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Screening and evaluation of ligninolytic dye decolourisation capacity of *Pleurotus ostreatus*

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Abstract

Ligninolytic enzymes as biocatalysts have the potential to replace conventional processes in several industries including the water purification industry especially in the treatment of dye effluent. Development of water treatment systems based on ligninolytic enzymes is a desirable option because ligninolytic enzymes can degrade dyes of diverse chemical structure and be used in a wide variety of industries. Studies were carried out to evaluate and screen for ligninolytic dye decolourisation capacity of *Pleurotus ostreatus* (*P.ostreatus*). Pure fungal cultures of *P.ostreatus* were screened for ligninolytic enzyme activity using solid phase decolourisation of aromatic food dyes. The assay was carried out on PDA plates with 100mg/l of individual commercial food dyes - Sunset Yellow, Orange C10 and Lemon Yellow. All the food dyes were effectively bleached or decolourised by fungal mycelia of *P.ostreatus* after 10 days of active growth in the dark at 25°C. The observation strongly suggests the presence of fungal peroxidase enzymes which play a role in the degradation of synthetic lignin or dyes. Lignin peroxidase (LiP) activity from crude enzyme extract was determined using the method of Tien and Kirk, 1988. An average LiP activity of 7.635U/ml was observed under Solid State Fermentation (SSF) by *P.ostreatus* in wheat bran/soya bean substrate (90:10) after 10 days of full substrate colonisation. In agreement with the results of other workers, the study indicates potential for *P.ostreatus* for industrial production of ligninolytic enzymes through solid state fermentation on locally available agricultural products.

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Introduction

Industries that use and manufacture dyes constitute a notable portion of economically important and environmentally significant commercial sectors in the world (Vishwakarma *et al*., 2012, Abdulla *et al*., 2000, Ahlawat *et al*., 2006). Dyes, both natural and synthetic, are used extensively in the food industry, pulp and paper industry, cosmetic industry and textile industry amongst many others (Dzulkalfi, 2012, Roushdy and Abdel-Shakour, 2011, Marina *et al*., 2010). A significant portion of these dyes is however, eventually discharged into the environment as industrial effluent (Ahlawat *et al*., 2006, Anliker, 1979). This scenario creates a serious need for effective methods of clean up or removal of these dyes from the environment. In 2012, world production of dyes was estimated to be around one million tons (Vishwakarma *et al*., 2012) and is continually on the increase thus directly compounding the effects caused by dye pollutants in the environment (GonCalves *et al*., 2000, and Singh *et al*., 2010).

In the food manufacturing industry, synthetic dyes are more widely used in place of natural dyes (Kiseleva *et al*., 2002). The availability of natural dyes is limited, but synthetic dyes can be manufactured at a larger scale and also possess other desirable attributes which include stability to light, temperature and resistance to microbial attack (Dzulkafli, 2012, Kiseleva *et al*., 2002). Synthetic food dyes are made of complex aromatic molecules and can be classified according to their chromophoric group as azo, anthraquinone, triarylmethane and phthalocyanine dyes (Liu *et al*., 2004). Azo dyes form the majority of dyes used in most manufacturing industries especially the food industry (Carliell *et al*., 1995, Chang *et al*., 2001). These dyes are recalcitrant xenobiotics which are resistant to most conventional degradation processes (Fu and Tiraraghavan, 2001). The methods available for treatment of industrial effluent containing dyes cannot reduce the pollutants to required levels; they are not effective and also tend to be costly. In addition, most dyes are known to be toxic and carcinogenic (Novotny *et al*., 2006, Kariminaae-Hamedaani *et al*., 2007). Their persistence in the environment is detrimental to living organisms and also affects the photosynthetic processes of aquatic plants (Vishwakarma *et al*., 2012). It is imperative therefore to develop methods that effectively remove dye pollutants from the environment.

Although numerous physicochemical methods have been used for a long time in the treatment of waste water containing dyes, they are less favoured to biological methods (Gharbani *et al*., 2008, Roushdy and Abdel-Shakour, 2011). Biological methods have been found to be more satisfactory, effective and efficient (Banat *et al*., 1996) and thus have received attention as a preferred alternative for treating effluent containing dyes. Several microorganisms including fungi (e.g. *Streptomyces spp, Aspergillus spp*), bacteria (e.g. *Proteus vulgaris, Streptococcus faecalis*), yeast (*Rhodotorula spp, Rhodosporidium spp*) and algae (e.g. *Chlorela spp, Spirulina spp*) are reported to be effective bioremediators (Roushdy and Abdel-Shakour, 2011, Banat *et al*., 1996, Azmi, 1998, Alhassani *et al*., 2007, Ghasemi *et al*., 2010). Fungi however have exhibited greater potential and as a result they have been intensively studied for environmental bioremediation processes (Maciel *et al*., 2010).

The white rot fungi, a group of diverse eco-physiological fungi which are composed of basidiomycetes and litter decomposing fungi, have received a lot of attention due to their capacity to degrade synthetic chemicals such as azo dyes (Marina *et al*., 2010, Dzulkafli, 2012, Maciel *et al*., 2010).They possess a ligninolytic enzyme system secreting lignin peroxidase (LiP), manganese peroxidase (MnP), and laccase (Lac) which degrade synthetic dyes and dye based effluent. White rot fungi have an excellent ability to reduce environmental pollution and thus are an impressive alternative to the currently existing approaches of waste water treatment (Selvam *et al*., 2003, Eichlerova, *et al*., 2007). Consequently, they have become a major area of research focus and a huge number of fungal strains have been investigated

for potential application in treatment of industrial waste water (Zeinab *et al*., 2013, Nadeem *et al*., 2014, Sadia and Asgher, 2011). Although various aspects on physiology, biochemistry, chemistry and genetics of the ligninolytic enzyme system of the white rot fungi have been studied, further research thrust is required for optimisation of parameters for sustainable commercialisation. The objective of this study was to screen for and evaluate ligninolytic enzyme activity in *P. ostreatus* mushroom as an initial step in a series of studies targeted towards the long term goal of biotechnological exploitation for large scale industrial and environmental application in our local circumstances.

Materials and methods

Strains and Preparation of Pure Cultures and Maintenance

The fungal strain used in this study was propagated from sterile tissue of fresh basidiocarps of oyster mushroom, *P. ostreatus* which were sourced from a local grower. A pure culture of this *P. ostreatus* was prepared using the tissue culture method. A fresh and tender fruiting body was longitudinally split into two and using a sterile blade; a small piece of the mushroom tissue was excised and aseptically transferred to sterile agar plates of Potato Dextrose Agar (PDA) (OXOID, Ltd Basingstoke Hampshire, England) containing 300mg/ml ampicillin (Sigma, USA). The antibiotic was incorporated in the media to inhibit bacterial growth – however PDA plates without antibiotic were also used. The petri plates were incubated in the dark at 25^{o}C. Fungal growth reached the edge of the plate after 10 days. Mycelium from the plates was aseptically transferred to PDA slants and again incubated in the dark at 25^{o}C for at least one week to obtain pure stock cultures that would be used in subsequent stages of this work.

Solid Phase Decolourisation Assay

The pure fungal cultures of *P. ostreatus* were screened for ligninolytic enzyme production using solid phase decolourisation of aromatic food dyes. The assay was carried out on PDA plates with100mg/l of individual commercial food dyes - Sunset Yellow, Orange C10 and Lemon Yellow. The agar plates were aseptically inoculated at the centre with 1cm^{2}mycelia plugs cut from actively growing fungal mycelia on agar plates. Inoculated petri plates with the same media but without the dyes and agar plates with the dyes but with no inoculum served as controls. The experiment was performed in triplicate for each culture. The plates were incubated in the dark at 25^{o}C and observed for results after the plates were fully colonised at 10 days.

Solid State Fermentation and Production of Enzymes

Solid state fermentation (SSF) for enzyme production was achieved on medium containing 36g wheat bran mixed with 4g of soybean flour (i.e. 90% bran and 10% soybean flour). The mixture was humidified with 0.1M sodium phosphate (NaH_2PO_4) buffer added at 60% v/w at pH 6.0. The medium was then sterilised by autoclaving at 120^{o}C for 60 minutes in 1l Erlenmeyer flasks. The sterile medium was aseptically inoculated with 1cm^{2} discs of actively growing fungal mycelia on PDA plates. The flasks were incubated in the dark at 30^{o}C for 10 days to achieve full colonisation.

Preparation of Samples and Extraction of Enzymes

After incubation, contents of the flask containing fully colonised SSF medium were dried in an oven for 24 hours at 40^{o}C. They were then ground into a powder in a coffee grinder for 2 minutes. The crude enzyme extract was obtained by soaking the ground medium in potassium phosphate buffer at pH 6.0 for 15minutes.This was followed by centrifugation at 6000 rpm for 15 minutes and filtration of the supernatant through Whatman No 1 filter paper to remove residual particles. The filtrate was stored in sterile glass bottles and stored in the refrigerator at 4^{o}C. This was regarded as the crude enzyme extract to be later used in subsequent enzyme activity assays.

Assay for Lignin Peroxidase Activity

Lignin peroxidase activity was determined by monitoring the H_2O_2 dependent oxidation of veratryl

alcohol to veratraldehyde according to Tien and Kirk, 1988. The assay mixture contained (in a final volume of 2.5ml), 1.8ml of crude enzyme extract, 0.1ml of 50mM veratryl alcohol, 0.5ml of 0.5M sodium tartrate buffer (pH 3.0) and 0.1ml of 10mM H_2O_2. Oxidation of the substrate at room temperature was measured by an absorbance increase at 310nm due to the formation of veratraldehyde. Controls without H_2O_2 were also used. One unit of enzyme activity (expressed as enzyme units per litre U/l) was considered as the amount of enzyme producing 1µmol of veratraldehyde per minute under the assay conditions with molar extinction ε 310 = 9300/M/cm.

Results and discussion

Fungal Mycelial Growth

The cultural methods used in this study for production of fungal mycelia from young and fresh fruiting bodies of *P. ostreatus* are routine and have been described by other workers, (Dhouib *et al.*, 2005,Shittu *et al.*, 2005). The purpose of producing pure fungal mycelium was to obtain active and vigorous cultures that would rapidly colonise the Solid State Fermentation substrate for efficient enzyme production. Fungal mycelia of *P. ostreatus* was successfully grown without contamination on both PDA media with and without antibiotic suggesting that strict practice of aseptic technique may eliminate the need for antibiotic use in the establishment of fungal cultures. Thick cottony white fluffy mycelia covering the entire petri dish were produced within 10 days (Fig. 1).These visual observations are consistent with the results of other workers who recommend PDA as one of the most preferred media for tissue culture and maintenance of *P. ostreatus* (Mehta and Bhandal, 1988, Rehana *et al.*, 2007, Maz Al *et al.*, 2012).

Fig. 1. Mycelia of *P ostreatus* growing in PDA plates on day 1 and after 10 days of incubation in the dark.

Aromatic Dye Decolourisation

The ability to decolourise dyes by the cultured *P. ostreatus* fungal mycelium was assayed on PDA plates containing aromatic food dyes; Sunset Yellow, Orange C10 and Lemon Yellow. These randomly selected food dyes, are commercially important dyes, with a wide range of uses both at household level and industrial scale. They, to a very significant extent, contribute to the challenges associated with waste water treatment and disposal (Marina *et al.*, 2010, Gianfreda *et al.*, 2003). The decolourisation experiments were performed in triplicate and assessed for visual disappearance of colour over a 10 day period (Fig. 2). All the food dyes Sunset Yellow, Orange C10 and Lemon Yellow were effectively decolourised by fungal mycelia of *P. ostreatus* after 10 days of active growth. The observation strongly suggested the presence of lignin modifying fungal peroxidase enzymes; laccase, manganese peroxidase and lignin peroxidase which play a role in the degradation of synthetic lignin or dyes (Roushdy and Abdel-Shakour, 2011).Our results indicated that the food dyes were degraded and decolourised under ligninolytic conditions - the excellent potential of oyster mushroom (*P. ostreatus*) in dye decolourisation was thus exhibited. Research in other laboratories has also demonstrated the application of *P. ostreatus* in dye degradation (Erkurt *et al.*, 2007,

Liu *et al*., 2004).This dye degrading property has earned *P. ostreatus* a place amongst strong candidates of industrially valuable enzyme producers (Maciel *et al*., 2010, Cho *et al*., 2009, Vikineswary *et al*., 2006) and it is indeed worthwhile exploiting it for commercial applications.

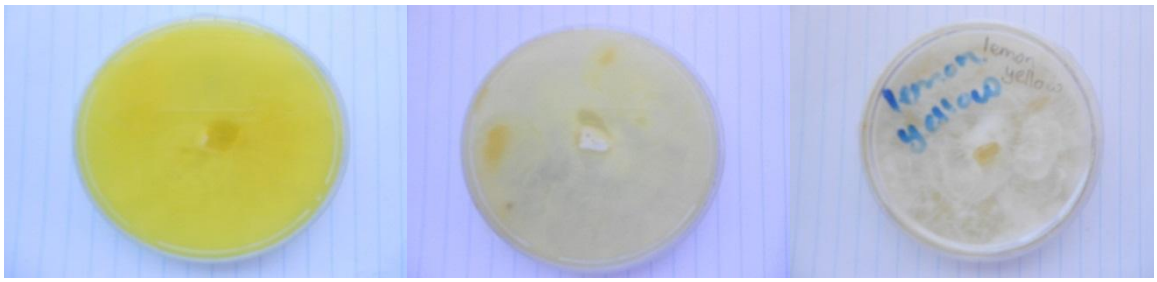

Fig. 2. Solid phase decolourisation assay of *P ostreatus* on Lemon Yellow food dye.

Enzyme Production in Solid State Fermentation

Oyster mushrooms can successfully grow on a wide range of ligninocellulosic substrates due to their ability to produce and secrete extracellular oxidative enzymes which are capable of degrading lignin thereby releasing nutrients for fungal growth (Mane *et al*., 2007, Ferdinandi *et al*., 2014). In this study, *P. ostreatus* produced thick cottony white fluffy mycelia colonising the entire wheat bran substrate within 10 days at 30^{o}C and secreting dye degrading ligninolytic enzymes under the given conditions (Fig. 3).

Fig. 3. Solid state fermentation of *P.ostreatus* on wheat bran and soya bean medium.

Other workers have previously reported a range of 12 to 41 days for complete mycelial colonisation in various *Pleurotus* species grown on different types of lignocellulosic substrates (Ferdinandi *et al.*, 2014, Mane *et al.*, 2007, Mshandete and Cuff, 2008). The results of our current study suggest that the *P. ostreatus* fungal mycelium was capable of effectively decolourising commercial food dyes and also successfully growing on SSF media containing wheat bran supplemented with soya bean flour. This observation implies that *P. ostreatus* was capable of synthesising and secreting dye degrading ligninolytic enzymes under solid state fermentation on an agricultural based substrate. Furthermore, the effective dye decolourisation action demonstrated significant potential application of this fungus in water purification and waste water treatment.

Lignin Peroxidase (LiP) Activity

Enzymatic digestion of lignocellulosic substrates is a complex secondary metabolic mechanism which is brought about by a mixture of several extra cellular enzymes of which lignin peroxidases are the most crucial. In this study, an average lignin peroxidase activity of 7.635U/ml was observed under SSF by *P. ostreatus* in wheat bran/soya bean substrate (9:1) after 10 days of full substrate colonisation. Although various LiP enzyme yields on a variety of substrate formulations have been reported by other authors (Parani and Eyini, 2012, Sadia and Asgher, 2011, Widiastuti *et al.*, 2008), our current results lie within the ranges reported by Ferdinandi *et al.*, 2014 and Zeinab *et al.*, 2013. The differences in enzyme yield could be due to the relative composition of the substrate polysaccharides and the supplements incorporated, the size of the substrates used, and probably the presence of natural inducers such as aromatic compounds (Papinutti and Forchiassin, 2007). Results of the enzyme activity assay demonstrated that *P. ostreatus* produced ligninolytic enzymes of which the presence of LiP was confirmed. The results of our study have collectively indicated that ligninolytic enzymes play a role in the degradation of dyes by *P. ostreatus*. The study has generated adequate information for the formulation of further scientific investigations on relevant parameters of ligninolytic enzyme production for possible scaling up and commercialisation.

Conclusion

The study demonstrated that *P. ostreatus* can be successfully propagated on locally available residue based agricultural substrates and has the ability to produce ligninolytic enzymes which are effective for degradation of ligninolytic compounds. Ligninolytic enzymes as biocatalysts have the potential to replace the conventional processes of several industries including the water purification industry especially in the treatment of dye effluent in this era where excessive use of chemicals is being discouraged. The biotechnological significance of these enzymes has led to a drastic increase in the demand for ligninolytic enzymes in recent times. The development of processes based on these ligninolytic enzymes is indeed an attractive solution due to their potential in degrading dyes of diverse chemical structure. Due to their versatility, they will find use in a wide variety of industries. Extensive exploitation of *P. ostreatus* will contribute significantly to bioremediation whilst promoting the local mushroom production industry for sustainable economic growth.

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