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Factors Affecting Laccase Production by *Pleurotus ostreatus* and *Pleurotus sajor-caju*

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Abstract: The highest laccase production by *P. ostreatus* and *P. sajor-caju* (0.190 and 0.612 U/ml) had been recorded on medium II. *P. sajor-caju* produced laccase higher than *P. ostreatus* on both medium I or II. Also the amount of laccase produced by *P. sajor-caju* and assayed by ABTS (0.468 and 0.612 U/ml) were more than that produced and assayed by DMP (0.357 and 0.467 U/ml) on medium I and II, respectively. The results revealed that the highest laccase (1.800 U/ml) was recorded on medium I supplemented with 200 μM $CuSO_4$ and assayed by ABTS after 21 days incubation. Comparing different three buffers used in laccase assay, it was clear that citrate buffer was the best one. Citrate buffer gave laccase (4.917 and 4.270 U/ml) on media I and II respectively. *P. sajor-caju* could remove 98.0% of 25 mg/L M.B after 7 days and 32.0% of 500 mg/L M.B after the same period. *P. sajor-caju* could removed more than 92% of M.B till concentration 150 mg/L, while it could removed 100% of Max. up to 200 mg/L after the same incubation period. The results revealed that as the dose of gamma radiation increased, the growth and laccase production by *P. sajor-caju* decreased gradually.

Key words: *Pleurotus* · Laccase · Gamma radiation · Cu^{2+} concentrations

INTRODUCTION

The genus *Pleurotus* comprises a group of edible ligninolytic mushroom with medicinal properties and important biotechnological and environmental applications. The cultivation of *Pleurotus* spp is an economically important food industry. Nutritionally, it has unique flavor and aromatic properties and it is considered to be rich in protein fiber, carbohydrates, vitamins and minerals *Pleurotus* spp are promising as medicinal mushrooms, exhibiting hematological, antiviral, antitumor, antibiotic, antibacterial, hypo-cholesterolic and organopollutants immunomodulation activities [1]. One of the most important aspects of *Pleurotus spp* is related to the use of their ligninolytic system for a variety of applications, such as the bioconversion of agricultural wastes into valuable products for animal feed and other food products and the use of their ligninolytic enzymes for the biodegradation of, xenobiotics and industrial contaminates [1]. Nowadays a large amount of compounds like lectins, poly-sacchariedes, polysacchariedespeptides, polysacchariedes-protein complexes have been isolated from mushroom, to many of these compounds have been found to have immunomodulatory, anticancer, antioxidant effects [2-5].

Pleurotus ostreatus, famous for its delicious taste and high quantities of proteins carbohydrates, minerals and vitamins as well as low fats, is the commercially important edible mushroom known as the oyster mushroom [6, 7]. *Pleurotus ostreatus* produces phenol oxidases (laccase) and Mn-oxidizing peroxidases (manganese peroxidases and versatile-peroxidase) as the main ligninolytic activities. However, it lacks lignin-peroxidases [8]. Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2). Laccase is widespread group of blue multicopper enzymes which can catalyze the oxidation of a variety of organic compounds, with concomitant reduction of molecular oxygen to water [9, 10]. Laccase have great potential in industrial applications. This enzyme lack substrate specificity and are thus capable of degrading a wide range of xenobiotics including industrial colored waste waters [11], oil and oil products [12], chlorinated compounds [13], polycyclic hydrocarbons [14,15], removing low concentrations of PPCPs from municipal wastewater [16]. Several fermentation parameters, such as temperature, pH, or

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oxygen aeration rate, play a role in laccase production [17-19]. Adding glucose and $MgSO_4.7H_2O$ resulting significantly more laccase production at 29°C level [9]. Better enzyme production was obtained in malt extract broth (MEB) or mineral salts malt extract broth (MSB-MEB) in comparison to mineral salts broth (MSB) alone [20]. Collines *et al.* [21], indicated that tryptophan as a provided by malt extract might be responsible for better enzymes yield. Extracellular ligninolytic and cellulolytic enzymes are regulated by heavy metals at the transcription level as well as during their catalytic action [22]. Cu^{2+} has been reported to be a strong laccase inducer in several species, among them *Trametes pubescens* [23] and *T. versicolor* [21].

One possible explanation for the observed differences between mono, di-and tricarboxylic acids results from literature data demonstrating at least two potential binding sites for anions in laccase. Firstly, anions may bind to the highly charged, coordinatively unsaturated trinuclear cupric cluster as it is known for halides or pseudo halides. Secondly, anions may also interact with protonated side chains of amino acids such as those of lysine, histidine or argnine. *T. versicolor* laccase, for example, has eight lysine residues and at least four of them are located on or near to the surface of the enzyme. Due to their size and electronic properties, di-and tricarboxylic acids may not to be able to enter the active site of laccase and preferably interact with the surface of the enzyme molecule, while monocarboxylic acids may be able to interact with the trinuclear cluster resulting in diffusion-controlled, slow inhibition type [24]. Shacking has variable effects on laccase production. *Rhizoctonia practicola* gives similar laccase production in shacking and stationary cultures. In *Pycnoporus cinnabarinus*, shacking increases laccase production, whereas in many other white rot fungi, it is suppressed [25, 26]. The substrate specificity of enzyme was determined by performing the assay with different substrates such as ABTS, 4-methyl alcohol. ABTS was the ideal substrate for laccase. For phenolic compounds, the enzyme showed only a little activity on alcohol (11%) and hydroquinone (25%) and no detectable activity on ferulic acid veratryl alcohol [9]. The ascomycete, *Botryosphaeria* sp, produced two extracellular constitutive laccases (PPoI and PPoII) active toward the substrates: 2, 2\-azino-bis (3-ethyl-benzothiazoline-6 sulfonic acid) (ABTS) and 2,6-dimethoxy phenol (DMP), respectively. The production of both laccase increased when the fungal isolate grown in the presence of veratryl alcohol and resulted in optimal laccase production (100-25 folds, respectively) at 40 mM [27]. Constantinovici *et al.* [28], studied the behavior at low doses of gamma-rays exposure (0.033-0.4 kGy) of horseradish peroxidase (HRP) and of two different purified fractions of apple (Jonathan cultivar) peroxidases (named APR_{1s} and APR_{2s}) and the results show that the irradiation inactivation mechanism takes place most probably through enzymatic structure disorganization following the translational conformational states appearing during increasing irradiation dose. The energy transfer from incidental radiation imposes the stochastic degradation of some amino acid residues, which, finally, leads to enzymatic architecture degradation.

This study aimed to investigate the suitable conditions for high production of laccase by the white rot fungi to be used for decolorization of dye which resemble a serious problem in Egypt. Using of laccase in decolorization of dyes is considered an environment friendly technique capable of remediate hazardous pollutants.

MATERIALS AND METHODS

Microorganisms: Four white rot fungi (WRF) were used. *Pleurotus ostreatus* CBS 411.71, *Pleurotus sajor-caju, Phanerochaete chrysosporium* BKM-F 1767 (ATCC 24725) and *Phanerochaete chrysosporium* American Type Culture Collection (ATCC 34541).

WRF Maintenance: The four WRF were maintained on PDA (Potato-Dextrose agar medium) [29], slants and plates. The slants were stored at 4°C till use. The WRF strains were cultivated on Malt agar plates [30], for 14 days at 30°C. Mycelium agar plugs (10 mm in diameter) cut along the edge of an active growing colonies were used as inoculum.

Production of Laccase Enzyme on Different Media: The four WRF basidiomycetes were used to inoculate 50 ml in 250 ml conical flask of the three different production media: Basal medium I ([30], N-Limited medium II [31] and Defined medium III [32]. Two discs (10mm in diameter) were used to inoculate each flask after sterilization of the media. For laccase production the media were supplemented with 600 μM $CuSO_4$. $5H_2O$. Three replicates were used for each strain in each medium. The flasks were incubated in dark without shacking at 30°C for 14 days. Five ml from each flask was centrifuged at 8000 rpm for 5 minutes and the supernatant was used for enzyme assay.

Enzyme Assay: Laccase assay [33] was carried out in 1 Cm quartz cuvatte. Reaction mixture of 1 ml contained 2 mM ABTS (2,2\, azino-bis (3-methyl benzo-thiazoline-6-sulphonic acid) diammonium salt or 1 ml DMP (2,6 dimethoxy phenol) products of Sigma-Aldrich USA. In Mcllvaine buffer (pH 5.0). To the assay mixture, 100 µl of centrifuged supernatant was added. The enzymatic activity was estimated in IU by monitoring the adsorbance change at 420 nm (ABTS), ε_{420} = 36 mM^{-1} Cm^{-1} by spectrophotometer at 30°C. OR 469 nm (DMP), ε_{469} = 27.5 $mM^{-1}Cm^{-1}$.

Effect of $CuSO_4$ Concentrations on Laccase Production: The two *Pleurotus* strains which produced laccase were inoculated into two medium (I and II) supplemented with different concentrations of $CuSO_4.5H_2O$ (zero, 100, 150, 200, 300, 400, 600, 900 µM) after sterilization of the medium.

Production of Laccase by *P. ostreatus* Supplemented with Different $CuSO_4$ Concentrations on Medium I and Incubated for Different Incubation Periods: *P. ostreatus* was inoculated into medium I supplemented with different concentrations of $CuSO_4.5H_2O$ and incubated under static or shacked conditions for 7 days incubation period and assayed by DMP and ABTS after 7, 14, 21 and 28 days incubation periods. Also, extracellular protein was determined according to Lowry *et al.* [34].

Production of Laccase by *P. sajor-caju* on Different Media at Different Incubation Periods: *P. sajor-caju* was used to inoculate media (I and II) supplemented with 200 µM $CuSO_4$ and incubated static at the dark for 7, 14, 21 and 28 days and assayed by ABTS.

Effect of Different Buffers on Laccase Produced by *P. sajor-caju* and Assayed by ABTS: *P. sajor-caju* was used to inoculate media (I and II) supplemented with 200 µM $CuSO_4$ and incubated stagnant for 14 days in dark. The laccase produced was assayed by ABTS at three buffers (Mcllvaine, acetate and citrate) pH 5.0.

Decolorization of Dye by *P. sajor-caju:* The ability of *P. sajor-caju* to remove methylene blue (M.B) and maxilon (Max.) (produced of Mombai, India) at different concentrations after inoculation of 2 discs (10mm in diameter) into the medium I supplemented with 200 µM $CuSO_4$ and incubated stagnant at dark for 7 days was determined. Three replicates were used for each concentration for each dye.

Effect of Gamma Irradiation on Growth of *P. sajor-caju:* *P. sajor-caju* grown on malt agar plates for 7 days were exposed to different doses (0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0 and 4.0 kGy) of gamma radiation (Indian cell, Co-60 at National Center for Radiation Research and Technology (NCRRT), Nasar City, Cairo, Egypt). The dose rate was 1kGy/ 12.5 minutes. Three plates were used for each dose. One disc (10mm in diameter) from each plate of each dose was used to inoculate malt agar plates at the center. The plates were incubated at 30°Cfor 6 days. The growth diameter was recorded.

Effect of Gamma Irradiation on Laccase Production by *P. sajor-caju:* Two discs (10 mm in diameter) from each plate of each dose were used to inoculate media (I and II). The media were supplemented with 200 µM $CuSO_4.5H_2O$. Three replicates were used for each dose to determine laccase activities. The flasks were incubated at 30°C for 14 days stagnant at dark.

RESULTS AND DISCUSSION

Production of Laccase Enzyme on Different Media: The ability of different white rot fungi (WRF) to produce laccase on different media have been studied. The WRF used in this experiment were *Pleurotus ostreatus*, *Pleurotus sajor-caju*, *Phanerochaete chrysosporium* 34541 and *Phanerochaete chrysosporium* 24725. These WRF were grown in three different media to study their abilities to support laccase production. *P. ostreatus* produced laccase on three media used as indicated in Table 1. The highest laccase production had been

Table 1: Production of laccase on different media by different white rot fungi (WRF)

	Medium (I) (U/ml)		Medium (II) (U/ml)		Medium (III) (U/ml)	
White rot fungi (WRF)	DMP	ABTS	DMP	ABTS	DMP	ABTS
P. ostreatus	0.1265	0.1656	0.14575	0.1908	0.1265	0.1656
P. sajor-caju	0.3575	0.4680	0.4675	0.6120	0.000	0.000
P. chrysosporium 34541	0.000	0.000	0.000	0.000	0.000	0.000
P. chrysosporium 24725	0.000	0.000	0.000	0.000	0.000	0.000

recorded on medium II. However, *P. sajor-caju* could produce laccase on medium I and II but failed to produce laccase on medium III. The two strains of *P. chrysosporium* failed to produce any laccase on any media. The highest laccase production by *P. ostreatus* and *P. sajor-caju* (0.190 and 0.612 U/ml) had been recorded on medium II. This medium was N-Limited medium contained 16 g/L glucose as a carbon source and 0.25g/L of Ammonium tartrate and 0.25g/L Yeast extract. This Limited-Nitrogen source media which contain a mixture of inorganic nitrogen source (Ammonium tartrate) and organic nitrogen source (Yeast extract) which was rich in amino acids and vitamins.

The ligninolytic enzymes have been seen to be regulated by the usable concentration of nitrogen in the media. The low nitrogen level can stimulate the ligninolytic enzyme production, whereas a high nitrogen level represses it [35]. The production of laccase enzyme can be increased by varying different physiochemical parameters such as in the presence of specific enzyme substrate/s (e.g., phenolic compounds) as adaptive response and the presence of certain inducers [36]. Production of laccase and MnP by *Trametes trogii* was increased by the addition of easily available carbon and nitrogen source to the media, such as malt extract and peptone [37]. Using different carbon sources at three concentration levels of 10, 25 and 40 g/L at which the pH was controlled at 4.0, 6.0 and 8.0 showed that the glucose and glycerol were different carbon sources compared to lactose. The best carbon source was 40 g/L glucose. With different nitrogen sources at three different levels when the pH was controlled at 4.0, 6.0 and 8.0 the results showed that the fungus produced the highest laccase activity with 0.22 g/L yeast extract 50 U/ml at pH 6.0 which was four times higher than that produced with ammonium tartrate and malt extract at the same pH 6.0 [38].The substrate specificity of enzyme was determined by performing the assay with different substrates such as ABTS, 4-methyl alcohol. ABTS was the ideal substrate for laccase [9].

Effect of $CuSO_4$ Concentrations on Laccase Production: Effect of different concentrations of $CuSO_4$ on laccase production by *P. ostreatus* and *P. sajor-caju* had been indicated in Fig. 1. The highest laccase production (1.620 and 1.850 U/ml) by *P. ostreatus* was on media I and II supplemented with 300 µM $CuSO_4$ respectively. However, *P. sajor-caju* produced the highest laccase (0.695 and 1.368 U/ml) on media (I and II) supplemented with 200 µM $CuSO_4$ respectively. *P. ostreatus* produced more laccase on medium II at none (control) and low concentration of $CuSO_4$ till 300 µM than the medium I. However as, the concentration of $CuSO_4$ increased, the laccase production by *P. ostreatus* was more on medium I, except at 900 µM $CuSO_4$. Cu^{2+} has been reported to be a strong laccase inducer in several species, among them *T. pubescens* [23]. Fonseca, *et al.* [39], studied the effect of copper on laccase production on different white rot fungi such as: *Ganoderma applanatum* F, *Peniophora* sp. BAFC 633, *Pycnoporus sanguineus* BAFC 2126 and *Coriolus versicolor f. antarticus* BAFC 266 and found that laccase secretion increased significantly ($p<0.001$) in cultures supplemented with Cu^{2+} for fungi used. Low laccase activity was detected in the absence of Mn^{2+} or Cu^{2+} in the culture. The presence of Mn^{2+} in the basal medium increased slightly the laccase activity levels, but the higher induction was obtained in presence of Cu^{2+} in the basal medium. The presence of ethanol in these cultures did not increase laccase activity. Maximal laccase activity and protein were obtained with 300µM $CuSO_4$ [30].

Production of Laccase by *P. ostreatus* Supplemented with Different $CuSO_4$ on Medium I and Incubated for Different Incubation Periods: Combination of more than one factor that could influence laccase production had been studied as indicated in Figs 2-6. To indicate the effect of incubation periods on laccase production by *P. ostreatus* on medium I supplemented with 200 µM $CuSO_4$, the results revealed that laccase was 0.558, 1.188, 1.800 and 0.760 U/ml after 7, 14, 21 and 28 days respectively when assayed by ABTS. However, laccase production by *P. ostreatus* when medium I supplemented with 300 µM $CuSO_4$was 0.720, 1.026, 0.580 and 0.470 U/ml after 7, 14, 21 and 28 days, respectively. Protein production by *P. ostreatus* on medium (I) supplemented with different concentrations of $CuSO_4$ and incubated for 14, 21 and 28 days the results was studied. The results show the highest extracellular protein secreted by *P. ostreatus* after 14 days has been recorded at 400µM $CuSO_4$ (312 µg/ ml) which equal that secreted by the control as indicated in Fig. 7. After 21 days the results revealed that the highest protein (357 µg/ ml) secreted by *P. ostreatus* on medium I supplemented with 150µM $CuSO_4$ as indicated in Fig. 8. While after 28 days the highest extracellular protein produced (302 µg/ ml) on medium I supplemented with 200µM $CuSO_4$ as indicated in Fig.9. *G. applanatum* response to stimulation was significantly different from 7th day ($p<0.001$) for all treatments, reaching the highest enzyme activity at 10th day (18,830Ug^{-1}), equivalent to 49.2-fold higher than the production without copper (383Ug^{-1}). *Peniophora* sp. achieved a significant enzyme activity increase from 7th culture day ($p<0.001$) with

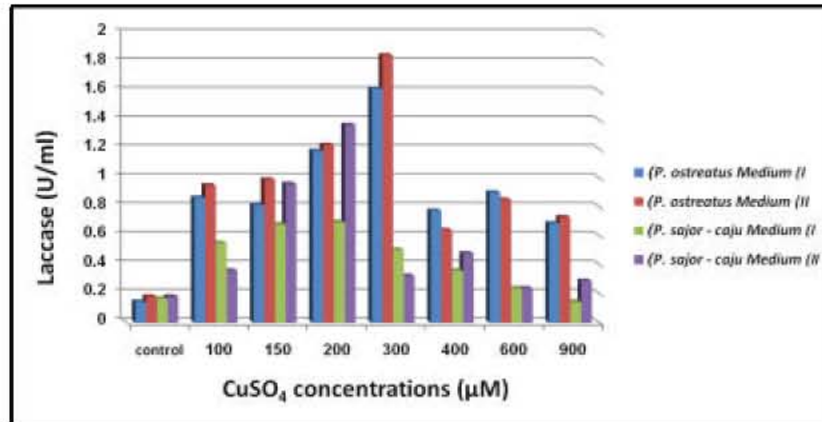

Fig. 1: Production of Laccase by *Pleurotus* spp. on different media supplemented with different $CuSO_4$ concentrations.

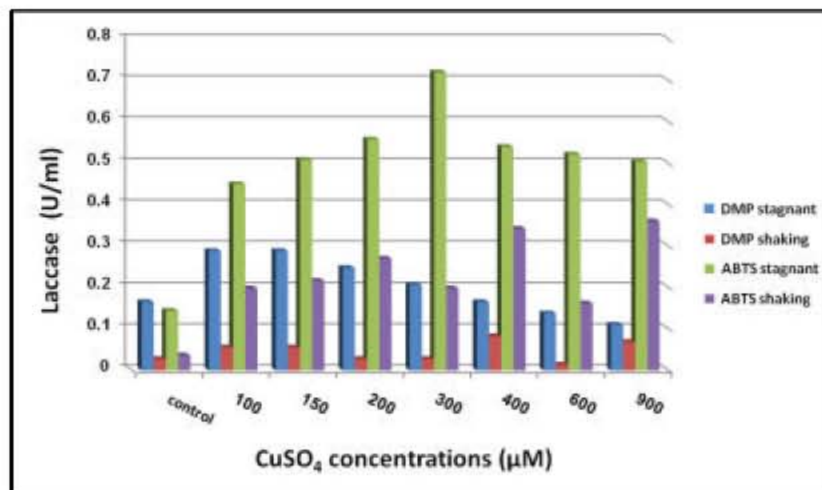

Fig. 2: Production of laccase on medium (I) by *P. ostreatus* supplemented with different concentrations of CuSO4 and incubated for 7 days.

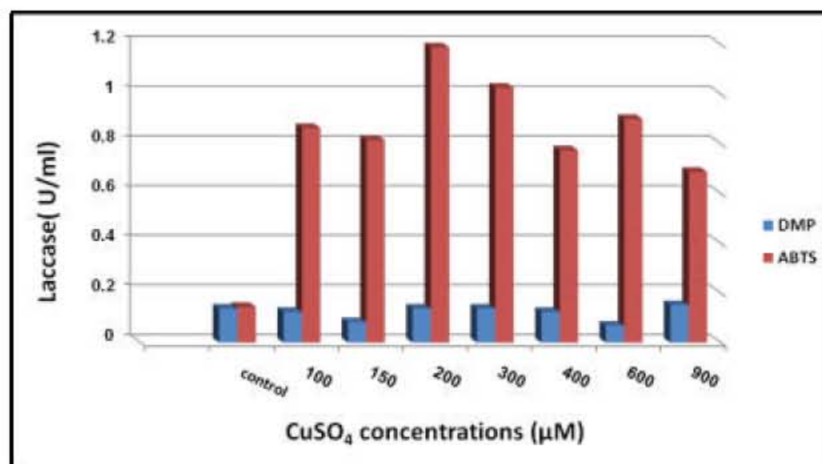

Fig. 3: Production of laccase on medium (I) by stagnant *P. ostreatus* supplemented with different concentrations of $CuSO_4$ and incubated for 14 days.

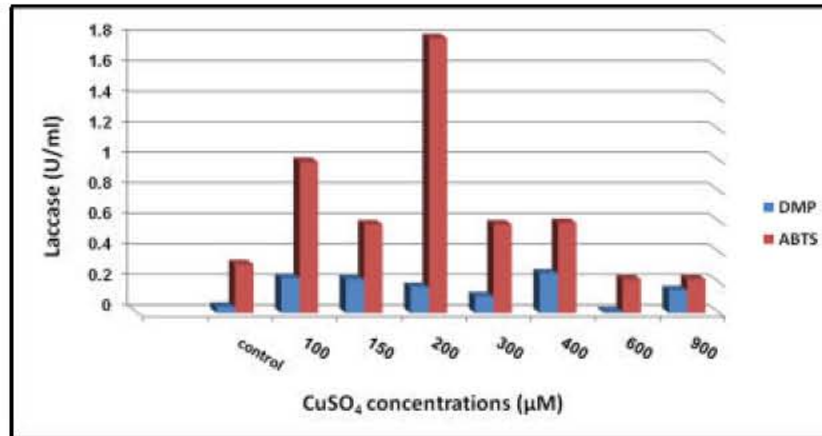

Fig. 4: Production of laccase on medium (I) by stagnant *P. ostreatus* supplemented with different concentrations of $CuSO_4$ and incubated for 21 days.

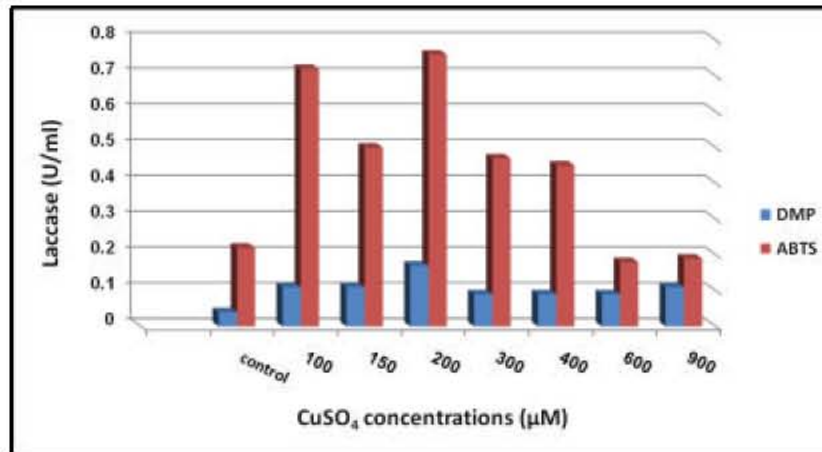

Fig. 5: Production of laccase on medium (I) by stagnant *P. ostreatus* supplemented with different concentrations of $CuSO_4$ and incubated for 28 days.

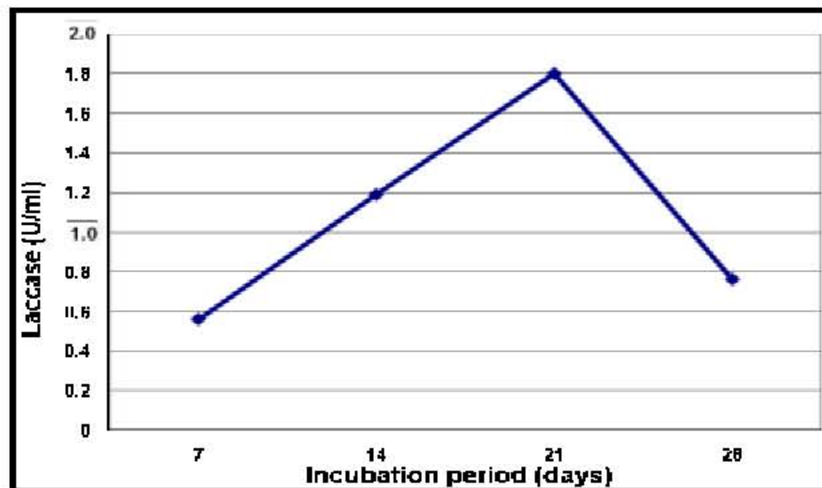

Fig. 6: Production of laccase on medium I by stagnant *P. ostreatus* supplemented with 200µM $CuSO_4$ and incubated for different incubation periods.

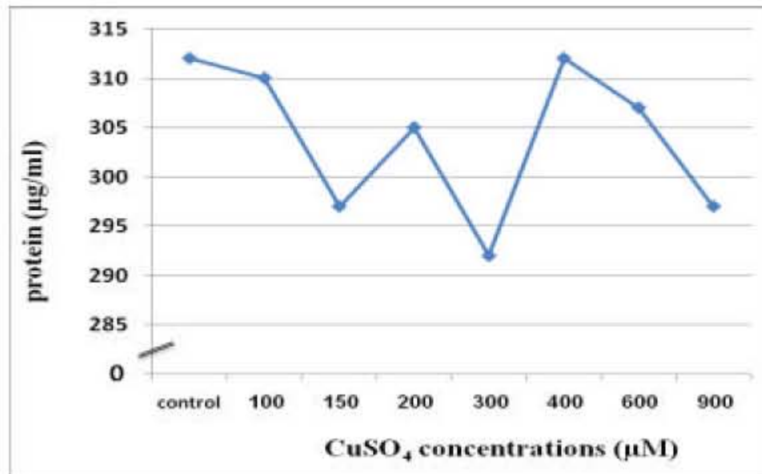

Fig. 7: Production of protein on medium (I) by *P. ostreatus* supplemented with different concentrations of $CuSO_4$ and incubated for 14 days.

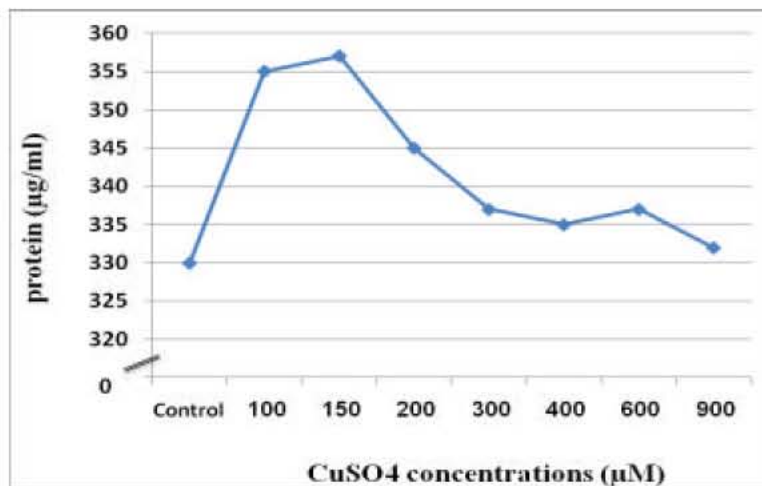

Fig. 8: Production of protein on medium (I) by stagnant *P. ostreatus* supplemented with different concentrations of $CuSO_4$ and incubated for 21 days.

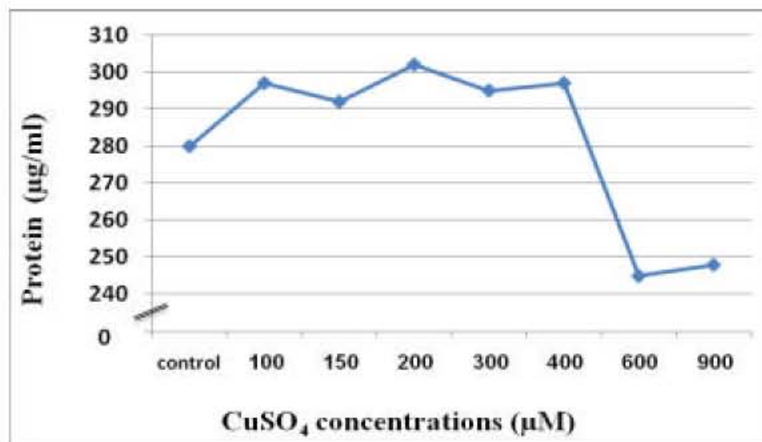

Fig. 9: Production of protein on medium (I) by stagnant *P. ostreatus* supplemented with different concentrations of $CuSO_4$ and incubated for 28 days.

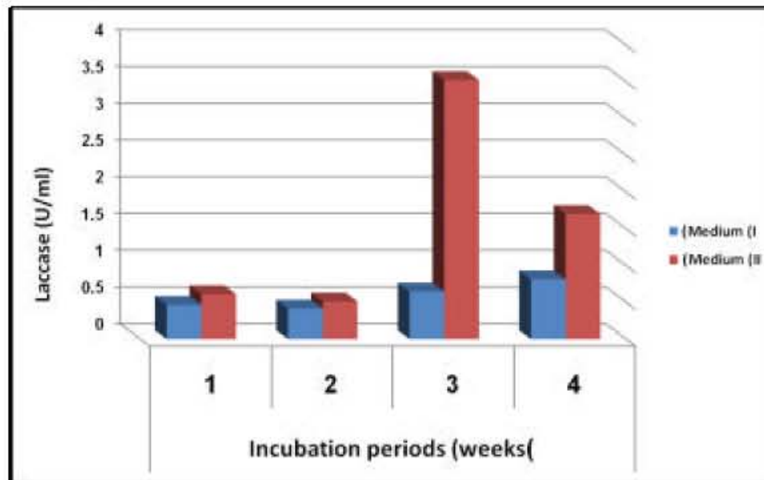

Fig. 10: Production of laccase on different media by stagnant *P. sajor – caju* at different incubation periods.

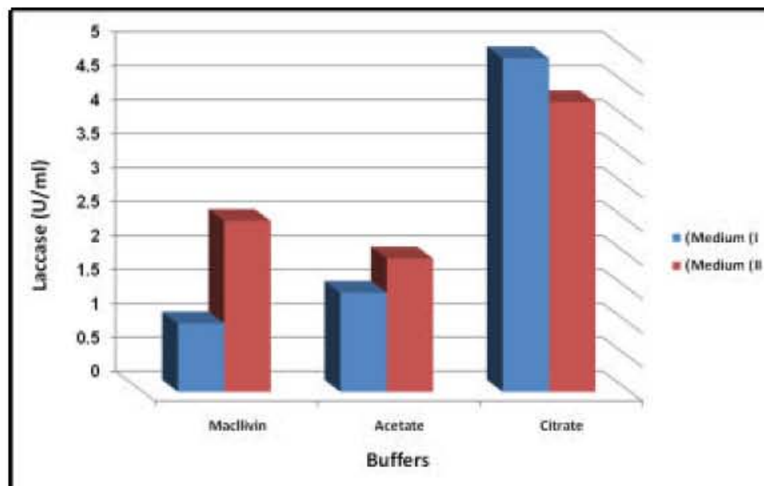

Fig. 11: Production of laccase by stagnant *P. sajor – caju* on different media and assayed by different buffers.

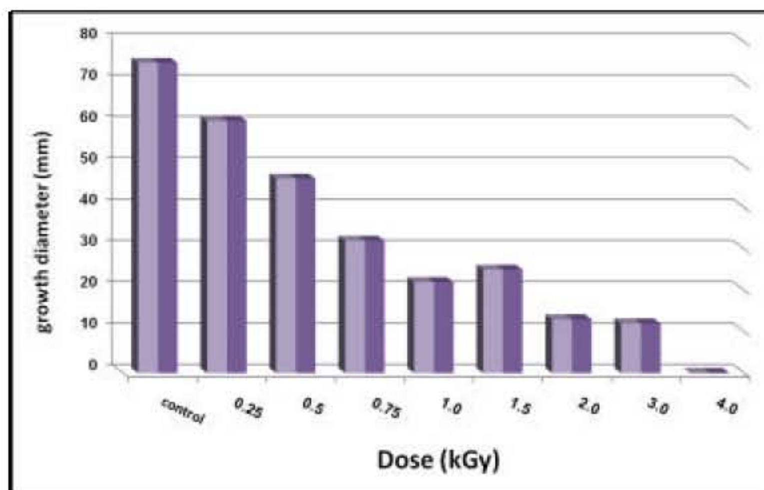

Fig. 12: Effect of gamma radiation on the growth of *P. sajor – caju*

the highest value at 10th culture day (11,462Ug^{-}), ^{1} equivalent to 19.7-fold higher than the production without copper (580Ug^{-1}). *P. sanguineus* showed maximum significant increase (p<0.001) at 14th day (27,132Ug^{-1}) 27.7-fold higher than without copper (978Ug^{-1}). *C. versicolor f. antarctius* laccase activity at 14th day (13,304Ug^{-1}) was 7.6-fold higher than without copper (1750Ug^{-1}) [39].

Production of Laccase by *P. sajor-caju* on Different Media at Different Incubation Periods: Production of laccase on different media (I and II) by stagnant culture of *P. sajo-caju* incubated for 7, 14, 21 and 28 days and assayed by ABTS was indicated in Fig.10. The results showed that laccase produced by *P. sajor-caju* on medium II was more than that produced on medium I. However, as the incubation period increased, laccase produced by *P. sajor-caju* increased, till it reached maximum productivity at 21 days (3.528 U/ml) and then began to decrease (1.703 U/ml) at 28 days incubation on medium II. But in case of medium I, increasing in laccase production contained till 28 days (0.814 U/ml). *P. sajor-caju* produced, the maximum laccase after 4 weeks incubation period i.e. need more time for maximum productivity on medium I, but still less than that produced on medium II. So, it was recommended to use medium II for laccase production to give high laccase production (3.528 U/ml) in less incubation period (21 days) by *P. sajor-caju.* Laccases were produced and characterized during the process of cultivation *Pleurotus sajor-caju* PS-2001 in stirred-tank bioreactor. High laccase activity peak 40 U/ml was reached on six day of cultivation [40].

Effect of Different Buffers on Laccase Produced by *P. sajor-caju* and Assayed by ABTS: Effect of using different buffers in laccase produced by *P. sajor-caju* on different media was shown in Fig. 11. Data indicated that medium II produced more laccase than medium I and it was clear that citrate buffer was the best one. Citrate buffer gave the highest laccase (4.917 and 4.270 U/ml) on media I and II respectively. Similar results had been reported by Ters *et al.* [24], who determined the most suitable carboxylic acid based buffer for *Trametes villosa* laccase production at a desired pH value and buffer strength. For example, citrate appears to be the ideal choice for assaying laccase at pH 4.0 and below, irrespective of the chosen buffer concentration. However, succinate buffer at concentrations up to 100 mM may be preferable at pH 5.0. Overall it can be said that carboxylic acids with more than one carboxy group are preferable buffer compounds for laccase assays not only because of

Table 2: Decolorization of methylene blue (M.B) dye by *P. sajor-caju* incubated for 7 days

Dye concentration (mg/L)	Removal %
25	98.00
50	96.00
75	94.00
100	93.00
150	92.10
200	72.50
300	50.00
500	32.00

Table 3: Decolorization of Maxilon (Max.) dye by *P. sajor-caju* incubated for 7 days

Dye concentration (mg/L)	Removal %
25	100
50	100
75	100
100	100
150	100
200	100
300	85
500	60

their relatively minor inhibitory effect but also because they inhibit instantly contrary to monocarboxylic acids. Apparently, time-independent inactivation was also observed with synthetic McIlvaine buffer, but the extent of inactivation was high (around 40%) independent of the chosen pH.

Decolorization of Dyes by *P. sajor-caju*: Investigating the ability of *P. sajor-caju* to decolorize the basic dye methylene blue (MB) was indicated in Table 2. The results revealed that as the concentrations of dye increased, the removal percentage decreased. *P. sajor-caju* could remove 98.0% of 25 mg/L M.B. after 7 days and 32.0% of 500 mg/L M.B. after the same periods. *P. sajor-caju* could remove more than 92.0% of M.B. till concentration 150 mg/L. However, the ability of *P. sajor-caju* to decolorize another dye, maxilon (Max.) has been studied as indicated in Table 3. *P. sajor-caju* could remove 100% of Max. up to 200 mg/L. From the previous results, it is clear that *P. sajor-caju* removed Max. more efficient than M. B. In another statement, M.B. was more recalcitrant to decolorization by *P. sajor-caju* than Max. However, *P. sajor-caju* removed 32.0% and 60% 500 mg/L of M.B. and Max., respectively. Methylene Blue is a thiazine cationic dye and has widespread applications, which include colouring paper, temporary hair colourant, dying cottons, wools and coating for paper stocks, it is also used in

Table 4: Effect of gamma radiation on laccase production by irradiated *P. sajor-caju*

Dose (kGy)	Media (I)	Media (II)
Control	1.80	0.41
0.25	0.83	0.32
0.50	0.45	0.33
0.75	0.41	0.38
1.00	0.44	0.19
1.50	0.36	0.16
2.00	0.45	0.22
3.00	0.56	0.16
4.00	0	0

microbiology, surgery and diagnostics and as a sensitizer in photo-oxidation of organic pollutants. Although is low toxicity, it can cause some specific harmful effects in humans such as heartbeat increase, vomiting, shocks, cyanosis, jaundice and tissue necrosis [41-46]. Zahangir-Alam *et al.* [47], investigated the optimization of decolorization of methylene blue (MB) dye by lignin peroxidase (Lip) enzyme produced by white rot fungus *Phanerochaete chrysosporium* using sewage treatment plant (STP) sludge as a major substrate was carried out in the laboratory. Optimization by one-factor-at-a-time (OFAT) and statistical approach was carried out to determine the process conditions on optimum decolorization of MB dye using Lip enzyme in static mode. The OFAT method indicated that the optimum conditions for decolorization of MB dye (removal: 14-40%) was at temperature 55°C, pH 5.0 with hydrogen peroxidase (H_2O_2) concentration 4.0 mM, MB dye concentration 20 mg/L and Lip activity 0.487 U/ml. The addition of veratryl alcohol to reaction mixture did not contribute any further increase in decolorization. The initial concentration of MB and the activity of Lip enzyme were further optimizing using response surface methodology (RSM). The colour and surface plots suggested that the optimum initial concentration of MB and Lip activity predicted were 15 mg/L and 0.687 U/ml, respectively for the removal 65%. The validation at the model showed that the decolorization process gave the higher removal of 90% in agitation mode compared to the static mode with 65% for 60 min of incubation time by Lip enzyme. D'Souza *et al.* [48], showed that Brilliant green was decolorized to the maximum by day 4 whereas reactive orange 176 (RO 176) was least decolorized. Among the synthetic blue dyes, aniline blue was decolorized almost totally by day 4. Poly R and crystal violet did not induce high laccase activity and their decolorization was also very low. On the other hand, trypan blue, methylene blue and Remazole Brilliant Blue R (RBBR) were decolorized by 60-70% but they did not induce laccase activity. About 60% decolorization of black liquor (used at 10% concentration) from paper and pulp mills were achieved by day 6. Molasses spent wash from distillery waste when used at 10% concentration was totally decolorized by day 6. Textile effluent B at this concentration was decolorized by 60% on day 2 and no further reduction in colour was observed.

Effect of Gamma Irradiation on Growth of *P. sajor-caju*: Effect of gamma irradiation on growth of *P. sajor-caju* was shown in Fig. 12. The results revealed that as the dose increased, the growth of *P. sajor-caju* decreased gradually. The reduction of growth and influence of enzymes production by gamma radiation had been recorded by other studied. These studies confirmed the findings of the present study as follow. Radiation reduced the viable count of bacteria and fungi. As dose increased the viable count decreased gradually [49-52]. Aziz and Mahrous [53] recorded that the dose required for complete inhibition of fungi ranged from 4.0 to 6.0 kGy. Abo-State *et al.* [54] found that gamma radiation reduced the viable count of *Aspergillus terreus* MAM-F23 and *A. fumigtus* MAM-F35 gradually as the dose increased. Doses 5.0 and 4.0 kGy reduced the viable count of *Aspergillus* MAM-F23 and MAM-F35 completely.

Effect of Gamma Irradiation on Laccase Production by *P. sajor-caju:* Studying the effect of gamma irradiation on laccase production by *P. sajor-caju* has been indicated in Table 4. Unfortunately, the results revealed that gamma radiation had a bad effect on laccase production as dose increased, laccase production decreased. Surprisingly, laccase production on medium II was less than that of medium I. To induce the lignocellulolytic mutants of *Pleurotus ostreatus*, the mycelia were irradiated by gamma-ray radiation to doses of 1-2 kGy. The strain was able to form the fruiting bodies and grew similarly to the control [55]. El-Batal and Abo-State [56], found enhanced productivity in CMCase, FPase, avicelase, xylanase, pectinase by gamma irradiation at dose 1.0 kGy with increased percent 8%, 20%, 10%, 4%, 31%,22% and 34% respectively as compared with unirradiated control. Also, the highest CMCase activity was recorded for *Fusarium neoceras* mutants No.1 and No.6 which exposed to 1 min UV-radiation, while the highest CMCase of *F. oxysorum* was mutant No.4 which exposed to 4 min UV-radiation [51]. Rajoka [57] reported 1.6 fold enhanced productivity of extracellular endoglucanase over the parent strain.

After the optimization, the FPase in *T. reesi* MCG77 mutant was increased by 2.5 folds compared to that *T. reesi* QM9414 mutant [58]. Abo-State *et al.* [54] reported that mutant No.4 of *Aspergillus* MAM-F23 which exposed to 0.5 kGy produced higher cellulases (CMCase 372 U/ml, FPase 64 U/ml and Avicelase 39 U/ml) than parent strain (CMCase, 305 U/ml, FPase 48 U/ml and Avicelase 29 U/ml). Bercu *et al.* [59], studied the gamma-rays irradiated *Pleurotus ostreatus* and found that no difference regarding the line shape or Spain Hamiltonian parameters was noticed between non-irradiated and ^{60}Co gamma-rays irradiated dry samples up to 9 kGy.

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