Effect of surfactants on lignin modifying enzyme activity of *Ganoderma* species KX879638 and *Lentinus* species KY006984 under solid state fermentation

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Lignin modifying enzyme (LME) activity [laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP)] was studied in two indigenously isolated white rot fungi, *Ganoderma* species KX879638 and *Lentinus* species KY006984 under solid state fermentation (SSF) of wheat bran and saw dust supplemented with various non-ionic polysorbate surfactants (Tweens) and Triton X-100. Both fungal species proved to be highly ligninolytic and promising laccase producers. Their laccase activity increased many folds due to supplementation of substrates with surfactants. Among surfactants, Tween 20 was the best, giving 39180 IU/g laccase in *Ganoderma* spp. and 37780 IU/g in *Lentinus* spp. on wheat bran corresponding to 3.8- and 10.6-fold increase over control condition. Triton X-100 was the best surfactant providing 29380 and 19877 IU/g laccase activity in *Ganoderma* spp. and *Lentinus* spp. on saw dust amounting to 2.9- and 3.9-fold increase over control condition, respectively. *Ganoderma* spp. always produced more laccase than *Lentinus* spp. on supplemented substrates. Between substrates, laccase production on supplementation was always more on wheat bran than saw dust. LiP and MnP activities were very low in comparison with laccase in both fungi under control conditions (1.92 to 4.07 IU/g and 0.98 to 2.07 IU/g, respectively) and upon treatment with surfactants; they did not show an appreciable change, thus, supplementation mediated ratio between laccase to LiP/MnP was larger.

Key words: White rot fungi, solid state fermentation, surfactants, laccase, lignin peroxidase, manganese peroxidase.

INTRODUCTION

White-rot fungi and their lignin modifying enzymes (LMEs) are considered promising for a wide range of industrial and environmental applications such as textiles, biological pulping, food industry, cattle feed production, production of biofuel and various other industrial products, and bioremediation (Kamm and Kamm, 2004; Rodríguez Couto and Toca-Herrera, 2006; Arora and Sharma, 2010; Osma et al., 2010; Fernandez-Fernandez...
et al., 2013; Kuhar et al., 2015; Sitarz et al., 2016; Senthivelan et al., 2016; Upadhay et al., 2016). These fungi are efficient lignin degraders and possess a very efficient enzymes system consisting mainly one or more of the three families of extracellular and non-specific LMEs, lignin peroxidases (LiP), manganese peroxidases (MnP) and laccases (Lac) (Gianfreda and Rao, 2004). More than 1,500 species of white-rot fungi have been reported to decompose lignin with little consumption of cellulose (Tian et al., 2012), but only some of them for example *Trametes*, *Phanerochaete*, *Pleurotus*, *Lentinus* (Elisashvili et al., 2008), *Ganoderma* (Adaskaveg et al., 1990; D’Souza et al., 1999; Zhou et al., 2013), *Phlebia* (Vares et al., 1994; Hatakka et al., 2003) and *Cerrena* species HYB07 (Yang et al., 2016) have been reported to show high potential for production of LMEs.

The increasing demand for LMEs, particularly, laccase which is in high demand in various industrial and biotechnological applications, necessitates the search for promising white rot fungi possessing excellent lignin-degrading systems (Chen et al., 2012; Fang et al., 2015; Fratocchi-Cardenas et al., 2016; Kandasamy et al., 2016).

Among white rot fungi, *Ganoderma* and *Lentinus* are two important genera able to grow on wood residue as well as other agro-lignocellulosic biomass materials and this makes them potential candidates for being employed in lignocellulose degradation. Several studies have been done in the past by various workers on different indigenously isolated species and strains of *Ganoderma* (Adaskaveg et al., 1990; Varela et al., 2000; Silva et al., 2005a; Songulashvili et al., 2006; Elissetche et al., 2007; Revankar et al., 2007; Elisashvili et al., 2009; Maria et al., 2009; Ding et al., 2012; Sitarz et al., 2013; Manavalan et al., 2013, 2015; Zhou et al., 2013; Sharma et al., 2015) and *Lentinus* (Leatham, 1985; Buswell et al., 1995; Crestini et al., 1996; Hatvani and Mecs, 2002; Boer et al., 2004; Pukahuta et al., 2004; Silva et al., 2005b; Lechner and Papinutti, 2006; Elisashvili et al., 2008) and they have reported that various species and strains within the same genus have different degradation pattern and ligninolytic potential. As reported for other white rot fungi (Elisashvili et al., 2008; Wong, 2009; Arora and Sharma, 2010), they found that all *Ganoderma* strains do not produce all LMEs at the same time, some secrete only one or two of them, and the types of enzymes produced are influenced by the type of strains, growth media and culture conditions.

Low cost ligninolytic enzyme production for large scale industrial application requires inexpensive and easily available lignocellulosic substrates (Papinutti et al., 2003) and a suitable fermentation system where conditions have been properly optimized for enhanced production of enzymes by selected fungal species (Kuhar et al., 2015). Many studies have shown that Solid State Fermentation (SSF) process is more suitable than submerged fermentation (SmF) for obtaining higher enzyme production by filamentous fungi (Viniegra-Gonzalez et al., 2003; Pandey, 2013) and the level of their LMEs production can be further enhanced by supplementing the fermentation medium with various aromatic inducers and metal ions (Elisashvili et al., 2010; Piscitelli et al., 2011; Manavalan et al., 2013; Yang et al., 2013, 2016; Kuhar and Papinutti, 2014) which regulate gene expression at the level of transcription (Elisashvili and Kachlishvili, 2009; Piscitelli et al., 2011). Supplementation of substrate with surfactants has also been explored for increasing enzymes production (Lestan et al., 1993; Zheng and Obbard, 2001; Urek and Pazarloglu, 2007; Zhang and Cheung, 2011; Usha et al., 2014). They increase the bioavailability of less soluble substrates for the fungus, improve the permeability of cell membrane leading to greater secretion of the extracellular enzyme, enhance enzyme stability and also prevent the denaturation of enzymes during hydrolysis by desorbing them from substrate (Helle et al., 1993; Anuja et al., 2004; Reddy et al., 1999; Zeng et al., 2006; Liu et al., 2008; Zhou et al., 2011).

In previous work by the authors, two isolates of tropical white-rot fungi collected from nearby forest produced substantial amount of ligninolytic enzymes in unoptimized SSF condition (Singh et al., 2014). Based on morphology and analysis of internal transcribed ribosomal sequence with ITS1 and ITS4 primers, the strains were identified as *Ganoderma* spp. and *Lentinus* spp. (GenBank accession number KX879638 and KY006984, respectively). Subsequently, the SSF conditions was optimized by using one-factor-at-a-time (OFAT) approach for enhancing LMEs production by both fungi on two very commonly used substrates in such studies, wheat bran and saw dust.

In the present study, laccase, MnP and LiP production capabilities under SSF of wheat bran and saw dust supplemented with various non-ionic polysorbate surfactants (Tweens) and Triton X-100 were evaluated.

**MATERIALS AND METHODS**

**Chemicals**

All chemicals used in the experiment were of analytical grade and of the highest purity available, procured from Sigma and Hi-Media Laboratories, Mumbai, India. Wheat bran and saw dust were collected locally and packed in a sealed plastic bag for further use in SSF.

**Screening of fungal isolates for laccase activity**

 Cultures of *Ganoderma* spp. KX879638 and *Lentinus* spp. KY006984 were maintained on 2% (g/L) malt extract agar (MEA) and incubated at 25°C for 8 to 10 days (Vasdev et al., 2005). Primary screening of the isolates was performed on 2% (w/v) malt extract agar plate containing 0.01% guaicol (Klikskinen et al., 2004). The presence of brick red color around the mycelium was considered as guaicol oxidizing laccase secreting organism (Figure 1). The cultures were maintained by periodic sub-culturing and were stored at 4°C.
Ligninolytic enzyme production under SSF

SSF for LMEs production was carried out in 250 ml Erlenmeyer flasks at the optimum conditions (optimized by one-factor-at-a-time approach). Production was carried out in the following conditions: carbon source, wheat bran/saw dust (5 g); nitrogen source, ammonium sulphate (0.05%); temperature, 25°C; pH 5.0; inoculum age, 8th day old culture; inoculum amount, 4 fungal discs (8 mm in diameter); and substrate to moisture ratio, 1:4 under aseptic conditions (Sharma et al., 2005; Deswal et al., 2012). The flasks were patted gently at their bottoms to shake the substrate for air exchange at regular intervals of 24 h after the onset of fungal growth.

Effect of surfactants on LMEs production

To evaluate the effect of surfactants on laccase production, non-ionic polysorbate surfactants (Tween 20, Tween 60 and Tween 80) and Triton X-100 were incorporated at 0.4% into the production medium after 48 h of inoculation. The control flasks were devoid of any surfactant. The fermentation was carried out for 7 days at 25°C.

Enzyme extraction

Enzyme extraction was done by suspending fungal fermented substrate in citrate buffer (pH 5.0, 100 mM) in fermented solid to liquid ratio of 1:10 and shaken gently for 30 min at 25°C. The extrudates were squeezed through muslin cloth for maximizing enzyme extraction and centrifuged at 10,000 rpm at 4°C for 10 min. The enzyme extract thus obtained was assayed for enzyme activity (Sharma et al., 2015).

Enzyme assays

Enzyme assay was performed using the supernatant obtained after centrifugation. Laccase activity was determined by monitoring spectrophotometrically at 470 nm using guaiacol as substrate (Diwaniyan et al., 2010). Manganese peroxidase (MnP) activity was determined in a reaction mixture containing 1 mM Di methoxy phenol (DMP) in sodium tartarate buffer (pH 4.5) using H2O2 as an oxidizing agent, and the absorbance was read at 469 nm (Martinez et al., 1996). Lignin peroxidase (LiP) activity was estimated by the method of Tien and Kirk (1988) which is based on oxidation of veratryl alcohol (3,4-dimethoxybenzyl alcohol) to veratryldehyde in the presence of H2O2, and absorbance was taken at 310 nm. To compare the enzyme activity of fungi grown under SSF on saw dust and wheat bran, all enzyme activities were expressed in international units per gram (IU/g). Triplicate experiments were performed for each enzyme assay.

RESULTS

LMEs activity in control conditions (without supplementation with surfactants)

Both fungi showed very high ligninolytic activities. Ganoderma spp. produced more LMEs than Lentinus spp. during SSF on both substrates (p<0.01). Its laccase activity on wheat bran and saw dust was 10,257 and 8971 IU/g which were much higher in comparison with Lentinus spp. (3555 and 5131 IU/g, respectively) (Figure 2a to c). Both fungi show very low activities of LiP and MnP (Figure 2). LiP activity by Ganoderma and Lentinus spp. was 4.07 and 2.22 IU/g on wheat bran and 2.45 and 1.92 IU/g on saw dust, respectively indicating that the former produces more LiP than the later, and also that wheat bran provided more LiP than saw dust. MnP activity, on the other hand, shows an opposite trend. Its secretion by both fungi was more on saw dust (2.07 and 1.92 IU/g) and less on wheat bran (0.98 and 1.25 IU/g). Further, Ganoderma spp. secreted more LiP than MnP both on what bran and saw dust, whereas Lentinus spp. secreted more LiP than MnP on wheat bran but on saw dust; its LiP and MnP secretion was similar.

LMEs production on supplementation of substrates with surfactants

Laccase activity

Supplementation of substrates with all surfactants
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Figure 2. Effect of surfactants on LMEs production by *Ganoderma* spp. and *Lentinus* spp. on wheat bran and saw dust under SSF.

tremendously increased laccase secretion by both fungi and the quantum increase was profoundly more on wheat bran than saw dust (Figure 2a). As witnessed in control condition, here also, *Ganoderma* spp. yielded more laccase than *Lentinus* spp. on both substrates irrespective of the type of surfactants used for supplementation. Its laccase activity on surfactant supplemented wheat bran and saw dust ranged between 35013 and 39180 IU/g and 22360 and 26083 IU/g, whereas for *Lentinus* spp. it was between 27573 and 37780 IU/g and 17723 and 19877 IU/g, respectively. Further, the quantum increase in laccase activity of the two fungi due to supplementation over their respective controls also varied widely. It was 2.5 to 2.9-fold and 3.4 to 3.8-fold on saw dust and wheat bran, respectively for *Ganoderma* spp., whereas 3.5 to 3.9-fold and 7.8 to 10.6-
fold for *Lentinus* spp. on the same substrates, respectively.

Among four surfactants, supplementation of wheat bran with Tween 20 resulted in the highest laccase activity both in *Ganoderma* spp. (39180 IU/g) and *Lentinus* spp. (37780 IU/g) and it was 3.8- and 10.6-fold higher in comparison with their respective control conditions. The order of other surfactants in enhancing laccase activity on this substrate in both fungi was Tween 60 (38127 and 34553 IU/g), Triton X-100 (37887 and 29380 IU/g) and Tween 80 (35013 and 27553 IU/g), respectively, and the minimum and maximum enhancement in laccase activity by them ranged between 3.4 and 3.7-fold for *Ganoderma* spp. and 7.8 and 9.7-fold for *Lentinus* spp.

Conversely, on saw dust, Triton X-100 supplementation resulted in the maximum laccase secretion by both fungi. It was 29380 IU/g for *Ganoderma* spp. and 19877 IU/g for *Lentinus* spp., thus giving 2.9- and 3.9-fold increase over respective control conditions. Tween 80 and 20 were next to Triton X-100 in enhancing the laccase activity of both fungi grown on saw dust. They were found to be almost equally effective in enhancing the laccase activity of *Ganoderma* spp. and *Lentinus* spp. by 2.6-2.7- and 3.8-3.9-fold, respectively. Tween 60, ranked second among the surfactants in enhancing laccase activity of both fungi on wheat bran, was found to be at the 4th position here, giving 2.5- and 3.5-fold increase in *Ganoderma* and *Lentinus* spp., respectively.

**LiP activity**

LiP secretion by both fungi was very low in control conditions (1.92 to 2.45 IU/g on saw dust and 2.22 to 4.07 IU/g on wheat bran), and declined further on supplementation with surfactants (0.80 to 1.57IU/g on saw dust and 1.61 to 2.84 IU/g on wheat bran; Figure 2b) barring one exceptional increase recorded in case of *Lentinus* spp. grown on Triton X-100 supplemented saw dust (2.53 IU/g). Nevertheless, in most of the treatments, LiP production by both fungi was more on wheat bran than saw dust. In terms of absolute quantity, the minimum and maximum percent decline in LiP production ranged between 11 and 60%. Quantitative decrease in LiP activity due to surfactants was comparatively more in *Ganoderma* spp. than *Lentinus* spp. and in the later one, decline was marginal (11 to 22%) on wheat bran but substantial (26 to 58%) on saw dust. Amongst surfactants, adverse effect of Tween 20, Tween 80 and Triton X-100 on LiP activity on wheat bran by both fungi was lesser in comparison with Tween 60. No such pattern was observed on saw dust for a particular fungus or surfactant.

**MnP activity**

MnP secretion in both fungi decreased (0.98 to 1.25 IU/g on wheat bran and 1.92 to 2.07 IU/g on saw dust) in comparison with control conditions. Substrate supplementation with surfactants did not affect much the absolute quantity of MnP produced by both fungi on both substrates (Figure 2c), though it increased in both fungi grown on saw dust supplemented with any of the surfactants (2.10 to 2.68 IU/g) as well as in *Ganoderma* spp. grown on Tween 20 and Triton X-100 supplemented wheat bran (1.58 and 1.94 IU/g, respectively), or showed a substantial decrease in *Lentinus* spp. grown on the wheat bran (0.33 to 0.76 IU/g) supplemented with any of the surfactants. MnP production by both fungi was always more on saw dust than wheat bran.

On saw dust, MnP activity in both fungi was almost comparable for all surfactant treatments; however, when viewed in terms of percent increase over respective controls, it was more for *Lentinus* spp. (19 to 40%) than *Ganoderma* spp. (02 to 22%).

The surfactants providing the highest MnP secretion in both fungi were Tween 20 and Triton X-100 on wheat bran and only Tween 20 on saw dust.

**DISCUSSION**

In this study, local isolate of both fungi, *Ganoderma* and *Lentinus* spp., were found to be highly liginolytic and promising laccase producers. Within a short fermentation period of optimized SSF conditions, they exhibited much higher level of laccase (5130 to 10257 IU/g), but very low and almost similar level of LiP (1.92 to 4.07 IU/g) and MnP (0.98 to 2.07 IU/g) (Figure 2a to c). The results are in conformity with similar findings made by other workers that some species of white rot fungi secrete only one or two LMEs (Elisashvili et al., 2008; Wong, 2009; Arora and Sharma, 2010). Such variations have also been reported in various *Ganoderma* spp. and strains exhibiting varying abilities to secrete LMEs; some species secrete all LMEs in good amount whereas others produced more laccase with either low levels of MnP and LiP or no LiP activities at all (Adaskaveg et al., 1990; D’Souza et al., 1999; De Souza Silva et al., 2005; Silva et al., 2005a; Ding et al., 2012; Zhou et al., 2013). Similar behaviour has also been reported in *Lentinus* spp. *Lentinula edodes* has been reported as laccase and MnP producers on solid media or during SSF on tree leaves (Hatvani and Mecs, 2002; Silva et al., 2005b; Elisashvili et al., 2008), whereas CCB-42 strain of this fungus was found to produce only MnP, with negligible amount of laccase and LiP on corn cob solid state cultures (Boer et al., 2004).

Revankar et al. (2007) reported 10,050 IU/g laccase yield from indigenously isolated *Ganoderma* spp. in SSF on wheat bran supplemented with additional carbon and nitrogen source which is comparable to our indigenous isolate of *Ganoderma* spp. A higher yield of laccase (37,423 IU/g) has been reported in *Ganoderma* spp. rckk-
Cultivation of both fungi under similar SSF conditions also revealed a wide difference between the two species in laccase production. The amount of laccase produced by *Ganoderma* spp. was always more than *Lentinus* spp. under control as well as surfactant treated conditions on both substrates (Figure 2a). In the present study, laccase activity in both fungi increased many fold due to supplementation of substrate with all surfactants. Further, for a particular fungus-substrate combination, the quantum increase in laccase activity by all surfactants differed very narrowly except in the case of *Lentinus* spp. saw dust combination where Tween 20 proved much better than others. Usha et al. (2014) also found all surfactants (Tween 20, Tween 80 and Triton X-100) effective in enhancing laccase activity in *Stereum ostrea* grown on wheat bran. However, they reported maximum laccase activity with Tween 80 followed by Tween 20 and Triton X-100, whereas in the present study it was Tween 20 that induced a maximum of 39180 IU/g laccase in *Ganoderma* spp. and 37780 IU/g in *Lentinus* spp. corresponding to 3.8- and 10.6-fold increase over control condition. Lestan et al. (1994) reported an increase in yield with both Tween 20 and 80 supplementation. In the present study, Tween 80 could provide laccase activity comparable to Tween 20 in both fungi on saw dust but not on wheat bran where it was comparatively inferior. Asther et al. (1987), however, reported no enhancement in ligninase production with Tween 80 in *Phanerochaete chrysosporium* INA-12.

Triton X-100 was found as the best surfactant for saw dust providing 29380 and 19877 IU/g laccase activity in *Ganoderma* and *Lentinus* spp., amounting to 2.9- and 3.9-fold increase over control conditions. When grown on Triton X-100 treated saw dust, the laccase activity of these species was 30% less in comparison with their laccase activity on Tween 20 treated wheat bran. In comparison with Triton X-100, Tween 20 treated saw dust could also induce equivalent amount of laccase activity in *Lentinus* spp., and slightly less in *Ganoderma* spp.

LiP and MnP activities were very low, in comparison with laccase, in both fungi under control conditions (1.92 to 4.07 IU/g and 0.98 to 2.07 IU/g, respectively) and supplementation with surfactants did not show any appreciable quantitative change in their activity (Figure 2b and c). As a result, ratio between laccase and LiP/MnP in surfactants treated conditions was much larger. In most of the cases, treatment of saw dust with surfactant improved MnP activity slightly but with a resultant decrease in LiP activity. On wheat bran, both MnP and LiP activities decreased slightly except in Tween 20 and Triton X treated conditions for *Ganoderma* spp. Usha et al. (2014) reported enhanced activity of MnP by surfactants in the order of Tween 80, Tween 20, and Triton X-100, but without any enhancement in LiP activity. Urek and Pazarlioglu (2007) also reported 2-fold increase in MnP activity on Tween 80 supplementation.

The results obtained in the study proved wheat bran as a better substrate than saw dust for laccase production in surfactant supplemented conditions and *Ganoderma* spp. always showed more laccase activity on this substrate (Figure 2a). Similar observations have been made in other studies (Revankar et al., 2007; Elisashvili et al., 2009; Songulashvili et al., 2007). LiP and MnP activities on wheat bran and saw dust respectively were always found to be higher in both fungi in control as well as surfactant treated conditions.

**Conclusion**

The indigenous strains of *Ganoderma* and *Lentinus* spp. were found to be excellent laccase producers with very low LiP and MnP activities under optimized SSF condition. Within a short fermentation period of 7 days on Tween 20 supplemented wheat bran, they produced 39180 and 37780 IU/g laccase, respectively. Their laccase production on saw dust was maximally enhanced by Triton X-100, but it was 30% lesser in comparison with Tween 20 treated wheat bran. Supplementation with surfactants increased laccase activity enormously in both fungi but without any consequent change in LiP and MnP activities. As a result, the ratio between laccase and LiP/MnP was always larger. These indigenous isolates of both fungi need to be further examined for their suitability for large-scale laccase production for various industrial-environmental applications.

**CONFLICT OF INTERESTS**

The authors have not declared any conflict of interests.

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