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Lignolytic enzymes (laccase and peroxidase) production by pink oyster mushroom, *Pleurotus djamor*

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ABSTRACT

This research work attempts the use of biogas digester liquid (BDL) as an inducer for lignolytic enzyme activity by *Pleurotus djamor,* when grown in SSF culture condition. BDL rich in polyphenols and micronutrients can induce lignin degrading enzymes such as laccase and peroxidase. Spraying 100 ml BDL/bag (1kg paddy straw)/day was found to increase yields of *Pleurotus djamor* in SSF and also reduced the time for various stages of mushroom growth such as pin head formation, fruiting body formation and first flush by 3-5 days. Of the 100ml BDL sprayed 70ml was collected as leachate and this showed high lignolytic enzyme (laccase 150 U/L and peroxidase 160U/L) activities in SSF and the enzyme activity coincided with the specific mushroom growth stages.

Key words: Biogas digester, Lignolytic enzyme, oyster mushroom, *Pleurotus djamor*

Pleurotus djamor is a white rot fungi, can grow on wide spectrum of lignocellulosic waste materials of agricultural origin such as paddy straw, wheat straw, maize straw, coir pith and cotton stalks, etc. They are capable of degrading complex aromatic biopolymer such as lignin, using their natural lignolytic enzyme (laccase, manganese peroxidase and lignin peroxidase) (Barh *et al.,* 2019). The degraded products are used as their energy source to produce protein rich edible fungal biomass (such as mushroom). Previous studies have demonstrated that simple nutrients and complex lignocellulosic agricultural substrates could affect the production of lignocellulolytic enzymes. *Pleurotus djamor* is an exotic wood-rotting edible mushroom also called as pink oyster and belongs to the family *Pleurotaceae*. It is an extremely fast growing mushroom that fruits easily on several of lignocellulosic substrates (Gupta *et al.,* 2018). Substrate formulation by combining rice straw, coco peat, and rice bran at 7:3:1 ratio initiated the higher yield with high biological efficiency of *P. djamor* in solid state fermentation (SSF). Biological efficiency of *P. djamor* and *P. eryngii* cultivated on safflower stalks (77.8% and 73.1, respectively) and stem of bean plant (78.2% and 67.0%, respectively) suggests the suitability of these lignocellulosic wastes as alternative substrate to sawdust and wheat straw for *P. eryngii* and *P. djamor* cultivation (Atila, 2017). Organic supplements such as pigeon pea flour, lentil flour and chickpea flour in SSF effectively enhance the yield (g/kg dry straw) and exhibited minimum days for spawn run, minimum days for first harvesting, maximum number of fruiting body per bag and maximum average weight of fruiting body (g/FB) of *Pleurotus djamor* (Singh *et al.,* 2017). All these being leguminous, the increase N and other nutrients could be implicated as the cause for higher yields. Higher yield performance of *Pleurotus florida* on

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various agro-industrial wastes reveals their use as growing substrate for commercial production of oyster mushrooms (Toppo and Chandravanshi, 2018).

Extracellular lignocellulolytic enzymes are produced during secondary metabolism of mushroom growth (Annepu *et al.,* 2018). Laccase is considered to be capable of degrading lignin together with lignin peroxidase and manganese peroxidise as reported by Youn *et al.* (1995). Laccase system in *P. djamor* is associated with the reproductive stage and is directly correlated with the growth and fruiting body formation and was detected from second day of incubation. The first activity peak (169.8-579.8 μM/g/min) was obtained on day 4 and the second peak of activity was observed during the initiation and development of fruiting bodies, with values ranging from 164.4 to 469.9 ìM/g/min (Salmones and Mata, 2015; Periasamy and Natrajan, 2004). The work of An *et al.* (2016a) cultivated *P. ostreatus* firstly on biomass substrate and the mycelia thus formed were used in submerged culture for lignocellulolytic enzyme production. In doing so they were able to achieve higher yields of laccase (543+21U/L) in the sequential solid state - submerged state method compared to conventional solid state and submerged state cultivation. Lignocellulolytic enzyme production can be enhanced by supplementing biomass substrates with inducers mainly polyphenols. The production of laccases by *P. sajor-caju* in the SSF was increased by use of agro-wastes *viz.,* coir pith waste, farm wastes of millets, banana leaves, pulses, oilseeds, cotton stalks, sugarcane trashes, cocoa leaf wastes, oil palm fibre wastes and spent coco peat (Thiribhuvanamala *et al.,* 2017a;b). Addition of ammonium tartrate (55 μM) as inducers in culture medium exhibited higher laccase activity by *Lentinula edodes* (shiitake) mushroom, reaching 251 U/mL of extract after 30 d of incubation (Cavallazzi *et al.,* 2005). Also adding inducers, such as copper and ferulic acid, enhanced the laccase production in SSF by *Pleurotus ostreatus* (Mazumder *et al.,* 2009). Using ABTS as chromogenic substrates on the agar plates is very useful for isolation laccase producing fungus (Alfarra, 2013). Kuhara and Papinutti (2014) revealed copper and ferulic acid as the best laccase inducers.

Malayil *et al.* (2016) for the first time reported the use of biogas digester liquid (BDL), containing polyphenols as inducer for production of lignin degradin enzymes. Spraying 100ml BDL/bag/day was found to increase yields of various *Pleurotus* species by 66–300%. BDL supplementation was also found to reduce the time for various stages of mushroom formation such as pin head formation, fruiting body formation and first flush was reported by Malayil *et al.* (2017). Inducing substances mainly aromatic or phenolic compounds related to lignin or derivatives stimulated lignocellulolytic enzymes production by white rot fungi. The type of induction material and the nature of the fungus play important roles in the expression of lignolytic enzymes. In view of these facts, the present study was carried out with an objective to evaluate the effect of biogas digester liquid (BDL) as an inducer for lignocellulolytic enzymes by *P. djamor* in SSF using paddy straw as growing substrate.

MATERIALS AND METHODS

Solid State Fermentation (SSF)

For SSF, pre-packed ready-to-fruit (r2f) mushroom bags, 1 kg weight inoculated with *P. djamor* and paddy straw as substrate were purchased from Bioscience Centre, Bangalore. These r2f bags had uniformly formed mycelia network and were at a ready-to-fruit stage (28d after inoculation). Two bags for water sprinkled controls and three bags for BDL sprinkled bags were studied (total five bags) in a metallic growth chamber lasting about 30d. The metallic chamber was made of mild steel, weld-mesh (25 × 25mm) covered by fine mesh (22’ 3mm; 8/22G) to keep the flies away. This chamber was in turn covered by closely woven jute-bags that were wetted with water every day to maintain humidity inside. The dimensions of metallic chamber was 0.6 × 1.2 × 0.6m (lbh), held 0.15m above ground on steel legs and could accommodate about 5 to 10 trays each carrying a one kg mushroom bag (Chanakya *et al.,* 2015; Ganguli and Chanakya, 1994). 100ml BDL sprayed per bag and 70 ml was collected as leachate. Lignocellulolytic enzymes (laccase and peroxidase) production by *P. djamor* in SSF was assessed by measuring the protein content and enzyme activities in leachate liquid at the alternate up to 30th day after the bags were opened.

Mushroom growth and harvest

The mushroom bags were opened after 28d of spawning when spawn run was completed. Spraying BDL/water on the bags leads initiation of the pin head stage along with the first flush. When the fruiting bodies developed they were harvested as first, second and third flush within an interval of 10-15days. The harvested mushrooms were weighed, washed and dried at in a hot air oven at 70+2 °C.

Sampling and enzyme assay

From the 100ml of BDL sprayed around 70ml leached out from the bags on daily basis (2L in 30 days). This was collected everyday and stored at "4°C as leachate collection. This mushroom leachate was assayed for enzymes such as laccase and peroxidase. The leachates collected from SSF were analyzed for various lignolytic enzymes such as laccase and peroxidase. The crude enzyme solution was obtained by centrifugation of the leachate at 5000rpm for 20min. Total protein in the crude enzyme extract was estimated using Lowry's method with BSA as standard (Sadasivam and Manickam, 1996).

Laccase activity

Laccase activity was determined spectrophotometrically by monitoring the oxidation of 50mM 2, 2-azino-bis-[3-ethyl benzothiazoline- 6-sulphonic acid] (ABTS) in 50mM acetate buffer pH 5. The reaction mixture contained 2mL of 50mM acetate buffer pH 5, 2mL of 1mM ABTS and 0.2mL enzyme solution. The blank contained the entire constituent except the active enzyme, which was replaced by 0.2ml of distilled water. Oxidation was recorded at 10min interval at 420nm up to 60min. One unit laccase activity was defined as the change in absorbance of the assay mixture at 420nm for 10min and the result was converted to ABTS oxidized per min to calculate unit activity. Laccase was expressed as enzyme unit per liter i.e. (µM/ min) /L.

Peroxidase activity

Peroxidase activity was determined by monitoring the oxidation of O-dianisidine by the enzyme at pH 6.5 and 35°C temperature. The reaction mixture contained 3.5mL phosphate buffer (pH 6.5), 0.2mL enzyme solution and 0.1mL O-dianisidine solution (1mg/mL). After adding 0.2mL of 0.2M H_2O_2, oxidation was recorded at 10min interval up to 60min at 430nm. Enzyme activity was expressed as the rate of increased absorbance per unit time per ìg of protein.

RESULTS AND DISCUSSIONS

Growth and yield of Mushroom

The use of BDL in SSF reduced the time for onset of various stages of mushroom growth such as days for pin head formation, fruiting body formation and first flushes (Table 1). The time taken for pin head formation for *P.djamor* in control was 30d and this time was reduced to 29d by using BDL. Fruiting body formation was initiated on 33d in control where as this was reduced to 32d in BDL sprayed bags. First flush was harvested on 35d in control bags where as in BDL sprayed it was on 33d.

BDL sprayed on substrate to maintain moisture content in pre-packed *P.djamor* bags increased the mushroom yield. Maximum yield (308.66g/kg wet wt. of substrate) was obtained from the BDL sprayed bags compared to control where it was (242g/kg wet wt. of substrate). The present study reveals that, total cropping duration of *P.djamor* was 61d in BDL sprayed bags and 58d in control. Malayil *et al.* (2016) reported maximum yield for *P. florida* in pre-packed

Table 1. Time taken for various growth stages (days)

	Pin head	Fruiting body	First flush	Second flush	Third flush	Total wt of the mushroom (g)
Control	30	33	35	58	-	242.00
BDL	29	32	33	51	61	308.66

mushroom bags sprayed with BDL compared to control bags sprayed with water. Spraying BDL increased the yield of *P. djamor* compared to control and also reduced the time for various stages of mushroom growth such as pin head formation, fruiting body formation and first flush in this study (Fig 1).

Production of laccase and peroxidase in SSF and SmF

Lignolytic enzymes (laccase and peroxidase) production by *P. djamor* was assessed by measuring enzyme activities in liquid leachate (SSF) on alternate days. Around 70 ml of leachate was collected after spraying 100 ml of biogas digester liquid on BDL sprayed bags and while 100 ml of water was used on control pre-packed mushroom bags.

Results in the present study shows that maximum laccase activity (150 U/L 24days) was achieved in BDL sprayed bags for *P.djamor* (Fig 2). BDL could be successfully used as a nutrient supplement for pre-packed mushroom bags. Also spraying BDL has the capability to initiate the production of higher lignin degrading enzymes such as Laccase and Peroxidase (Malayil *et al.,* 2017). Maximum Laccase activity was reported by Malayil *et al.* (2017) in BDL sprayed bags for *Hypsizgus ulmarius*. The first peak of activity for BDL sprayed bags was achieved on 8d and the second peak of activity was observed on 24d that coincided with both first flush and third flush when the mushrooms were ready to harvest and similar pattern of laccase production was observed for control as well. Laccase production in *P. djamor* is associated with the vegetative stage of mushroom and it has direct correlation with the growth and fruiting body formation (Periasamy and Natrajan, 2004; Salmones and Mata, 2015).

In the current study peroxidase activity (Fig. 3) was found to be higher in BDL supplemented leachate (170 U/L, 14 day) when compared to control (160U/L, 14 day). Peroxidase activity was reported to be higher in BDL supplemented leachate when compared to control for *P. florida* (Malayil *et al.,* 2016a; b) and *Hypsizgus ulmarius* (Malayil *et al.,* 2017) in SSF with paddy straw as substrate. This reveals that BDL has some compounds that are responsible for induction and increase production of lignolytic enzymes. Peroxidase production in BDL sprayed bags was high on 8d and 14d coinciding with pin head formation stage. This shows that

(a) (b) (c)

Fig. 1. Mushroom (*P. djamor*) growth and Yield (a-c) a: Pin head stage, b: Fruiting body stage and c: First flush

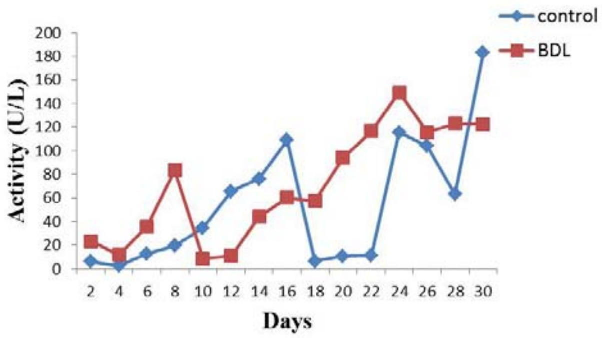

Fig. 2. Laccase activity

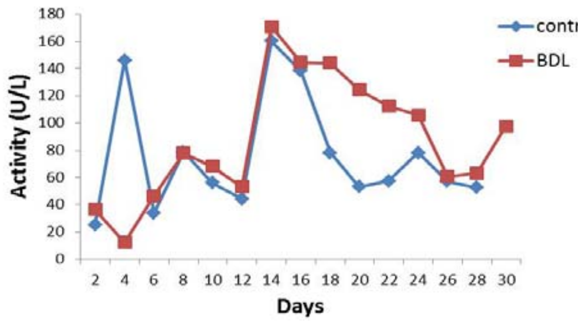

Fig. 3. Peroxidase activity

supplementing BDL to mushroom bags induced lignolytic enzyme production and simultaneously resulted in higher yield.

CONCLUSION

Addition of 100 ml of BDL on a daily basis to the pre-packed (*P. djamor*) mushroom bags allowed to maintain moisture while its constituents was found to increase mushroom yields compared to control. Further, leachate collected from BDL supplemented mushroom bags showed higher lignolytic enzyme (Laccase 150 U/L and Peroxidase 160U/L) activities in SSF (per kg wet wt. of substrate).

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