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Screening of Fungi Producing Ligninolytic Enzymes

by Plate test Technique

Zecarias Ghebreslasie[1], Duangporn Premjet[1, 2] and Siripong Permjet[3]*

[1]Department of Agricultural Science, Faculty of Agriculture, Natural Resources, and Environment, Naresuan University, Muang, Phitsanulok, 65000 Thailand
[2]Center for Agricultural Biotechnology, Faculty of Agriculture, Natural Resources, and Environment, Naresuan University, Muang, Phitsanulok, 65000 Thailand
[3]Department of Biology, Faculty of Science, Naresuan University, Muang, Phitsanulok, 65000 Thailand
[*]Corresponding author. E-mail: siripongp@nu.ac.th

Abstract

A total of 61 fungi isolate were subjected for screening of ligninolytic enzyme by dye decolorization plate test. The results presented the diversity of 8 orders belonging to 17 families, 30 genera and 25 unidentified cultures. Seven families of Agaricales, three families of Polyporales, two families of Russulales and one family represent for each of the orders, Boletales, Cantharellales, Phallales, Tremellales and Xylariales. The plate test perceived seven categorical groups as: strains decolorizing a single dye implies for secreting only one of the three main enzymes ; a) Phenol red for LiP, b) Azure B for MnP c) RBBR for Lacc; strains decolorizing two of the organic colors relays for secreting only two enzymes as; d) Phenol red and Azure B, e) Phenol red and RBBR, f) Azure B and RBBR; and g) strains which decolorize three organic dyes produce three of the ligninolytic enzymes.

Keywords : *Phenol red, Azure B and RBBR, Decolorization, Fungi*

1. Introduction

The diversity of the kingdom fungi was the target of great academic and industrial interest, particularly Macromycetes, Ascomycetes and Basidiomycetes which are expansively studied in various aspects[13]. Screening of fungi for their potential ligninolytic applications demonstrated the resistance and metabolic effects of ligninolytic enzymatic activities in almost all corners of the world [1, 5, 6, 18] were 315 fungal strains in Tunisia [8], 156 strains in Mexico[13, 27], 63 strains in Indonesia[10], 200 strains in Brazil[22, 30], 220 strains in Australia[23] 296 strains in Thailand[29], and 57 strains in United kingdom[14] were investigated by dye decolorization assay [6,11,12,17]. In addition, dye assay helps to understand the physic-biochemical features and deepen the core knowledge of promising ligninolytic enzymes for industrial and biotechnological

applications to reduce effect of chemical process and dirty fuel applications by adopt green technology[4, 24]. Many efforts were done to apply, design and develop the application of the major ligninolytic enzymes; Lignin peroxidase (LiP), manganese dependent peroxidase (MnP) and Laccase, and found positive impact in degradation of lignin and environmental toxic, recalcitrant pollutants such as pesticides, polychlorinated biphenyls, organochlorines, DDT, and various synthetic dyes [2, 3,5,26,]. Therefore, the purpose of this experimentation was to screen indigenous Thailand fungi for selection the highest ligninolytic producer for degrading recalcitrant pollutants like polychlorinated biphenyls (PCBs).

2. Materials and methods

2.1 Chemicals

All the chemicals used were analytical and reagent graded. Synthetic dyes Remazol Brilliant Blue R (RBBR), Azure B. and Phenol red were purchased from Sigma and Fluka, L-Asparagine and NH_4NO_3 from Acros-organic Company. The organic dyes were overnight UV sterilized and all medium were autoclaved prior to use.

2.2 Fungal strains

Sixty one fungal strains were preserved in Biotechnology research unit Faculty of Science, Naresuan University. Pure mycelium were obtained after aseptically transferred to PDA and incubated at 25°c for extensive mycelia growth. Reference strains *Phanerochaete chrysosporium* (NBRC 31249*)* and *Trametes versicolor* (NBRC6422) were purchased from the culture collection division, Biological Resource Center (NBRC) National Institute of Technology and Evaluation, Chiba, Japan.

2.3 Plate assay

Plate assay was the most common technique used to perform screening of fungal isolates for detecting their ability to grow and decolorize the organic dyes. The screening media was prepared, in a liter of basal medium 1.0g of yeast extract and 25.0mg of thiamine. HCl and 1ml of trace element[31] and 24gl^{-1} of PDA, 20gl^{-1} of agar, 10$gl^{-1}$of glucose and NH_4NO_3 and L-asparagine with an amount of 2.4 mM and 24mM for low and high nitrogen media, respectively[19,31,]. The pH of the screening media was adjusted to 5.5 [15, 17, 26]. 0.8cm cork plug of active mycelium from a periphery of fresh culture of petri dish was taken and inoculated at 25°c in triplets in 9cm diameter plate, medium of high and low nitrogen supplemented with three separate organic dyes of Azure B (0.003%), Phenol red (0.003%) and Remazol Brilliant Blue R (0.006%) [16, 21]. Finally inoculants inspected until they totally colonized and decolorized otherwise for 15 days. The extent of clear zone growth zone diameters were quantitatively measured via scientific ruler and visualized in contrast with Un-inoculated plates as a negative control. The decolorization rate per day was expressed as average rate of clear zone to growth zone for 15 days.

3. Result and discussion

In this experimentation a diversity of eight orders belonging to 17 families, 30 genera and 25 unidentified cultures of mushrooms were investigated to dye decolorization plate test. 13 genera from Agaricales, 7 genera of Polyporales, 5 genera of Russulales and one specie for

each of the orders, Boletales, Cantharellales, Phallales, Tremellales and Xylariales were studied. Orders Agaricales and Polyporales, represent 24.59% and 18.03% of the fungal strains respectively. Primary screening of 61 fungal strains was carried out by adopting dye decolorization to confirm ligninolytic enzyme production. Decolorization and growth rate were recorded and analyzed in two nitrogen medium supplemented with synthetic dyes. Positive isolates started decolorization activities in 2nd to 5th day of inoculation and reached maximum diameter in 7th day in Phenol red and RBBR but 11th day in Azure B. Finally considering on their reaction to decolorization test, all the strains fall in to eight groups as follows: A) Isolates with negative decolorization test: 12 isolates from four orders and six unidentified strains showed slow and retarded growth and no sign of decolorization over 15 days in two mediums and the three organic dyes. Isolates *Lepiota pseucdohelvecla Boletus appendiculatus, Dictyophora indusiata*, *Oligoporus caesius*, *Trichaptum biforme, Lentinus similis* and some unidentified strains did not produce ligininolytic enzymes; manganese peroxidase, lignin peroxidase and laccase [7, 10,11]. B) Positive plate test: single dye decolorization: 22 out of 61, 36.06% of the isolates were able to degrade either phenol red, Azure B. or RBBR which infers production of a single ligninolytic enzyme [19, 28] (table1). Order Agaricales dominated the phenolic oxidation out of which five families and seven genera show color change when grown (metabolize) phenol red. Fast decolorization rate was noticed even with slow mycelia growth of the isolates.

Table 1. Fungal diversity with decolorization potential of single dye.

Order	Family	Scientific name	Phenol red		Azure B.		RBBR	
			H.N	L.N	H.N	L.N	H.N	L.N
Agaricales	Agaricaceae	*Leucocoprinus bresadolae**	2.3	0.2	-	-	-	-
	Clavariaceae	*Scytinopogon* sp.	0.8	0.7	-	-	-	-
	Cortinariaceae	*Inocybe lanuginosa*	0.6	-	-	-	-	-
	Hydnangiaceae	*Laccaria laccata* *	1.9	1.6	-	-	-	-
Cantharellales	Ganodermatacea	*Ganoderma lucidum*	1.1	0.9	-	-	-	-
		Lenzites sp.*	2.2	2.3	-	-	-	-
Russulaceae	Stereaceae	*Stereum complicatum*	1.5	-	-	-	-	-
Unidentified	_	PH13044B*	2.4	-	-	-	-	-
	_	PH13049(2)	0.4	0.4				
	_	PH13053	1.6	1.0	-	-	-	-
	_	KLCU*	2.1	0.7	-	-	-	-
Agaricales	Agaricaceae	*Leucocoprinus cepaestipes*	-	-	0.7	1.2	-	-
Russulales	Russulaceae	*Russular alboareolata*	-	-	0.5	0.6	-	-
Unidentified		PH13044*	-	-	1.4	1.0	-	-
		PH223	-	-	1.0	1.0	-	-
Agaricales	Lycoperdaceae	*Cyathus straiatus*	-	-	-	-	-	1.5
Agaricales	Amanitaceae	*Amanita cokeri* *	-	-	-	-	1.0	1.3
		Hygrocybe calyptraefoemis	-	-	-	-	0.8	-
Polyporales	Fomitopsidaceae	*Ganoderma australes**	-	-	-	-	1.1	1.3
Unidentified		K228	-	-	-	-	0.5	-

Numerical values is average ratio of clear zone to growth zone in diameter rounded to one decimal place, - stands to no visible clear zone, *= good performance of decolorization

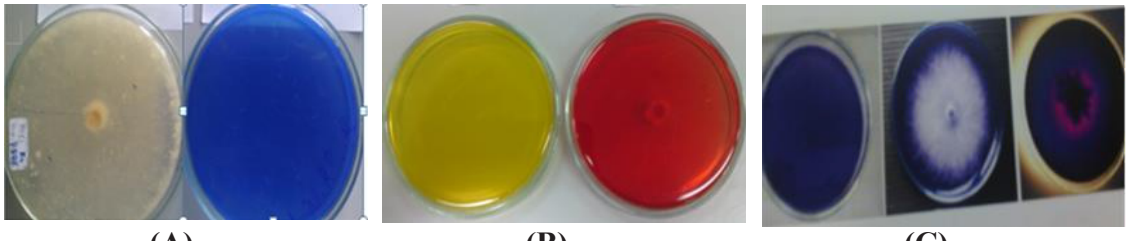
Leucocoprinus bresadolae, Scytinopogon sp., Scytinopogon sp, Ganoderma lucidum, Lenzites sp., Stereum complicatum start their growth at 2nd day of inoculation and decolorize phenol red fully (9cm) diameter from 3th - 7th day of their incubation time. RBBR, a light blue synthetic dye was decolorized by fungal mycelia more commonly due to common existence of Laccase in most of the isolates. Ganoderma austral (TM2) was one of the strongest degrader of RBBR with an average decolorization rate of 1.14cm diameter per day as compared to control strains *Phanerochaete chrysosporium* which is 0.54cm diameter per day. C) Positive plate test for two dyes: 19 isolates (31.14%) decolorize only two dyes, and fall in to three categorical properties. Isolates which decolorized both Azure B and RBBR (6 strains), both Phenol red and RBBR (11 strains) and both Phenol red and Azure B (2 strains) (Table2). *P. chrysosporium* was cited as strong degrader in many researches but *Psilocybe coprophila* (PH201) of Agaricales and *Grifola gigantea* (NKS10) of Ganodermataceae were among the leading two dye decolorizers. D) colonies which show a metabolic oxidation of the three synthetic dyes either totally decolorize or show a halo of color change as in RBBR from light blue to cloudy brown or no color (Fig 1.A),

Table 2. Fungal isolates with decolorization potential of two synthetic dyes.

Order	Family	Average clear zone/ growth zone for 15 days (cm)						
		Scientific Name	Phenol red		Azure B.		RBBR	
			H.N	L.N	H.N	L.N	H.N	L.N
Agaricales	Lycoperdaceae	*Calvatia craniformis*	-	-	1.0	1.0	1.0	-
Russulales	Stereaceae	*Stereum guaspartum*	-	-		0.4	0.8	0.4
Xylariales	Xylariaceae	*Daldinia concentrica*	-	-	0.3	0.8	1.0	1.1
Unidentified	-	PH13045C*	-	-	0.7	1.0	1.8	1.3
	-	PH101	-	-	_	0.3	-	0.6
	-	1001	-	-	1.0	1.1	0.3	0.8
Agaricales	Agaricaceae	*Entolomata sp*	0.6	0.9	-	-	0.9	1.0
	Lycoperdaceae	*Psilocybe coprophila**	2.1	1.8	-	-	1.6	-
	Tricholomataceae	*Mycena* sp.	0.7	0.6	-	-	0.6	0.6
Polyporales	Fomitopsidaceae	*Fomitopsis dochmius*	0.7	0.6	-	-	-	0.7
	Ganodermataceae	*Grifola gigantea**	0.7	0.9	-	-	1.1	1.0
Russulales	Russulaceae	*Russula fellea*	1.0	-	-	-	0.1	-
Cantharellales	Cantharellaceae	*Creterellus cornucopioides*	0.9	1.6	-	-	1.1	-
Unidentified	-	PH35	1.6	-	-	-	-	0.1
-	-	K812	1.3	0.7	-	-	-	1.3
-	-	PRK2	0.4	0.5	-	-	1.2	1.0
-	-	TV	0.5	0.3	-	-	0.8	0.7
Unidentified		PH19	1.8	-	-	0.6	-	-
		1118	0.9	0.8	0.9	0.9	-	-

Numerical values is average ratio of clear zone to growth zone in diameter rounded to one decimal place, - stands to no visible clear zone, *= good performance of decolorization

Phenol red from light yellow to red (Fig 1.B) and Azure B from dark blue to pink or clear it to white radiation (Fig1.C). Observation of color change was done by comparing with a control media supplemented with dyes without inoculant strain as indicated below. Decolorization of three synthetic dyes stand for production of three of the ligninolytic enzymes [11, 25]. In this decolorization test eight isolates and two control strains were found to show positive result and highest decolorization activity was achieved by order Agaricales of the family, Tricholomataceae, *Megacollybia platyphylla* (PRK16) and K127, PH176 of Unidentified strains. These isolates show decolorization activities in three organic dyes in both high and low nitrogen media in the specific culture duration, 15days (table3). The Average clear zone growth zone ratios was expressed and overall, result in decolorization of 3 organic dyes, and two media of low and high nitrogen indicates that Phenol red (93.3%), RBBR (78.3%) and Azure B. (56.7%) of the isolates decolorize them. The range of decolorization was from zero to 9cm in diameter were 22 isolates in Phenol red.



(A) **(B)** **(C)**

Fig.1 Plate test of Megacollybia platyphylla (PRK16) decolorizing three synthetic dyes (A) against control RBBR (B) against Phenol red (C) against Azure B.

Table 3. Potential isolates with Positive plate test for degrading three of the organic dyes

Order	Family	Scientific name	Phenol red		Azure B.		RBBR	
			H.N	L.N	H.N	L.N	H.N	L.N
Polyporales	*Phaerochaetaceae*	*Phaerochaete chrysosporium**	-	0.5	0.7	0.8	0.8	0.7
	Polyporaceae	*Trametes versicolor**	0.1	0.5	0.9	0.9	0.8	0.7
Agaricales	Tricholomataceae	*Megacollybia* sp.*	0.7	0.7	0.2	0.6	1.0	1.0
Polyporales	Ganodermataceae	*Ganoderma* sp.	0.7	0.5	-	0.2	1.2	1.2
		Polyporus sulphureus	0.3	0.4	-	0.2	1.4	1.2
Tremellales	Exidiaceae	*Pseudohydnum* sp.	0.9	-	0.5	0.7	-	0.6
	-	176*	0.5	0.7	0.4	0.6	0.5	0.8
Unidentified	-	PH176*	0.5	0.9	0.4	0.6	0.5	0.8
	-	K127**	0.3	0.5	0.9	0.9	0.9	0.8
	-	NO.51	-	0.6	-	0.2	0.9	0.1

Numerical values is average ratio of clear zone to growth zone in diameter rounded to one decimal place, - stands for no visible clear zone, *= good performance of decolorization

and 4 isolates in Azure B in high nitrogen seen to decolorize the disk fully (table 5). Comparing the two screening media high nitrogen is favorable to be metabolized as the colonies express nitrogen to metabolize in both Phenol red and RBBR but Azure B was decolorized more in low nitrogen by percentage of fungal population under study as it is replaced in instead of metabolized.The isolates which are able to decolorize phenol red, RBBR and Azure B are compared with each other and with the control strains, were effect of nitrogen was clearly noticed (figure2.A & B) and most of the strains found to grow favorably in high nitrogen. The clear zone growth zone ratio obtained in 15 days culture time and found that strains *Megacollybia platyphylla* (PRK16), *Pseudohydnum getatinosum* (PH106), No. 51 and PH 176 from unidentified species were best decolorizeres in both low and high nitrogen in three of the organic dyes and identified as future promising isolates for biodegradation of recalcitrant anthropogenic products like polychlorinated biphenyls (PCBs) and other organo-pollutants as they are expected to produce highly concentrated ligninolytic enzymes[29].

Table 5. Comparing decolorization of the three synthetic dyes by the fungal isolates.

Characteristic feature	Phenol red		RBBR		Azure B.	
	H.N.	L.N.	H.N.	L.N.	H.N.	L.N.
Number of positive strain (n)	31 (22)	25 (15)	23 (18)	24 (15)	15 (4)	19 (7)
Percentage of total (%)	51.7 (35)	41.7 (36)	38.3 (30)	40(25)	20.6 (6)	31.6 (11.7)

% was expressed by number of isolates decolorized each dye to total number of isolates, 61 and ( ) indicates number of isolates decolorizing full (9cm) in diameter.

Figure 2. Histogram comparing effect of nitrogen to degrade 3 dyes

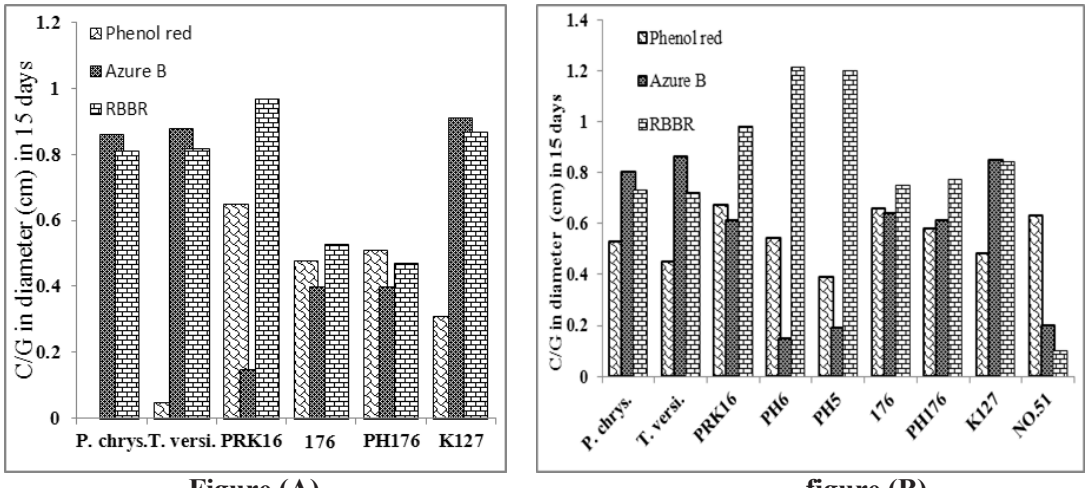


Figure (A) figure (B)

Figure 2. Decolorization rate of Potential isolates to degrade 3 dyes against control Phanerochaete chrysosporium in (A) high nitrogen; (B) Low nitrogen.

4. Conclusion

Quantitative screening using different dye indicators was found effective strong positive technique for large number of mushroom population to clear up about their extracellular ligninolytic activities. 49 isolates showed a positive decolorizing activity and their rate of decolorization was comparably favorable with those previously reported as the fastest dye-degrading *T. versicolor* and *P. chrysosporium*. Over half of the isolates (52.8%) showed a decolorization potential over *P. chrysosporium* in RBBR and 18.9% in Azure B. The intensity and patterns of decolorization was varied (scattered, diffused, deposited and radiated) even among same genus which indicates family, genus, and species diversity among isolates. Azure B was found to inhibit growth of strains unless combination of enzymes (coexistence) was found. Isolates *Megacollybia platyphyll, Laccaria laccata, Lenzites spe*., and KLCU, PH176, PRK 25, and 1001 from unidentified stains were novel degraders each group by performing strong activity than the control strains *P. chrysosporium* and *T. versicolor*.

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