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Decolourisation studies of azo dyes by locally isolated fungi for application as bioremediation agent.

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Decolourisation studies of azo dyes by locally isolated fungi for application as bioremediation agent.

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Declaration

I hereby declare that this thesis is based on my original work except for quotation and citation, which have been duty acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UNIMAS or other institutions.

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List of Abbreviations

cm	Centimetre
DMW	Dry mycelium weight
g/ml	Gram per millitre
GMM	Glucose minimal medium
Lac	Laccase
LiP	Lignin peroxidase
MEA	Malt extract agar
mm^2	Millimetre square
MnP	Manganese peroxidase
nm	Nanometer
RBBR	Remazol Brilliant Blue R
rpm	Revolution per minute
u/ml	Unit per millilitre
w/v	Weight per volume
µl	Microlitre
%	Percent
℃	Degree Celcius

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DECOLOURISATION STUDIES OF AZO DYES BY LOCALLY ISOLATED FUNGI FOR APPLICATION AS BIOREMEDIATION AGENT.

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ABSTRACT

The four fungal isolates namely *Marasmius cladophyllus*, *Cerrena unicolor*, *Cunninghamella bainieri* and *Trichoderma harzianium* were used in the study of dye decolourisation. Orange G was chosen as a representative of azo dyes in the decolourisation study. The fungal isolates were used in Orange G decolourisation study in both solid and liquid culture conditions. The study was carried out to test for the ability of locally isolated fungi in dye degradation. It is also hoped that the ligninolytic enzymes can be identified and characterized at the end of experiment. The results showed that *M. cladophyllus* MS8 could decolourise Orange G completely in solid GMM agar on day 7. Spectrophotometer analysis revealed that *M. cladophyllus* MS8 could remove 56.33% of Orange G in liquid GMM on the 16th day of decolourisation experiments. The enzyme assays conducted showed that laccase, manganese peroxidase and lignin peroxidase were responsible for the Orange G decolourisation. Maximum percentage of Orange G decolourisation was recorded under optimal experimental condition with the present of dye at a final concentration of 0.02% (w/v) and by two fungal plugs as inoculums. The Orange G was decolourised rapidly initially in the 3 days pre-grown culture but only 40.90% of percentage of decolourisation was achieved. RBBR was decolourised by *M. cladophyllus* MS8 effectively with 98.56% of color removal on the 8th day. These therefore show the interesting potential of fungal isolate *M. cladophyllus* MS8 for dye decolourisation.

Keywords: Orange G, decolourisation, *Marasmius cladophyllus*, enzymes.

ABSTRAK

Empat jenis kulat iaitu Marasmius cladophyllus, Cerrena unicolor, Cunninghamella bainieri *dan* Trichoderma harzianium *telah dgunakani dalam kajian degradasi pewarna. Orange G telah dipilih sebagai wakil pewarna azo. Kesemua kulat ini digunakan dalam kajian penyahwarnaan di dalam media larutan serta pepejal agar. Kajian ini telah dijalankan untuk menguji keupayaan kulat asingan tempatan dalam degradasi pewarna. Ia juga diharapkan menunjukkan jenis-jenis enzim ligninolitik yang terlibat dalam degradasi agar dapat dikenalpasti. Keputusan yang diperolehi telah menunjukkan bahawa* M. cladophyllus *MS8 berupaya untuk menyahwarnakan Orange G dengan sepenuhnya dalam pepejal agar dalam tempoh 7 hari. Analisis spektrofotometer menunjukkan* M. cladophyllus *MS8 mampu menghilangkan Orange G sebanyak 56.33% dalam larutan GMM pada hari ke-16. Kajian aktiviti enzim lignolitik menunjukkan lakase, mangan perokxidase dan lignin peroksidase bertanggungjawab dalam menyahwarnakan Orange G. Kepekatan pewarna sebanyak 0.02% (w/v) dengan menggunakan dua plug kulat telah mencapai peratusan penyahwarnaan yang maksimum. Orange G dapat dinyahwarnakan dengan lebih cepat apabila kulat telah dikultur selama 3 hari dalam larutan media sebelum pertambahan pewarna tetapi hanya 40.90% penyahwarnaan tercapai. Pewarna RBBR dapat dinyahwarnakan oleh* M. cladophyllus *MS8 dengan efektif dan mencatat peratusan dekolorisasi sebanyak 98.56% pada hari ke-8. Ini telah menunjukkan potensi* M. cladophyllus *MS8 yang menarik dalam penyahwarnaan pewarna.*

Kata kunci: Orange G, penyahwarnaan, Marasmius cladophyllus, *enzim*

1.0 Introduction

Azo dyes are widely used in the textile, paper and pharmaceutical industries. Chagas and Durrant (2001) mentioned that azo dyes are the compounds with one or more aromatic structures that may have sulphonic groups carried with them and with one or more azo bonds (-N= N-). A number of studies have shown that these azo dyes are toxic and carcinogenic. Asthmatic and allergic reactions can be triggered when the azo dyes are accidentally taken into human body and come into contact with drugs such as the aspirin and benzoic acids (Moorthi *et al.*, 2006). These colorants are not easily removed from the conventional treatment of sewage water system and therefore utilization of dyes is controlled and effluents are firstly treated before discharging into aquatic and terrestrial areas.

The release of industrial dye effluent has created a problem to the environment. The discharge of this industrial wastewater into the river has greatly affected the aquatic life as the azo dyes are toxic and carcinogenic (Chenini *et al.*, 2011). These dyes are difficult to be degraded naturally and are not removed completely through conventional methods. According to Eichlerova *et al.* (2006), there are few physico-chemical methods such as the adsorption, precipitation and chemical degradation can be applied to break down the azo dyes. However, those methods mentioned earlier are expensive which make them less applicable. Thus, there is a need to develop a cost effective yet efficient method to break down the azo dyes in the wastewater. The white-rot fungi seem to fulfill these criteria and can be used as a bioremediation agent. Some researchers also reported that the fungal endophytes are also able to decolourise azo dyes but there are only limited studies on this field.

The white-rot-fungi have the ability to degrade a wide range of synthetic dyes through an oxidative, free radical mechanism and a highly non-specific ligninolytic system (Chagas & Durrant, 2001). Enzymes produced from these fungi are able to function in many of the oxidation and reduction reactions producing highly reactive oxygen compounds (Chagas & Durrant, 2001). Due to its non-specific system in degrading lignin or structurally related compounds, it is thus able to degrade a wide range of pollutant substances resembling lignin and its derivative.

White-rot fungi are able to degrade the toxic azo compounds through the ligninolytic enzyme activities into non-toxic compounds. It is hoped that the best fungal isolate in degradation of azo dyes can be selected. There are two main aims in the decolourisation study of azo dyes by fungal isolates. Firstly, this study is carried out to test for the ability of locally isolated fungi in degradation of azo dyes. Secondly, it is to determine and characterize the ligninolytic enzymes activity of the fungi during the biodegradation of azo dyes.

The study of dye decolourisation by the locally isolated fungi is divided into two phases which are the study in the solid and liquid state. The solid agar and liquid glucose minimal media (GMM) are used in the study of dye decolourisation. In solid state study, it is believed that the halo will be formed in the agar plate whereby clear zone can be seen which indicates the ability of dye decolourisation by the locally isolated fungi (Eichlerova *et al.*, 2006). In liquid state study, the research focuses on the fungal isolate that is able to show optimum decolourisation of azo dyes. The enzyme assay which are to be carried out is also believed that it is actually the three main ligninolytic enzymes namely the laccase, manganese peroxidase and lignin peroxidase that degrade the azo dyes.

2.0 Literature review

2.1 Industrial dye effluent

The use of azo dyes mainly in the textile industries is currently in a rise. India is the second largest country that produces large amount of cotton yarn and silk and also the third largest producer of cotton and cellulose fiber in the world (Balakrishnan *et al.*, 2008). The increase in population in India allows the people to get involved in the textile industries. Despite the increase in industrial production in textile sector, the environment is greatly polluted by vast amount of wastewater generated from dyeing process. According to Balakrishnan *et al.* (2008), dyeing is a process that combines the bleaching and coloring steps and this step alone has generated large volume of dye wastewater. Improper management of dye contaminated wastewater and the resistant of azo dyes to the natural methods have given rise to the contamination of the water source. For instance in Nigeria, the main drinking water source is from the estuaries and inland and is often contaminated with the industrial dye effluent (Kanu *et al.*, 2011). Unfortunately, the rivers are always the chosen sites to discharge the dye contaminated wastewater. This has greatly polluted the receiving water bodies due to the discharge of the treated and untreated or partial treated effluent. All the fisheries, life forms and environment of the aquatic are greatly affected by the increase in discharge of different forms of pollutants. The Nigerians have limited water sources to be used and the polluted water bodies further worsened the situation.

2.2 Fungal endophytes

Endophytes were initially defined as any organisms that found within the plant (Ghimire & Hyde, 2008). It is later described as organisms that live within the plant organs and colonize the internal plant tissues but they do not pose threats to the host. These endophytes can be fungi or bacteria that live in the tissues of living plants and have limited or no pathogenicity to the plants. The endophytes and the plants (hosts) have close mutualism interaction where both gain benefits from each other (Molina *et al.*, 2012). For example, there is a nutrient cycling between the fungus and the hosts and enhanced photosynthetic capacity of cell and tissues due to endophyte infection.

Recently there are few research groups that are interested in finding out more about the endophytes in biotechnological processes by studying their bioactive compounds produced (Molina *et al.*, 2012). These include their production of bioactive secondary metabolites such as alkaloids, flavonoids, phenolic acids, steroids and others which can be applied in agrochemicals, antibiotics, immunosuppressants antioxidant and anticancer agent. However, there are limited studies and research done on the fungal endophytes in the decolourisation of textile dyes. According to Bhagobaty and Joshi (2012), fungal endophytes are considered as a new source for the harvest of their enzymes with different potentialities.

2.3 Ligninolytic enzymes

The white-rot basidomycetes have been reported to be a potential bioremediation agent in decolourisation of azo dyes. This main contributor to the degradation of azo dyes is the extracellular ligninolytic enzymes mainly the laccase, manganese peroxidase and lignin peroxidase. According to Wesenberg *et al.* (2003), secretion and production of these enzymes is induced by scarce level of nutrients such as the nitrogen and carbon. Production of laccase can be further enhanced by agitation in submerged culture. However, this hinders the synthesis of peroxidases but they can be greatly produced at high oxygen tension.

The laccase enzyme is classified under the oxidase group (Torres *et al.*, 2012). They are the glycoproteins that synthesized by the white-rot fungi, plants and microorganisms to degrade the lignin structure. According to Maciel *et al.* (2010), laccase acticity has also been found in few phyla of fungi such as the Ascomycota, Zygomycota and especially the phylum Basidiomycota. They are able to recognize the aromatic structure of the substrates and hydrolyse the phenolic hydroxyl groups into radical form and this initiate the breaking of the long chain lignin structure (Torres *et al.*, 2012). The laccase activity is further enhanced by the mediator which is the ABTS {2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)}.

Manganese peroxidase (MnP) falls in the oxidoreductase group and is expressed in almost all of the white-rot fungi. It is able to break down the lignin (Maciel *et al.*, 2010). MnP is found in most of the basidiomycetes and is more widely spread than the LiP (Hofrichter, 2002). MnP oxidize Mn^{2+} ions into Mn^{3+} ions, a reactive compound that will form Mn^{3+}-chelate organic acid complex (Maciel *et al.*, 2010). MnP is able to oxidize Mn^{2+} ions into

Mn^{3+} ions in the absence of H_2O_2 unlike LiP where H_2O_2 is required for oxidation to take place.

Lignin peroxidase (LiP) is also an oxidoreductase and was initially found in the basidiomycete *Phanerochaete chrysosporium* in 1983 (Maciel et al., 2010). It is also found in several species of white-rot basidiomycetes and actinomycetes. LiP is the hemeprotein that is produced extracellularly with a high redox potential and requires H_2O_2 at low optimum pH in order for LiP activity to take place. There are various reduced substrates including polymeric substrates that can be oxidized by the high redox potential and enlarged substrate range of LiP (Maciel *et al.*, 2010). These characteristics have made LiP being studied extensively in bioremediation in the industrial processes. Unlike MnP, LiP needs H_2O_2 in order to start off the catalysis. LiP has lower substrate specificity and hence is able to degrade many different lignin-like substrates or the non-lignin like structures. LiP can therefore be a potential degradation agent in many different industrial processes.

2.4 Bioremediation / Mycoremediation

Soil and water pollution by the azo dyes are currently in a rise and is hazardous to the living things and the environment. There is a need to develop a less expensive and efficient solution towards this issue and bioremediation seems to fit the requirements. Bioremediation can be defined as the process of microorganisms or fungi to break down the contaminants and return the soil or water to their original conditions. Biodegradation is the process taken place by the microorganism in metabolizing the organic contaminants and converting them to less harmful compounds (Magan, Fragoeiro, & Bastos, 2010). Bioremediation is therefore developed to speed up the naturally occurring biodegradation process by optimizing certain conditions.

Mycoremediation refers to the degradation of organic pollutants by utilizing the filamentous fungi. The three ligninolytic enzymes are potential in modifying the chemical structures of synthetic dyes.

There are few reasons to support for the extensive research in bioremediation. Firstly, it is a cost-effective technique of reducing harmful pollutants than the physical-chemical means. This is because bioremediation is able to treat the contaminated soil and groundwater without excavation and this helps to maintain the soil texture. Moreover, it does not require a lot of energy besides will not threaten the natural environment. Other than that, fungi are more tolerant to the fluctuating environmental conditions compared to the bacteria. They are able to survive in harsh condition such as in the high concentration of chemical pollutants and synthesize extracellular enzymes that enhance the rate of bioremediation. The ability of filamentous fungi to oxidize a variety of compounds makes them preferably as bioremediation agent rather than the bacteria (Magan *et al.*, 2010).

3.0 Materials and methods

3.1 Culture preparation

The fungal isolates *Marasmius cladophyllus* MS8, *Cunninghamella bainieri*, *Trichoderma harzianum* and *Cerrena unicolor* were tested for their dye decolourisation ability. These fungi were obtained from the Molecular Genetic Laboratory, UNIMAS Fungal Collection. All of the fungal strains were maintained on the malt extract agar (MEA) for seven days at 27^{o}C until extensive mycelia are seen. This was used as working culture for solid and liquid state of dye Decolourisation study as proposed by Eichlerova *et al.* (2006). In solid state decolourisation study, glucose minimal (GMM) agar was used. The GMM agar used per litre contains: 1g of K_2HPO_4, 10 mg of $ZnSO_4.7H_2O$, 5 mg of $CuSO_4.5H_2O$, 0.5 g of $MgSO_4.7H_2O$, 10 mg of $FeSO_4.7H_2O$, 0.5 g of KCl, 10 g of glucose, 3 g of $NaNO_3$ as the sole source of nitrogen and 20 g of agar (Carvalho *et al.*, 2009). The pH of the GMM agar was firstly adjusted to 5.5 before autoclaved at 121^{o}C for 15 minutes. In dye decolourisation study in liquid state, the fungal strains were grown on the liquid GMM that has the similar contents as in GMM agar but without the addition of agar.

3.2 Stock culture preparation

After seven days of cultivation, 5mm^2 of the agar plug containing fungi was cut and transferred to 20% glycerol stock solution and stored at -20^{o}C. This stock culture must be prepared as a backup preparation of fungal working culture so that inoculums can still be obtained for other sets of experiment.

3.3 Dye solution preparation

The synthetic dye chosen for this experiment was Orange G with maximum absorbance wavelength of 478nm (Eichlerova *et al.*, 2006). The dye solution was prepared based on the method proposed by Annuar *et al.* (2009) whereby the dye powder was dissolved with autoclaved distilled water and used as a stock dye solution of 1.0 % (w/v). The dye concentration used in both solid and liquid state of dye decolourisation study was fixed at a concentration of 0.02 % (w/v).

3.4 Solid state Decolourisation study

Four fungal strains *C. unicolor*, *M. cladophyllus MS8*, *C. bainieri* and *T. harzianium* were examined for their ability to decolourise Orange G in the glucose minimal medium (GMM) agar plates. The solid state decolourisation was carried out based on the slight modification of the method from Eichlerova *et al.* (2006). A $5mm^2$ in diameter of agar plug was cut from a seven days old working culture and transferred onto the GMM agar plate with final dye concentration made at 0.02 % (w/v). The sample was done in duplicate and an abiotic control was made without addition of the inoculums. After that, all plates were incubated for 14 days in the dark at room temperature. The decolourisation activity on solid was analyzed by the measurement of radial growth (the diameter of the colonies) and the zone of color change (diameter of the decolourised zone) on the agar plate which were recorded at three days interval. All measurements are repeated three times to obtain an accurate result. The fungal strain that shows the best percentage degradation of Orange G was chosen to be analyzed for its enzyme activities in the liquid state decolourisation study and in the optimization study.

3.5 Liquid state decolourisation study

The four fungal strains were also examined for their ability to decolourise Orange G based on the modified method of Eichlerova *et al.* (2006). The same GMM contents were used but without the addition of agar were used as growing culture for the four fungal strains. The 200ml Erlenmeyer flask supplemented with Orange G was inoculated with two agar plugs of $5mm^2$ from working culture. It was then incubated in static at 37^oC for 15 days in the dark. At interval of 3 days that is on the 0^{th}, 3^{th}, 6^{th}, 9^{th} ,12^{th} and 15^{th} day, the flask were harvested by centrifugation at 6000 rpm for 10 minutes at $4^{\circ}C$ to separate the fungal mycelium from the culture medium. The supernatant collected was used to quantify dye decolourisation by UV-visible spectrophotometer at 478nm which is the maximum absorbance wavelength of Orange G. The samples were prepared in duplicate and a control, supernatant made without the addition of inoculums was also prepared. Percentage of dye decolourisation was calculated using the formula as shown below (Senthilkumar *et al.*, 2011).

Percentage of dye decolourisation (%) =

$$\frac{\text{Initial absorbance} - \text{absorbance at particular time interval}}{\text{Initial absorbance}} \times 100\%$$

Based on the results obtained from the solid and liquid state of decolourisation, the best dye degrading fungal isolate was selected for the study of its ligninolytic enzyme assay in the liquid state decolourisation. In this enzyme assay study, similar method as mentioned above was used but at an incubation period of 20 days. The dye decolourisation analysis and enzyme assay were carried out on 4 days interval for 20 days which is on the 0^{th}, 4^{th}, 8^{th}, 12^{th}, 16^{th} and 20^{th} day of incubation.

3.5.1 Weight of dry fungal biomass

The growth of the selected fungus in the liquid dye medium was also monitored by measuring the amount of fungal biomass produced. The content of each flask was centrifuged at 6000 rpm for 10 minutes to separate the fungal mycelium with the culture medium. In order to obtain the dry mycelium weight of fungal biomass, a glass petri plate was used to dry the fungal biomass. The glass plate and its cover were weighed prior to be used to dry the fungal biomass at 80^{o}C until constant weight was recorded (Chagas & Durrant, 2001). The dry mycelium weight is expressed in terms of weight (g) of biomass per volume (ml) of culture (Erkurt *et al.*, 2007).

3.6 Analysis of the ligninolytic enzyme activity

The fungus was screened for the production of ligninolytic enzymes namely lignin peroxidases (LiP), manganese peroxidase (MnP) and laccase (Lac). A standard reaction mixture was prepared for each enzyme assay prior to be used. The average readings for all three enzyme activities were taken on each flask and the experiment was done in duplicate. One unit of Lac, MnP and LiP is defined as 1.0 µmol of substrates oxidized per minute (Chagas & Durrant, 2011). Enzyme activities were calculated based on the formula as shown below (Annuar *et al.*, 2009):

$$\text{Enzyme activity, U/ml} = \frac{Absorbance/minutes}{\varepsilon \, x \, d} \times \frac{Reaction\ volume}{Sample\ volume} \times 10^3$$

ε = Extinction coefficient for each substrate ($cm^{-1}\ M^{-1}$)

d = cuvette diameter

3.6.1 Laccase Assay

The laccase enzyme activity was assayed by the oxidation of ABTS (2,2-Azino-bis-(ethylbenzthiazoline-6-sulphonate) and the absorbance increase at 420nm (ε = 36,000 cm^{-1} M^{-1}) was monitored (Saparrat & Guillen, 2005). The standard reaction mixture for laccase assay contains the following of: 400 µL of sodium acetate buffer at pH 5.0, 400 µL of ABTS, 400 µL of crude enzymes.

3.6.2 Managanese Peroxidase (MnP) Assay

MnP activity was determined by the oxidation of 2,6-dimethoxyphenol (DMP) based on the method described by Lopez *et al.* (2004) at absorbance reading of 469 nm (ε = 49,600 cm^{-1} M^{-1}). The standard reaction mixture for MnP assay contains the following of: 675 µL of 0.2 M sodium tartarate buffer at pH 4.5, 67.5 µL of 0.02 M DMP, 13.5 µL of 0.1 M $MnSO_4$, 1.35 µL of 0.1 M H_2O_2, 150 µL of crude enzyme, 592 µL of ultrapure water.

3.6.3 Lignin Peroxidase (LiP) Assay

Based on the study of Kalmis *et al.* (2008), the activity of LiP was measured spectrophotometrically at 310nm (ε = 9300 cm^{-1} M^{-1}) when veratyrl alcohol (VA) is oxidized to veratraldehyde (VAD) in the presence of H_2O_2. The standard reaction mixture for LiP assay contains the following of: 125 µL of 2mM VA/Dimethoxybenzyl, 625 µL of 50 mM sodium tartarate buffer at pH 2.5, 250 µL crude enzymes, 250 µL of 500 mM of H_2O_2.

3.7 Optimization study on physicochemical conditions

The dye decolourisation ability of the selected fungus was further optimized in terms of dye concentration (w/v) and the size of inoculums used and also pre-cultivation. These parameters were manipulated to analyze the efficiency of fungal decolourisation at different conditions so as to obtain the optimum conditions for the bioremediation of azo dyes. In this optimization experiment, the two parameters were applied only on the liquid GMM culture. Static cultivation of the inoculated liquid GMM was carried out at room temperature in the dark for a period of 20 days and was observed for decolourisation. The fungal strain used was the selected fungal isolate that works best in decolourisation of Orange G from the previous solid and liquid state decolourisation study.

3.7.1 Initial dye concentration

The dye concentration was set at 0.03 and 0.04% (w/v) to find out the best concentration for maximum decolourisation of Orange G. The percentage of inoculums was kept constant in this experiment. Orange G was used and the percentage of inoculums is set by inoculating with two fungal plugs. Static cultivation was applied to the inoculated liquid GMM at room temperature in the dark for a period of 20 days. Every four days interval, the solution was harvested from the flasks with different concentration by centrifugation at 6000 rpm for 10 minutes to remove the fungal mycelium. The absorbance reading of the supernatant was measured at the 478nm which is the maximum wavelength of Orange G by spectrophotometer. The experiment for each different concentration was done in triplicate and a control is prepared without the fungal inoculums.

3.7.2 Size of inoculums

The percentage of inoculums was set by using different number of fungal plugs to analyze for the optimum decolourisation of Orange G. The number of fungal plug was set at one, three and four. The dye concentration was kept constant which is at 0.02% (w/v) (concentration used in the default experiment). The inoculated liquid GMM was incubated at room temperature in static condition in the dark for 20 days. Every four days interval, the solution was taken out from the three flasks and centrifuged at 6000 rpm for 10 minutes to obtain the supernatant. It was then used for spectrophotometry analysis at absorbance wavelength of 478nm. The experiment was done in triplicate and a control was prepared without the inoculums.

3.8 Different growth systems on optimized physicochemical parameters

The optimized parameters obtained from the previous experiment were applied in the study of the Orange G decolourisation according to the method proposed by Diwaniyan *et al.* (2010). The 250ml Erlenmeyer flask with 20ml sterile liquid GMM broth was inoculated with two fungal disks from seven-day old working culture in MEA plate. After three days of fungal growth in liquid GMM, the medium was supplemented with Orange G at a final concentration of 0.02 % (w/v). The time for the addition of the dye was considered to be day. The fungal culture was harvested on the 5^{th}, 10^{th} and 12^{th} day of incubation for 12 days by centrifugation at 6,000 rpm for 10 minutes to separate the supernatant from fungal mycelium. The supernatant was monitored at 478nm using UV spectrophotometer to detect for any decolourisation of Orange G. Another set of experiment was prepared similarly to the method described previously but without the step of three days pre-grown culture in liquid GMM. On