



PURIFICATION AND STUDY OF THE PROPERTIES OF LACCASE FROM LOCAL ISOLATE OF THE MEDICAL FUNGUS *GANODERMA RESINACEUM*

Warkaa Khalaf Hussein* and Shimal Younis Abdul- Hadi

Department of Life Sciences, College of Education for Pure Sciences, University of Mousl, Iraq.

Abstract

Local isolation of the genus *G. resinaceum* of medicinal importance was obtained from one of the farms located on the Tigris River in the Rashidiyeh region. Laccase enzyme was purified from the filtrate of the fungal farm by a series of steps that included ammonium sulfate precipitation by 75% saturation, in which the specific activity of the enzyme accelerated and reached 17.18 units / mg, followed by enzyme dialysis for 24 hours and resulted in increased specificity to 34.44 units / mg. The ion exchange step using DEAE-cellulose achieved a specific efficacy of 143.46 units / mg. As for the last step of purification, which was carried out using gel filtration chromatography using Sephadex G-100, the specific enzyme efficacy increased to 406.28 units / mg and the number of purification times reached 62.31 times with an enzymatic yield of 30.67%. The results of determining the properties of the purified enzyme resulted in obtaining one 52 kd molecular package at electrophoresis on the acrylamide gel, and the process of detecting the enzyme purity resulted in obtaining one single bundle of the purified enzyme from the two ion exchange and gel filtration steps, which were evidence of purification. The enzyme has a degree of homogeneity. The optimum pH of enzyme activity (6), while the pH of proven enzyme activity has a range of (5-7). The optimum temperature for enzyme activity was (60) ° C, while the degree of thermal stability ranged between (20-60) ° C. And it appeared that the enzyme has the ability to maintain (98%) of its original efficacy when storing at laboratory temperature for a period of (7) days.

Key words : Laccase, Purification, Characterization, Medical Mushroom, *Ganoderma*.

Introduction

Laccase is one of the enzymes of the peroxidase family containing Multi copper oxidases, and is known as Benzenedial: Oxygen oxidoructases. This enzyme has a high ability to break down low molecular weight compounds such as poly phenyl It was discovered by the scientist Yoshida in 1883 AD in the resinous secretions of the Japanese plaint tree *Rhus vernicifera*. Laccase is found in various neighborhoods and occupy the forefront among these organisms, especially the Bazidia fungi (Forootanfar and Faramarzi, 2015) and we also find it in insects such as (*Drosophila*, *Papilio*, *Tenebrio*, *Musca calliphora*) In bacteria such as *Azospirillum*, *Bucillus subtilis*) (and in various plants such as pears, apples, potatoes, soybeans, turnips, etc. More recently it has been

proven in lichens (Laufer *et al.*, 2009) The role of laccases in fungi is based mainly on analysis of lignin from stem wood in plants for the purpose of facilitating colonization by mushrooms in addition to many other functions in fungi and that possess them, and has obtained a large number of studies for its role in the biological treatment of environmental pollutants) in addition to its entry into the Many industrial applications such as making paper, fabric, furniture, cosmetics, fixing juices, removing the bitter taste from it, improving baking dough, making soaps, as well as making anti-tumor drugs, making them enzymes that drew attention to purifying them from organisms and studying their biochemical properties There are many ways To separate and purify the enzymes that were purely a lot of interest by the researchers (Singh *et al.*, 2011), and the importance of the fungus and the enzyme,

**Author for correspondence* : E-mail: warkaakha86@gmail.com

which are facts proven in the scientific references

Materials and Methods

Purification of laccase

The laccase enzyme was purified from the raw enzymatic extract that was separated from the local isolate *G. resinsaceum*. The purification process began by precipitation with ammonium sulfate at a saturation rate of 75% and the raw extract was discarded by the centrifuge at a temperature of 4 ° C and a speed of 9000 Revolution/minute For a period of 15 minutes, followed by the separation step using dialysis tubes where the enzymatic precipitate was washed using a buffer solution (Sodium acetate) with a pH of 5 and this step lasted 24 hours with the exchange of the buffer solution every 8 hours, after which the filter was performed on the enzyme using a DEAE _ Cellulose exchanger using A glass column with dimensions (2.5 x 20 cm) and the process of balancing and recovery was done using the same buffer solution at a flow velocity of 30 ml / hour at a rate of 3 ml / part. The recovery and balancing process using the buffer solution itself and at a flow velocity of 30 ml / hour at a rate of 3 ml / part. The separated parts were collected for the next step of purification, which is the gel filtering step using Sephadex G-100 gel. The recovery and balancing process was carried out using the buffer solution of sodium acetate at a rate of 30 parts / An hour and 30 ml / part. After the purification process was completed, the separated parts were collected and gave enzymatic activity in order to study the properties of the purified enzyme

Characterization of enzyme Method

Molecular weight and purification level of purified lactase enzyme from fungus isolation were determined by using a horizontal plate-relay (Bio-RAD) electrophoresis gel on polyacrylamide gel and with the presence of the sodium-Dodecyl-Sulfate gel. Experiments were conducted to determine the following enzyme properties, which are the optimum pH of efficacy and stability, the optimum temperature for the efficacy and stability of the enzyme, and the effect of the storage

period.

Results and Discussion

1. Laccase Enzyme Purification

The process of purification of the laccase enzyme from the medical fungus *G. resinsaceum* was carried out in its fine detail with a series of steps that deplete a long time. The first step resulted in the concentration of the raw enzyme with ammonium sulfate, which was used with a saturation rate of 75%, in which the specific efficacy of the raw enzyme increased from 6.52 units / mg to 17.18 units / Mg. The output of the enzymatic outcome reached 46.14% and the number of purification times reached 2.63 times. Table 1 shows the accuracy and clarity of the results.

The enzyme concentration step with ammonium sulfate was followed by a dialysis step for 24 hours, which resulted in an increase in the specific efficacy of the enzyme amounting to 34.44 units / mg, accompanied by an increase in the number of purification times to 5.28 times and an enzymatic yield of 40.12%.

To achieve an important step forward in the direction of purification of the enzyme, I used DEAE-Cellulose exchange chromatography topped Fig. 1 three protein peaks in this step, the second summit in which only contained an enzymatic activity after measuring the enzymatic activity in the recovered parts of the column using the recovery solution contained between (20-32). The specific activity of the enzyme reached the highest value at the separated part (26) 143.46 units / mg and the number of purification times 22 times, while the enzymatic outcome was 34.82%.

In order to complete the purification steps, the last step was taken with a recent step using gel filter chromatography (Sephadex G-100). Fig. 2 describes the results of the step that showed a single protein peak in which the enzymatic activity was associated and that appeared in the recovered parts of the column between (6-20) and above. At section 16, the specific activity of the enzyme was 406.28 units / mg, and the number of purification times increased to 62.31 times, and the

Table 1: Step to purify laccase enzyme from the isolation of *G. resinsaceum*.

Purification steps	The size (ml)	Enzymatic activity unit/ml	Protein mg/ml	Ovre all effectiveness	Qualitative effectiveness unit/mg	Fraction number	Enzymatic outcome
The raw enzyme	100	9.27	1.42	927	6.52	1	100
Sedmintation with ammonium sulfate	30	14.26	0.83	427.8	17.18	2.63	46.14
Dialysis	20	18.60	0.54	372.0	34.44	5.28	40.12
Ion exchanger DEAE-Cellulose	15	21.52	0.15	322.8	143.46	22.00	34.82
Gel feltration Cephdex G-100	10	28.44	0.07	284.4	406.28	62.31	30.67

enzymatic yield reached 30.67%.

An important detail that must be mentioned is the

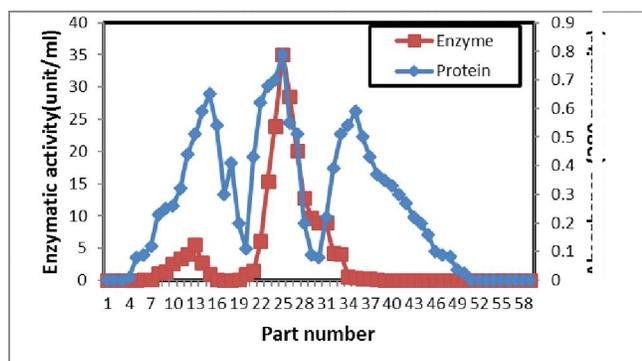


Fig. 1: Purification of laccase enzyme using DEAE Cellulose exchanger in a column of dimensions (2.5 × 20) cm. Balancing and recovery was carried out with the solution of sodium acetate buffer at a concentration of 10mM at a flow rate of 30 ml / hour at a rate of 3 ml / part.

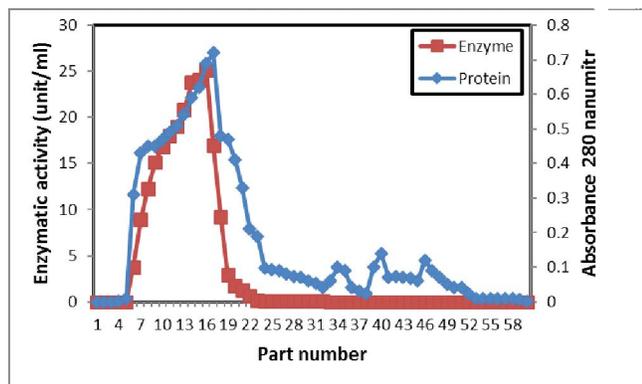


Fig. 2: Laccase Enzyme Purification Using Gel Filtration Chromatography in Sephadex G-100 Column in a Dimensional Column (1.5 × 40) cm. Balancing and recovery was carried out with the solution of the sodium acetate buffer at a concentration of 10mM at a flow velocity of 30 ml / hour at a rate of 3 ml / part.

concentration of the enzyme with ammonium sulfate, which is characterized by its rapid solubility, as the proteins stagnate depending on the quality and concentration of the salts used. The reason for the binding is due to the parity of the proteins charges by the charges carried by the ammonium sulfate, and as a result of this tie, they accumulate and separate in a phenomenon called external salting.

A study conducted by it (Ding *et al.*, 2012) revealed that the laccase enzyme was purified from the fungus *G. lucidum* by a series of steps that started concentrating the enzyme with ammonium sulfate at a rate of saturation of 40% the specific activity in this step reached 10.96 units/mg and the number of purification times 2.0 as for

the enzymatic outcome it reached 90.39%. Whereas, the next step using the DEAE ion exchanger achieved a specific efficacy that reached 21.13 units / mg and the number of purification times increased to 3.87 times with an enzymatic yield of 37.66% and the purification steps ended with gel filtration using Sephacryl S-100 in which the specific efficacy reached 39.25 units / mg and the number increased The enzyme purification times to 7.19 Once with an enzymatic yield of 30.65%.

2. Determination of purifying enzyme properties

1. The molecular weight of the enzyme

Different methods are used to know the molecular weight of the enzyme, which depends on its physical characteristics. In this study, the method of electrical migration on the multiple acrylamide gel was used in the presence of sodium dodecyl sulfate SDS, the movement of the protein complex with SDS depends on its size. The image taken for the migration result, as shown in Figure 3, showed the appearance of one single 52 kD molecular package compared to Marker ladder volumetric evidence. The results achieved are close to the results published in a research paper by (More *et al.*, 2011), in which the researchers reported that the molecular weight of the Laccase enzyme purified from the isolation of the oyster mushroom *Pleurotus sp.* It was 40 kD when estimated by electrophoresis method on acrylamide gel and in the presence of SDS.

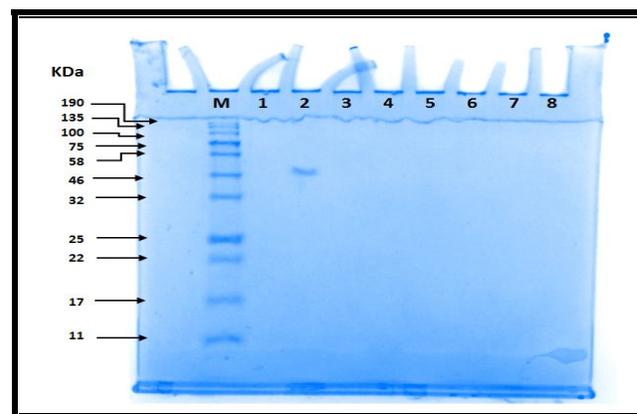


Fig. 3: Molecular Weight of Laccase-Purifying Enzyme by isolate *G. resinacum* by Electrophoresis Method on Multiple Acrylamide Gel.

2. The degree of enzyme purity

One of the most important steps that occupies the center stage in characterizing the enzyme is making sure that the enzyme is purified to the point of homogeneity and free of proteins, so this goal was taken into consideration and each of the purified enzyme was

removed from the *G. resinaceum* according to the last step and the raw enzyme on the polyacrylamide gel, and in A scene that facilitates sight. The results, as in Figure 4, resulted in one bundle of the purified enzyme from the two purification steps (ion exchange and gel filtration), while several bundles of the raw enzymatic extract and 6 bundles of the purified enzyme appeared from the first step of purification, which took place by focusing with ammonium sulfate. The emergence of one bundle of the purified enzyme is conclusive evidence that the enzyme was purified for the purpose of homogeneity, while the appearance of more than one bundle of the raw and concentrated enzyme indicates the presence of a mixture of proteins with different molecular weights. It should be noted that there are many methods for detecting the purity of enzymes extracted from fungi.

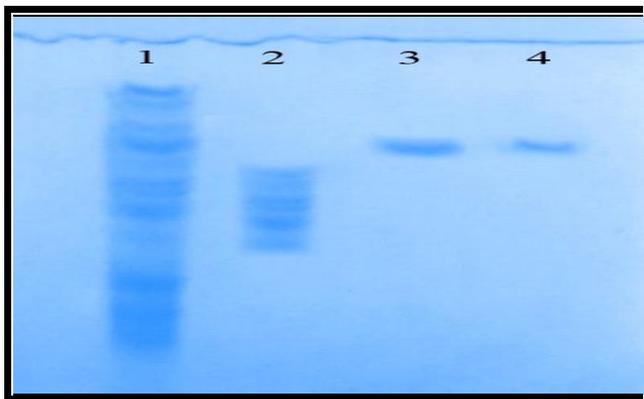


Fig. 4: Detection of Purified Laccase Enzyme from the Isolation of *G. resinacum* by Electrophoresis Method on Multiple Acrylamide Gel.

Where it represents:

- 1: The raw enzyme.
- 2: The enzyme from the sedimentation step with ammonium sulfate.
- 3: The purified enzyme from the ion exchanger step.
- 4: The purified enzyme from gel filtration chromatography step.

Among the citations within the same experiment was what was published in the journal *Appl. Microbiol. Biotechnol* by the researchers (Nagai *et al.*, 2002) when purifying the laccase enzyme from the *Lentinula edodes* mushroom, which was proven to be pure to the level of homogeneity by the method of electrophoresis on the multiple acrylamide gel in terms of the appearance of one package of the purified enzyme.

3. Optimal pH

Each enzyme has a specific range of pH where the activity of the enzyme is most effective and is called the optimal pH of the activity. To determine the optimal pH of the activity of the purified laccase enzyme, the enzymatic

reaction was carried out using different pHs that ranged between (4-10). The results in Fig. 5 confirmed that the optimum pH for enzyme activity was 6, which gave the highest efficacy of 23.54 units / mL. After that, there was a gradual decrease in the pH height towards the basement, so that no activity of the enzyme was recorded at pH 10.

The results can be explained by the presence of electrical charges of the R groups near the active site of the enzyme at different pH values that create ion bonds or the effects of attraction or repulsion as possible which increases the conjugation of the enzyme with the base material and vice versa.

The results obtained were supported by a study found during the research in the scientific references of the researchers (Tian *et al.*, 2012), as it indicated that the pH of the activity of the laccase enzyme purified from the fungus *Pleurotus nebrodensis*

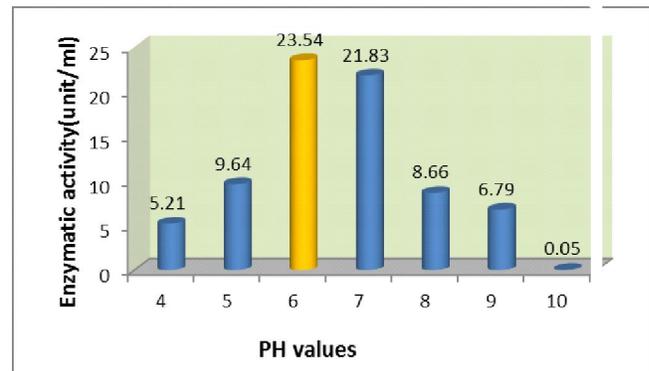


Fig. 5: The optimal pH curve for the effectiveness of the purified laccase enzyme from the isolation of the medicinal mushroom.

4. Optimum pH for potency stability

Of the properties of enzymes in general, their effectiveness has been proven with specific pH, and it is a very important characteristic in purifying the enzymes that are used commercially. From this standpoint, the experiment sheds light on the optimal pH knowledge for the stability of the laccase enzyme. The results shown in Fig. 6 showed that the enzyme showed clear stability in the acidic pH near to parity that ranged between (5-7), as it kept 97.5% of its activity at pH 5, while maintaining 96.8% of its enzymatic activity at exponent. PH 6 The percentage of retained enzymatic activity was 93% at neutral pH 7. The effectiveness of the enzyme was halved by increasing the pH as it reached 50% effectiveness at pH 8 and the activity recorded a sharp drop at pH 10 when it reached what Maintained by the enzyme from the efficacy of 4.4%. Upon reviewing the relevant research, it was possible (Fang *et al.*, 2015) to conclude

that the laccase enzyme purified from the fungus *G. lucidium* 77002 has the advantage of proving its enzymatic activity at a range of pH ranging from (5-7). Within the same field.

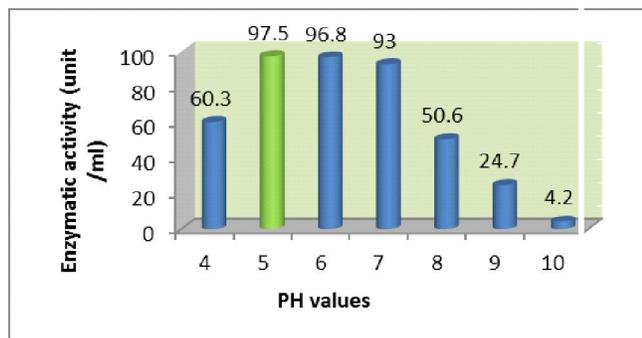


Fig. 6: The optimal pH curve to stabilize the effectiveness of the purified laccase enzyme from the isolation of the medicinal mushroom *G. resinacum*.

5. The optimum temperature for the laccase activity

One of the most important properties of the enzyme is its sensitivity to temperature, and each enzyme has a specific temperature in which it operates to its maximum effectiveness, and by tracking a range of temperatures ranging between (20-80 ° C) the results are crucial to the order in Fig. 7 and it is noted that there is a gradual increase in The activity of the enzyme by increasing temperatures up to the optimum temperature of 60 ° C, which gave an efficiency of 33.7 units / ml, while the activity tended to decrease when the reaction temperature rose to 70 ° C and reached 6.5 units / ml accompanied by continued high temperatures, a sharp drop in effectiveness It reached 1.4 units / ml at a temperature of 80 ° C which is the highest temperature used in the experiment.

The result reached was different from his (Park and Park, 2008), as the maximum efficacy of the purified laccase enzyme from the isolation of *Fomitella fraxinea* reached its peak at 70°C.

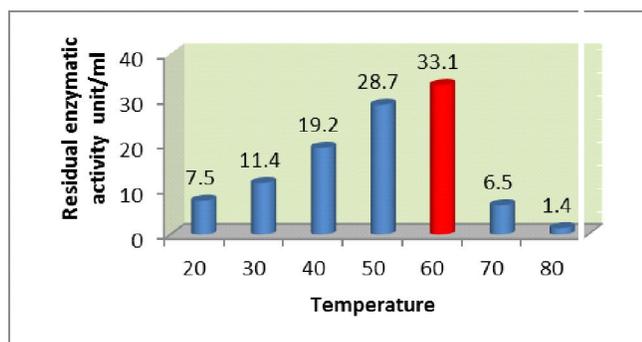


Fig. 7: The optimum temperature curve for the effectiveness of the purified laccase enzyme from the isolation of the medicinal mushroom *G. resinacum*.

6. Thermal stability of the enzyme laccase

In addition to the fact that the enzymes are sensitive to different temperatures and operate within a certain range and reach their maximum effectiveness as mentioned previously, the temperature in which the effectiveness of the enzyme is established varies greatly with the different fungal isolates extracted and purified from the enzyme. Based on the results reached in Fig. 8, it is clear that the activity of the enzyme is stable at a range of temperatures ranging between (20-80) ° C for one hour, as the enzyme maintained its full effectiveness at a temperature of 20 and 30 ° C and maintained 98% of Its effectiveness is at a temperature of 50 ° C and the retention ratio decreased by half at a temperature of 70 ° C while the temperature of 80 ° C resulted in a sharp drop in the activity of the enzyme reaching 3.6%.

This characteristic of enzymes, excluding others, explains that high temperatures led to a change in the shape of the protein and a change in the active site that resulted in the enzyme not interacting with the base material under high temperatures.

The results that were conducted to investigate the degree of thermal stability of the laccase enzyme came from different fungal isolates according to the fungi type and the conditions of purification and extraction. Among the literature published in this field is what he reached (Wang *et al.*,2018), as it was stated that the laccase enzyme purified from the fungus white rot *Trametes sp. MA_XO1* is characterized by thermal stability as it maintained its full enzymatic effectiveness at a range of temperatures between (40-30)°C and retain 92% Of its efficacy at a range of temperatures (60-50)°C, while the enzymatic activity decreased to 85% at a temperature of 70% and the enzyme did not show any enzymatic activity at the temperatures of 80°C and above.

7. The effect of storage on the effectiveness of the laccase enzyme

The retention of the enzyme has to be proven to be

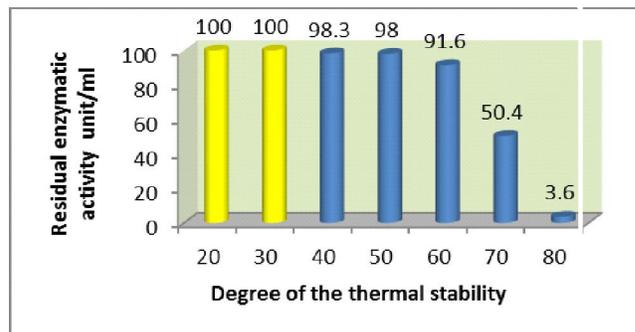


Fig. 8: Curved Thermal Stability of the Enzyme Purified Laccase from the Isolation of *G. resinaceum*.

effective on the time period of storage, whether in laboratory or cooling conditions. The most important results achieved after measuring the enzymatic activity for each time period as shown in Fig. 9 that the enzyme retained 98% of its effectiveness at storage for a period of 7 days and with the continuation of the storage period for a period of 15 days did not lose the enzyme only a little of its original activity where the effectiveness was Enzymatic 96%. The remaining enzymatic activity decreased to 88% at storage for a period of 30 days, the value reached 75% after 45 days have passed since storage, and the enzymatic activity decreased by 50% after 60 days of sadness in laboratory conditions.

The results obtained can be explained by the interference of several factors that lead to the retention of the enzyme, including buffer solutions used in purification and pH, as well as temperatures, which lead to variation in the results of very important.

The results published by (Dedeyan *et al.*, 2000) when comparing its procedure to show the effect of different storage periods on the effectiveness of the laccase enzyme purified from the fungus *Marasmius quercophilus*) indicate that it has the ability to maintain its enzymatic activity for 5 months at room temperature (30)°C.

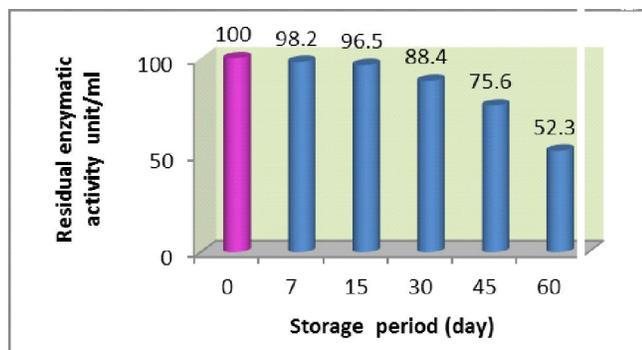


Fig. 9: Effect of the storage period on the efficacy of purified laccase enzyme from isolate of medicinal mushroom *G. resinacum*.

References

Dedeyan, B., A. Klonowska and S. Tagger *et al.* (2000). Biochemical and molecular characterization of a laccase from *Marasmius quercophilus*. *Appl. Environ. Microbiol.*, **66**(3): 925-929. doi:10.1128/aem.66.3.925-929.2000

Ding, Z. & Peng, Lin & Chen, Youzhi & Zhang, Liang & Shi,

Guiyang & Zhang, Kechang. (2012). Production and characterization of thermostable laccase from the mushroom, *Ganoderma lucidum*, using submerged fermentation. *Afr. J. Microbiol. Res.*, **6**: 10.5897/AJMR11.1257.

Fang, Z. & Liu, Xiaoman & Chen, Liyuan Shen, YU & Zhang, Xuecheng & Fang, Wei & Wang, Xiaotang & Bao, Xiaoming & Xiao, Xiaotang & Bao, Xiaoming & Xiao, Yazhong (2015). Identification of a laccase Glas 15 from *Ganoderma lucidum* 77002 and its application in bioethanol production David Wilson. *Biotechnology for biofuels*, 8.54.10.1186/s13068-015-0235-x.

Foorootanfar, H. and M.A. Faramarzi (2015). Insights into laccase producing organisms, fermentation states, purification strategies, and biotechnological applications. *Biotechnol. Prog.*, **31**: 1443–1463. doi: 10.1002/btpr.2173

Laufer, Z., R.P. Beckett, F.V. Minibayeva, S. L  uthje and M. Bottger (2009). Diversity of laccases from lichens in suborder Peltigerineae. *Bryologist*, **112**: 418–426.

More, S., K. Renuka, M. Pruthvi, S. Swetha, Malini and V. More (2011). Isolation, Purification, and Characterization of Fungal Laccase from *Pleurotus* sp. *Enzyme research*, 248735. 10.4061/2011/248735

Nagai, M., S. Toshitsugu, H. Watanabe, K. Saito, M. Kawata and H. Enei (2002). Purification and characterization of an extracellular laccase from the edible mushroom *Lentinula edodes*, and decolorization of chemically different dyes. *Applied microbiology and biotechnology*, **60**: 327-35. 10.1007/s00253-002-109-2

Park, K.M. and S.S. Park (2008). Purification and characterization of laccase from basidiomycete *Fomitella fraxinea*. *J. Microbiol. Biotechnol.*, **18**(4): 670-675.

Saha, B.C. (2002). Production, purification, and properties of xylanase from a newly isolated *Fusarium proliferatum*. *Process Biochemistry*, **37**: 1279-1284. *Sci. Biotechnol.*, **10**(4): 309–326 .

Singh, G., A. Bhalla, P. Kaur, N. Capalash and P. Sharma (2011). Laccase from prokaryotes: a new source for an old enzyme. *Rev. Environ.*

Tian, G.T., G.Q. Zhang, H.X. Wang and N.G. Tzi (2012). Purification and characterization of a novel laccase from the mushroom *Pleurotus nebrodensis*. *Acta biochimica. Polonica.*, **59**: 407-12. 10.18388/abp.2012_2130

Wang, Q.D. and L.Z. Changwei (2018). Characterization of laccase from a novel isolated white rot fungus *Trametes* sp. MA-X01 and its potential application in dye decolorization. *Biotechnology & Biotechnological Equipment*, 1-9. 10.1080/13102818.2018.1517028.