

Solid-state production of laccase by *Pleurotus* sp. applied in glyphosate degradation

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Abstract— N-phosphonomethyl glycine (glyphosate) is the derivative of the amino acid glycine. Glyphosate is a broad-spectrum herbicide. Glyphosate is translocated via phloem system and moves from the roots to the growing parts of the plants. Many researches proved that lignin-modifying enzymes LME including laccases have ability to degrade glyphosate. *Pleurotus* sp. was cultured during 12 days in the media with coir dust, sesame residue, urea, saccharose, K_2HPO_4 and minerals. After 12 days, the media contain laccase with the activity of 0.227U/g. Moreover, *Pleurotus* sp. also grew in the media which contain glyphosate and made a decrease in this content from 50mg/kg to 10.5mg/kg after 12 days.

Index Terms— glyphosate degradation, *Pleurotus*, laccase.

1 INTRODUCTION

With the advance in technology to clear grass or control pest and plant diseases, we are using the chemicals which are the one of many reasons for the agricultural pollution and soil contamination. Laccases with its advantages have being used in many researches to degrade xenobiotic which are difficult to naturally decompose. Laccases were studied in decomposition of herbicides or pesticides such as atrazine, DDT, phenyl urea, 2,4,6-trichlorophenyl p-nitrophenyl, p-benzoquinone, ... [1,3], halogenated pesticide, 2,4-D, dioxin [2], ... and glyphosate [7].

Manuscript Received on March 15th, 2017, Manuscript Revised on November 01st, 2017.

The authors are grateful to the Department of Science and Technology of Ho Chi Minh City for financial support.

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Glyphosate is the common herbicide used for controlling weeds in the riverside, unused fields, gardens. Glyphosate acts as an inhibitor of 5-enolpyruvylshikimate-3-phosphatesynthase (EPSPs, E.C.2.5.1.19) enzymes, which are present in the chloroplast of most plant species. EPSPs are important in shikimic acid pathway to synthesize aromatic amino acids necessary for protein formation [6]. Glyphosate is also the toxin for fish, some species of earthworms, water invertebrates and many kinds of land animals [4]. In human, glyphosate affects gut bacteria through the shikimic acid pathway, is a toxic on nervous system [10], livers, kidneys [9] and is a carcinogenicity [4].

In nature, lignin-modifying enzymes LME from some species of fungi including Basidiomycetes have ability to degrade glyphosate. LMEs include 3 main enzymes, lignin peroxidase, manganese peroxidase and laccase. Maria del Pilar Castillo and John Strenstro (2009) proved that laccases were able to degrade 90% of glyphosate after 24h in the studies that are in vitro [7].

Pleurotus sp., is belong to Basidiomycetes division, has ability to produce extracellular enzymes to degrade glyphosate [7]. They are able to grow their mycelia in the liquid or solid media and synthesize laccases. Besides, straw (argowastes) was used to culture some species belong to Basidiomycetes for laccase production [3]. In fact, in order to apply glyphosate degradation of laccase from *Pleurotus* sp. to the areas with glyphosate soil contamination, their growth and synthesis of laccase should be surveyed in solid-state fermentation.

2 MATERIALS AND METHODS

2.1 Fungal strain – Chemicals

White rot fungi *Pleurotus* sp. were provided from Department of Biochemistry, Faculty of Biology and Biotechnology, University of Science, VNU-HCM. The chemicals used for

laccase assay were purchased from Sigma and Merck. Glyphosate 480SL is the product was imported by An Giang Plant Protection Joint Stock Company.

2.2 Media and Laccase production

The fungus was maintained on potato dextrose agar (PDA) with 2% yeast extract. After 7 days incubated at room temperature, the fungus was transferred to the 4 different solid media with unchanged coirdust substrate, changed more nutrient composition and supplemented minerals (Table 1). These experiments were carried out in a Φ 25mm test tube that contains 12.5g solid media or a flask 500ml that contains 70g solid media.

TABLE 1
COMPOSITION OF THE SOLID CULTURE MEDIA

Media	Coir dust (%)	Sesame Oil residue (%)	Urea (%)	Saccharose (%)	K ₂ HPO ₄ (%)	Minerals ^a
Control medium	94.43	4.72	0.19	0.47	0.19	0
Modified medium 1	91.78	7.00	0.26	0.70	0.26	0
Modified medium 2	91.78	7.00	0.26	0.70	0.26	x
Coir dust	100	0	0	0	0	x

^aComposition of the minerals per kilogram solid medium: CuSO₄ (0.125g), MgSO₄ (0.205g), NH₄Cl (2g), KH₂PO₄ (3g), NaCl (5g), Na₂HPO₄ (15.13g).

2.3 Growth of the fungal mycelia in the different solid culture media

In the Φ 25mm tubes, the growth of *Pleurotus* sp. mycelia was followed up by measuring the spread of mycelia in centimeter unit. The data were shown in this report are the ratio of the length of the observed mycelia and the height of the media in the tube.

2.4 Extraction of crude laccase from *Pleurotus* sp.

After 4,8,12, 16 days for growth of the fungus, acetate buffer 0.1M pH 4.5 was added to the solid media. This mixture was shaken 150rpm/30 min, filtered through filter cotton, re-filtered through filter paper, this filtrate was used as the crude enzyme.

2.5 Degradation of glyphosate by laccase from *Pleurotus* sp.

Glyphosate 480SL was mixed with 100g suitable solid medium (which chosen from Table 1 with high level of synthesis of laccase) to reach

the glyphosate concentration of 50ppm (50mg/kg). *Pleurotus* sp. was cultured in this medium to produce laccase and then degrade glyphosate, after 12 days, 500ml distilled water was used to obtain glyphosate sample. The filtrate was used to determine the glyphosate content before and after degraded by *Pleurotus* sp.

Quantitative of glyphosate in the media before and after 12 days for culturing *Pleurotus* sp. was carried out by GC/MS/MS by Center of Analytical Services and Experimentation HCMC followed Ref. Journal of Chromatography A, 886 (2000) 207 - 216

2.6 Laccase Assay

Laccase activity was determined by the oxidation of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) to the blue-green compound strongly absorbed at 405 nm. "One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 405nm of one per minute per milliliter of reaction mixture under the aforementioned condition [5]." The reaction mixture consisted of 900 μ l acetate buffer 0.1mM, pH 4.5, 50 μ l enzyme sample (diluted enzyme sample), 50 μ l ABTS. The reaction was started by adding ABTS and absorbance was read at 0 and 5 minutes [5].

2.7 Data analysis

In the experiments to find the best media for laccase production, the laccase activity values were shown are the result of 6 repeated times and the values from the experiments of the growth of fungal mycelia are the result of 3 repeated times. The data were analyzed by ANOVA for a single factor and t-test.

3 RESULTS AND DISCUSSION

3.1 Laccase production from *Pleurotus* sp. cultured in different solid media

Culture *Pleurotus* sp. in Φ 25mm tube size

Pleurotus sp. was cultured in the 4 different solid media. Each test tube contains 12.5 g medium. After 4, 8, 12 and 16 days of incubation, the crude enzyme samples were used for laccase assay. The results were shown in Table 2.

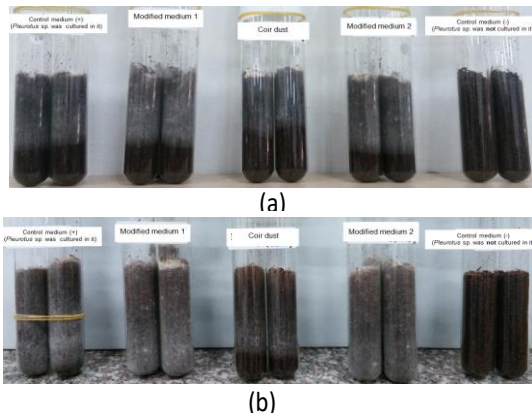


Fig. 1. Mycelial growth of *Pleurotus* sp. after 8 (a), and 12 (b) days of incubation

TABLE 2
LACCASE ACTIVITY (U/G) PRODUCED BY *PLEUROTUS* SP. AFTER 4, 8, 12 AND 16 DAYS OF INCUBATION IN 4 MEDIA IN THE $\Phi 25$ MM TUBE SIZE

Time of incubation	Control medium	Coir dust	Modified medium 1	Modified medium 2
4 days	0.012±0.004	0.017±0.006	0.017±0.008	0.014±0.006
8 days	0.036±0.009	0.064±0.009	0.083±0.022	0.080±0.016
12 days	0.053±0.008	0.080±0.010	0.115±0.015	0.162±0.023
16 days	0.041±0.010	0.099±0.002	0.077±0.010	0.067±0.005

Pleurotus sp. had an ability to produce laccase in all of media used in this survey. The laccase activity values were no remarkable differences on day 4 among the media, they varied from 0.012 to 0.017. The laccase activity increased with the time of incubation. From day 8, *Pleurotus* sp. made high activity of laccase in modified medium 1 and 2 than in control and coir dust media. The difference of activity values on day 8 in modified medium 1 and 2 did not reach statistical significance. However, because the growth of *Pleurotus* sp. gradually reached stability, laccase activity on day 12 in modified medium 2 was higher than modified medium 1 and this difference of the 2 values reached statistical significance. On the 16th day, the values of laccase activity decreased in all kinds of media lightly. That means the 12th day is the optimum time, at that time the media contained laccase with the highest activity.

Corresponding to laccase assay, the mycelial growth of *Pleurotus* sp. was investigated too. The results were shown in Table 3.

The results in Table 3 and Fig.1 showed that

modified medium 1 and 2 were suitable for the growth of *Pleurotus* sp., so the mycelia were fully-grown after 12 days of incubation.

If the mycelia grow well in modified media (especially modified medium 2), laccase activity will reach the highest value comparing with the left media. In accordance with the fact that laccase is the product of the secondary metabolism of different fungi, therefore, the cultivation is suitable for the fungal growth, the more fungus biomass there was and the higher laccase activities they was obtained.

TABLE 3
MYCELIAL GROWTH^B OF *PLEUROTUS* SP. AFTER 4, 8, 12 DAYS OF INCUBATION IN 4 MEDIA WITH $\Phi 25$ MM TUBE SIZE

Time of incubation	Control medium	Coir dust	Modified medium 1	Modified medium 2
4 days	0.274±0.056	0.207±0.016	0.255±0.056	0.278±0.050
8 days	0.648±0.023	0.567±0.015	0.731±0.014	0.717±0.015
12 days	0.978±0.031	0.821±0.004	0.967±0.058	1.000±0.000

^BThe data were shown are the ratio of the length of the observed mycelia and the height of the media in the tube

Modified medium 2 was used for scale-up production of laccase (500ml flask with 70g medium)

Culture Pleurotus sp. in a 500ml-flask size contained 70g medium

The values of mycelial growth in modified medium 1 and 2 were also no remarkable differences (Fig.2). However, laccase activity on day 12 in modified medium 2 was higher than in modified medium 1 (Table 4). Moreover, standard deviation values showed that the mycelial growth and laccase activities were stable after 12 days of incubation. Therefore, this is a stationary phase of the growth of this fungus. The results also proved that the important role of minerals (especially metal ions) to the production of laccase [11]. It found that the presence of Cu^{2+} resulted in stimulation of laccase production by this strain. Modified medium 2 with the presence of minerals made laccase activity of 21% higher than in modified medium 1.

TABLE 4
LACCASE ACTIVITY AFTER 8 DAYS OF INCUBATION (U/G)

Time of incubation	Modified medium 1	Modified medium 2
8 days	0.078 ±0.041	0.078 ±0.024
12 days	0.187 ±0.013	0.227 ±0.019

The composition of modified medium 2 was suitable for laccase production from *Pleurotus* sp., made an activity of 0.227U/g. Therefore, modified medium 2 was used for the survey glyphosate degradation of laccase from *Pleurotus* sp.

3.2 Laccase production from *Pleurotus* sp. in modified medium 2 contained 50mg/kg glyphosate

Laccase is able to degrade glyphosate [7]. Therefore, *Pleurotus* sp. has ability to degrade glyphosate only if it can grow and produce laccase in the media which contain glyphosate. As described above, the mycelial growth and the laccase activity were stable after 12 days of incubation. Thus, this time of incubation was selected to investigate the effect of glyphosate on the growth and laccase production from *Pleurotus* sp.

TABLE 5
LACCASE ACTIVITY AFTER 12 DAYS OF INCUBATION

Medium	Modified medium 2	Modified medium 2 + glyphosate
Laccase activity (U/g)	0.342 ±0.026	0.273 ±0.059

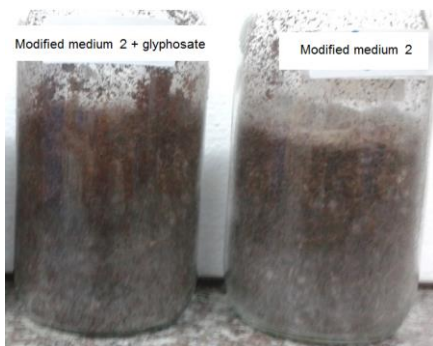


Fig. 2. Mycelial growth of *Pleurotus* sp in the 500ml flasks for 12 days

Laccase activity values from culturing *Pleurotus* sp. in modified medium 2 contained glyphosate were shown in Table 5.

Fig. 2 showed that the mycelial growth of this fungus in modified medium 2 with glyphosate was slower than modified medium 2 without glyphosate. Similarly, the laccase activity in the medium with glyphosate was 20% lower than in the medium without glyphosate. Thus, glyphosate made a decrease in the growth and laccase production of this fungus in modified medium 2.

3.3 Glyphosate degradation by laccase from *Pleurotus* sp.

Pleurotus sp. was cultured in the modified

medium 2 contained 50mg/kg glyphosate was able to produce laccase which degrades glyphosate. What is the percent decrease of glyphosate content after 12 days of incubation? The results were shown in Table 6.

TABLE 6
GLYPHOSATE CONTENT IN THE MODIFIED MEDIUM 2 AFTER 1 AND 12 DAYS OF INCUBATION

	Glyphosate content (mg/kg)	% decrease of glyphosate
M1	50.7	0%
M2	10.5	79.29%
M3	26.3	48.13%

In which:

M1: (Modified medium 2 + glyphosate), glyphosate extract was collected on day 1.

M2: (Modified medium 2 + glyphosate + *Pleurotus* sp.), glyphosate extract was collected on day 12.

M3: (Modified medium 2 + glyphosate), glyphosate extract was collected on day 12.

The results in Table 6 showed that *Pleurotus* sp. made an increase the degradation of glyphosate in culture medium after 12 days of incubation (M2) comparing with the sample without fungus (M3). The percent decrease of glyphosate in two samples were 79% and 48%, respectively.

4 CONCLUSION

Pleurotus sp. was able to grow on the media which contain coir dust, sesame oil residue, urea, sucrose, K_2HPO_4 and minerals. The addition of minerals acts the important role in the synthesis of laccase of *Pleurotus* sp. and its activity is 0.227U/g (the activity in one gram of solid medium) after 12 days of cultivation. Moreover, *Pleurotus* sp. also grew on the media which contain glyphosate of 50g/kg during 12 days, synthesized laccase of 0.273U/g and made the decrease in glyphosate content, from 50mg/kg to 10mg/kg.

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Khảo sát môi trường bán rắn nuôi cấy *Pleurotus* sp. sinh tổng hợp laccase ứng dụng phân hủy glyphosate

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Tóm tắt— N- phosphonomethyl glycine (glyphosate) là một dẫn xuất của glycine. Glyphosate là loại thuốc diệt cỏ phổ rộng, tác dụng lên hệ rễ thông qua việc di chuyển trong mạch libe và được vận chuyển một cách nhanh chóng trong thực vật. Nhiều nghiên cứu đã chứng minh hệ enzyme phân hủy lignin trong đó có laccase có khả năng phân hủy glyphosate. *Pleurotus* sp. được nuôi cấy 12 ngày trong môi trường chứa mật ứa, bã dầu mè, urê, đường mía, K₂HPO₄ và thành phần khoáng có khả năng sinh tổng hợp laccase có hoạt tính 0,227U/g môi trường. Sau khi bổ sung 50.7mg/kg glyphosate vào môi trường để nuôi cấy *Pleurotus* sp. trong 12 ngày thì hàm lượng glyphosate giảm còn 10.5mg/kg.

Từ khóa— *Pleurotus*, laccase, phân hủy glyphosate