. Production of Enzymes

The following steps were performed for the production of enzymes.

2.6.1. Sterilization of Biomass

Separately, 5 g of each kind of substrate was taken into 250 mL conical flasks that had already been prepared and stored for the experiment. Each substrate-containing flask received 5 mL of water before being autoclaved for 15 min to sterilize them.

2.6.2. Inoculation of Substrates

All selected substrates were inoculated by adding 3 mL of the spore suspension of *A. niger*. Then, these inoculated flasks were kept in an incubator by adjusting the temperature to 35 ± 1 °C for 4–5 days. *A. niger* can grow well at the temperatures of 6 to 47 °C and pH ranges of 1.4 to 9.8, however, its ideal temperature range is 35 to 37 °C [18].

2.6.3. Extraction of Enzymes

All experimental flasks were filled with 50 mL of distilled water and placed in the benchtop shaking incubator, model TOU-50, at 120 rpm for an hour. Using Whatman No. 1 filter paper or muslin cloth, the biomass was individually filtered before being placed into falcon tubes. The 50 mL filtrate in these falcon tubes was then centrifuged by the laboratory centrifuge 5702 RH, at 4400 rpm for 30 min. Following centrifugation, the pellet was discarded, and the supernatant was used as a crude enzyme in further tests.

2.7. Determination of Enzyme Activity

Laccase activity was assayed, using guaiacol G5502 (manufactured by Sigma-Aldrich), as a substrate, available in the Biochemistry Lab, Department of Biochemistry and Biotechnology, University of Gujrat, Pakistan. According to previous studies, guaiacol was oxidized for the laccase test [28]. The reddish-brown color that resulted from the oxidation of guaiacol by laccase is used to assess the enzyme's activity. The enzyme activity was measured using a UV-VIS double-beam spectrophotometer, UVD-2950, at 450 nm wavelength. A spectrometer and a photometer are the components of a spectrophotometer. The photometer measures light intensity, while the spectrometer generates light of any wavelength. The liquid or sample is positioned between the spectrometer and the photometer because of the spectrophotometer construction. The photometer generates a voltage signal for the display and calculates the amount of light that enters the sample. The voltage signal also fluctuates when the light absorption varies. A light source, digital display, monochromator, wavelength sector (to transmit a chosen wavelength), collimator (for straight light beam transmission), photoelectric detector, and cuvette (to hold the sample) make up the basic spectrophotometer equipment.

The reaction mixture contained 3 mL of 10 mM sodium acetate buffer at pH 5, 1 mL of 2 mM guaiacol as the substrate, and 1 mL of the enzyme extract [21]. A blank was also prepared containing 1 mL of distilled water instead of the enzyme extract. Then, the activity of the enzyme in all biomass substrates was checked and expressed in U/mL, that defined one unit of enzyme activity as the amount of enzyme required for oxidizing one micromole guaiacol per minute. The change in absorbance was monitored, that show the activity of laccase by using a UV spectrophotometer at 450 nm wavelength [28]. The laccase activity in U/mL was calculated using this formula:

$$E.A = A \times V / t \times e \times v$$

where:

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

t = Incubation time
e = Extinction coefficient for guaiacol (0.6740 μM/cm).
The substrate that had the maximum absorption was selected for further experimentation.

2.8. Optimization via Response Surface Methodology (RSM)

In the present experiment, a critical analysis was made to explore the impact of different factors that can affect the production of laccase, by using response surface methodology (RSM). The different parameters investigated were temperature, pH, inoculum size, biomass size, moisture level, and incubation days for growth. All factors were crucial for the expression of laccase. The trials (number of replicates n = 3) were performed at varying temperatures (30 °C, 37 °C, 45 °C, 52 °C), at pH ranging from 2 to 9.5, with the moisture level ranging from 20 to 125%, and with different substrate sizes and inoculum sizes by varying incubation time. The phosphate buffer was used at pH 8, citrate buffer at pH 5.5 and carbonate buffer at pH 9.5. The combinations of these experiments were executed to explore the impacts of particular conditions on the production of laccase. The laccase activity was monitored under standard assay.

2.9. Coded and Non-Coded Values for RSM

Coded values for all variables from −1 to +1, and non-coded values for each variable are given in Table 1.

Table 1. Coded and Non-Coded Values.

Value Type	**Variables**	**Values**				
Coded	**All Variables**	**$-\alpha$**	**−1**	**0**	**+1**	**$+\alpha$**
Non-coded	pH	2	4	5.5	8	9.5
	Temperature (°C)	30	37	40	45	50
	Moisture Level	20	40	60	80	100
	Inoculum Size (mL)	2	4	6	7	8
	Incubation Time	1	3	5	7	9

2.10. Partial Purification via Ammonium Sulphate Precipitation

The initial step for the purification of proteins is an ammonium sulphate precipitation. This valuable technique empowers the rapid and bulk precipitation of cellular proteins. The filtered broth was subjected to ammonium salt precipitation for the recovery of the extracellular enzyme protein. A salting-in and salting-out technique was used in the ammonium sulphate precipitation process [29]. The filtrate was 70% saturated with ammonium sulphate, before being incubated at room temperature with gentle stirring for 60 min and then centrifuged by the centrifuge 5702 at 10,000 rpm for 15 min. After that, 1 mL of the pellet was added to the test tube together with 3 mL of the sodium acetate buffer pH 5 and 1 mL of guaiacol for the enzyme assay. After 30 min in the incubator, the absorbance of these test tubes was measured at 450 nm. The enzyme with the highest absorbance was taken for further purification.

2.11. Dialysis

Dialysis is a process that involves removing and exchanging tiny molecules from a solution using a specialized semi-permeable membrane that is made up of cellulose acetate. The enzyme that had precipitated was put in a dialysis bag that was submerged in a phosphate buffer. The Spectra/ Por Biotech Grade dialysis tube was shaken overnight in an incubator. To get purer enzyme and to get rid of ammonium sulphate, the phosphate buffer was routinely adjusted.

2.12. Column Chromatography

Gel-filtration chromatography is an extensively used technology for enzyme purification. Silica was employed as a matrix. The column of 100 mL was first thoroughly cleaned, then pure sand and sephadex G-100 were added. After covering the sand in the column with silica gel and locking the column knob, it was pre-equilibrated with 10 mM phosphate buffer at pH 5. Then, 10 mL of the dialyzed enzymes were added to the chromatographic column, gently opening the knob. The activity of the enzyme was tested using an enzyme assay, after the filtered enzyme fractions were administered to falcons on a constant basis, at a steady flow rate of 1 mL/min. Observations of the column flow were made after 60 min. Due to the fact that the initial stage entailed the evacuation of unbound material through a column, no laccase activity was observed. Maximum laccase activity was recorded in the following phase, along with a sharp protein peak, and a slight reduction of laccase activity was seen in the last stage, but was not taken into consideration. Figure 1 depicts the laccase purification procedure.

Figure 1. Laccase purification through column chromatography.

2.13. Characterization

The purified laccase was characterized through kinetic studies. The thermal stability of laccase was determined by oxidation of the guaiacol substrate at optimum a temperature and pH. The optimal parameters for laccase were recorded by performing an enzyme assay.

2.13.1. Effect of Temperature

Enzyme was characterized for a suitable temperature. For this purpose, crude enzyme was taken at different temperatures, ranging from 20 °C to 50 °C, with the same incubation time, to get the optimal temperature for the laccase.

2.13.2. Effect of pH

This effect was investigated at varied pHs, followed by an enzyme assay, to check for the optimal pH for enzyme activity. Different buffers with a varied pH, i.e., sodium acetate buffer (pH 2.0–5.0), phosphate buffer (pH 6.0–8.0) and sodium carbonate buffer (pH 9.0–10.0), were used to explore the impact of pH on enzyme activity.

2.14. Statistical Analysis

Statistical analysis was performed using the Minitab 17 software. The R-square value, which measures model fitness, was evaluated after the RSM readings were analyzed. [30].

3. Results and Discussions

3.1. Screening of Biomass for Laccase Production

Biomass screening was the initial phase in the production and optimization process. The synthesis of the laccase enzyme was then tested on all of the gathered substrates, using guaiacol. After five days of solid-state fermentation incubation, wheat straw demonstrated the highest laccase activity, at 2.551 U/mL, in the enzyme assay used to measure enzyme activity. Previous studies have shown that the *Trichoderma harzianum* strain HZN10 can generate laccase from wheat bran (63 U/g and 9.6 mg/g protein) during solid-state fermentation. The production of laccase as a whole was strongly influenced by both the yeast extract and the wheat bran, according to the results of statistical optimization by RSM, employing a central composite design (CCD) [30].

It has been noted that microorganisms require some time to settle before displaying noteworthy activity [31]. Agitation improves the dissolved oxygen in the culture media, and offers appropriate mixing, mass, and heat transmission. Insufficient oxygen in the culture media typically inhibits microbial growth at lower agitation speeds, whereas higher agitation speeds can also result in decreased enzyme production [32]. Higher agitation rates create shear forces between the microbial cells that are suspended in the culture media, and the production declines as a result of cell damage brought on by cell collision. Shear forces can have a variety of impacts on fungus cells. By causing harm to the external and internal cell structures, varying fungal growth, and altering yield formation, it can affect the morphology of the fungus [33]; however, the pace of growth may vary due to the varied chemical structures of various substrates. The microbial growth is initially shown to increase in the log phase, but it subsequently drops as a result of nutritional exhaustion in the media, and this dropping phase is referred to as the death phase. Other parameters, such as the nutrient content, temperature, pH, culture state, and media composition, also affect the enzyme production. In line with our findings, wheat bran demonstrated the highest laccase activity when tested with *Pleurotus ostreatus* and *Ganoderma* sp. [34,35]. The synthesis of laccase is significantly impacted by the various substrate types. The substrate made of wheat straw was similarly shown to be the most effective for producing laccase by *A. niger*, with full substrate colonization occurring on the fifth day of incubation.

Composition of Biomass for Laccase Production

The biochemical makeup of rice straw and wheat straw is typical of lignocellulosic residue derived from agriculture. It comprises, on average, 30–45% cellulose, 20–25% hemicellulose, 15–20% lignin, and several other minor organic compounds [36]. About 40–50% of cellulose and 25–35% of hemicellulose can be found in sugarcane bagasse. The remainder is made of lignin, wax, etc. [37]. The lignocellulosic substance in the peanut shell has a complex fibrous structure and is made up of cellulose (44.8%), hemicellulose (5.6%), and lignin (36.1%) [38]. In terms of corn husk, its chemical makeup had 34–41% hemicellulose, 31–39% cellulose, 2–14% lignin, 3–7% ash, 10–18% extractives, and components that were water-soluble [39]. The outer layers of the wheat kernel are contained in the wheat bran, which is mostly made up of the insoluble components AX, cellulose, starch, protein, β-glucan, and lignin [40]. Guava leaves (GLs) are a great source of several macro- and micronutrients that are good for health, as well as bioactive substances. They include 103 mg ascorbic acid, 3.64% ash, 0.62% fat, 18.53% protein, 12.74% carbs, and 1717 mg gallic acid equivalents (GAE)/g, total phenolic components. They also contain 82.47% moisture [41]. Rice bran is very nutrient-dense due to its chemical composition, which includes 12–17% protein, 13% fat, 34–54% carbs, 6–14% fiber, and 8–18% ash [42]. Grass is entirely organic because it is a naturally occurring material. Basic elements, including carbon, oxygen, nitrogen, and phosphorus, make up its composition. It also has cellulose and chlorophyll, which are both components of photosynthesis. Water and lignin are the two basic elements in grass. Figure 2 displays the screening graph for biomass.

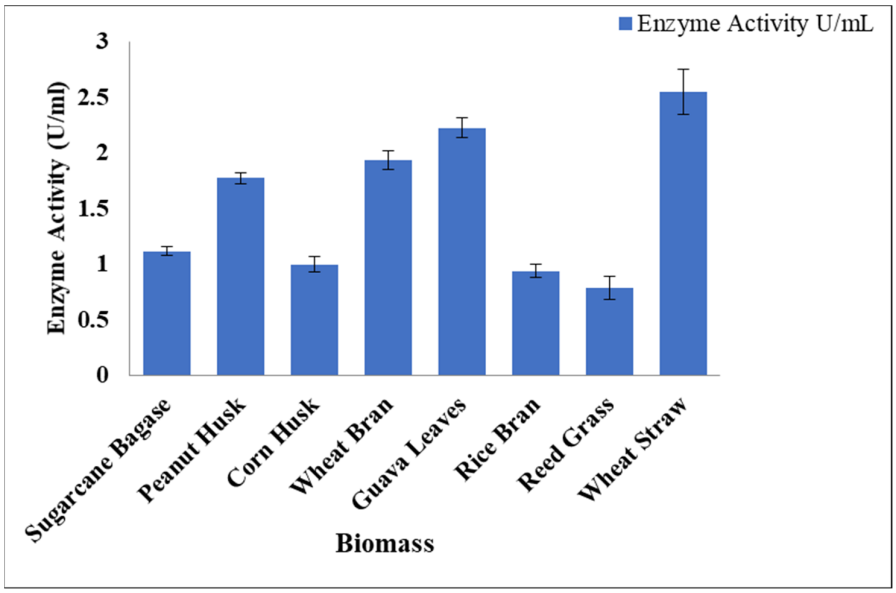

Figure 2. Screening of biomass for laccase production.

3.2. Optimization through Response Surface Methodology (RSM)

After the selection of maximum activity substrate, the optimization of different parameters, i.e., temperature (°C), pH, inoculum size (mL), biomass size (g), incubation time (days) and moisture level (%), was done by a central composite design (CCD), using response surface methodology (RSM). The summary of all these experiments is given in Table 2.

Table 2. Optimization via RSM trials.

Sr #	Substrate Size (g)	pH	Temperature (°C)	Inoculum Size (mL)	Moisture Level (%)	Incubation Time (Days)	Enzyme Activity U/mL Observed	Predicted
1	4	2	45	5	60	5	1.926	2.021
2	4	5.5	37	5	60	5	1.634	1.564
3	2	5.5	45	2	100	5	1.218	1.224
4	2	5.5	45	7	20%	5	1.319	1.311
5	6	5.5	45	7	100	8	2.454	2.254
6	6	5.5	45	2	20	5	3.206	3.328
7	4	5.5	50	4	60	5	1.954	1.955
8	6	9.5	45	2	100	5	1.858	1.785
9	2	8	50	2	20	8	0.932	1.011
10	6	4	50	7	20	3	2.274	2.255
11	4	5.5	45	5	60	5	2.336	2.345
12	2	5.5	45	7	100	5	1.959	1.975
13	4	4	30	8	60	8	2.628	2.644
14	4	8	30	3	125	1	1.696	1.664
15	4	5.5	40	1	60	5	2.521	2.445
16	7	8	50	4	60	3	2.285	2.221
17	4	8	30	5	50	8	1.566	1.550
18	4	4	30	5	60	1	2.049	2.110
19	1	4	50	5	60	8	0.555	0.996
20	4	5.5	45	3	60	5	2.701	2.785

3.2.1. Optimization of Incubation Time

The results from RSM show that the optimized incubation time is 5 days, when maximum laccase activity is 3.206 U/mL, gained from target substrate. In previous research *Aspergillus flavus* production of laccase over time was observed. At the 12th day of culture,

the highest laccase production (17.39 IU/mL) was noted. According to Fortina et al. (1996), *Botrytis cinerea* produced significant amounts of laccase (9.8 IU/mL) in about 5–7 days, whereas other fungi needed 12–30 days to complete the process. The peak laccase activity in *Ganoderma sp.* was seen on day 10 of incubation [29]. After 12 days under stationary conditions, *Pleurotus florida* was said to have produced a significant amount of laccase (4.60 IU/mL) in a malt extract broth [43].

3.2.2. Optimization of Substrate Size and Moisture Level

According to RSM data, the optimal substrate size is 6 g with a 20% moisture level, where the target substrate exhibits the highest levels of laccase activity. According to Srinivasan et al. [44], laccase synthesis was thought to be stimulated by the presence of cellulose and lignocellulosic residues. Increased laccase activity in comparison to growth on glucose may be caused by the presence of water-soluble components from lignocellulosic residues, such as p-coumaryl alcohol, coniferyl alcohol, sinapyl alcohol, and other aromatic compounds [45].

3.2.3. Optimization of Inoculum Size

According to RSM data, a 2 mL inoculum size was optimal, where the target substrate showed the highest level of laccase activity. In previous research, five discs, each measuring 14 mm in diameter, were found to be the ideal inoculum size for *Penicillium martensii* NRC 345 laccase synthesis, and any further increase in inoculum size resulted in a decrease in the amount of enzyme creation [46].

3.2.4. Optimization of Temperature and pH

According to the findings of the RSM, the optimal temperature and pH for maximizing the laccase activity of the target substrate are 45 °C and 5.5, respectively. *Penicillium martensii* NRC 345 produced laccase at its maximum level when the temperature was 30 °C. In previous study findings, *Agaricus heterocystis* laccase activity was found to be best at pH levels of 5.5. The ideal pH and temperature for the enzyme activity were discovered to be 5.0 and 70–80 °C, respectively [47].

3.3. Ammonium Sulphate Precipitation

To recover the extracellular enzyme, the supernatant of the fermented culture was saturated with 70% ammonium sulphate and centrifuged at 10,000 rpm for 15 min. The acquired crude filtrate was then exposed to the purification process. More protein and salt interactions were observed at 70% based on salting-in and salting-out strategies [48]. It was seen that after precipitation, the enzyme activity of laccase was increased to 5.4502 U/mL. In previous studies, a 70-fold purification of laccase from *stereum ostrea* was reported by Viswanath et al. [49], using ammonium sulphate precipitation and Sephadex G-100 column chromatography. By using ammonium sulphate precipitation with 40–70% saturation and DEAE cellulose chromatography with a purification fold of 1.34 and 3.07, laccase was purified from fruiting bodies [50]. When salting in, the solubility was enhanced by adding more salt, but the increased ionic strength reduced the solubility of the protein when salting out. Table 3 is showed the purification index of laccase.

At various stages of purification, different quantities of the laccase enzyme were extracted, and the total enzyme activity for the entire volume was estimated from the enzyme activity. These numbers are incredibly useful, because they told us how much purification increased after each step.

3.4. Statistical Analysis

The results of RSM have been catalyzed using Minitab 17. In each graph, enzyme activity (U/mL) was plotted against two parameters. The contour plots and surface plot graphs were plotted each for two parameters against enzyme activity (U/mL) in the following combinations:

(a) Substrate Size and pH
(b) Substrate Size and Temperature
(c) Substrate Size and Inoculum Size
(d) Substrate Size and Incubation Days
(e) Substrate Size and Moisture Level
(f) pH and Temperature
(g) pH and Inoculum Size

Table 3. Purification index of laccase.

Purification Step	Volume (mL)	Total Enzyme Activity (U)	Yield (%)	Purification Fold
Cell-free Supernatant	250	801.5	100	1
$(NH_4)_2SO_4$ Precipitation	100	544.8	69.5	1.34
Dialysis	25	224.3	48.03	1.83
Column Chromatography	10	112.4	30.72	4.07

3.5. Substrate Size and pH

Figure 3 shows a 3D surface plot between substrate size and pH. The plot of the enzyme activity (U/mL) has been shown on the *z*-axis, and its interaction has been shown with substrate size on the *y*-axis and pH on the *x*-axis. The peaks in this plot indicate maximum enzyme activity. The plot shows that the production of laccase increases when we used 6.0 g quantity of substrate. Similarly, the increasing laccase activity was obtained at pH 5.0. Previously, it was seen that the favorable range of pH for laccase production is 5.5 to 7.7 [51]. Thus, with respect to substrate size, it was seen that a of pH 5.5 gives maximum activity of laccase. This plot also indicates that for the production of laccase, we need a substrate size in the range of 5 g to 7 g, because in this plot the enzyme activity was increasing at 5 g of substrate and the best yield was obtained at 6 g of substrate. Therefore, more enzyme was produced at a lower pH and a higher concentration of substrate size.

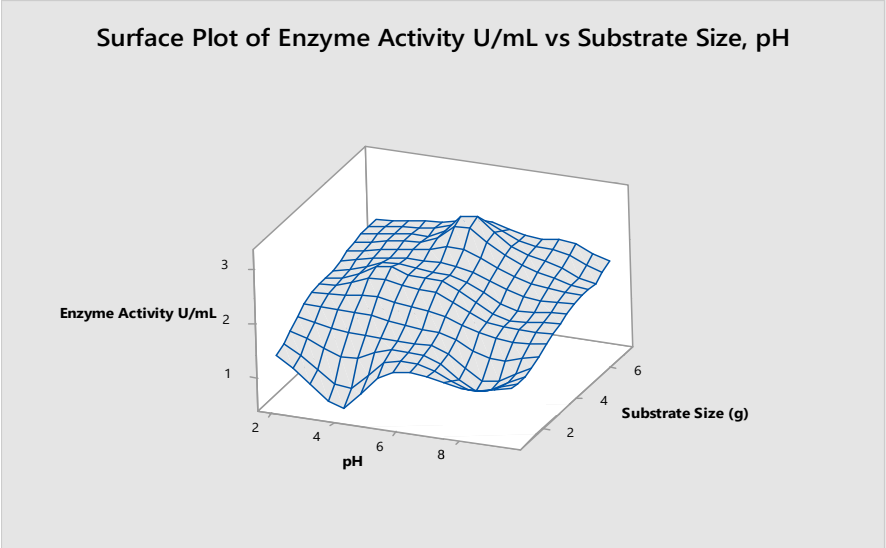

Figure 3. Surface plot of Enzyme Activity (U/mL) vs. Substrate Size and pH.

3.6. Substrate Size and Temperature

This 3D surface plot also indicates the interaction of enzyme activity (U/mL) with size of the substrate and temperature. The peaks in the 3D surface plot show that maximum enzyme activity was observed at 40 °C on the *x*-axis, while the concentration of the substrate was 4 g for the highest laccase activity on the *y*-axis. The study conducted by [52] indicates that the optimum range of laccase activity was at 30 °C to 60 °C. Any further rise in the temperature results in a decrease in enzyme activity. Therefore, in the present study, the optimum temperature was 40 °C. The change of substrate and different types of strains can deviate the results. The enzyme activity was plotted on the *z*-axis and has been shown in Figure 4.

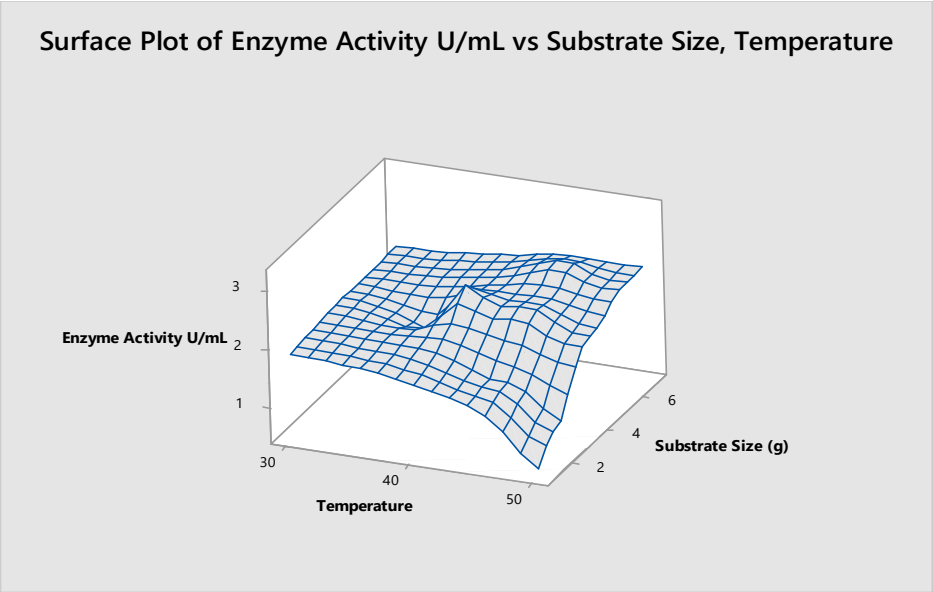

Figure 4. Surface plot of Enzyme Activity (U/mL) vs. Substrate Size and Temperature.

3.7. Substrate Size and Inoculum Size

This 3D surface plot also explains the interaction of enzyme activity with substrate size and inoculum size, as shown in Figure 5. It is explained with the help of the peaks. Two peaks have been shown in the plot. The enzyme activity (U/mL) on the *z*-axis was increased with the inoculum size of 2 mL on the *x*-axis and substrate size of 6 g on the *y*-axis. It also gives another high peak when the inoculum size was 8 mL and substrate size was 4 g. During optimization, it has been observed that when the inoculum size was 2 mL and substrate size was 6 g, the maximum laccase activity was obtained. However, with the passage of time, when the inoculum size was increasing 8 mL and substrate size was 4 g, it also produced a high laccase activity. Previous studies have shown that a high inoculum size does not give maximum activity, but in the present study, the deviation has been seen due to a different fermentative organism.

3.8. Substrate Size and Moisture Level

In Figure 6, the 3D surface plot shows the interaction of the two parameters on enzyme activity. These two parameters were substrate size on the *y*-axis and moisture level on the *x*-axis, while enzyme activity was plotted on the *z*-axis. The peak shows that the best enzyme activity is achieved at 6 g of substrate size and 30% moisture level in this study.

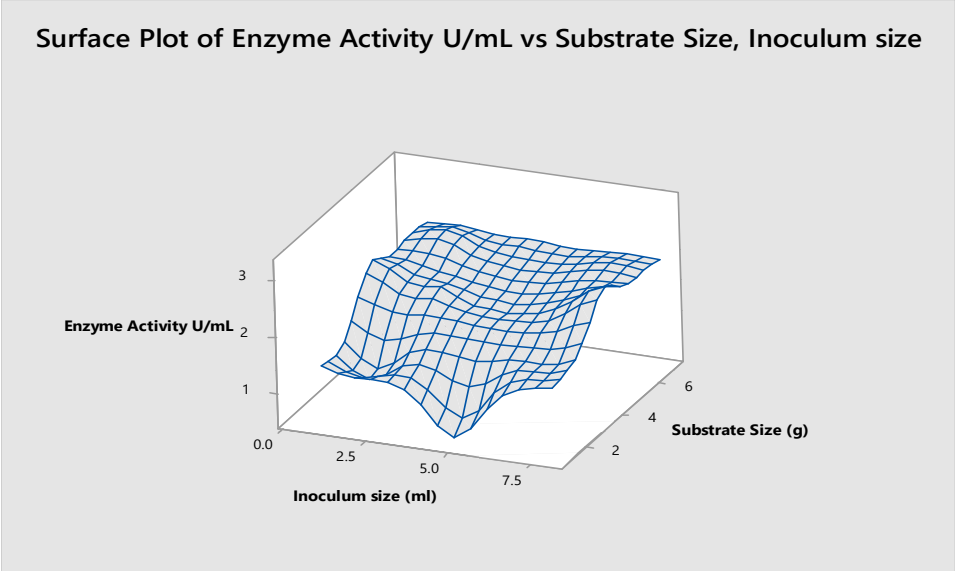

Figure 5. Surface plot of Enzyme Activity (U/mL) vs. Substrate Size and Inoculum Size.

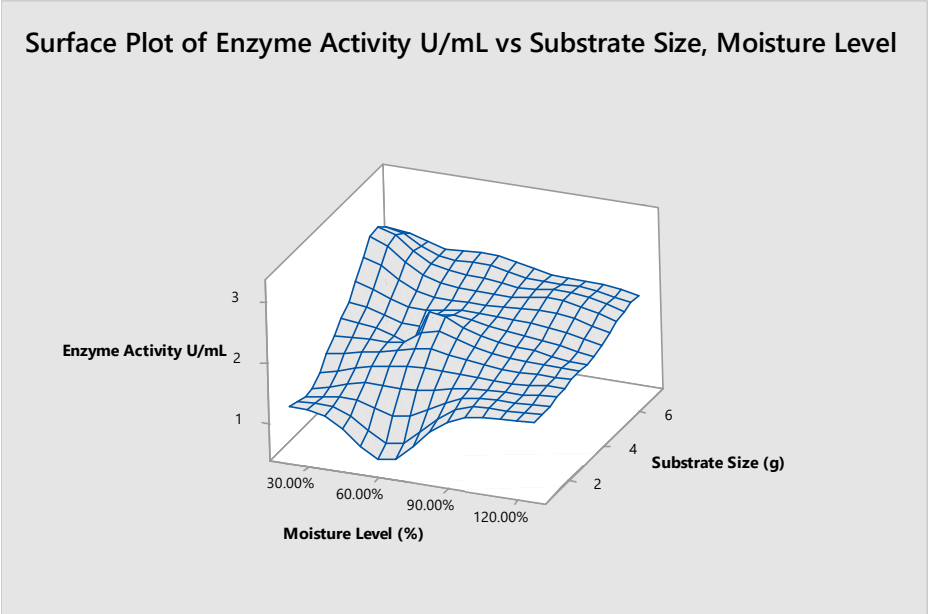

Figure 6. Surface plot of Enzyme Activity (U/mL) vs. Substrate Size and Moisture Level.

3.9. Substrate Size and Incubation Days

This 3D surface plot indicates the effect of incubation time and substrate size on enzyme activity. The incubation time was plotted on the *x*-axis, substrate size was plotted on the *y*-axis, while enzyme activity was plotted on the *z*-axis. In this plot, the enzyme activity was achieved at its maximum level on the fifth day of the incubation, corresponding to the substrate size of 6 g (Figure 7). The present study also indicates that increasing the incubation time causes a decrease in the activity of laccase. Different studies have suggested varied incubation times depending on the type of fermentative organism and substrates [53].

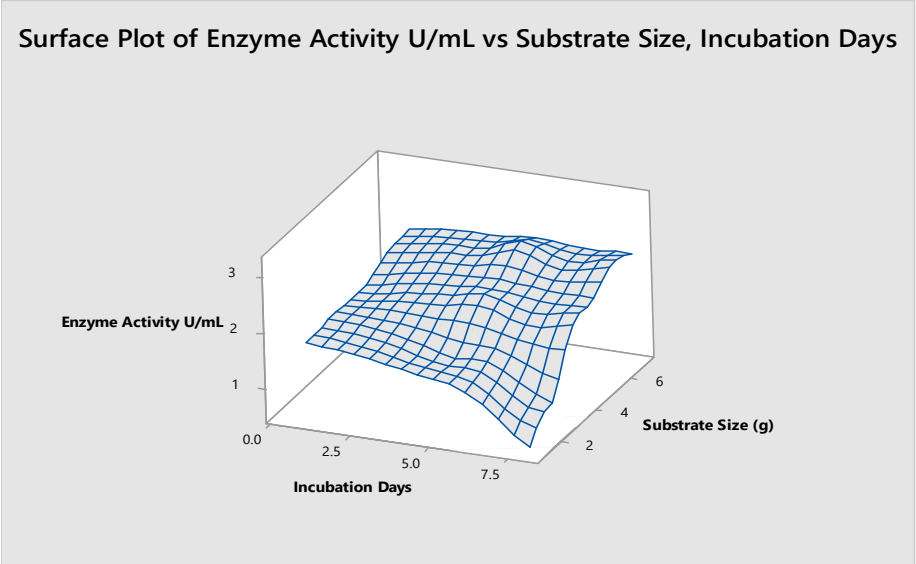

Figure 7. Surface plot of Enzyme Activity (U/mL) vs. Substrate Size and Incubation Days.

3.10. pH and Temperature

The 3D surface plot in Figure 8 shows the effect of pH and temperature on enzyme activity. The peaks in this plot show that the maximum laccase activity was obtained at 40 °C. The three peaks were obtained with reference to the temperature and pH. The first peak was shown at 30 °C, with pH 5.5. The second highest peak was conducted at 40 °C, with pH 7 and the third peak was seen at 47 °C, with pH 6. So, the high laccase activity was obtained at 40 °C with pH 7.

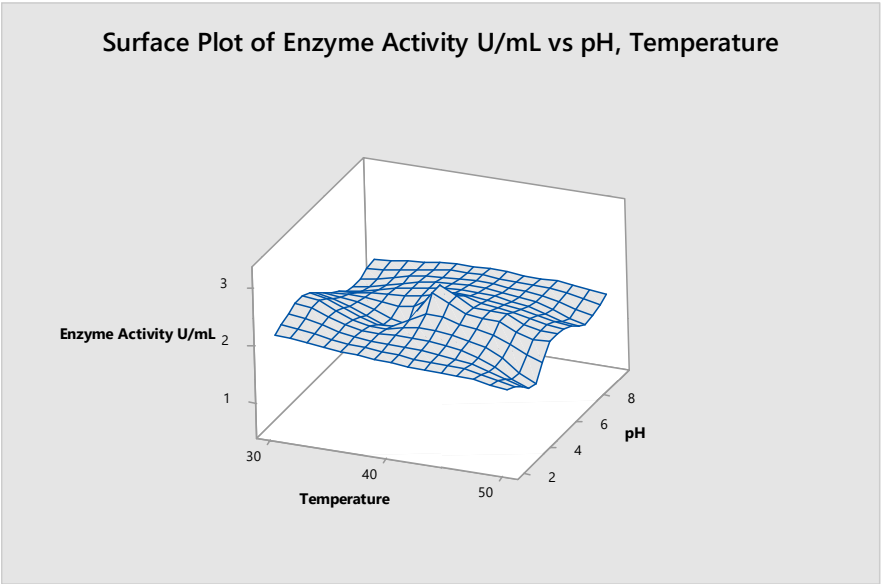

Figure 8. Surface plot of Enzyme Activity (U/mL) vs. pH and Temperature.

3.11. pH and Inoculum Size

The 3D surface plot in Figure 9 indicates the interaction of pH and inoculum size with enzyme activity, in the form of peaks. Two peaks were obtained in this study that suggest the maximum enzyme activity. The first peak was obtained at pH 4, when the inoculum size was 8 mL and the other highest peak was obtained at pH 5.5, with the inoculum size of 3 mL.

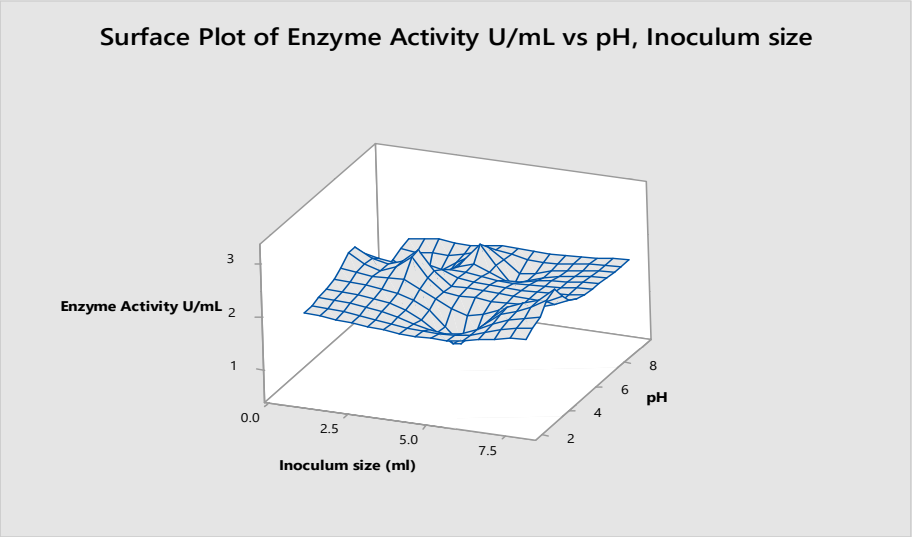

Figure 9. Surface plot of Enzyme Activity (U/mL) vs. pH and Inoculum Size.

3.12. Interpretations of Regression Surface Analysis

Three-dimensional surface plots represent that substrate size, pH, temperature, moisture level and incubation days showed maximum contribution for the production of laccase. The R^2 value was 100%, that showed the model fitness. The following equation can be used to determine the production of laccase enzyme:

$$\text{Enzyme Activity U/mL} = -3.146 - 1.785\ \text{Substrate Size}$$
$$+ 0.6242\ \text{pH} + 0.05011\ \text{Temperature}$$
$$+ 0.7733\ \text{Inoculum Size} + 5.800\ \text{Moisture Level}$$
$$- 0.8797\ \text{Incubation Days}$$
$$- 0.02954\ \text{Substrate Size} \times \text{Substrate Size} - 0.008113\ \text{pH} \times \text{pH}$$
$$- 0.000161\ \text{Temperature} \times \text{Temperature}$$
$$- 0.003549\ \text{Inoculum Size} \times \text{Inoculum Size}$$
$$- 0.05356\ \text{Moisture Level} \times \text{Moisture Level}$$
$$+ 0.02120\ \text{Incubation Days} \times \text{Incubation Days}$$
$$+ 0.006917\ \text{Substrate Size} \times \text{pH}$$
$$+ 0.006613\ \text{Substrate Size} \times \text{Temperature}$$
$$- 0.01782\ \text{Substrate Size} \times \text{Inoculum size}$$
$$- 0.4165\ \text{Substrate Size} \times \text{Moisture Level}$$
$$+ 0.003508\ \text{Substrate Size} \times \text{Incubation Days}$$
$$- 0.000084\ \text{pH} * \text{Temperature} - 0.01551\ \text{pH} \times \text{Inoculum size}$$

Because the R-square value was 1 and suggested a high correlation between these parameters for laccase production, when two-way interactions between different parameters were analyzed, all of the interactions between distinct parameters displayed significant values. Table 4 of the regression surface analysis is provided.

Table 4. Regression surface analysis: Enzyme Activity versus Substrate Size, pH, Temperature, Inoculum Size, Moisture Level, and Incubation Days.

Analysis of Variance					
Source	**DF**	**Adj SS**	**Adj MS**	**F-Value**	***p*-Value**
Model	19	7.79432	0.410228	0.000	0.000
Linear	6	0.40646	0.067744	0.000	0.000
Substrate Size	1	0.05847	0.058473	0.000	0.000
pH	1	0.01027	0.010266	0.000	0.000
Temperature	1	0.09015	0.090151	0.000	0.000
Inoculum Size	1	0.03451	0.034515	0.000	0.000
Moisture Level	1	0.00796	0.007958	0.000	0.000
Incubation Days	1	0.29379	0.293788	0.000	0.000
Square	6	0.89527	0.149212	0.000	0.000
Substrate Size × Substrate Size	1	0.04984	0.049838	0.000	0.000
pH × pH	1	0.07747	0.077475	0.000	0.000
Temperature × Temperature	1	0.37892	0.378921	0.000	0.000
Inoculum size × Inoculum size	1	0.01497	0.014965	0.000	0.000
Moisture level × Moisture level	1	0.00000	0.000000	0.000	0.000
Incubation Days × Incubation Days	1	0.51024	0.510241	0.000	0.000
2-Way Interaction	7	1.34118	0.191597	0.000	0.000
Substrate Size × pH	1	0.00540	0.005404	0.000	0.000
Substrate Size × Temperature	1	0.17158	0.171578	0.000	0.000
Substrate Size × Inoculum size	1	0.12129	0.121290	0.000	0.000
Substrate Size × Moisture level	1	0.08448	0.084481	0.000	0.000
Substrate Size × Incubation Days	1	0.00440	0.004400	0.000	0.000
pH × Temperature	1	0.00071	0.000711	0.000	0.000
pH × Inoculum size	1	0.02710	0.027096	0.000	0.000
Error	0				
Total	19	7.79432			

R-square: 100%.

3.13. Characterization of the Laccase Enzyme

The filtered enzyme was subjected to characterization at different temperatures. It was seen that the enzyme activity was increased by increasing the temperature. The range of the temperature was 20 °C–50 °C. The maximum activity was recorded to be 5.110 U/mL at 45 °C. The majority of research recommends an initial pH that is adequate for enzyme production between 4.5 and 6.0 [10], despite the scarcity of knowledge on the effects of the pH and temperature on laccase production. The ideal temperature for laccase formation was between 25 °C and 30 °C [54]. The activity of the enzyme was decreased when growing fungi at temperatures over 30 °C [55]. However, the results deviated depending on the substrate, different strains and growth conditions. The temperature optimization has been shown in Figure 10.

The optimal pH for maximum laccase activity was obtained at pH 5.5. It was also seen that above this pH, enzyme activity was observed to decline. Previous research revealed that pH 4.0–6.0 is usually considered a stable pH for fungal laccase. The alternation in pH depends on the fungal strain. *T. trogii* gave a high activity at pH 3.0, while *Stereum* gave stale laccase at pH 6.0 [56]. The pH characterization has been given in Figure 11.

This figure shows the optimal pH for enzyme activity. Enzyme activity was assayed at different pHs. The optimum pH for the enzyme activity was found 5.5. Other assay conditions, such as the incubation period and substrate concentration, were kept constant.

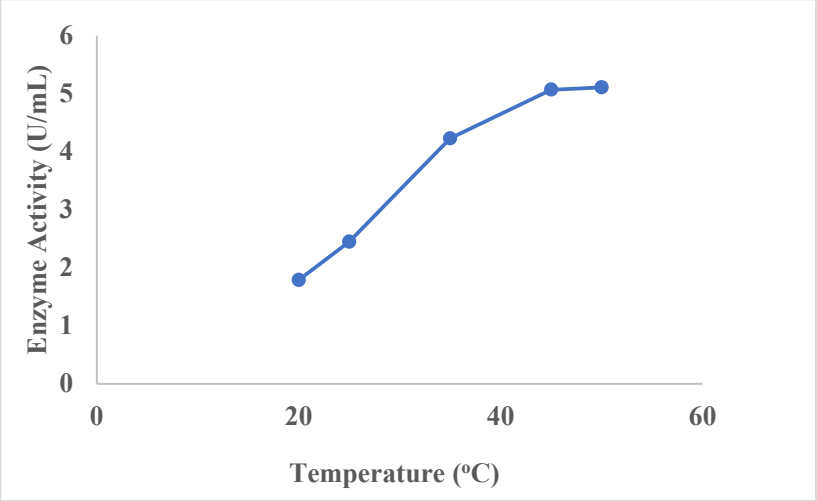

Figure 10. Enzyme activity (U/mL) at different temperatures.

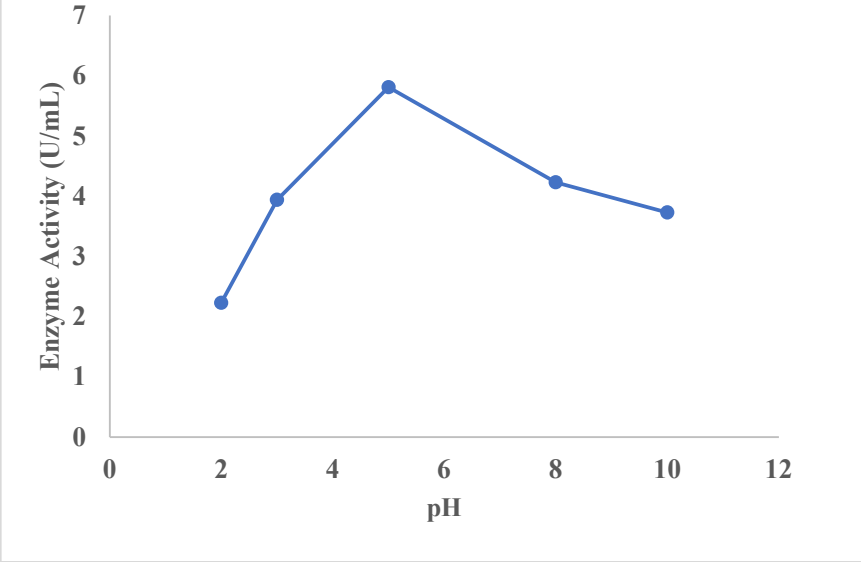

Figure 11. Laccase activity (U/mL) at different pH values.

4. Conclusions

The study's objective was to use an indigenous strain of *A. niger* to generate laccase. The use of agro-industrial-based waste as substrates adds value to the sector and raises the knowledge of recycling and energy usage. The ubiquitous availability of laccase is ensured by its frequent occurrence in a variety of fungal genera. Particularly, excellent laccase makers are the wood-rotting basidiomycetes, often known as white-rot fungi. The goal of the study was to develop laccase from *A. niger*, while also choosing low-cost, readily accessible high-yielding substrates. For this purpose, a variety of substrates were employed, with wheat straw producing the best results. To examine the effects of different parameters, this wheat straw was subjected to RSM. A pH of 5.5, temperature 45 °C, inoculum size 2 mL, substrate size 6 g and 5 days of incubation were selected as the best conditions for optimization. By forming these conditions, the enzyme activity of 3.206 U/mL was obtained. The enzyme was produced in bulk and partially purified by ammonium sulphate precipitation and dialysis. After this purification process, the enzyme activity was increased to 5.4502 U/mL. Further purification was also done by column chromatography, that increased the enzyme activity to 9.618 U/mL. The purified enzyme was used for characterization and to analyze the effects of pH, temperature and size of the inoculum. The purified enzyme activity was maximal at 45 °C and at pH 5.5. In addition to

being useful for the utilization of waste materials, this technique has industrial applications in the dairy, textile, paper and pulp, waste-water treatment, and dye decolorization sectors. Recent discoveries may lead to more effective techniques and plans for enzyme extraction and production. Future research focuses on the potential industrial applications of safe laccase mediators. Furthermore, by investigating genes in an appropriate host, significant levels of laccase synthesis can be accomplished.

Author Contributions: Conceptualization, Z.A., S.H., U.A.; methodology, Z.A., M.Y.R. and F.A.; software, F.A. and W.K.; validation, W.K. and F.A.; formal analysis, W.K., F.A. and M.Z.; investigation, W.K., F.A. and M.A.; resources, S.H., Z.A.; data curation, W.K., U.A. and M.O.A.; writing—original draft preparation, S.H. and W.K.; writing—review and editing, W.K. and F.A.; visualization, Z.A., S.H. and M.A.; supervision, Z.A., S.H. and A.A.-F.; project administration, W.K.; funding acquisition, M.O.A. and A.A.-F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Princess Nourah bint Abdulrahman University Researchers Supporting Project Number PNURSP2023R251, Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Baldrian, P. Fungal laccases-occurrence and properties. *FEMS Microbiol. Rev.* **2006**, *30*, 215–242. [CrossRef] [PubMed]
2. Wang, F.; Xu, L.; Zhao, L.; Ding, Z.; Ma, H.; Terry, N. Fungal Laccase Production from Lignocellulosic Agricultural Wastes by Solid-State Fermentation: A Review. *Microorganisms* **2019**, *7*, 665. [CrossRef] [PubMed]
3. Statistics Sweden. Design Your Questions Right: How to Develop, Test, Evaluate and Improve Questionnaires 2004. Available online: http://www.scb.se/statistik/_publikationer/OV9999_2004A01_BR_X97OP0402.pdf (accessed on 5 June 2020).
4. Kantharaj, P.; Boobalan, B.; Sooriamuthu, S.; Mani, R. Lignocellulose degrading enzymes from fungi and their industrial applications. *Int. J. Curr. Res. Rev.* **2017**, *9*, 1.
5. Hoegger, P.J.; Kilaru, S.; James, T.Y.; Thacker, J.R.; Kües, U. Phylogenetic comparison and classification of laccase and related multicopper oxidase protein sequences. *FEBS J.* **2006**, *273*, 2308–2326. [CrossRef]
6. Messerschmidt, A.; Huber, R. The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin. Modelling and structural relationships. *Eur. J. Biochem.* **1990**, *187*, 341–352. [CrossRef]
7. Kunamneni, A.; Ballesteros, A.; Plou, F.J.; Alcade, M. Fungal laccases—A versatile enzyme for biotechnological applications. In *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*; Mendez-Vilas, A., Ed.; Formatex: Badajoz, Spain, 2007; pp. 233–244.
8. Bourbonnais, R.; Paice, M.G.; Reid, I.D.; Lanthier, P.; Yaguchi, M. Lignin oxidation by laccase isozymes from Trametes versicolor and role of the mediator 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. *Appl. Environ. Microbiol.* **1995**, *61*, 1876–1880. [CrossRef]
9. Leontievsky, A.; Myasoedova, N.; Pozdnyakova, N.; Golovleva, L. 'Yellow' laccase of Panus tigrinus oxidizes non-phenolic sub-strates without electron-transfer mediators. *FEBS Lett.* **1997**, *413*, 446–448. [CrossRef]
10. Thurston, C.F. The structure and function of fungal laccases. *Microbiology* **1994**, *140*, 19–267. [CrossRef]
11. Li, K.; Xu, F.; Eriksson, K.-E.L. Comparison of fungal laccases and redox mediators in oxidation of a nonphenolic lignin model compound. *Appl. Environ. Microbiol.* **1999**, *65*, 2654–2660. [CrossRef]
12. Lenz, J.; Hölker, U.; Höfer, M. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. *Appl. Microbiol. Biotechnol.* **2004**, *64*, 175–186. [CrossRef]
13. Castilho, L.R.; Polato, C.M.; Baruque, E.A.; Sant'Anna, G.L., Jr.; Freire, D.M. Economic analysis of lipase production by Penicillium restrictum in solid-state and submerged fermentations. *Biochem. Eng. J.* **2000**, *4*, 239–247. [CrossRef]
14. Viniegra-González, G.; Favela-Torres, E.; Aguilar, C.N.; de Jesus Rómero-Gomez, S.; Dıaz-Godınez, G.; Augur, C. Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem. Eng. J.* **2003**, *13*, 157–167. [CrossRef]
15. Roy, R.V.; Das, M.; Banerjee, R.; Bhowmick, A.K. Comparative studies on rubber biodegradation through solid-state and submerged fermentation. *Process. Biochem.* **2006**, *41*, 181–186. [CrossRef]
16. Forootanfar, H.; Faramarzi, M.A. Insights into laccase producing organisms, fermentation states, purification strategies, and biotechnological applications. *Biotechnol. Prog.* **2015**, *31*, 1443–1463. [CrossRef] [PubMed]

17. Dittmer, N.T.; Suderman, R.J.; Jiang, H.; Zhu, Y.-C.; Gorman, M.J.; Kramer, K.J.; Kanost, M.R. Characterization of cDNAs encoding putative laccase-like multicopper oxidases and developmental expression in the tobacco hornworm, Manduca sexta, and the malaria mosquito, Anopheles gambiae. *Insect Biochem. Mol. Biol.* **2003**, *34*, 29–41. [CrossRef]
18. Palacios-Cabrera, H.; Taniwaki, M.H.; Hashimoto, J.M.; De Menezes, H.C. Growth of Aspergillus ochraceus, A. carbonarius and A. niger on culture media at different water activities and temperatures. *Braz. J. Microbiol.* **2005**, *36*, 24–28. [CrossRef]
19. Arora, D.S.; Sharma, R.K. Ligninolytic Fungal Laccases and Their Biotechnological Applications. *Appl. Biochem. Biotechnol.* **2009**, *160*, 1760–1788. [CrossRef]
20. Majeau, J.-A.; Brar, S.K.; Tyagi, R.D. Laccases for removal of recalcitrant and emerging pollutants. *Bioresour. Technol.* **2010**, *101*, 2331–2350. [CrossRef]
21. El Monssef, R.A.A.; Hassan, E.; Ramadan, E.M. Production of laccase enzyme for their potential application to decolorize fungal pigments on aging paper and parchment. *Ann. Agric. Sci.* **2016**, *61*, 145–154. [CrossRef]
22. Afreen, S.; Anwer, R.; Singh, R.; Fatma, T. Extracellular laccase production and its optimization from Arthrospira maxima catalyzed decolorization of synthetic dyes. *Saudi J. Biol. Sci.* **2018**, *25*, 1446–1453. [CrossRef]
23. Surwase, S.V.; Patil, S.A.; Srinivas, S.; Jadhav, J.P. Interaction of small molecules with fungal laccase: A Surface Plasmon Resonance based study. *Enzym. Microb. Technol.* **2016**, *82*, 110–114. [CrossRef] [PubMed]
24. Sethi, B.; Satpathy, A.; Tripathy, S.; Parida, S.; Singdevsachan, S.K.; Behera, B. Production of ethanol and clarification of apple juice by pectinase enzyme produced from Aspergillus terreus NCFT 4269.10. *Int. J. Biol. Res.* **2016**, *4*, 67. [CrossRef]
25. Kumar, A.; Gautam, A.; Dutt, D. Biotechnological Transformation of Lignocellulosic Biomass in to Industrial Products: An Overview. *Adv. Biosci. Biotechnol.* **2016**, *07*, 149–168. [CrossRef]
26. Habib, N.; Niaz, M.; Akhtar, S.; Shahid, S. In silico phylogenetic analysis of fungal lipase genes and harnessing the inherent potential of Aspergillus niger IBP2013 for extracellular triglycerol acyl-hydrolase production under solid state fermentation. *Pak. J. Bot.* **2018**, *50*, 2019–2029.
27. Powell, K.A.; Renwick, A.; Peberdy, J.F. (Eds.). *The Genus Aspergillus: From Taxonomy and Genetics to Industrial Application*; Springer Science & Business Media: Berlin/Heidelberg, Germany, 2013; Volume 69.
28. Kalra, K.; Chauhan, R.; Shavez, M.; Sachdeva, S. Isolation of laccase producing *Trichoderma* spp. and effect of pH and temperature on its activity. *Int. J. Chemtech. Res.* **2013**, *5*, 2229–2235.
29. Yang, J.-T.; Wu, C.-E.; Li, Y.-Y.; Jia, S.-Q.; Fan, G.-J.; Peng, F.-R. Identification and Purification of an Allergic Glycoprotein from Ginkgo biloba Kernel. *Agric. Sci. China* **2011**, *10*, 631–641. [CrossRef]
30. Bagewadi, Z.K.; Mulla, S.I.; Ninnekar, H.Z. Purification and immobilization of laccase from Trichoderma harzianum strain HZN10 and its application in dye decolorization. *J. Genet. Eng. Biotechnol.* **2017**, *15*, 139–150. [CrossRef]
31. Koser, S.; Anwar, Z.; Iqbal, Z.; Anjum, A.; Aqil, T.; Mehmood, S.; Irshad, M. Utilization of Aspergillus oryzae to produce pectin lyase from various agro-industrial residues. *J. Radiat. Res. Appl. Sci.* **2014**, *7*, 327–332. [CrossRef]
32. Seth, M.; Chand, S. Biosynthesis of tannase and hydrolysis of tannins to gallicacid by Aspergillus awamori-optimisation of process parameter. *Process Biochem.* **2000**, *36*, 39–44. [CrossRef]
33. Znidarsic, P.; Pavko, A. The morphology of filamentous fungi in submerged cultivations as a bioprocess parameter. *Food Technol Biotechnol.* **2001**, *39*, 237–252.
34. El-Batal, A.I.; Abd-Algawad, M.H.; Abdelbaky, N.M. Enhancement of some natural antioxidants activity via microbial bio-conversion process using gamma irradiation and incorporation into gold nanoparticles. *World Appl. Sci. J.* **2012**, *19*, 1–11.
35. Revankar, M.S.; Desai, K.M.; Lele, S.S. Solid-state Fermentation for Enhanced Production of Laccase using Indigenously Isolated *Ganoderma* sp. *Appl. Biochem. Biotechnol.* **2007**, *143*, 16–26. [CrossRef] [PubMed]
36. Carvalheiro, F.; Fernandes, T.; Duarte, L.C.; Gírio, F.M. Wheat Straw Autohydrolysis: Process Optimization and Products Characterization. *Appl. Biochem. Biotechnol.* **2008**, *153*, 84–93. [CrossRef] [PubMed]
37. Sun, J.X.; Zhao, X.F.S.H.; Sun, R.C. Isolation and characterization of cellulose from sugarcane bagasse. *Polym. Degrad. Stab.* **2004**, *84*, 331–339. [CrossRef]
38. Rubin, E.M. Genomics of cellulosic biofuels. *Nature* **2008**, *454*, 841–845. [CrossRef]
39. Mendes, C.A.D.C.; Adnet, F.A.D.O.; Leite, M.C.A.M.; Furtado, C.R.G.; Sousa, A.M.F.D. Chemical, physical, me-chanical, thermal and morphological characterization of corn husk residue. *Cellul. Chem. Technol.* **2015**, *49*, 727–735.
40. Hemery, Y.; Rouau, X.; Lullien-Pellerin, V.; Barron, C.; Abecassis, J. Dry processes to develop wheat fractions and products with enhanced nutritional quality. *J. Cereal Sci.* **2007**, *46*, 327–347. [CrossRef]
41. Shabbir, H.; Kausar, T.; Noreen, S.; Rehman, H.U.; Hussain, A.; Huang, Q.; Gani, A.; Su, S.; Nawaz, A. In Vivo Screening and Antidiabetic Potential of Polyphenol Extracts from Guava Pulp, Seeds and Leaves. *Animals* **2020**, *10*, 1714. [CrossRef]
42. Saunders, R.M. Rice bran: Composition and potential food uses. *Food Rev. Int.* **1985**, *1*, 465–495. [CrossRef]
43. Sivakumar, R.; Rajendran, R.; Balakumar, C.; Tamilvendan, M. Isolation, screening and optimization of production medium for thermostable laccase production from *Ganoderma* sp. *Int. J. Eng. Sci. Technol.* **2010**, *2*, 7133–7141.
44. Srinivasan, C.; Dsouza, T.M.; Boominathan, K.; Reddy, C.A. Demonstration of laccase in the white rot basidiomycete Phanerochaetechrysosporium BKM-F1767. *Appl. Environ. Microbiol.* **1995**, *61*, 4274–4277. [CrossRef] [PubMed]
45. Schlosser, D.; Grey, R.; Fritsche, W. Patterns of ligninolytic enzymes in Trametes versicolor. Distribution of extra-and in-tracellular enzyme activities during cultivation on glucose, wheat straw and beech wood. *Appl. Microbiol. Biotechnol.* **1997**, *47*, 412–418. [CrossRef]

46. Elshafei, A.M.; Hassan, M.M.; Haroun, B.M.; Elsayed, M.A.; Othman, A.M. Optimization of Laccase Production from Penicillium martensii NRC 345. *Adv. Life Sci.* **2012**, *2*, 31–37. [CrossRef]
47. Ranimol, G.; Venugopal, T.; Gopalakrishnan, S.; Sunkar, S. Production of laccase from Trichoderma harzianum and its application in dye decolourisation. *Biocatal. Agric. Biotechnol.* **2018**, *16*, 400–404.
48. Shrestha, P.; Joshi, B.; Joshi, J.; Malla, R.; Sreerama, L. Isolation and physicochemical characterization of laccase from Ganoderma lucidum-CDBT1 isolated from its native habitat in Nepal. *Biomed. Res. Int.* **2016**, *2016*, 3238909. [CrossRef]
49. Sumathi, T.; Viswanath, B.; Lakshmi, A.S.; SaiGopal, D.V.R. Production of Laccase by *Cochliobolus* sp. Isolated from Plastic Dumped Soils and Their Ability to Degrade Low Molecular Weight PVC. *Biochem. Res. Int.* **2016**, *2016*, 9519527. [CrossRef]
50. Khammuang, S.; Sarnthima, R. Laccase activity from fresh fruiting bodies of *Ganoderma* sp. MK05: Purification and Remazol Brilliant Blue R decolorization. *J. Biol. Sci.* **2009**, *9*, 83–87. [CrossRef]
51. Dhakar, K.; Pandey, A. Laccase Production from a Temperature and pH Tolerant Fungal Strain of *Trametes hirsute* (MTCC 11397). *Enzym. Res.* **2013**, *2013*, 869062. [CrossRef]
52. Nishizawa, Y.; Nakabayashi, K.; Shinagawa, E. Purification and characterization of laccase from white rot fungus Trametes sanguinea M85-2. *J. Ferment. Bioeng.* **1995**, *80*, 91–93. [CrossRef]
53. Edae, T.; Alemu, M. Selection and optimization of lignocellulosic substrate for laccase production from *Pleurotus* species. *Int. J. Biotechnol.* **2017**, *8*, 38–48.
54. Pointing, S.B.; Jones, E.B.G.; Vrijmoed, L.L.P. Optimization of laccase production by *Pycnoporus sanguineus* in submerged liquid culture. *Mycologia* **2000**, *92*, 139–144. [CrossRef]
55. Lang, E.; Gonser, A.; Zadrazil, F. Influence of incubation temperature on activity of ligninolytic enzymes in sterile soil by Pleurotus sp. and Dichomitus squalens. *J. Basic Microb. Int. J. Biochem. Physiol. Genet. Morphol. Ecol. Microorg.* **2000**, *40*, 33–39.
56. Kumar, R.; Kaur, J.; Jain, S.; Kumar, A. Optimization of laccase production from Aspergillus flavus by design of experiment technique: Partial purification and characterization. *J. Genet. Eng. Biotechnol.* **2016**, *14*, 125–131. [CrossRef] [PubMed]