Biotechnological Approach for Lignin Peroxidase (LiP) Production from Agricultural Wastes (Rice Husk) by *Cunninghamella elegans*

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Abstract: Peroxidases are essential enzymes in biodegradation of lignin which have been investigated intensively in fungi. Six fungal isolates were isolated from rice husk and screened for their activities to produce an extracellular lignin peroxidase enzyme (LiP). One isolate (identified as *Cunninghamella elegans*) was found to be the most potent one. The maximum LiP productivity was under static condition (LiP activity= 15800 U L^{-1}), at pH 6 (LiP activity= 14200 U L^{-1}), 30°C (LiP activity= 15300 U L^{-1}) and after 14 days incubation period (LiP activity= 14300 U L^{-1}). LiP Purification protocols involved several steps including firstly, the precipitation of LiP with 80% ammonium sulphate with a fold of 2.43 and enzyme activity of 89500 U L^{-1}. Secondly, LiP was further purified using Sephadex G-100 fractionation with a purification fold of 2.76 and enzyme activity of 112200 U L^{-1}. Finally, the molecular mass of purified LiP was estimated at 50 kDa. using SDS-PAGE technique.
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1. Introduction

The increasing expansion of agro-industrial activity over the last 40 years has led to the accumulation of a large quantity of lignocellulosic residues all over the world (Villas-Boas *et al*., 2002). Agriculture has played and will continue to play an important economic role in Egypt. Egyptian agriculture is traditionally based on crop productions, particularly cotton, wheat, vegetables, rice and bean. By-products derived from these sub-sectors have and will continue to provide large quantities of valuable nutrients to sustain livestock, particularly ruminant production in Egypt. However, they are known mostly containing lignocellulosic materials which are a complex structure of lignin. Zanirun (2008) reported that removal of lignin is important in order for enzymatic hydrolysis to occur as it acts as a barrier to most of agricultural wastes. Lignin is a complex aromatic amorphous polymer, most commonly derived from wood and is an integral part of plant cell walls and is the second most abundant biopolymer after cellulose, employing 30 % of non-fossil organic carbon and constituting from a quarter to a third of the dry mass of wood (Boerjan *et al.*, 2003). It is relatively hydrophobic and aromatic in nature and is known for its heterogeneity in lacking a defined primary structure (D'Souza Ticlo, 2008). It is an essential part of the plant cell wall, imparting rigidity and protecting the easily degradable cellulose from attack by pathogens (Lankinen, 2004). Eriksson *et al*. (1990) also stated that lignin is a three-dimensional heterogeneous polymer stored in the plant cell wall of all vascular plants. It is the most abundant renewable aromatic biopolymer in the biosphere. Owing to its recalcitrant nature, lignin is remarkably resistant to degradation by most microorganisms, an important factor limiting the rate of degradation of lignocellulosic materials. Studies on lignin biodegradation are important for possible biotechnology application, since lignin polymers are major obstacles to the efficient of utilization of lignocellulosic materials in a wide range of industrial processes. The lignocellulosic biomass from plants is a renewable source of food, energy and chemicals. It accounts for more than 60% of the total biomass production (Kuhad *et al.*, 1997). Lignocellulosic waste material is produced in huge amounts in agriculture, forestry and in the pulp and paper industry. The use of this waste is an important way to recycle carbon to energy and food. The lignocellulosic material of plants consists of three main components, namely cellulose, hemicelluloses and lignin.

Ligninase or lignin peroxidase (LiP) is gaining importance for its biotechnological application due to its integral role in the biodegradation of lignin, lignin related aromatic compounds and the potential use in industrial processes such as biopulping, biobleaching and bioremediation. (LiP) has been extensively studied and has been reported that its production was by several fungal isolates (Zanirun, 2008).

Due to its complicated structure and non-hydrolysable bonds, lignin is more difficult to break

down than cellulose or hemicelluloses. The molecular mass of lignin is about 100 kDa which prevents its uptake inside the microbial cell. Thus, the biological degradation of macromolecular lignin must occur through the activity of extracellular enzymes (Argyropoulos and Menachem, 1997). In 2004, Kareem and Akpan reported that the use of agricultural by-products as substrate for enzyme production was cheap and could facilitate large scale production of industrial enzymes in the tropics. Obligate and facultative fungi from lignocelluloses substrates are an important source of lignin-degrading enzymes (Bucher *et al.*, 2004; Pointing and Hyde, 2000). Fungi play a mostly saprophytic role to release nutrients which can again be used by plants and animals as organic sources for metabolism (Raghukumar, 2004). This role involves the degradation and mineralization of lignocellulosic substrates and fungi that do so are termed as lignicolous or lignin-degrading fungi (D'Souza Ticlo, 2008). The lignin degrading fungi initiate the process of lignin degradation using a specialized set of enzymes, i.e., the lignin-degrading enzymes, which include the heme-containing peroxidases.

A lignocellulosic substrate (wheat straw and hemp woody core) promotes the production of LiP of *P. chrysosporium* under culture conditions, in which, N and C are non-limiting (Kapich *et al.*, 2004). Peroxidases catalyze the oxidation of substrates using H_2O_2 as a final electron acceptor. All peroxidases share the same catalytic mechanism consisting of three steps. First, the ground state ferric peroxidase reacts with H_2O_2 to yield two electron oxidized intermediate known as the compound I. Then, compound I is reduced by an exogenous substrate in one electron reaction to another enzyme intermediate called compound II, which is subsequently reduced back to the ground state by another substrate molecule, also, in one electron reaction (Verdin *et al.*, 2006).

Under natural conditions fungi are able to grow on many substrates difficult to exploit by more rapidly growing organisms such as bacteria. Growth on complex substrates is based on the secretion of extracellular enzymes (Lankinen, 2004).

Guo (2001) and Shen *et al.* (2005) isolated the LiP enzyme by precipitation with ammonium sulphate and the enzyme was further purified by fractionation on three steps of chromatography, cation exchange, hydrophobic interaction and anion exchange to obtain an electrophoretically homogenous pectinase. The molecular weight of the purified enzyme was estimated by SDS-PAGE.

2. Material and Methods

2.1. Rice husk preparation and cultivation

The rice husk was obtained from Mansoura governorate of Egypt. It was homogenized using sterile mortar and pestle and passed through sterile stainless steel sieves of 200 µm and 100 µm in succession, followed by washing with sterile distilled water. The particles were then plated on potato dextrose agar (PDA) supplemented with 10% antibiotic solution (contained 1 gm of penicillin in a combination with 1 gm of streptomycin sulphate in 100 ml of sterile distilled water) to prevent bacterial growth (D'Souza Ticlo, 2008). The plates were then incubated at 25°C for 4-7 days. After the end of incubation period, six fungal isolates were isolated and purified.

2.2. Media preparation for enzyme production

The culivation medium (production medium) of the fungal isolates contained 4.5% (w/v) rice husk, 1.5% yeast extract, 1% glucose, 0.25% NH_4Cl, 0.05% thiamine chloride, 0.2% KH_2PO_4, 0.2% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, and 0.05% KCl. Tap water was used for preparation of the medium and the pH was adjusted to 6. Erlenmeyer flasks (250 ml) containing 100 ml of medium were inoculated with 1 cm square agar piece ($8x10^6$ spores/ml) from an actively growing fungal isolates on PDA and incubated at 25°C for 7 days (Dayaram and Desgupta, 2008).

2.3. Enzyme extraction

The enzyme extraction was carried out according to the method described by Dayaram and Desgupta (2008). Briefly, cultures were harvested after 7 days, filtered and clarified by cooling centrifugation at ~12000 x*g* for 20 min at 4°C to remove the mycelia and enzyme activity was assayed.

2.4. Lignin peroxidase activity assay

Lignin peroxidase (LiP) was determined by monitoring the increase in absorbance (A_{310}) due to the oxidation of Veratryl alcohol to veratraldehyde at 37°C (Tien and Kirk 1988). The reaction mixture (2.5 ml) contained 500 µl enzyme extract (previously prepared as mentioned above), 500 µl H_2O_2, 500 µl Veratryl alcohol solution and 1.0 ml sodium tartarate buffer, pH 3.0 and LiP activity was calculated from the molar extinction coefficient (ε) of 9300 mM cm^{-1} (D'Souza Ticlo, 2008). LiP was expressed as enzyme units per liter (U L^{-1}), where one unit of enzyme activity is defined as the amount of enzyme oxidizing 1 µmol of substrate per minute. One fungal isolate was selected as the most potent isolate and sub-cultured to be identified.

2.5. Identification of the fungal isolate

The most potent fungal isolate was characterized and identified at Regional Center of Mycology and Biotechnology (Al-Azhar university, Cairo, Egypt) for further identification. The identification method was based on the fungal identification as described by Domsch *et al.* (1993) and Samson *et al.* (2000).

2.6. Factors affecting LiP productivity

The effects of pH, agitation, incubation time and temperature on the production of the crude enzyme were studied. In each experiment the optimum conditions deduced from the previous experiments were considered.

2.7. Protein estimation

Protein concentration was determined according to Bradford (1976) method. To 30 µl of sample, 900 µl of Bradford's reagent (Bio-Rad, CA, USA) were added and incubated at room temperature for 30 min. The absorbance was recorded spectrophotometrically at 595 nm. Bovine serum albumin (Sigma) was used for standard curve plotting.

2.8. Ammonium sulphate fractionation

Ammonium sulphate was added slowly to the culture filtrate with stirring at 4°C to 80% saturation. The mixture was stored overnight at 4°C, to allow the protein to precipitate. The solution was then centrifuged at 17000 x*g* at 4°C for 30 min. The precipitate was dissolved in distilled water to a final volume 10 ml (D'Souza Ticlo, 2008).

2.9. Dialysis and concentration of the crude LiP enzyme

The culture filtrate was placed in dialysis bags, cut off 12 kDa (Sigma), and dialyzed against 0.2M citrate-phosphate buffer, pH 6 for three successive times until no longer of ammonium sulphate in the buffer, then concentrated by osmosis using sucrose (D'Souza Ticlo, 2008).

2.10. LiP enzyme purification

The dialyzed protein preparation was applied onto a column (Pharmacia column, 2.5 x 50 cm) packed with Sephadex G-100. This was equilibrated in 0.2M citrate-phosphate buffer pH 6, then eluted with the same buffer. The fractions O.D. was monitored at A_{310} and LiP activity was measured. LiP purity was confirmed by SDS-PAGE. Preparation of the column and the fractionation procedure was carried out as mentioned by Scopes (1994).

2.11. Determination of LiP molecular mass

LiP molecular mass was estimated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Weber and Osborn (1969).

3. Results

3.1. Microorganisms

Six fungal isolates were used in teh preliminary experiment to screen their efficiency for the production of the lignin-degrading enzymes. Only one isolate was selected for further identification due to its highest enzyme productivity. The isolate was identified as *Cunninghamella elegans* as shown in Fig (1).

3.2. Production and activity of LiP enzyme

The production of ligninolytic enzyme by *Cunninghamella elegans* was investigated in rice husk containing medium i.e. Production medium. Optimization of medium was regularly studied for further characterization purposes.

3.2.1. Optimization of cultivation medium for LiP production

Results shown in Fig.2 revealed that LiP activity reached the maximum productivity on day 14 of growth (10.800 U L^{-1}) at 25°C. Below and above this particular incubation period, a remarkable drop in the enzyme yield was noticed. LiP productivity at different pH values was determined. LiP showed the highest activity peak at pH 6 (14.200 U L^{-1}). However, the activity decreased gradually in the alkaline side. The maximum LiP activity found at 30°C (15.300 U L^{-1}). The noticeable difference between static and shaking conditions (12.3 x*g*) on production of LiP was found (Fig.2). Since, static state was more effective (15.800 U L^{-1}) than shaking one (8.400 U L^{-1}).

3.2.2. Concentration of LiP by ammonium sulphate fractionation

The highest activity of LiP protein preparation was obtained by precipitation at 80% ammonium sulphate (Table, 1).

3.2.3. Purification of LiP enzyme by column chromatography

Results revealed that only one peak was found. This step resulted in increasing purification folds of LiP enzyme up to 2.76. A summary of purification steps is represented in Table 2.

3.2.4. Molecular mass determination of LiP

As shown in Fig. (3), the purified LiP was analyzed by SDS-PAGE. A single protein band was

observed and the molecular mass of LiP was estimated at 50 kDa.

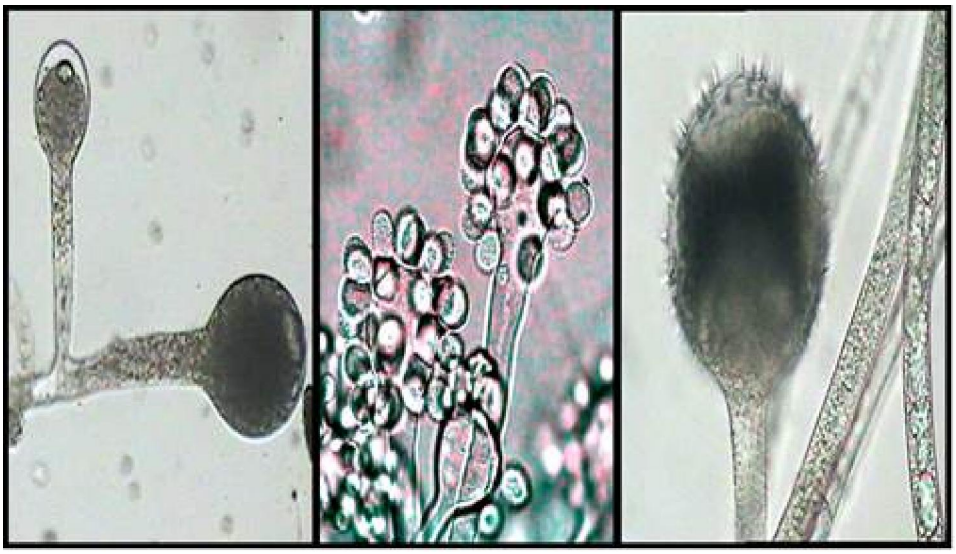

Figure 1. Lignin peroxidase producing *Cunninghamella elegans*.

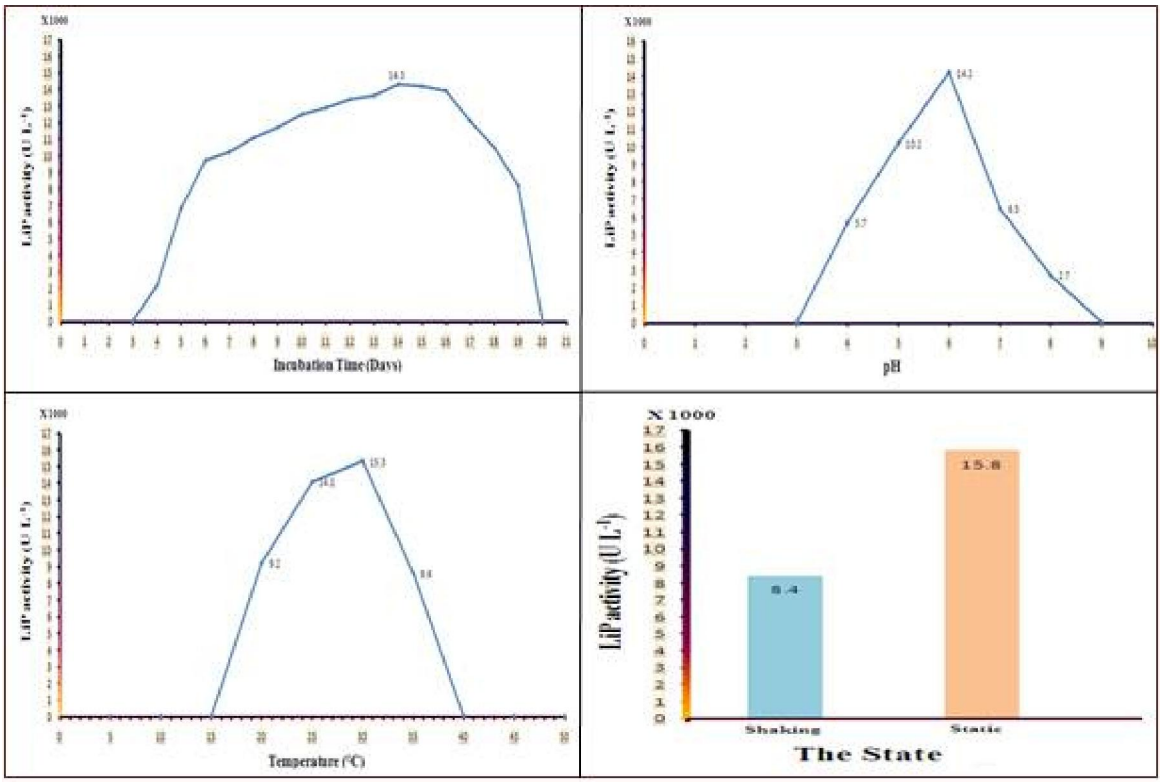

Figure 2. LiP production by *Cunninghamella elegans* at different incubation times, pH values, temperatures as well as at static and shaking states.

Table 1. Ammonium sulphate fractionation

$(NH_4)_2SO_4$ Conc. (%)	Protein Content (mg/L)	LiP activity (Unit/L)	Sp. Activity Units/mg protein
0.0	350	15900	45.43
20	610	32400	53.11
40	680	58100	85.44
60	720	70600	98.06
80	840	89300	106.31
100	800	76700	95.88

Table 2. A summary of purification steps of LiP produced by *Cunninghamella elegans*

Step No.	Purification Step	Volume (L)	Enzyme activity	Protein Content	Specific activity	Purification Folds	Yield (%)
1	Cell free filtrate	0.5	16000	370	43.24	1.0	100
2	Amm. Sulph. 80%	0.1	89500	853	104.92	2.43	55.9
3	Concentration by	0.01	112200	955	117.49	2.72	70.1
4	Sephadex G-100	0.15	115400	967	119.34	2.76	72.1

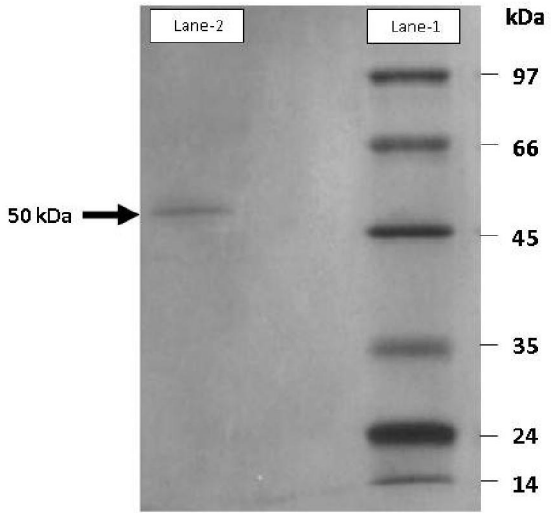

Figure 3. SDS-PAGE pattern of purified LiP produced by *Cunninghamella elegans* showing molecular mass standards (Lane-1) and molecular mass of LiP from Sephadex G-100 (Lane-2)

4. Discussions

To reduce environmental damage from weathering and coal burning, biotechnological processes are needed to convert hard coal or lignin to clean, cost-effective energy sources or other useful materials. A microbial, enzymatic or enzyme-mimetic technology that can take place at moderate temperatures and pressures (Fakoussa, 1992) would have great advantages compared to the current physical and chemical coal conversion technologies. Biocatalytic particles are also smaller than conventional catalytic particles and thus more efficient. In this study, the potential of producing LiP has been investigated. *Cunninghamella elegans* was found to be the most potent isolate between six fungal isolates for producing LiP. The maximum LiP productivity was under static condition at pH 6, 30°C and after 14 days of incubation period. LiP

Purification was isolated firstly by the precipitation with ammonium sulphate. Its enzyme activity was 89500 U L^{-1}. Then, LiP was purified by gel permission chromatography and its activity increased to 112200 U L^{-1}. The molecular mass of LiP was estimated, by SDS-PAGE, at 50 kDa. During the past decade, fungi were studied in relation to their ability to degrade recalcitrant organo-wastes (Field *et al*., 1993; Vyas *et al*., 1994; Demir, 2004). These studies were done in parallel with those on a complex enzymic system that allows fungi to degrade lignin (Barr and Aust, 1994; Costa *et al*., 1994). Peroxidases are essential enzymes in biodegradation of lignin and lignite which have been investigated intensively in fungi (Yang *et al*., 2005). In our study, it was observed that LiP activity appeared to reach the maximum in the culture fluid, when *Cunninghamella elegans* was grown in specially designed rice husk broth. Growth and enzyme production in static flasks were superior when compared to that found in shaked cultures, therefore, static conditions were chosen for routine cultivation. LiP activity in the culture fluid was detectable after 14 days. Kareem and Akpan (2004) reported that the use of agricultural by-products as a substrate for the enzyme production was cheap and could facilitate large scale production of industrial enzymes. LiP activity was determined by monitoring the oxidation of veratryl alcohol to veratraldehyde at 37°C as indicated by an increase in A_{310}. Chia (2008) stated that the discovery of LiP, veratryl alcohol has played a pivotal role in the study of the lignin biodegradation process. Veratryl alcohol is used as an assay for enzyme activity due to the easily detectable absorbance of the produced veratraldehyde. LiP is capable of oxidizing non-phenolic compounds with a relatively high redox potential of the oxidized enzyme intermediates (Schoemaker and Piontek, 1996). The mechanism of action of LiP was discovered from studies of methoxylated benzenes, which were oxidized by LiP to unstable molecules called cation radicals. The cation radical 1,4-dimethoxibenzene, decomposes in the reaction with H_2O, producing methanol and benzenequinone (Chia 2008). Fakoussa and Hofrichter (1999) reported that the pH range for LiP was between 2.0 and 5.0 with an optimum somewhere between 2.5 and 3.0. In our study, the *Cunninghamella elegans* LiP is different from other fungal LiP, since the optimum pH of *Cunninghamella elegans* LiP was 6.0. However, the *Cunninghamella elegans* LiP had a wider range of pH. At pH 8.0, the LiP activity was still retained. The optimum temperature was between 25 and 30°C. It was highest at 30°C, and rapid inactivation occurred above 35°C. These results on extracellular LiP agree fairly well with those reported by other researchers (Zanirun *et al*., 2009 and Yadav *et al*., 2010). The subunit molecular mass range of white-rot fungal LiP was 38– 47 kDa and that of peroxidases was 38–50 kDa (Fakoussa & Hofrichter 1999). *Cunninghamella elegans* LiP had a subunit molecular mass of 50 kDa, that was within this range. Peroxidase activity was detected in PAGE by selective staining. The optical absorption spectra of enzyme showed that *Cunninghamella elegans* peroxidase had a distinct band. Yadav *et al*., (2010) isolated LiP from a fungal culture filtrate with a molecular mass of 48 kDa estimated by SDS-PAGE. Further studies are in progress for the practical application of *Cunninghamella elegans* LiP. Meanwhile, future study using this isolate would concentrate upon finding the breadth of pollutants that can be further exploited to develop a cost effective bioremediation process.

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