High production of laccase by *Ganoderma lucidum* 447 in submerged cultivation on ethanol production residue supplemented with Cu^{2+}

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The white rot fungus (WRF) *Ganoderma lucidum* 447 was shown to produce laccase in submerged cultivation on two media based on glucose and ethanol production starch free residue (EPSFR) coming from wheat bran. A very high yield of laccase production of 149,600 U l^{-1}, corresponding to about 1.81 g enzyme protein l^{-1}, was obtained with the EPSFR medium supplemented with 3mM of Cu^{2+} ions. It is suggested that an EPSFR based medium in the presence of copper as an inducer, may be relevant for economic production of laccase at an industrial level.

Key words – *Ganoderma lucidum* – laccase – microelements – submerged cultivation – ethanol production residue

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Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a component of the ligninolytic system of white-rot fungi (Munoz et al. 1997), and may find applications in biosensors, pulping, textile dyes, detoxification of polluted water and other biotechnological procedures (Brenna & Bianchi 1994, Reid & Paice 1994, Ghindilis et al. 1995, Martirani et al. 1996). *Ganoderma lucidum* is a white-rot fungus (WRF) and D'Souza et al. (1999) reported that a strain of *G. lucidum* produced two isoforms of laccase excreted from the mycelium. A fruiting body associated laccase was also characterized by Wang & Ng (2006).

Laccase is generally produced at low concentrations by fungi grown on basal media (Vasconcelos et al. 2000), but higher concentrations are obtained by addition of various supplements, in particular metal ions (Lee et al. 1999).

Cu^{2+} and Mn^{2+} ions are important activity and/or biosynthesis modulators of ligninolytic enzymes, in particular laccases, and are naturally present in the environment (Wariishi et al. 1988, Baldrian & Gabriel 2002). Cu^{2+} regulates the transcription of laccase (Baldrian & Gabriel 2002). The promoter regions of the genes encoding for laccase contain various recognition sites specific for xenobiotics and heavy metals (Sannia et al. 2001). The addition of low concentrations of copper to the cultivation media of laccase producing fungi generally stimulated enzyme production (Assavanig et al. 1992). Palmieri et al (2000) found, for example, that the addition of 150 μM copper sulfate to the cultivation media of some strains can result in a fifty-fold increase in laccase activity as compared to a basal medium. White-rot fungi are diverse in their responses to inducers tested for laccase. The addition of certain inducers can increase the concentration of a specific

laccase or induce the production of new isoforms of the enzyme (Robene-Soustrade & Lung-Escarmant 1997). Some inducers interact variably with different fungal strains (Eggert et al. 1996). Therefore, laccase production has been found to be highly dependent on the conditions of cultivation of the fungal strains (Heinzkill et al. 1998).

The purpose of the present investigation was to study the possibility to use ethanol production starch free residue (EPSFR), from wheat bran, to produce laccase with *G. lucidum* 447. This strain was selected from a previous study as a good producer of laccase (Songulashvili et al. 2007).

Ethanol can be made from different feedstocks, such as corn, barley and wheat (Lal 2008). Wheat bran, produced worldwide in large quantities as a by-product of the wheat milling industry, constitutes an important but underestimated source of sugars. Saccharification of starch and non-starch carbohydrates of wheat bran can be made by chemical or/and enzymatic treatment (Palmarola-Adrados et al. 2005), to generate sugars for ethanol production, mainly by subsequent yeast fermentation. Ethanol production residues (EPR) obtained after the implementation of this type of process could be used as an agricultural amendment or fuel, but are generally not considered comercially valuable. Therefore, the use of EPR for the production of high value added substances as an enzyme could be an interesting alternative to the use of this waste.

Materials and Methods

Organism and pre cultivation conditions

Ganoderma lucidum (W.Curt:Fr.) 447. isolated from Mount Carmel, Israel was stored on wort agar medium, in the culture collection of Institute of Evolution, University of Haifa, Israel (HAI) (Wasser et al. 2002). The fungus was grown on a rotary shaker at 140 rpm and 26±1°C in 250 mL conical flasks containing 100 mL of the following standard medium (g l^{-1}): glucose 10; peptone 2; KH_2PO_4 0.8; Na_2HPO_4 0.2; $MgSO_4$ $7H_2O$ 0.25 and yeast extract 4 (pH 6.0). After nine days of cultivation, mycelial pellets were harvested and homogenized three times for 20s, at 1 min intervals, with a Waring Commercial® laboratory blender operating at low speed. Mycelial homogenates (10 mL) were used to inoculate the flasks containing the different media mentioned below.

Cultivation conditions and EPSFR substrate

The medium described above was chosen for the cultivation of the strain on glucose, while the same medium, with EPSFR concentration of 50 g l^{-1} instead of glucose, was used as the lignocellulosic growth substrate. EPSFR came from a Vodka production factory located in the Republic of Georgia. An exo-α-amylase industrial preparation (TERMAMYL®) was used to process wheat bran to sugar solution used for yeast fermentation. Before enzyme processing, wheat bran was composed mainly of nonstarch polysaccharides (~ 58%), lignin (~ 3%), starch (~ 19%) and crude protein (~ 18%). Non-starch polysaccharides were for the most part composed of ~ 70% α-arabinoxylans, ~ 24% cellulose and ~ 6% β-(1,3)(1,4)-glucan. About 90% of starch was saccharified after the TERMAMYL® treatment. The treated wheat bran was used as so for yeast fermentation. After yeast fermentation and distillation to remove ethanol, the medium was filtered. The filtration residue constituted the EPSFR and contained mainly cellulose, hemicelluloses, lignin, protein and yeast insoluble residues. EPSFR served as a composite substrate for *G. lucidum*.

Concentrations of 1 and 3mM of Cu^{2+} or Mn^{2+} ions were added in the media for the evaluation of effect of microelements on laccase production. The sources of microelements were $CuSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot H_2O$.

After 3, 5, 7, 9, 11, and 14 days of cultivation, samples (1 mL) were taken from flasks and the solids were separated by centrifugation (13000 × g; 5 min) at 4°C. The supernatants obtained after the biomass separation were analyzed for enzyme activity.

Enzyme assay

Laccase activity was determined by monitoring the A_{420} change related to the rate of oxidation of 1 μmol 2,2′-azino-bis-(3-ethyltiazoline-6-sulfonate) (ABTS) in 25 mM Na-acetate buffer (pH 3.8). One unit of laccase activity was defined as the amount of enzyme, which leads to the oxidation of 1 μmol of substrate per minute. The specific activity of lac-

case, obtained from the glucose and EPSFR media, were calculated by total protein. Conversion of laccase activity in unit gram per pure enzyme protein was done according to the data of Songulashvili et al. (2008).

Biomass protein estimation

After 14 days of fungi cultivation, the mycelia with the fermented substrates were separated by centrifugation (5800 × g; 15 min) at 4°C. The centrifugation pellet was dried at 60°C until constant weight, and total nitrogen was determined according to the Kjeldahl procedure with Nessler reagent after pre-boiling samples in 0.5% solutions of trichloroacetic acid for 15 min to remove the non-protein content. Protein content was calculated as the total nitrogen multiplied by 4.38 (Songulashvili et al. 2007). To receive data on protein gain, protein contents in inoculated substrates were subtracted from the values obtained in final biomasses.

All experiments were performed at least twice using three replicates. The data presented in the table correspond to mean values.

Results

Production of laccase by *G. lucidum* 447 growing on glucose medium

G. lucidum 447 cultivated in the 1% glucose medium or with the lignocellulosic EPSFR grew equally well, in the form of small pellets, at all the investigated Cu^{2+} and Mn^{2+} ion concentrations. Supplementation with Mn^{2+} ions had no effect on laccase production and hence these results are not shown.

In the glucose medium, the highest level of accumulation of laccase by *G. lucidum* 447 was obtained with supplementation of 3mM Cu^{2+} at day 0 of cultivation attaining an activity approximately four hundred times higher than in the control, 19,040 (after 14 days of cultivation) and 50 U l^{-1}, respectively (Table 1). Much lower accumulation of laccase by *G. lucidum* 447 was shown on this medium when 1mM Cu^{2+} ions was added at day 0 or after day 3 of cultivation, accounting for 1,115 and 1,088 U $l^{-1,}$ respectively (Table 1). Moreover, the activity of laccase increased only 3 fold (168 U l^{-1}), comparatively to control medium, when 3mM Cu^{2+} ions was added at day 3 of cultivation. Highest laccase specific activity (341,218U g^{-1} protein) on glucose medium was detected after addition of 3mM Cu^{2+} ions at the beginning of cultivation (Table 1). When Cu^{2+} was added at day 5 or 7 of cultivation the laccase levels attained were comparatively close to those obtained with a control medium. The data presented in Table 1 clearly show the advantage of Cu^{2+} ions as a stimulator of laccase production by *G. lucidum* 447.

Production of laccase by *G. lucidum* 447 growing on EPSFR medium

Mn^{2+} had no stimulating effect on laccase production in the EPSFR medium and hence these results are not shown. The highest level of laccase activity on this medium (149,600 U l^{-1}), corresponding to about 1807 mg enzyme protein l^{-1}, was obtained after 10 days cultivation when supplementation with 3mM Cu^{2+} ions was at day 0. Laccase activity was two times higher than in the control EPSFR medium (73,440 U l^{-1}). Lower laccase activity (from 100,160 to 122,400 U l^{-1}), however, comparatively 37–66% higher than in control EPR medium, was obtained in all the other cases of addition of Cu^{2+} ions at different stages of cultivation (Table 1). The highest specific activity of laccase (554,074 U g^{-1} total proteins) was shown when 3mM Cu^{2+} ions were supplemented at day 0 of cultivation in the EPSFR medium. Details on the kinetics of laccase accumulation are given in Fig. 1. The peak of the laccase activities obtained with *G. lucidum* 447 was shown at day 10 as in control EPSFR medium and the same medium supplemented with different concentration of Cu^{2+} ions (Fig. 1).

Discussion

Fungal laccases have received much attention in recent years due to their ability to oxidize a plethora of phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants (Rodriguez & Toca 2006). A point frequently raised regarding the use of enzymes in biotechnological processes is, however, their cost of production (Piscitelli et al. 2010). Laccases obtained from native fungal sources have been often identified as inappropriate for large-scale use, mainly due to low production yields and excessive costs of downstream processing if

Table 1 Effect of microelements on laccase production by *Ganoderma lucidum* 447 in submerged fermentation of glucose and EPSFR media.

Compound added	Day of addition	Glucose medium			EPSFR medium		
		Protein	Laccase		Protein	Laccase	
		g l^{-1}	U l^{-1}	U g^{-1}	g l^{-1}	U l^{-1}	U g^{-1}
Control†		0.0015	$50^{(5)*}$±2	33,333	0.24	$73,440^{(10)}$±340	306,000
1mM of Cu^{2+}	0	0.0213	$1,115^{(14)}$±17	52,347	0.26	$118,320^{(10)}$±680	455,077
3mM of Cu^{2+}	0	0.0558	$19,040^{(14)}$±29	341,218	0.27	$149,600^{(10)}$±540	554,074
1mM of Cu^{2+}	3	0.0325	$1,088^{(10)}$±11	33,477	0.26	$100,160^{(10)}$ ±420	385,230
3mM of Cu^{2+}	3	0.0031	$168^{(10)}$±7	54,193	0.26	$122,400^{(10)}$ ±390	470,769
1mM of Cu^{2+}	5	0.0017	$82^{(5)}$±4	48,235	0.26	$100,640^{(10)}$ ±510	387,076
3mM of Cu^{2+}	5	0.0017	$51^{(3)}$±3	30,089	0.27	$103,360^{(10)}$ ±490	382,814
1mM of Cu^{2+}	7	0.0020	$57^{(3)}$±2	28,687	0.25	$100,640^{(10)}$ ±530	402,560
3mM of Cu^{2+}	7	0.0023	$82^{(3)}$±5	35,652	0.24	$106,080^{(10)}$ ±410	442,000

†No microelement was added to the basal medium with glucose and EPSFR.
*The numbers in parentheses indicate the day of peak activity.

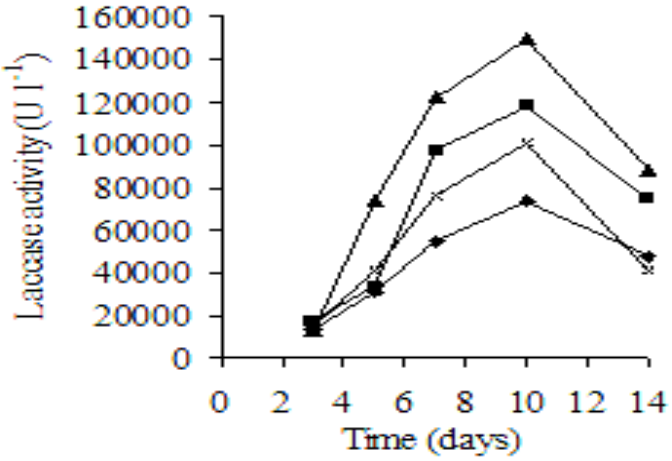

Fig. 1 – Profile of activity of laccase by *Ganoderma lucidum* 447 in submerged fermentation of EPSFR medium: (♦) control, (■) Control+1mM Cu^{2+}, (▲) Control+3mM Cu^{2+}, (×) Control+1mM Cu^{2+} after day 5.

applicable. Use of recombinant technology has been advocated as a choice approach for solving these problems (Piscitelli et al. 2010). Heterologous expression of laccases has been reported in bacteria, yeasts, filamentous fungi and plants. Yields of heterologous laccases are in some cases in the range of hundreds of milligram enzyme protein per liter, figures largely above as was obtained with most of the native strains. Disadvantages to use recombinant technology for producing laccases have, however, been pinpointed (Jönsson et al. 1997) and this is why we should not ignore the resources of the fungal natural heritage. On several occasions it has been shown that screening of collections of fungi from the environment has highlighted strains particularly effective for various enzymatic and other high value added productions (Jaouani et al. 2003, Arboleda et al. 2008). It was shown here that the use of a native strain growing on a cheap medium based on a waste, with the addition of Cu^{2+} as a microelement, may successfully compete with the use of recombinant strains.

Many studies have shown the regulatory effect of microelements (Kerem & Hadar 1993, Camarero et al. 1996, Baldrian & Gabriel 2002, Elisashvili et al. 2006). For example, Palmier et al. (2001) demonstrated that increased activity of laccase was proportional to the concentration of added Cu^{2+} ions for *Pleurotus ostreatus* grown under submerged fermentation. Maximum inducing effect was obtained with a $CuSO_4$ concentration around 0.15 mM.

Similar results were reported by Assavanig et al. (1992) for a *Trichoderma* strain.

In the present study it was shown that, the supplementation of accurate concentration of Cu^{2+} ions at appropriate time had a crucial effect on laccase accumulation by *G. lucidum* 447 growing on glucose or EPRSF (Table 1). The effect of Cu^{2+} appeared as specific because supplementation with Mn^{2+} ions had no effect on laccase production. Food industry wastes have been recognized as potential substrates for producing high value added compounds (Songulashvili et al. 2006) Cereal corps including wheat, rye, barley and corn are used all over the world to produce alcohol. Yet, processing of this type of material generates large quantities of waste from which no clear opportunities of utilization are currently available. Direct burning of the waste to obtain energy, agricultural use, or in animal nutrition is conceivable. However, before coming directly to these uses, an intermediate processing of these residues to obtain products with high added value by fungal fermentation, could be a fruitful economic step. On the other hand, the increase in fungal protein content of the final residue if proven safe should be an interesting asset for valorization of the waste as a single cell protein for cattle food (Gabriel et al. 1981).

It was concluded that a yield of around 150,000 laccase $U.l^{-1}$, corresponding to 1,807 mg enzyme l^{-1} culture can be obtained with standard submerged cultivation of *G. lucidum* on an EPR. This value compares with, and often surpasses yields obtained with engineered strains (Piscitelli et al. 2010). EPR could be also utilized for fungal production of laccases with different specificities using different *Ganoderma* strains.

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