LIGNINOLYTIC ENZYMES OF THE FUNGUS ISOLATED FROM SOIL CONTAMINATED WITH COW DUNG

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ABSTRACT

Lignin is the complex polymer and very few microorganisms are able to degrade it. The study aimed at isolating lignin degrading fungi from soil contaminated with cow dung using ligninolytic screening media. The study was able to isolate one ligninolytic fungus by using lignin media. The strain was screened for production of ligninolytic enzymes using Rhemazol Brilliant blue R (RBBR) dye, 2,2-azino-bis (3- ethylbenzthiazoline)-6-sulfonate (ABTS) and guaiacol in a semi-solid medium. The isolate decolourized RBBR dye, ABTS and guaiacol in a semi solid media. The strain was further tested for lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) enzymatic activities in liquid media. It was found that; fungal filtrate had maximum LiP, MnP and Lac activities of 1.44, 0.30 and 0.28 U/ml. Therefore, it was concluded that soil contaminated with cow dung manure, is a good source of isolating ligninolytic fungi which can be used for different biotechnology applications including bioremediation of polluted soils and bio fuels.

Key words: Cow dung, lignin peroxidase, Manganese peroxidase, Laccase

INTRODUCTION

Lignocellulose is the major structural component of woody and non woody plants such as grass and represents a major source of renewable organic matter and consists of lignin, hemicellulose as well as cellulose (Taherzadeh and Karimi 2008, Sasek *et al*. 1998). Lignocellulose materials are difficult to biodegrade thus it may take more than a year to fully decompose under natural condition, (Kumar *et al*. 2012). Presence of lignin in the environment has an adverse impact on an ecosystem. There is a need to find out an eco-friendly and cost effective method for decomposition of these hard materials in a short span of time (Mtui 2012).

Lignin degrading fungi are species that have ability to secrete extracellular ligninolytic enzymes. Fungi are among the microorganisms that are known to produce such enzymes (Maciel *et al*. 2010). They are considered to be the most promising group of microorganisms because they produce ligninolytic enzymatic complex composed of several enzymes including cellulases, lignin peroxidase, manganese peroxidase and laccase (Tien and Kirk 1984). They are the only organisms that are known to have evolved complex enzymatic machinery to degrade lignin, the non-hydrolysable part of wood, to any extent (Revankar and Lele 2006).

Ligninolytic enzymes are extracellular oxidative enzymes that are secondary metabolic products, differing in chemical compositions and are often species specific (Mtui 2014, Dhouib *et al*. 2005). These are biocatalysts that are responsible for degradation of lignin and cellulosic materials fall into two categories namely: peroxidases and oxidases (Christopher,

2014). Ligninolytic enzymes utilise hydrogen peroxide (H_2O_2) as co-substrates, most are heme associated enzymes with an exceptional broad substrate spectrum that include organic and inorganic compounds. (Olukunle and Oyegoke 2016, George *et al*. 2000). These enzymes have been shown to degrade not only lignocellulose, but also recalcitrant environmental pollutants such as crude oil wastes, textile effluents, organochloride agrochemicals and pulp effluents (Mtui and Nakamura 2004). The enzymes have attracted a wide range of industrial and environmental applications including pulping, de-inking, decolourization and detoxification of textile wastes, wastewater treatment, bioremediation of polluted soils and bio fuels (Mtui 2012, Maciel *et al*. 2010, Machando *et al*. 2005).

Currently, debates and significant gaps in the lignocellulolytic fungal enzymes' research have been focused on bioprospecting of fungi with novel biodegradative enzymes, use of novel inducers to enhance production and gene cloning to screen for new generation of enzymes (Mtui 2012). Fungi from soil contaminated with cow dung are considered to be more robust in degrading ligninolytic compounds than their counterparts, mainly because cow dung increase geo-technical properties of the soil like bulk-density, dry density, water holding capacity, porosity, infiltration (Devi and Kumar 2012). Nevertheless, they are adapted to harsh environments such as high amount of indigestible lignin and waste from the cow. Therefore this study aimed at isolating and screening of fungi from the soil contaminated with cow dung manure and culturing for the production of ligninolytic enzymes.

MATERIALS AND METHODS

Soil sample collection

The soil sample used was collected at cow dung-contaminated sites at a depth of about 10-15cm in Ubungo Kibangu, in Dar es salaam, Tanzania. The soil was air dried, mixed thoroughly to increase homogeneity, put in airtight plastic bag and carried out to the department of Molecular Biology and Biotechnology (MBB), University of Dar es Salaam. The soil was stored at 4 ^{o}C for seven days at MBB before further analysis.

Isolation of ligninolytic fungi

An amount of 1.0 gm of soil sample was serially diluted up to 10^{-4} with sterilized normal saline solution. Pour plate technique was used, diluted suspensions of 0.1 ml were applied and spread over a selective medium (lignin medium) containing 3 % (w/v) agar, 0.4 % (w/v) alkaline lignin and ampicillin (50 µg/ml) and incubated for 7 days at room temperature. Repeated sub culturing using lignin medium was done to get pure culture for easy identification. The fungal mycelium was observed while still on plate. The strain was identified by morphological characteristics include color and growth pattern. Microscopic characteristics were examined under the microscope include spore formation and color (Olukunle and Oyegoke 2016).

Screening for ligninolytic enzymes production using Rhemazol Brilliant blue -R dye (RBBR) decolourization assay.

A petri dish containing 2 % (w/v) malt extract agar with 0.05 g RBBR (Sigma) per 100 ml medium mixture was used for screening lignolytic enzymes production. The plates were inoculated with 5-mm-diameter agar plug from 7 days-old mycelium of the test strain grown in lignin media. Plates were incubated at 30 ^{o}C in the dark until they were totally colonized for a

maximum period of 7 days (Okino *et al*. 2000).

Screening for ligninolytic enzymes using α-naphthol and pyrogallol in solid media

A 7-mm- diameter agar plug from a 7 days old mycelium of the test strain was utilized to inoculate plates with sugarcane baggase powder 0.2 g, guaicol 0.01 g and agar culture medium 1.6 g per 100 ml of distilled water. Plates were incubated for 7 days at 30 ^{o}C. The drop test was then carried out in the same plate using: a) α-naphthol solution and b) pyrogallol solution to detect laccases and peroxidases, respectively. The results were evaluated on the basis of colour changes - purple for laccases and yellowish-brown for peroxidases (George *et al*. 2000, Okino *et al*. 2000)

Cultivation of fungi for ligninolytic enzyme activities

Kirk's modified medium was used for fungal cultivation (Tien and Kirk 1984). The isolate was cultivated in submerged culture medium for ligninolytic enzyme expression then assayed for peroxidase (LiP) (Nyanhongo *et al*. 2002), manganese peroxidase (MnP) (Dhouib *et al*. 2005), and laccase (Lac) (Dhouib *et al*. 2005). Cultures were collected daily over a period of 12 days and supernatants from cultivations kept into eppendorf tubes after centrifuging at 10,000 rpm for 10 minutes. Each enzyme assay was followed on a UV-visible spectrophotometer (Thermospectronic, Great Britain) (Mtui and Masalu 2008).

Determaination of ligninolytic enzyme activities from submerged culture

The enzymatic activities were measured according to the principle explained by Bouguer-Beer-Lambert's law (Skoog *et al*. 1998). The law states that, absorbance is proportional to the concentration of the colour in the transparent solution. Therefore, the rate of appearance or disappearance of light absorbing product or substrates can be followed quickly and conveniently with a spectrophotometer.

The catalytic activity Z, then corresponds to the absorbance change per minute.

$$Z = \frac{\Delta A.V.1000}{\varepsilon.d.\Delta t}$$

Where, V = assay volume in litres
t = time in minutes

The unit of Z is micromoles per minute and corresponds to international unit U, which is the activity of an enzyme that under optimized conditions catalyze the conversion of 1µ mole of substrate per minute.

Determination of Lignin peroxidase (LiP) activities

The isolate was cultured at 30 ^{0}C in 250 ml Erlenmeyer flasks containing 25 ml of the modified medium as described by Nyanhongo *et al*. (2002). Lignin peroxidase activity was followed spectrophotometrically at 310 nm, through the oxidation of veratryl alcohol to veratryl aldehyde, whose molar absorptivity is 9300 M-1cm-1 of the product (Tien and Kirk, 1984). The reaction mixture contained 300 µl veratryl alcohol (8mM), 600 µl sodium tartrate buffer (0.5 M, pH 4.5 at 30 ^{0}C), 100 µl mycelial liquid fraction, and 1850 µl distilled water. The mixture was then incubated for two minutes at 30^{0}C and the reaction was initiated by addition of 150 µl H_2O_2 (5 mM). The absorbance was immediately measured in one-minute intervals after addition of H_2O_2.

Determination of Manganese peroxidase (MnP) activities

The isolate was cultured at 30^{o}C in static conditions in 250 ml Erlenmeyer flasks containing 25 ml of the modified Kirk

medium (Dhouib *et al*. 2005). In this method guiacol was used as substrate, and then the increase in absorbance at 465 nm due to oxidation of guiacol was measured, absorption coefficient ε_{465} = is 12,100 $M^{-1}cm^{-1}$. The reaction mixture contained 300 µl sodium succinate buffer (0.5 M, pH 4.5 at 27 ^{o}C), 300µl guaiacol (4 mM), 600 µl manganese sulphate (1 mM), 300 µl mycelial liquid fraction and 1200 µl distilled water. The mixture was then incubated for two minutes at 30 ^{o}C and the reaction was initiated by addition of 300 µl hydrogen peroxide (1mM). The absorbance was measured immediately at 465 nm in one-minute intervals after addition of hydrogen peroxide.

Determination of Laccase (Lac) activities

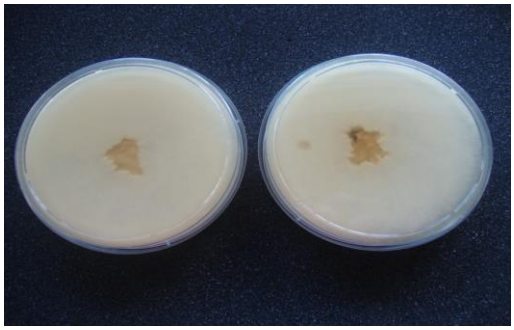
The isolate was grown at 30^{o}C with rotary shaking (125 rpm) in 250ml Erlenmeyer flasks containing 25ml of the modified Kirk medium (Dhouib *et al*. 2005). The laccase activity was determined via the oxidation of ABTS as the substrate (Bourbonnais *et al*. 1995). This method was based on the oxidation of the substrate 2,2'-azino–bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS). The rate of ABTS oxidation was followed spectrophotometrically at 420 nm using molar absorptivity of 36,000 M^{-1} cm^{-1} for product. The reaction mixture contained 600 µl sodium acetate buffer (0.1M, pH 5.0 at 27 ^{o}C), 300 µl ABTS (5 mM), 300 µl mycelia liquid fraction and 1400 µl distilled water. The mixture was then incubated for 2 min at 30 ^{o}C and the reaction was initiated by addition of 300 µl hydrogen peroxide. Then the absorbance was measured immediately in one-minute intervals after addition of hydrogen peroxide.

RESULTS AND DISCUSSION

Isolation of ligninolytic fungi

In this study, fungal strain from soil contaminated with cow dung manure was successfully isolated. The fungal mycelium was observed while still on plate. The strain was identified by morphological characteristics include color and growth pattern. Microscopic characteristics were examined under the microscope include spore formation and color. The strain was identified to belong to genus Trichoderma (Olukunle and Oyegoke, 2016). The selective media used contain only lignin as source of carbon; therefore, whichever fungi grew could have been lignin degrading fungi. The cow dung manure soil was selected as a source of fungi because it contains partially or non-degraded cellulose with high lignin content after passing through the digestion system (Devi and Kumar, 2012). It has been shown that animal faeces provide the soil high nitrogenous material suitable for fungi proliferation (Olukunle and Oyegoke, 2016).

a



b

Figure 1: Pure fungal isolate isolated from the cow dung manure soil obtained after repeated sub culturing on lignin medium (a) the forward view of the plate (b) the back view of the plate.

Screening for ligninolytic enzymes production using Rhemazol Brilliant blue -R (RBBR) dye decolourization and Guaicol oxidation assays on solid media.

After 7 days of incubation, strain showed appearance of halo in RBBR culture media resulting from decolourization/oxidation of RBBR dye by the fungal strain (Figure 2). Oxidation of synthetic polymeric dyes such as RBBR has been attributed to catalysis of ligninolytic enzymes that including peroxidases and oxidases (George at al., 2000; Mtui and Masalu 2008). Some authors have related decolourization to peroxidases namely Lignin Peroxidase (LiP) and Manganese Peroxidase (MnP) (Sivakumar *et al*. 2010). Other studies have revealed that laccase (lac) played an important role in decolourization of RBBR dye (Machando *et al*., 2005, Marciel *et al*. 2010). Furthermore, oxidation of polymeric dyes is an indication of a potential to degrade xenobiotic compounds, indicative method for multienzymatic system also can be used as a tool for xenobiotic biodegradation studies (Elishavilli *et al*. 2008, Machando *et al*. 2005). The assay has been used since the eighties as a rapid screening method for detection of ligninolytic ability in fungal system (Mtui 2012). In the plate test, the production of laccase and peroxidase were detected using α-naphthol and pyrogallol, respectively. These results further demonstrate the complexity of associated with the degradation of lignin and its derivatives. The results of this study support previous studies that plate test is an efficient and simple method for bioprospecting fungi with novel lignolytic enzymes for industrial application purposes (Masalu, 2004).

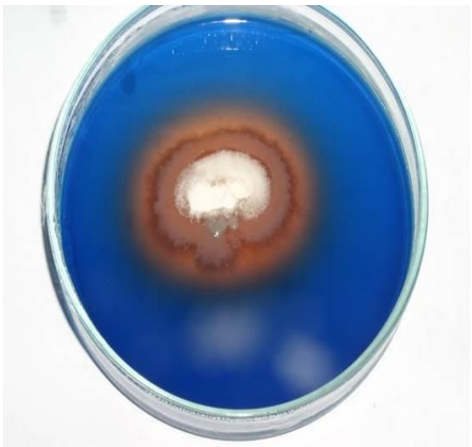


Figure 2: A halo in the plate results from Rhemazol brilliant blue-R (RBBR) dye decolourization by fungal strain on solid medium after seven days of incubation. The dye was completely decolourized after 14 days.

Ligninolytic enzyme activities from submerged fermentation

Fungal filtrate exhibited lignolytic enzyme activities that were increase with time until

reach maximum and thereafter decrease. The maximum Lignin peroxidase (LiP), Manganese Peroxidase (MnP) and Laccase (Lac) activities were 1.44, 0.30 and 0.28 U/ml, respectively, in the submerged culture fermentations (Figure 3). The results obtained in this study showed the Trichordema sp is able to produce all three types of lignin degrading enzymes namely LiP, MnP and Lac. It has been reported previously that among the well-known ligninolytic enzymes producer (basidiomycetes) very few strain have found to produce all the three major classes of ligninolytic enzymes (Levin *et al*. 2001, Doralice and Regina 2001). The presence of all major ligninolytic enzymes in Trichoderma sp underlines its degradation potential for lignin, cellulose and other organic compounds (Da Silva 2010, Vaithanomsat *et al*. 2010). The strain exhibited low amount of all enzymes, contrary to previous study on white rot fungi (*F. flavus*) which had shown to have 70 U/ml, 10 U/ml and 8 U/ml for LiP, MnP and Lac respectively (Mtui and Nakamura 2008). It is suggesting that improvement of cultural growth conditions and addition of potential inducers might increase enzymes production (Akpinal and Urek 2014).

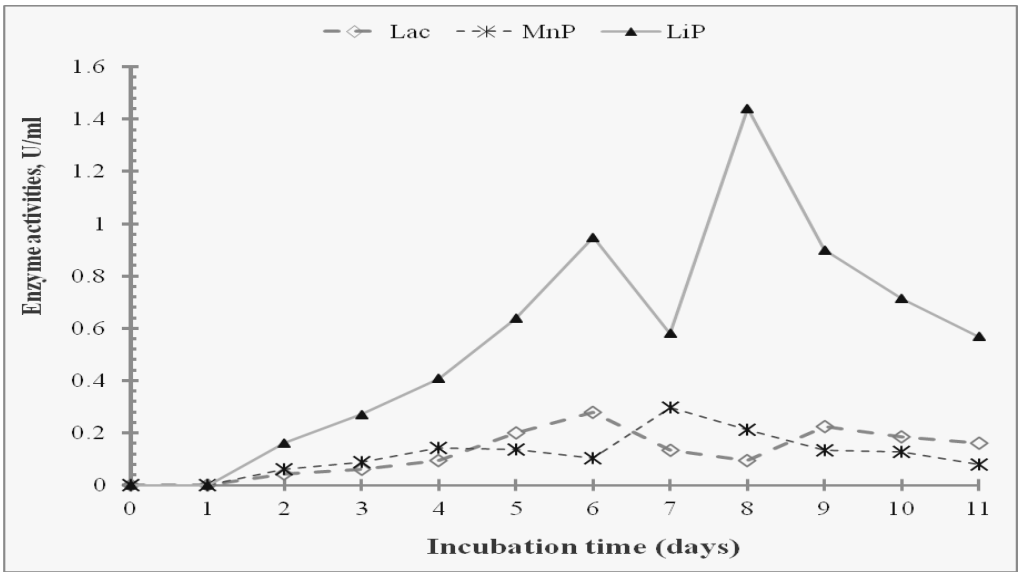


Figure 3: Time course for ligninolytic enzyme activities of the Trichoderma sp in the submerged culture fermentation grown in Kirk's medium.

The amount of LiP (1.44 U/ml) produced by the strain is higher than the amount of LiP (1 U/ml) produced by marine basidiomycete strain, *L. sulphurae* which reported previous by Mtui and Masalu (2008). The results indicating that t)he strain is good LiP producer, suggesting for gene cloning specifically on this essential lignin degrading enzyme. LiP enzyme has been shown to catalyse synthetic lignin, that there is clear correlation between lignin mineralization and LiP (Liew *et al*. 2011). Contrarily to this study, high amount of LiP (44.84 U/ml) was detected in Cladosporium sp under optimized conditions (Da Silva *et al*. 2010) suggesting that high level of

ligninolytic enzyme production can be obtained under optimized growth conditions of the fungi. In the present study, the strain showed Lac maximum production on day 6 with an activity of 0.28 U/ml in the submerged culture fermentation. This support previously observation that Lac is produced under growth limiting conditions nitrogen in particular which however has a negative impact on the enzyme yields (Valle *et al*. 2014, Okino *et al*. 2001). Results are also in agreement with other studies where low laccase activities (0.42 U/ml) was detected in most native fungi (Christopher *et al*. 2014) and where no laccase activities were detected in 19 fungi strains (Sumandano *et al*. 2015). Therefore improved productivity through cloning of lac genes and their heterologous expressing are inevitable. The study also showed maximum MnP production on day 7 of incubation with an activity of 0.30 U/ml in the submerged culture fermentation. The results support previous observation that MnP an activity is widely spread in different species but the production is not as higher than Lac and LiP production (Elisashvilli 2008). It was previously reported that MnP can only be highly produced if inducer like manganese ion is supplemented in culture media (Akpinal and Urec 2012).

It was further noted that, enzymes activities were fluctuating and started declining gradually after day eight, this could be attributed to several reasons; glucose from the broth was converted to organic acids that might decrease the pH of the culture medium thus inhibit cell growth and enzyme activities (Kanwal and Reddy 2012). It might be due to accumulation of nitrogen waste/toxicity of metabolic products such as NO_3, NO_2 and NH_3 that has been noted to be produced in other studies elsewhere (Kanwal and Reddy, 2011) and lastly enzyme degradation by proteases that is also released into the broth which digests proteins in solutions and addition of protease inhibitor in the reaction mixture would solve the problem (Akpinal and Urek 2014). The results obtained here are in consistent with previous findings of Patrick *et al*, (2011), who obtained similar results with fluctuations in the enzymatic activities before they attained the maximum level.

These findings provide baseline information on ligninolytic fungi (Trichoderma sp) isolated from cow dung manure contaminated sites. It has been revealed that the source is potential for isolating ligninolytic fungi which can further be cloned to create potential genomic library for different biotechnology applications such as wastewater treatment, bioremediation of polluted soils and bio fuels. Future studies will focus towards optimization conditions for maximum production of the enzyme as well as application of the fungi in bioremediation processes.

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