BIODEGRADATION OF DICHLOROPHENOLS BY BJERKANDERA ADUSTA AND LENTINUS SQUARROSULUS AND EXTRACELLULAR LIGNINOLYTIC ENZYME ACTIVITIES.

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Abstract

Dichlorophenols are a group of toxic compounds that have been widely used as biocides. Fifty fungal strains were screened for their potential to tolerate with 2,3-dichlorophenol, 2,5-dichlorophenol and 3,4-dichlorophenol on solid medium supplemented with 2% malt extract (MEA). Growth rate (mmday⁻¹) was determined at three concentrations (0.10, 0.15 and 0.2 mM) of all the three dichlorophenols. From the fifty fungal strains only Bjerkandera adusta and Lentinus squarrosulus were able to tolerate all the three dichlorophenols (DCPs). These white-rot fungi (WRF) were chosen for liquid medium studies for the biodegradation of DCPs and ligninolytic enzyme activity at 0.10 mM concentration over the period of 20 days. Both varieties completely degrade all the three DCPs. Aryl Alcohol Oxidase (AAO) is the main oxidase enzyme in B. adusta while laccase plays important role in L. squarrosulus. MnP (Manganese peroxidase) showed good activity with both varieties. The DCPs biodegradation potential of the WRF positively correlated with their potential to express ligninolytic enzymes such as laccase, AAO, MnP and lignin peroxidase (LiP). These fungal strains were capable to degrade DCPs and could be used for bioremediation applications on large scale.

Abbreviations:- AAO- Aryl alcohol oxidase, DCP- dichlorophenol, HPLC- high performance liquid chromatography, LiP- lignin peroxidase, MnP- manganese peroxidase, NPM- nutrient poor medium, NRM- nutrient rich medium, VA- veratryl alcohol, WRF- white rot fungi

Introduction:-
Chloroorganic contaminants are present in the environment due to their extensive use in agriculture and industry. Chlorophenols (CPs) are a group of toxic compounds that have been widely used as biocides to control bacteria, fungi, algae, mollusks, insects, slime and other biota. CPs that enter non-target upland, wetland, and aquatic environments associate with colloidal and particulate matter and, if not photodegraded, eventually settle onto surface soils (Shiu et al. 1994). The chlorinated phenols have been recognized one of the major environmental problems because they have been widely found in surface water and in wastewater, in particular, in pulp mill effluents (Schellenberg et al. 1984). Chlorinated phenols are considered to be relative persistent in the environment and they are highly toxic. The number of chloro substituents on the phenolic ring affects the optimum pH for peroxidases (Dec and Bollag 1990). There are 19 compounds of chlorophenols of three monochlorophenol, six isomeric substances each of dichlorophenol, trichlorophenol, as well as three isomeric substances tetrachlorophenol, and one fully chlorinated pentachlorophenol.
Chlorophenols are introduced into the environment through various human activities such as waste incineration, uncontrolled use of wood preservatives, pesticides, fungicides and herbicides, as well as via bleaching of pulp with chlorine and the chlorination of drinking water (Contrerasa et al. 2003). CPs are one of the most dangerous classes of environmental pollutants that have been produced in thousands of tons annually by the pulp and paper and agrochemical industries (Steiert et al. 1985). CPs with few chlorine atoms are used mainly as starting materials for the synthesis of higher chlorinated phenols or chlorophenol-derived products, such as the chlorophenoxyacetic acid herbicides. Because of the toxicity of CPs, techniques for their removal are urgently needed. Currently, many physical and chemical methods are available for decontaminating CPs. But, considering the high costs of these procedures, attention has been focused on oxidative coupling of CPs. The oxidative process gives rise to less soluble, higher molecular weight polymers; these can consequently be removed by sedimentation or filtration (Bollag et al. 2003).

Biodegradation of chlorophenols is dependent on number and position of chlorine substituents; the rate of degradation decreases with increase in number of chlorine substituents. Metabolism of CPs is not unusual in fungi. WRF are a group of filamentous wood decay fungi which have ability to metabolize lignin and the polysaccharide components of wood. WRF are also effective in destroying xenobiotics. Azo- and other textile dyes, polyaromatic hydrocarbons, amino-, nitro- and chloroorganic compounds, including chlorophenols, and other xenobiotics, were found to be substrates for these fungi and their extracellular enzymes (Field et al. 1993; Cameron et al. 2000; Pointing 2001). Extracellular production of ligninolytic enzymes is responsible for the biodegradation of CPs by fungi (Mileski et al. 1988; Reddy et al. 1988; Valli and Gold 1991). WRF capable of degrading CPs attracts considerable attention as an effective biotreatment tool in aqueous effluents (Michizae et al. 2001). The degradation pathways of several CPs by the WRF P. chrysosporium have been determined in vitro (Valli and Gold 1991; Joshi and Gold 1993; Reddy and Gold 2000). The bioremediation method depends on selecting the right microbes in the right place with the right environmental factors for degradation to occur.

Several studies are currently focused in the feasibility of using WRF for treatment of phenolic compounds which represent toxicity and an environmental concern. WRF have been shown to be potent degraders of a broad variety of recalcitrant xenobiotic compounds including polycyclic aromatic hydrocarbons (Aranda et al. 2010), polychlorinated biphenyls (PCBs), dioxins, DDT (Reddy and Mathew 2001) and industrial dyes (Faraco et al. 2009). Among xenobiocenviron mental concern, several monoaromatic compounds have been shown to be degraded by the Phanerochaete chrysosporium. 2,4-DCP (Valli and Gold 1991), 2,4,5-trichlorophenol (Joshi and Gold 1993) and pentachlorophenol (Mileski et al. 1988) were shown to be efficiently degraded by P. chrysosporium, where the fungus produce lignin peroxidase and manganese peroxidase (Grey et al. 1998). Extracellular ligninolytic enzymes are secreted from WRF and produced during a fermentation process and possess the ability to break down bonds within organic compounds and catalyze their transformation into less toxic and more biodegradable forms (Hatakka 2001). The main extracellular enzymes participating in lignin degradation and biodegradation of xenobiocencompounds are heme-containing lignin peroxidase (LiP, EC 1.11.1.14), MnP (EC 1.11.1.13) and Cu-containing laccase (EC 1.10.3.2) (Hatakka 2001). In addition, enzyme involved in hydrogen peroxide production such as AAO (EC 1.1.3.7) is considered to belong to the ligninolytic system (Hatakka 2001).

The objectives of this study were to accelerate the biodegradation of DCPs by two WRF. The ligninolytic enzyme pattern secreted during degradation of DCPs has been determined.

Materials and methods:-

Cultures:-
Mycelial cultures of various saprophytic, ligninolytic and mycorrhizic fleshy fungi were selected from the “Mushroom Gene Bank” of Directorate of Mushroom Research, Solan (Himachal Pradesh, India). Name of fifty different mycelial cultures are given in Table-1.

Chemicals:-
Analytical grade 2,3-DCP, 2,4-DCP, 3,4-DCP (MW 163.0 gmol\(^{-1}\)), Methanol (MW 32.04 gmol\(^{-1}\)) and Ortho Phosphoric Acid (OPA, MW 98 gmol\(^{-1}\)) were purchased from Merck. 2,2‘-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS, MW 548.7 gmol\(^{-1}\)) was obtained from Sigma. Veratryl alcohol (VA, MW 168.19 gmol\(^{-1}\)) was purchased from Aldrich. All other chemicals used were of analytical grade.
Screening of fungi:
2,3-DCP, 2,4-DCP, 3,4-DCP (0.1, 0.15, 0.2 mM) were individually added in 2% MEA containing 20 gl⁻¹ of malt extract and 20 gl⁻¹ of agar powder after autoclaving. Plates were inoculated with 8-10 days old mycelial culture bit (5 mm in diameter) in the centre and incubated at 25°C±1°C except Auricularia mesentrica, Volvariella spp., Calocybe indica, Lentinus squarrosulus, Ganoderma sp., Bjerkandera adusta and Phanerochaete chrysosporium were incubated at higher temperature (at 30°C±2°C). Radial mycelial growth was taken every 48 h and average radial growth was calculated. In control mycelium spread was complete, no further observations were recorded. The percentage inhibition in mycelial growth was calculated by comparing mycelial growth with DCPs treated plates and control. Reduction (%) in mycelial growth was calculated as follows:

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\% \text{inhibition} = \frac{\text{Growth (mm/day) control} - \text{Growth (mm/day) in treated plates}}{\text{Growth (mm/day) control}} \times 100
\]

High Performance Liquid Chromatography (HPLC) analysis of DCPs:-
The biodegradation studies of DCPs were carried out in the nutrient rich medium (NRM) containing 2 g of ammonium tartrate, 10 g of glucose, 1 g of KH₂PO₄, 1 g of yeast extract, 0.5 g of MgSO₄·7H₂O, 5 g of KCl, 1 ml of solution containing trace elements per liter of medium and nutrient poor medium (NPM) containing 10 g of glucose, 2 g of KH₂PO₄, 0.2 g of yeast extract, 0.1 g of peptone, 1 ml of solution containing trace elements per liter of medium. Solution of trace elements containing 2 g of KH₂PO₄, 10 mg of Na₂B₄O₇·10H₂O, 7 mg of ZnSO₄·7H₂O, 5 mg of FeSO₄·7H₂O, 1 mg of CuSO₄·5H₂O, 1 mg of (NH₄)₆Mo₇O₂₄·4H₂O, 1 mg of MnSO₄ dissolved in 100 ml of water (Espindola et al. 2007). The pH was adjusted to 7 before autoclaving at 120°C for 20 min. and 0.1 mM (16.3 μgml⁻¹) of 2,3-DCP, 2,5-DCP and 3,4-DCP were individually added after autoclaving. 150 ml Erlenmeyer flasks containing 50 ml of the liquid medium were inoculated with 5-8 days old 5 mycelium bits (5 mm in diameter). Three replicates of both media were incubated in static condition in BOD incubator and shaking condition in a rotatory shaker at 150 rpm at 30°C. The cultures were harvested on 5th, 10th, 15th and 20th day of incubation. Each sample was centrifuged (10,000 x g for 15 min) at 4°C. The supernatant of liquid culture was used for quantitative estimation of DCPs. Quantitative estimation of DCPs was done by reverse phase lichrosphere 5 μM, RP-18 columns (125 x 4mm ID). The apparatus consists of one LC-200 series pump, one UV/VIS detector LC-295 and one sample injector L-7350. All components are from Perkin Elmer 1022. Methanol, water and OPA in different concentrations (40:60:05, 50:50:05, 60:40:05) were used as mobile phase and finally 60:40:05 mobile phase was selected for HPLC. The flow rate was 1ml/minute. The wavelength for DCPs was 280 nm and 10 µl sample was injected in column after cleaning with syringe filters (0.45 μm). The concentration of DCPs was quantified using standard curve from the known substances against the area. Control was served without culture and with DCPs for calculated actual amount degraded by selected varieties.

Extracellular ligninolytic enzyme essay:-
The production of extracellular enzymes with or without DCPs, were carried out in NRM and NPM, same medium and procedure used for HPLC. Medium without DCPs used as control. Each sample was centrifuged (10,000 x g for 10 min) at 4°C. The enzymatic reactions were determined using a double beam Perkin Elmer Lambda 12 UV/VIS spectrophotometer. All the enzyme activities were measured at room temperature (20±2°C). The enzymatic activity was expressed as International units (U) defined as the amount of enzyme required to produce 1 μmol product min⁻¹ and expressed as UI⁻¹.

Protein concentration was determined following Bradford method (Bradford 1976). Protein content in the sample was determined from standard curve and the amount of protein μgml⁻¹ was calculated. Laccase activity was measured following the oxidation of ABTS (ε₅₂₃₅ 36,000 M⁻¹ cm⁻¹). The assay mixture contained 100 mM sodium acetate buffer pH 5 and 5 mM ABTS (Han et al. 2005). AAO activity was assayed as the oxidation of Veratryl alcohol to veratreraldehyde at 310 nm (9,300 M⁻¹ cm⁻¹) and activity was measured with 5 mM VA in 100 mM sodium phosphate buffer at pH 6 (Varela et al.1999). LiP activity was measured by the oxidation of 2 mM VA to veratraldehyde (ε₃₁₀ 9,300 M⁻¹ cm⁻¹) in 100 mM sodium tartrate buffer (pH 3) in the presence of 0.4 mM H₂O₂ (30%) (Camarero et al. 1999). MnP activity was determined by the production of a Mn³⁺ tartrate complex (ε₃₂₃₅ 6,500 M⁻¹ cm⁻¹) from 0.1 mM MnSO₄ in 100 mM sodium tartrate buffer pH 4.5 with 0.1 mM H₂O₂ (30%) (Cohen et al. 2001).
Statistical analysis:-
All the experimental analysis was carried out in triplicates. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey’s HSD Test using SAV v.9.1.3 program. Differences at p<0.05 were considered to be significant.

Results:-
Screening of fungi for DCPs tolerance on solid medium:- Fifty fungal strains of various, saprophytic, ligninolytic and mycorrhizic fleshy fungi representing different group of fungi were selected to screen for their potential to tolerate DCPs on solid medium. From the fifty fungal strains only B. adusta, L. squarrosulus and P. chrysosporium were showed best tolerance with all DCPs. P. citrinopileatus, P. djamor, P. playtypus, G. flaccossus and T. hirsutus also showed good mycelia growth with all DCPs at all three concentrations. However, A. bisporus, C. comatus, C. hortanse, Descolia sp., Marasmius sp. and Tremella sp. gave very poor tolerance. Mycelial growth was drastically reduced at lower to higher concentration. 3,4-DCP was more toxic than 2,3-DCP and 2,5-DCP. B. adusta showed better tolerance in comparison to L. squarrosulus. Reduction (%) in mycelia growth by DCPs is shown in Table-1.

B. adusta and L. squarrosulus were selected on the basis of initial screening. Both varieties proved to be the most effective among the tested fungi in tolerance of DCPs. B. adusta is non-edible while L. squarrosulus is edible mushroom grow extensively on saw dust. B. adusta is known their ability to degrade many xenobiotic compounds. Both varieties are WRF and produced ligninolytic enzymes (Tripathi et al. 2012).

Biodegradation of DCPs by B. adusta and L. squarrosulus:-
Three isomers of DCP (2,3-, 2,5-, 3,4-) were selected for this study and 0.1 mM (16.3 μg/ml) concentration was used for all DCPs. NPM gave better results in comparison to NRM and under shaking condition, DCPs were fast degrade than static condition. 3,4-DCP was more toxic than 2,3-DCP and 2,5-DCP. The toxicity of the chlorinated phenols is dependent on the number and position of the chlorine substituents in the benzene ring (Kishino and Kobayashi 1994). The toxicity decreases as the number of the chlorine substituents decreases. The PCP was found to be the most toxic of chlorinated phenols. In the PCP all five available substituent positions are occupied by chlorine atom. Both varieties were fully degraded all DCPs in 20 days in both media and condition. 2,3-DCP and 3,4-DCP were fast degraded by B. adusta while 2,5-DCP was fast degraded by L. squarrosulus (Table-2). Biodegradation or mineralization of chlorophenols is also dependent on number and position of chlorine substituents; the rate of biodegradation decreases with increase in number of chlorine substituents. However the scientists have not found agreement on the effect of substituent position on biodegradation (Annachhatre and Gheewala 1996). Fig.1 shows the time course of all three DCPs biodegradation by B. adusta and L. squarrosulus.

dichlorophenol (2,3-DCP):- 2,3-DCP was easily degrade by both varieties in both media and conditions. Retention time (RT) of 2,3-DCP is 7.31 min.

In B. adusta one metabolite was common in both media and conditions (RT-4.12 min). One another metabolite was found in NRM shaking condition (RT-2.36 min) on 10th and 15th day. In NPM one metabolite was formed under both conditions (RT-5.71 min) and one another minor metabolite was formed under shaking condition (RT-3.31 min). Except one common metabolite (RT-4.12 min) rest metabolites were not detected on 20th day.

In L. squarrosulus NPM was better than NRM. Metabolites were not detected in both media and conditions up to 20 days (Fig.2).

dichlorophenol (2,5-DCP):- 2,5-DCP was not observed by both varieties on 20th day (RT-8.51 min). In NRM static condition rate of biodegradation was slow. L. squarrosulus fast degrade 2,5-DCP in comparison to B. adusta.

In B. adusta metabolites were not detected in NRM under both conditions and NPM under static condition. Two metabolites were observed in NPM shaking condition on 15th day and these metabolites has a RT of 3.52 and 5.49 min respectively. Metabolites were degraded with 2,5-DCP on 20th day.

In L. squarrosulus one metabolite was detected in NRM shaking and NPM both conditions on 15th day (RT-8.23 min). This metabolite was degrade with 2,5-DCP on 20th day (Fig.3).
dichlorophenol (3,4-DCP):- 3,4-DCP was degraded by both varieties, media and conditions in 20 days. Biodegradation was noticeably slow in NRM under static condition in comparison to other medium and condition. B. adusta could faster degrade 3,4-DCP than L. squarrosulus. RT of 3,4-DCP is 9.32 min. One metabolite was common in both varieties, media and conditions except in L. squarrosulus in NRM static condition this metabolite was not detected. This metabolite had a RT of 2.23 min.

In B. adusta four metabolites were detected in both media and conditions and RT of these metabolites are 2.23, 2.89, 4.91 and 5.42 min respectively. All metabolites were degraded up to 20 days in NRM under both conditions while in NPM minor peak of one metabolite (2.23 min) was observed in both conditions and rest metabolites were degraded with 3,4-DCP.

In L. squarrosulus metabolites were not detected in NRM under static condition. Two metabolites were detected in static condition of NPM and shaking condition of both media one was common (2.23 min) and another had a RT of 8.01 min. One more metabolite was detected in NPM static condition and this metabolite was also detected in B. adusta (2.89 min). Selected HPLC chromatograms are shown in Fig.4.

Extracellular ligninolytic enzymes in the presence of DCPs:- Ligninolytic enzymes are believed to be responsible for degradation or biodegradation of xenobiotic compounds due to their non specific nature for the substrate.

Extracellular protein in B. adusta, was not observed on 5th and 10th day in NRM shaking condition and NPM both conditions but on 20th day, this media and conditions gave highest protein, 34.5, 30.9, 31.9 µgml⁻¹ respectively in control. Extracellular protein was best with 3,4-DCP in NRM static condition (24.6 µgml⁻¹) on 10th day followed by with 2,5-DCP in NPM static condition and 2,3-DCP in NRM static condition respectively on 15th day (20.3, 19.0 µgml⁻¹). In L. squarrosulus, amount of protein was best with 3,4-DCP in NRM shaking condition on 5th day (21.1 µgml⁻¹). It was seen that the amount of protein was different with DCPs in both varieties (Table-3). The extracellular protein was obtained from both the strains; however the crude protein content of L. squarrosulus was comparable to L. tigrinus (Adejumo and Awosanya 2005).

Laccase activity was very less in B. adusta with all DCPs. In control activity was observed only in NRM static condition. Laccase activity reached a significant level only with 3,4-DCP (5.7 Ul⁻¹) and 2,5-DCP (4.2 Ul⁻¹) in NRM static condition on 20th day. It indicates that laccase activity is inducible in B. adusta and it requires nutrient rich condition for its secretion. In L. squarrosulus laccase activity was higher in comparison to B. adusta. With 2,3-DCP and 2,5-DCP laccase activity was best under static condition of both media on 10th day. In the presence of 3,4-DCP laccase activity was observed in NRM static condition (179.0 Ul⁻¹) on 10th day followed by in NPM and NRM shaking condition respectively on 5th day (169.2, 152.7 Ul⁻¹). 3,4-DCP showed best laccase activity in comparison to 2,3-DCP and 2,5-DCP. It was seen that presence of DCPs increase the activity of laccase (Table-4).

AAO activity in B. adusta was better under shaking condition of both media. 2,3-DCP, 2,5-DCP and in control AAO activity was best in NRM under shaking condition while 3,4-DCP showed better activity in NPM under shaking condition on 15th and 20th day. It indicates that AAO activity is directly related to nutritional and incubation conditions. L. squarrosulus gave very poor AAO activity in comparison to B. adusta. Significant activity was found in control on 15th day in NRM shaking condition (19.8 Ul⁻¹) followed by with 2,3-DCP on 20th day and in control on 10th day in NPM shaking condition respectively (18.3, 16.7 Ul⁻¹; Table-5).

LiP activity in B. adusta was good with 2,5-DCP on 15th day in NRM shaking condition (18.2 Ul⁻¹) followed by in control on 10th day in NRM static condition (16.5 Ul⁻¹). LiP activity was not more significant and some times LiP activity could not be detected in the culture supernatant, but it appeared during enzyme purification (Heinfling et al. 1998a). In L. squarrosulus LiP activity was better than B. adusta and best activity was observed with 2,5-DCP in NPM shaking condition on 5th day (20.4 Ul⁻¹; Table-6).

MnP activity in B. adusta was higher in control in NRM static condition on 15th day (215.9 Ul⁻¹). In all selected DCPs 2,3-DCP gave best MnP activity in NPM shaking condition on 15th day (118.3 Ul⁻¹). L. squarrosulus gave higher MnP activity in comparison to B. adusta. In the presence of DCPs best activity was observed with 2,3-DCP on 5th day under shaking condition and on 15th day under static condition of NRM. MnP from both varieties suggested that a corresponding enzymatic activity might be responsible for the biodegradation of DCPs (Table-7).
Discussion:-

Different approaches have been used to study fungal biodegradation of chlorinated phenols: intact cultures of fungi (Alleman et al. 1992; Mileski et al. 1988; Reddy et al. 1998; Valli and Gold 1991), separation of mycelium and culture filtrate separately (Armenante et al. 1994), free and immobilized enzymes (Iimurya et al. 1996; Roy-Arcand and Archibald 1991; Ruggiero et al. 1989; Ruttiman-Johnson and Lamar 1996; Shuttleworth and Bollag 1986). These studies revealed that lignin-degrading enzyme systems are usually responsible for the CP transformation or degradation. The most extensive and diverse research was conducted with the Phanerochaete chrysosporium, chosen because it has been the most studied model of ligninolytic fungi. Temperate fungal species offer more advantages, provided they have extracellular enzymes capable of DCPs transformation. 2,4-DCP (Valli and Gold 1991), 2,4,5-trichlorophenol (Joshi and Gold 1993) and pentachlorophenol (Mileski et al. 1988) were shown to be efficiently mineralized by P. chrysosporium under conditions, where the fungus produces LiP and MnP. These enzymes have originally evolved for degradation of lignin and the reactions they perform are non-specific.

All selected DCPs showed variations in enzymatic activity in both varieties, media and conditions. In B. adusta laccase activity was not significant and some studies have been reported that laccase is not present (Heinfling et al. 1998a; Belcarz et al. 2005). Supplementation and incubation conditions were affected the enzymatic activity and activity was varying with compound to compound in both media and conditions. Some WRF like Volvariella volvacea gave good laccase activity under high nitrogen condition when medium was supplemented with Cu but in the absence of Cu activity was not found and under low nitrogen condition laccase activity was very poor (Chen et al. 2003). Laccase was main oxidase enzyme in L. squarrosulus. Laccase is the most active enzyme for CP transformation in some other WRF like Coriolus versicolor (Ullah et al., 2000a). In cultures of T. versicolor, laccase is reported to be the dominant ligninolytic enzyme involved in CPs degradation (Kadhim et al. 1999; Leontievsky et al. 2002b; Roy-Arcand and Archibald 1991; Ullah et al. 2002b). 3,4-DCP gave best laccase activity in comparison to 2,3-DCP and 2,5-DCP in L. squarrosulus. Production of laccase depends on appropriate growth media for the fungus, with different isomers of enzyme being expressed under different growth conditions. The reactivity of CPs with laccase depends on the number and position of substituted chlorines on the aromatic ring. The lower degree of substitution of the CPs, the more reactive molecule with ortho and para substituted CPs being transformed by laccase more readily than those with meta substitution (Kadhim et al. 1999). AAO activity was better in B. adusta in comparison to L. squarrosulus. In B. adusta, 2,3-DCP and 2,5-DCP gave best AAO activity in NRM shaking condition while 3,4-DCP gave best activity in NPM shaking condition and MnP activity was good with 2,5-DCP in comparison to 2,3-DCP and 3,4-DCP in NRM under both conditions and NPM static condition. Bjerkandera spp. biosynthesize veratryl alcohol and veratraldehyde de-novo from glucose, together with anisaldehyde, 3-chloroanisaldehyde and 3,5-dichloro-anisaldehyde and their respective aryl alcohols (de Jong et al. 1994). It is interesting to note that the AAO of Bjerkandera spp. has a much lower affinity for VA than the AAO of the Pleurotus spp. Thus VA is well protected against unwanted AAO mediated oxidation (de Jong et al. 1994). AAO is the main oxidases enzyme in B. adusta while laccase plays important role in L. squarrosulus for biodegradation of DCPs. In some WRF both enzymes were observed during lignin degradation like P. ostreatus has shown that the concerted action of laccase and AAO, produces significant reduction in the molecular mass of soluble lignosulphonates (Marzullo et al. 1995). AAO is H2O2-generating enzyme in basidiomycetes, such as P. sajor-caju, P. ostreatus, P. eryngii, B. adusta and P. chrysosporium which has been purified and their properties characterized (Okamoto and Yanase 2002). LiP was better in L. squarrosulus in comparison to B. adusta. LiP is a true peroxidase and the kinetic of enzyme intermediates have been studied in detail and a heme peroxidase which is secreted extracellularly at the onset of secondary metabolism, triggered by nitrogen limitation, in P. chrysosporium, but it can also be produced in nitrogen-sufficient conditions by other WRF (Collins et al. 1997). Under ligninolytic conditions, degradation of chlorophenols is initiated by oxidative dechlorination of the substrate to its corresponding p-quinone, catalyzed by two extracellular peroxidases, LiP and MnP (Reddy and Gold 2000; Duran et al. 2002). In both selected varieties MnP was very important enzyme for DCPs biodegradation. MnP was found to be responsible for transformation of chlorophenols (Leontievsky et al. 2002). CPs are also substrates for MnP and LiP that requires hydrogen peroxide and Mn(II) and for reaction of MnP (Milestone et al. 1992) and veratryl alcohol for reaction of LiP (Reddy and D’souza 1994). This peroxidase oxidizing Mn3+ (MnP) is described for the first time in B. adusta (Heinfling et al. 1998b). MnP catalyzed polymerization of aromatic compounds was reported in pentachlorophenol and ferulic acid and synthesis of DHP by MnP from B. adusta in 70% aqueous acetone (Iwahara et al. 2000). MnP activity was higher in L. squarrosulus in comparison to B. adusta. Purified or crude MnP has been used in cell-free systems (in vitro) and shown to oxidize not only lignin, chlorolignins and synthetic lignin compounds, but also Humic Substances (HS) from brown coal, and HS synthesized from catechol, nylon, PAH, chlorophenols, nitroaromatic compounds and arsenic-containing warfare agents (Steffen 2003).
Metabolism of CPs is not unusual in fungi. Many WRF such as Ganoderma lucidum and Polyporus can synthesize chlorophenols, and some can release large quantities of organic chlorides, including halomethanes, which are thought to be utilized as substrate cofactors for ligninolytic pathways (Beynon et al., 1981; Oberg et al., 1997; Teunissen and Field, 1998; Watling and Harper, 1998). The ligninolytic enzymes secreted by WRF may be involved in the first oxidative dechlorination step in the degradation of several chlorinated phenol. Several chlorophenols have also been shown to be oxidized by laccases (Roy-Arcand and Archibald, 1991). The present study describes a procedure to produce high amounts of extracellular MnP activity with mycelium of the B. adusta and L. squarrosulus. Both varieties gave good activity of MnP. Activity varies with DCPs, medium and their incubation conditions. Among the four ligninolytic enzymes, AAO in B. adusta, laccase in L. squarrosulus and MnP in both varieties, seemed to be responsible for the biodegradation of DCPs. These results show that enzymatic activity of ligninolytic enzymes were affected by the presence of DCPs biodegradation which can be important in the elimination of the toxicity of different xenobiotics. Further investigation will have to identify metabolites and their role as well as the metabolic pathway of DCPs biodegradation.

**Fig.1:-** Time course removal of DCPs by B. adusta and L. squarrosulus. Columns represents mean of three replicates with SD.
Fig.2: HPLC chromatogram of 2,3-DCP on 10\textsuperscript{th} day in B. adusta and on 15\textsuperscript{th} day in L. squarrosulus and further peaks are unknown metabolites, which generated during biodegradation of 2,3-DCP by both varieties in both medium and conditions.
Fig. 3:- HPLC chromatogram of 2,5-DCP on 15th day and further peaks are unknown metabolites, which generated during biodegradation of 2,5-DCP by both varieties in both medium and conditions.
Fig.4: HPLC chromatogram of 3,4-DCP on 15th day and further peaks are unknown metabolites, which generated during biodegradation of 3,4-DCP by both varieties in both medium and conditions.
Table 1: Reduction (%) in mycelial growth of mushrooms by three different concentrations (mM) of DCPs (mean ± SD; n = 3)

| Varieties                  | 2,3-DCP | 2,5-DCP | 3,4-DCP |
|----------------------------|---------|---------|---------|
|                            | 0.1     | 0.15    | 0.2     | 0.1     | 0.15    | 0.2     | 0.1     | 0.15    | 0.2     |
| Agaricus bisporus          | 81.8 ± 0.13 | 100     | 100     | 81.8 ± 0.48 | 100     | 100     | 72.7 ± 0.21 | 100     | 100     |
| Agrocybe aegerita          | 20.8 ± 0.21 | 37.5 ± 0.17 | 58.3 ± 0.32 | 33.3 ± 0.34 | 50.0 ± 0.47 | 62.5 ± 0.15 | 58.3 ± 0.36 | 87.5 ± 0.16 | 100     |
| A. badia                  | 23.1 ± 0.04 | 42.3 ± 0.05 | 61.5 ± 0.13 | 23.1 ± 0.91 | 34.6 ± 0.86 | 46.2 ± 0.63 | 69.2 ± 0.52 | 100     | 100     |
| Agrocybe sp.               | 31.8 ± 0.17 | 54.5 ± 0.07 | 63.6 ± 0.75 | 40.9 ± 0.35 | 59.1 ± 0.92 | 72.7 ± 0.72 | 36.4 ± 0.37 | 45.4 ± 0.43 | 68.2 ± 0.26 |
| Amanita caesarea           | 28.5 ± 0.42 | 54.3 ± 0.15 | 68.6 ± 0.91 | 48.6 ± 0.75 | 62.8 ± 0.45 | 82.8 ± 0.58 | 65.7 ± 0.29 | 77.1 ± 0.52 | 82.8 ± 0.52 |
| Auricularia mesentrica     | 43.6 ± 0.06 | 59.5 ± 0.11 | 100      | 48.4 ± 0.05 | 56.3 ± 0.61 | 76.2 ± 0.11 | 64.3 ± 0.15 | 96.0 ± 0.67 | 100     |
| Bjerkandera adusta         | 7.2 ± 0.32 | 26.9 ± 0.24 | 35.8 ± 0.43 | 7.6 ± 0.17 | 23.8 ± 0.65 | 32.6 ± 0.85 | 12.7 ± 0.63 | 25.0 ± 0.42 | 38.9 ± 0.74 |
| Calocybe indica            | 33.3 ± 0.11 | 72.4 ± 0.06 | 100      | 9.5 ± 0.39 | 52.4 ± 0.57 | 71.4 ± 0.06 | 52.4 ± 0.85 | 66.6 ± 0.19 | 100     |
| Chlorophyllum hortense     | 72.2 ± 0.32 | 100      | 100     | 72.2 ± 0.88 | 100      | 100     | 83.3 ± 0.99 | 100     | 100     |
| Coprinus comatus           | 90.0 ± 0.07 | 100      | 100     | 90.0 ± 0.25 | 100      | 100     | 100      | 100     | 100     |
| Coprinus sp.               | 50.0 ± 0.26 | 80.0 ± 0.03 | 100 | 30.0 ± 0.31 | 60.0 ± 0.87 | 70.0 ± 0.36 | 70.0 ± 0.74 | 90.0 ± 0.55 | 100     |
| Descolia sp.               | 100      | 100      | 100     | 100      | 100      | 100     | 100      | 100     | 100     |
| Flammulina sp.             | 26.5 ± 0.08 | 94.1 ± 0.78 | 100 | 47.1 ± 0.54 | 70.5 ± 0.90 | 88.2 ± 0.44 | 41.1 ± 0.43 | 94.1 ± 1.32 | 100     |
| F. velutipes               | 90.6 ± 0.17 | 100      | 100     | 6.3 ± 0.63 | 68.7 ± 0.43 | 87.5 ± 0.96 | 56.2 ± 0.48 | 100     | 100     |
| Ganoderma sp.              | 38.9 ± 0.24 | 52.8 ± 0.24 | 62.5 ± 0.17 | 12.2 ± 0.74 | 53.7 ± 0.48 | 68.7 ± 0.16 | 43.7 ± 0.29 | 68.7 ± 0.72 | 100     |
| Gleophyllum sp.            | 42.8 ± 0.17 | 57.1 ± 0.37 | 71.4 ± 0.88 | 20.0 ± 0.86 | 31.4 ± 0.60 | 54.2 ± 0.26 | 25.7 ± 0.74 | 48.6 ± 0.47 | 65.7 ± 0.38 |
| Gomphus flaccossus         | 11.1 ± 0.01 | 46.7 ± 0.64 | 100      | 11.1 ± 0.30 | 16.7 ± 0.37 | 33.3 ± 0.77 | 11.1 ± 0.53 | 46.7 ± 0.59 | 100     |
| Hypholoma fasciculare      | 30.0 ± 0.04 | 55.0 ± 0.76 | 75.0 ± 0.64 | 50.0 ± 0.66 | 65.0 ± 0.76 | 100      | 50.0 ± 0.25 | 70.0 ± 0.11 | 100     |
| Kuehneromyces sp.          | 22.2 ± 0.09 | 44.4 ± 0.09 | 88.9 ± 0.32 | 16.7 ± 0.34 | 33.3 ± 0.84 | 55.5 ± 0.61 | 72.2 ± 0.19 | 72.2 ± 0.45 | 100     |
| Lentinula edodes           | 51.1 ± 0.55 | 94.4 ± 1.21 | 100      | 38.9 ± 0.18 | 55.6 ± 0.29 | 72.2 ± 0.84 | 50.0 ± 0.72 | 72.2 ± 0.45 | 100     |
| Lentinus squarrosulus      | 16.7 ± 0.21 | 25.0 ± 0.76 | 58.3 ± 0.48 | 16.7 ± 0.77 | 33.3 ± 0.64 | 44.4 ± 0.32 | 11.1 ± 0.16 | 27.8 ± 0.92 | 55.6 ± 0.92 |
| Macrolepiota proceria      | 62.5 ± 0.17 | 100      | 100     | 37.5 ± 0.04 | 100      | 100     | 100      | 100     | 100     |
| M. rhacoides               | 46.7 ± 0.13 | 60.0 ± 0.25 | 66.7 ± 0.51 | 13.3 ± 0.16 | 33.3 ± 0.85 | 66.7 ± 0.47 | 66.7 ± 0.25 | 100     | 100     |
| Marasmius sp.              | 100      | 100      | 100     | 20.0 ± 0.74 | 100      | 100     | 100      | 100     | 100     |
| Mycena sp.                 | 18.7 ± 0.03 | 50.0 ± 0.43 | 100      | 12.5 ± 0.36 | 31.2 ± 0.17 | 50.0 ± 0.76 | 18.7 ± 0.49 | 100     | 100     |
| Pleurotus florida          | 53.3 ± 0.18 | 100      | 100     | 16.7 ± 0.52 | 33.3 ± 0.11 | 48.7 ± 0.59 | 20.0 ± 0.74 | 23.3 ± 0.60 | 100     |
| Species                        | 16.1 ± 0.71 | 32.2 ± 0.17 | 51.6 ± 0.29 | 12.9 ± 0.75 | 51.6 ± 0.51 | 61.3 ± 0.93 | 29.0 ± 0.92 | 51.6 ± 0.58 | 61.3 ± 0.26 |
|-------------------------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| P. citrinopileatus            | 40.0 ± 0.05 | 93.3 ± 0.89 | 100         | 20.0 ± 0.39 | 53.3 ± 0.87 | 80.0 ± 0.17 | 66.7 ± 0.15 | 80.0 ± 0.97 | 100         |
| P. coremia sp. 1              | 41.8 ± 0.07 | 100         | 100         | 27.3 ± 0.30 | 33.3 ± 1.17 | 45.5 ± 0.25 | 24.8 ± 0.73 | 51.5 ± 0.77 | 100         |
| P. coremia sp. 2              | 38.1 ± 0.15 | 60.0 ± 0.34 | 76.2 ± 0.71 | 38.1 ± 0.75 | 52.4 ± 0.36 | 66.7 ± 0.36 | 47.6 ± 0.22 | 69.5 ± 0.51 | 95.2 ± 0.79 |
| P. coremia sp. 3              | 53.6 ± 0.04 | 61.5 ± 0.48 | 87.2 ± 0.74 | 38.5 ± 0.27 | 61.5 ± 0.83 | 74.4 ± 0.72 | 35.9 ± 0.50 | 48.7 ± 0.48 | 97.4 ± 0.46 |
| P. cornucopiae                | 17.6 ± 0.48 | 29.4 ± 0.22 | 41.2 ± 0.43 | 23.5 ± 0.54 | 35.3 ± 0.52 | 94.1 ± 0.58 | 52.9 ± 0.36 | 64.7 ± 0.66 | 82.4 ± 0.53 |
| P. cystidiosus sub sp.        | 8.3 ± 0.03  | 45.8 ± 0.83 | 50.0 ± 0.27 | 37.5 ± 0.17 | 58.3 ± 0.64 | 70.8 ± 0.47 | 50.0 ± 0.73 | 66.7 ± 0.13 | 83.3 ± 0.36 |
| P. citrinopileatus            | 7.9 ± 0.14  | 21.0 ± 0.26 | 36.8 ± 0.85 | 15.8 ± 0.11 | 34.2 ± 0.71 | 60.5 ± 0.49 | 28.9 ± 0.87 | 52.6 ± 0.19 | 100         |
| P. flabellatus                | 21.1 ± 0.23 | 35.6 ± 0.74 | 100         | 7.8 ± 0.38  | 25.0 ± 0.69 | 47.2 ± 0.02 | 15.0 ± 0.33 | 46.7 ± 0.57 | 100         |
| P. membranaceus 1             | 33.3 ± 0.17 | 55.6 ± 0.08 | 66.7 ± 0.94 | 22.2 ± 0.20 | 52.8 ± 0.77 | 72.2 ± 0.79 | 30.6 ± 0.29 | 52.8 ± 0.48 | 75.0 ± 0.91 |
| P. membranaceus 2             | 50.0 ± 0.09 | 62.5 ± 0.29 | 72.5 ± 0.58 | 45.0 ± 0.16 | 57.5 ± 0.46 | 67.5 ± 0.53 | 40.0 ± 0.52 | 52.5 ± 0.32 | 100         |
| P. opuntiae                   | 40.0 ± 0.16 | 48.6 ± 0.53 | 60.0 ± 0.72 | 22.9 ± 0.38 | 54.3 ± 0.91 | 65.7 ± 0.83 | 37.1 ± 0.19 | 54.3 ± 0.79 | 65.7 ± 0.27 |
| P. ostreatus                  | 27.1 ± 0.73 | 59.6 ± 0.47 | 100         | 17.5 ± 0.33 | 24.7 ± 0.53 | 37.9 ± 0.96 | 27.7 ± 0.05 | 48.8 ± 0.63 | 100         |
| P. platythus                  | 10.0 ± 0.15 | 23.3 ± 0.88 | 40.0 ± 0.47 | 13.3 ± 0.29 | 36.7 ± 0.37 | 63.3 ± 0.58 | 26.7 ± 0.14 | 40.0 ± 0.47 | 50.0 ± 0.74 |
| P. sajor-caju                 | 41.8 ± 0.27 | 100         | 100         | 27.3 ± 0.74 | 33.3 ± 0.33 | 45.5 ± 0.61 | 24.8 ± 0.73 | 51.5 ± 0.72 | 100         |
| P. sapidus                    | 22.4 ± 0.13 | 56.9 ± 0.95 | 100         | 4.7 ± 0.09  | 29.4 ± 0.74 | 38.2 ± 0.49 | 22.9 ± 0.25 | 44.1 ± 0.22 | 100         |
| Phanerochaete chrysosporium   | 8.2 ± 0.26  | 28.5 ± 0.36 | 42.3 ± 0.34 | 10.0 ± 0.28 | 30.7 ± 0.81 | 43.6 ± 0.61 | 16.6 ± 0.22 | 28.9 ± 0.18 | 40.5 ± 0.39 |
| Phellinus badius              | 12.5 ± 0.05 | 43.7 ± 0.72 | 62.5 ± 0.25 | 25.0 ± 0.57 | 50.0 ± 0.65 | 75.0 ± 0.27 | 50.0 ± 0.84 | 75.0 ± 0.15 | 100         |
| Stropharia rugoso annulata    | 38.1 ± 0.08 | 61.9 ± 0.48 | 90.5 ± 1.56 | 38.1 ± 0.38 | 52.4 ± 0.73 | 61.9 ± 0.09 | 57.1 ± 0.43 | 76.2 ± 0.09 | 100         |
| Trametes cinnabarinus         | 33.3 ± 0.19 | 55.5 ± 0.46 | 100         | 44.4 ± 0.86 | 55.5 ± 0.48 | 88.9 ± 1.19 | 38.9 ± 0.12 | 100         |
| Trametes hirsutus             | 13.8 ± 0.36 | 42.8 ± 0.17 | 57.1 ± 0.47 | 25.7 ± 0.39 | 40.0 ± 0.93 | 54.3 ± 0.48 | 22.8 ± 0.74 | 47.1 ± 0.82 | 65.7 ± 0.57 |
| Tremella sp.                  | 72.7 ± 0.14 | 100         | 100         | 70.9 ± 0.90 | 94.5 ± 0.25 | 100         |
| Volvariella sp.               | 20.0 ± 0.04 | 50.0 ± 0.49 | 100         | 22.5 ± 0.73 | 37.5 ± 0.19 | 100         |
| Volvariella volvacea          | 46.6 ± 0.57 | 69.9 ± 0.71 | 100         | 15.2 ± 0.26 | 48.3 ± 0.45 | 57.6 ± 0.32 | 44.9 ± 0.22 | 76.7 ± 0.68 | 100         |
Table 2: Biodegradation of DCPs (16.3 μg ml⁻¹) followed by HPLC (mean ± SD; n = 3)

| Day | NRM | 2,3-DCP | L. squarrosulus |
|-----|-----|---------|-----------------|
|     | Static | Shaking | Static | Shaking | Static | Shaking | Static | Shaking |
| 5   | 3.8 ± 0.90 b | 6.3 ± 1.17 | 5.2 ± 1.04 ab | 6.2 ± 1.05 | 3.7 ± 0.92 b | 5.3 ± 1.05 | 3.8 ± 1.08 b | 5.4 ± 0.92 ab |
| 10  | 9.2 ± 0.95 c | 10.1 ± 1.05 bc | 10.3 ± 1.25 bc | 10.7 ± 1.06 bc | 9.3 ± 0.90 c | 11.8 ± 1.11 b | 9.8 ± 1.11 c | 12.3 ± 0.90 a |
| 15  | 14.2 ± 1.01 b | 16.3 ± 0.00 a | 16.3 ± 0.00 a | 16.3 ± 0.00 a | 14.7 ± 1.05 ab | 15.4 ± 0.95 ab | 15.4 ± 0.95 ab | 16.3 ± 0.00 a |
| 20  | 16.3 ± 0.00* | 16.3 ± 0.00 | 16.3 ± 0.00 | 16.3 ± 0.00 | 16.3 ± 0.00 | 16.3 ± 0.00 | 16.3 ± 0.00 | 16.3 ± 0.00 |

Note: In each row different letters means significant difference (p<0.05)
* Compound was not detected through HPLC
### Table 3:- Extracellular protein (µg ml⁻¹) in B. adusta and L. squarrosulus with or without DCPs (mean ± SD; n = 3)

| S.No. | Medium | Condition | B. adusta | L. squarrosulus |
|-------|--------|-----------|-----------|---------------|
|       |        | Day Interval |          | Day Interval |
|       |        | 5 10 15 20 |          | 5 10 15 20   |
| 2,3-DCP | NRM    | Static | 2.3 ± 0.44 de 10.7 ± 0.77 e 19.0 ± 0.15 de 18.8 ± 0.40 cd | 18.9 ± 0.15 b 19.9 ± 0.74 a 14.1 ± 0.68 b 8.7 ± 0.87 d |
|       |        | Shaking | 1.6 ± 0.34 ef 5.0 ± 0.82 h 12.0 ± 0.87 ef 7.0 ± 0.38 h | 12.7 ± 0.79 c 18.4 ± 0.39 ab 11.2 ± 0.71 cd 6.6 ± 0.32 f |
|       | NPM    | Static | 2.3 ± 0.73 de 8.4 ± 0.70 fg 10.6 ± 0.88 de 8.2 ± 0.65 gh | 2.8 ± 0.24 g 7.3 ± 0.61 fg 8.0 ± 0.89 efg 7.6 ± 0.70 def |
|       |        | Shaking | 1.6 ± 0.60 ef 6.7 ± 0.53gh 14.4 ± 0.79 ef 9.0 ± 0.46 gh | 3.7 ± 0.10 fg 9.0 ± 0.64 ef 13.3 ± 0.36 b 3.9 ± 0.46 g |
| 2,5-DCP | NRM    | Static | 12.3 ± 1.02 a 16.1 ± 0.80 c 18.7 ± 0.84 a 12.5 ± 0.71 e | 5.9 ± 0.43 e 5.0 ± 0.50 hi 12.7 ± 0.74 bc 11.7 ± 0.67 bc |
|       |        | Shaking | 4.1 ± 0.88 cd 13.6 ± 0.67 d 14.2 ± 0.83 cd 19.3 ± 0.54 c | 5.1 ± 0.58 ef 8.6 ± 0.39 f 9.1 ± 0.72 efg 16.3 ± 0.43 a |
|       | NPM    | Static | 12.1 ± 0.59 a 18.5 ± 0.50 b 20.3 ± 0.68 a 11.8 ± 0.47 ef | 3.2 ± 0.44 g 6.6 ± 0.18 gh 7.3 ± 0.28 g 8.5 ± 0.50 de |
|       |        | Shaking | 7.7 ± 0.68 b 9.8 ± 0.96 ef 9.8 ± 1.01 fg 9.9 ± 0.45 i | 3.7 ± 0.80 fg 4.5 ± 0.14 i 8.0 ± 0.76 efg 12.8 ± 0.22 b |
| 3,4-DCP | NRM    | Static | 9.0 ± 0.59 b 24.6 ± 0.84 a 18.1 ± 0.86 b 16.8 ± 0.52 d | 8.2 ± 0.55 d 7.8 ± 0.61 fg 19.1 ± 0.76 a 11.9 ± 0.87 bc |
|       |        | Shaking | 3.4 ± 0.78 cde 9.2 ± 0.64 ef 6.8 ± 0.47 b 16.7 ± 0.78 d | 21.1 ± 0.51 a 17.4 ± 0.78 bc 9.5 ± 0.43 de 9.5 ± 0.55 c |
|       | NPM    | Static | 1.5 ± 0.54 ef 7.8 ± 0.80 fg 14.1 ± 0.98 ef 9.4 ± 1.08 g | 9.3 ± 0.72 d 8.3 ± 0.49 fg 9.1 ± 0.25 efg 7.5 ± 0.37 def |
|       |        | Shaking | 4.1 ± 1.20 cd 6.8 ± 0.90 gh 8.0 ± 1.44 cd 12.6 ± 0.81 e | 9.4 ± 0.49 d 13.5 ± 0.29 d 8.0 ± 0.52 efg 6.7 ± 0.98 ef |
| Control | NRM    | Static | 4.4 ± 0.88 c 7.9 ± 0.44 fg 12.1 ± 0.35 c 16.9 ± 0.62 d | 3.9 ± 0.46 fg 10.7 ± 1.11 e 11.2 ± 0.77 efg 11.4 ± 0.36 bc |
|       |        | Shaking | nd nd 12.1 ± 0.42 f 34.5 ± 0.49 a | 8.3 ± 0.38 d 16.2 ± 0.82 c 19.4 ± 0.44 a 12.6 ± 0.54 bc |
|       | NPM    | Static | nd nd 3.4 ± 0.35 f 30.9 ± 0.15 b | 3.3 ± 0.52 g 4.6 ± 1.00 i 7.5 ± 0.23 fg 6.1 ± 0.64 f |
|       |        | Shaking | nd nd 5.8 ± 0.19 f 31.9 ± 1.13 b | 3.4 ± 0.96 g 7.4 ± 0.43 fg 9.3 ± 0.54 def 6.3 ± 0.61 f |

Note: In each column different letters means significant difference (p<0.05)
nd- not detected
### Table 4:
Extracellular laccase production (U/l) in B. adusta and L. squarrosulus with or without DCPs (mean ± SD; n = 3)

| S.No. | Medium | Condition | B. adusta | L. squarrosulus |
|-------|--------|-----------|-----------|-----------------|
|       |        |           | Day Interval | Day Interval |
|       |        |            | 5 | 10 | 15 | 20 | 5 | 10 | 15 | 20 |
| 2,3-DCP | NRM | Static | nd | nd | nd | nd | 21.6 ± 0.31 j | 108.5 ± 0.35 d | 59.1 ± 0.35 d | 34.6 ± 0.15 de |
|         |      | Shaking | nd | nd | nd | nd | 96.7 ± 0.69 c | 47.7 ± 0.89 g | 4.9 ± 0.741 | 3.7 ± 2.63 i |
|         | NPM  | Static | nd | nd | nd | 2.6 ± 0.82 bc | 28.3 ± 1.02 i | 129.9 ± 2.37 c | 88.3 ± 0.92 b | 83.7 ± 0.78 a |
|         |      | Shaking | nd | nd | nd | 3.2 ± 0.50 bc | 61.7 ± 1.07 e | 28.0 ± 0.81 i | 24.9 ± 0.93 h | 17.8 ± 1.44 g |
| 2,5-DCP | NRM | Static | nd | nd | 1.9 ± 0.70 b | 4.2 ± 0.71 ab | 17.6 ± 0.40 k | 75.4 ± 0.15 e | 43.1 ± 0.29 f | 18.6 ± 0.21 g |
|         |      | Shaking | nd | nd | nd | 2.5 ± 0.96 cd | 44.7 ± 1.55 f | 6.6 ± 0.30 l | nd | nd |
|         | NPM  | Static | nd | nd | nd | nd | 23.4 ± 0.46 j | 63.3 ± 0.74 f | 55.0 ± 1.55 e | 46.6 ± 0.96 c |
|         |      | Shaking | nd | 1.9 ± 0.20 b | nd | nd | 36.5 ± 0.15 g | 18.6 ± 0.91 jk | 22.0 ± 1.72 i | 33.9 ± 0.49 e |
| 3,4-DCP | NRM | Static | nd | nd | nd | 5.7 ± 0.62 a | 78.7 ± 0.53 d | 179.0 ± 0.62 a | 82.6 ± 0.26 c | 20.4 ± 0.15 fg |
|         |      | Shaking | nd | nd | nd | 3.6 ± 1.21 bc | 152.7 ± 0.08 b | 20.6 ± 1.17 j | 8.5 ± 0.65 | 19.2 ± 0.49 fg |
|         | NPM  | Static | nd | nd | nd | nd | 76.9 ± 1.36 d | 144.9 ± 0.91 b | 98.9 ± 0.90 a | 85.0 ± 1.16 a |
|         |      | Shaking | nd | nd | nd | 1.2 ± 0.50 de | 169.2 ± 0.92 a | 37.6 ± 1.82 j | 32.9 ± 1.65 g | 22.7 ± 1.74 f |
| Control | NRM | Static | 3.3 ± 1.30 a | 5.4 ± 0.70 a | 2.3 ± 0.21 a | 1.5 ± 0.57 de | 16.6 ± 0.32 k | 29.1 ± 0.29 i | 18.8 ± 0.47 j | 12.7 ± 0.76 h |
|         |      | Shaking | nd | nd | nd | nd | 44.0 ± 1.04 f | 28.1 ± 1.81 i | 16.9 ± 0.55 j | 10.6 ± 0.32 h |
|         | NPM  | Static | nd | nd | nd | nd | 28.0 ± 0.81 i | 45.0 ± 1.04 g | 57.2 ± 0.51 de | 64.0 ± 1.65 b |
|         |      | Shaking | nd | nd | nd | nd | 32.2 ± 0.84 h | 16.6 ± 1.28 k | 32.7 ± 0.82 g | 37.7 ± 2.18 d |

Note: In each column different letters mean significant difference (p<0.05)
nd - not detected
Table 5: AAO activity (Ul⁻¹) in *B. adusta* and *L. squarrosulus* with or without DCPs (mean ± SD; n = 3)

| S.No. | Medium | Condition | 5 | 10 | 15 | 20 | 5 | 10 | 15 | 20 |
|-------|--------|-----------|---|----|----|----|---|----|----|----|
|       |        |           | 13.8 ± 0.30 i | 16.9 ± 0.56 j | 3.3 ± 0.06 d | 9.0 ± 0.41 c | 12.0 ± 0.78 b | 5.8 ± 0.57 b |
|       |        | NRM       | 14.5 ± 0.10 f | 15.7 ± 0.47 f | 4.8 ± 0.19 f | 8.2 ± 0.45 c | 11.8 ± 0.55 e | 5.6 ± 0.61 b |
|       |        | Shaking   | 22.2 ± 0.86 a | 17.6 ± 1.00 a | 21.9 ± 0.23 k | 6.9 ± 0.79 a | 2.0 ± 0.62 fg | nd |
|       |        | NPM       | 4.2 ± 0.06 k | 14.3 ± 0.95 i | 21.9 ± 0.23 k | 6.9 ± 0.79 a | 2.0 ± 0.62 fg | nd |
|       |        | Shaking   | 15.2 ± 0.87 ef | 33.9 ± 0.70 f | 38.4 ± 0.61 g | 4.8 ± 0.76 bc | 4.7 ± 0.95 e | 8.7 ± 0.32 c | 18.3 ± 0.74 a |
|       |        | NRM       | 3.9 ± 0.76 fg | 6.7 ± 0.50 j | 9.9 ± 0.66 j | 4.8 ± 0.71 n | nd |
|       |        | Shaking   | 22.7 ± 0.81 a | 154.7 ± 2.17 a | 131.9 ± 1.82 c | 2.5 ± 0.85 de | 4.6 ± 0.58 e | 2.1 ± 0.11 f | 1.6 ± 0.10 d |
|       |        | NPM       | 5.5 ± 0.87 jk | 9.0 ± 0.60 jk | 27.9 ± 0.50 j | 1.3 ± 0.21 ef | 2.4 ± 0.26 f | 2.7 ± 0.15 ef | nd |
|       |        | Shaking   | 5.1 ± 0.61 f | 11.0 ± 0.25 hi | 16.1 ± 0.29 hi | 35.8 ± 0.72 h | 5.6 ± 0.42 ab | 13.5 ± 1.19 b | 9.4 ± 0.32 c | 5.4 ± 0.87 b |
|       |        | NRM       | 7.5 ± 0.66 e | 11.1 ± 0.66 hi | 22.8 ± 0.40 g | 8.4 ± 0.96 m | nd |
|       |        | Shaking   | 7.2 ± 0.65 e | 17.0 ± 0.58 e | 37.5 ± 0.75 e | 69.5 ± 0.80 e | nd |
|       |        | NPM       | 2.0 ± 0.36 h | 13.2 ± 0.68 fg | 22.8 ± 0.84 g | 8.5 ± 0.52 m | 1.5 ± 0.38 e | 2.4 ± 0.43 f | nd |
|       |        | Shaking   | 10.3 ± 0.99 d | 48.2 ± 0.59 b | 112.4 ± 0.73 c | 88.3 ± 0.66 d | nd |
|       |        | NRM       | 2.3 ± 0.40 gh | 11.9 ± 0.70 gh | 18.1 ± 0.59 h | 33.0 ± 0.65 i | 6.2 ± 0.65 ab | 8.2 ± 0.45 c | 3.6 ± 0.55 e |
|       |        | Shaking   | 4.3 ± 0.25 f | 44.4 ± 0.74 c | 111.7 ± 1.36 c | 163.1 ± 1.46 a | nd |
|       |        | NPM       | 15.5 ± 1.05 c | 9.7 ± 0.47 i | 6.1 ± 0.38 k | 4.5 ± 0.25 n | 3.8 ± 0.79 cd | 4.7 ± 1.44 e | nd |
|       |        | Shaking   | 18.9 ± 0.64 b | 25.5 ± 0.99 d | 41.6 ± 0.45 d | 57.5 ± 0.95 f | 3.6 ± 0.72 cd | 16.7 ± 1.00 a | 11.8 ± 0.50 b | 5.6 ± 0.61 b |

Note: In each column different letters means significant difference (p<0.05)
nd- not detected
### Table 6: LiP activity (U/l) in *B. adusta* and *L. squarrosulus* with or without DCPs (mean ± SD; n = 3)

| S.No. | Medium | Condition | **B. adusta** | | | **L. squarrosulus** | |
|-------|--------|-----------|---------------|---|---|---------------|---|
|       |        |           | Day Interval |       | Day Interval |       | |
|       |        |            | 5 | 10 | 15 | 20 | 5 | 10 | 15 | 20 | |
| 2,3-DCP | NRM | Static | nd | 4.8 ± 0.96 d | nd | 10.3 ± 0.85 c | 7.6 ± 0.67 c | nd | 7.4 ± 0.81 d | 18.4 ± 1.10 a | nd | |
|        |        | Shaking | nd | 2.3 ± 0.57 e | 10.3 ± 0.85 c | 7.6 ± 0.67 c | nd | 7.4 ± 0.81 d | 18.4 ± 1.10 a | nd | |
|        | NPM | Static | nd | 4.8 ± 0.68 d | nd | 10.3 ± 0.85 c | 7.6 ± 0.67 c | nd | 7.4 ± 0.81 d | 18.4 ± 1.10 a | nd | |
|        |        | Shaking | nd | 4.8 ± 0.68 d | 6.7 ± 0.93 de | 4.2 ± 0.90 d | 14.2 ± 0.91 b | 18.5 ± 0.61 a | 11.7 ± 0.57 b | 5.9 ± 2.0 bc | |
| 2,5-DCP | NRM | Static | nd | 1.4 ± 0.61 ef | 2.9 ± 0.75 f | 15.8 ± 1.31 a | nd | 1.3 ± 0.21 f | nd | nd | |
|        |        | Shaking | nd | 10.4 ± 0.84 b | 18.2 ± 0.52 a | 9.6 ± 1.12 b | nd | nd | nd | 9.4 ± 0.58 a | |
|        | NPM | Static | nd | nd | nd | 2.4 ± 0.76 e | 7.8 ± 0.46 c | 13.5 ± 0.70 b | 7.5 ± 0.89 cd | 5.5 ± 0.49 c | |
|        |        | Shaking | 5.1 ± 0.61 b | 4.3 ± 0.67 d | nd | nd | 20.4 ± 1.10 a | nd | 8.6 ± 0.20 c | nd | |
| 3,4-DCP | NRM | Static | nd | 1.9 ± 0.31 e | 7.1 ± 0.35 de | 15.0 ± 0.74 a | 3.4 ± 0.67 d | nd | nd | nd | |
|        |        | Shaking | nd | nd | nd | 8.3 ± 0.92 d | 15.5 ± 0.81 a | nd | nd | 8.3 ± 1.00 c | 2.9 ± 0.21 de |
|        | NPM | Static | nd | 9.8 ± 0.12 bc | 7.5 ± 0.90 de | nd | 7.3 ± 0.81 c | 12.4 ± 1.21 b | 7.6 ± 0.83 cd | 3.4 ± 0.10 d | |
|        |        | Shaking | nd | 8.5 ± 0.20 c | 14.1 ± 0.81 c | 5.2 ± 0.1 d | nd | 8.0 ± 0.74 d | 19.7 ± 0.55 a | nd | |
| Control | NRM | Static | nd | 16.5 ± 0.60 a | nd | nd | nd | nd | nd | 6.2 ± 0.50 bc | |
|        |        | Shaking | nd | nd | 6.3 ± 0.95 e | nd | nd | 3.9 ± 0.70 e | 11.9 ± 1.41 b | 7.6 ± 0.45 ab | |
|        | NPM | Static | 10.0 ± 0.85 a | 1.1 ± 0.62 ef | nd | nd | nd | 7.3 ± 0.66 d | 5.8 ± 0.75 d | 2.9 ± 0.30 de | |
|        |        | Shaking | 5.6 ± 0.81 b | 4.5 ± 0.15 d | nd | nd | nd | 10.6 ± 0.53 c | 7.0 ± 0.67 cd | 1.4 ± 0.21 ef | |

Note: In each column different letters mean significant difference (p<0.05)
nd- not detected
Table 7: MnP (U1) in B. adusta and L. squarrosulus with or without DCPs (mean ± SD; n = 3)

| S.No. | Medium | Condition | B. adusta | L. squarrosulus |
|-------|--------|-----------|-----------|-----------------|
|       |        |           | Day Interval | Day Interval     |
|       |        |           | 5 | 10 | 15 | 20 | 5 | 10 | 15 | 20 |
| 2,3-DCP | NRM     | Static    | nd | 33.8 ± 0.81f | 14.8 ± 0.81 h | nd | 34.6 ± 2.37 b | 37.6 ± 2.25 bi | 339.6 ± 6.27 a | 86.6 ± 2.97 h |
|        |         | Shaking   | nd | nd | nd | 3.9 ± 0.74 f | 381.7 ± 7.05 a | 180.7 ± 7.91 e | 108.7 ± 4.00 g | 64.1 ± 1.95 i |
|       | NPM     | Static    | nd | 10.9 ± 0.75 i | 18.8 ± 1.27 e | 36.5 ± 2.66 b | 249.1 ± 5.03 a | 289.1 ± 4.75 d | 235.1 ± 4.07 b |
|        |         | Shaking   | nd | 25.1 ± 0.95 g | 118.3 ± 1.05 b | 34.9 ± 1.33 c | 34.1 ± 2.95 b | nd | nd | 192.8 ± 5.20 c |
| 2,5-DCP | NRM     | Static    | 12.3 ± 0.95 e | 48.5 ± 0.60 c | 34.6 ± 0.84 f | 26.6 ± 1.76 d | 34.0 ± 1.75 b | 36.2 ± 1.05 hi | 207.6 ± 2.65 f | 160.3 ± 2.15 e |
|        |         | Shaking   | 16.1 ± 0.78 cd | 37.0 ± 0.85 e | nd | nd | 33.6 ± 1.61 b | nd | nd | nd |
|       | NPM     | Static    | 7.3 ± 0.66 f | 17.3 ± 1.79 h | 9.2 ± 0.15 i | nd | 34.8 ± 1.44 b | 197.9 ± 5.82 d | 253.8 ± 5.12 l | 309.6 ± 7.46 a |
|        |         | Shaking   | 9.8 ± 0.56 e | 13.9 ± 0.47 ij | 17.8 ± 0.65 h | nd | 35.0 ± 0.82 b | 7.7 ± 0.46 j | nd | nd |
| 3,4-DCP | NRM     | Static    | nd | 37.2 ± 1.27 e | 44.6 ± 0.06 e | 17.9 ± 1.01 e | 35.9 ± 2.00 b | 44.6 ± 1.13 hi | 306.5 ± 3.65 c | 138.4 ± 1.50 f |
|        |         | Shaking   | nd | 11.8 ± 0.55 j | 3.49 ± 1.46 f | 26.7 ± 1.46 d | 25.6 ± 0.80 cd | 34.7 ± 1.82 i | 53.2 ± 2.50 h | 37.7 ± 2.20 k |
|       | NPM     | Static    | nd | 4.2 ± 0.45 k | 8.2 ± 0.55 i | nd | 32.9 ± 1.95 bc | 47.2 ± 1.78 h | 251.5 ± 3.84 e | 171.8 ± 3.53 d |
|        |         | Shaking   | nd | 15.4 ± 1.64 hi | 33.1 ± 0.62 fg | 33.9 ± 2.21 c | nd | nd | nd | 48.6 ± 1.04 j |
| Control | NRM     | Static    | 45.0 ± 1.30 a | 105.7 ± 0.81 a | 215.9 ± 3.02 a | 215.7 ± 3.35 a | 359.9 ± 2.00 b | 44.6 ± 1.13 hi | 306.5 ± 3.65 c | 138.4 ± 1.50 f |
|        |         | Shaking   | 17.6 ± 0.66 c | 66.6 ± 0.70 b | 75.1 ± 0.26 d | 47.9 ± 0.60 b | 37.8 ± 1.88 b | 209.8 ± 4.70 c | 350.6 ± 4.80 a | 104.3 ± 1.87 g |
|       | NPM     | Static    | 25.8 ± 0.30 b | 43.4 ± 1.07 d | 106.8 ± 0.64 c | 48.7 ± 1.13 b | 32.8 ± 2.26 bc | 165.4 ± 5.42 f | 253.9 ± 4.76 e | 303.8 ± 2.54 a |
|        |         | Shaking   | 15.1 ± 2.51 d | 37.4 ± 1.70 e | 31.2 ± 0.87 g | 33.9 ± 2.47 c | nd | 86.5 ± 0.86 g | 31.8 ± 2.89 i | 36.9 ± 1.70 k |

Note: In each column different letters means significant difference (p<0.05)
nd- not detected

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