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Changes in biochemical composition of fruiting substrates by the activities of Reishi Mushroom (*Ganoderma* spp.)

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Abstract
The present investigation was conducted at the Mushroom Research Laboratory, Department of Plant Pathology, Department of Medicinal and Aromatic Plant Science, and Department of Soil Science, College of Agriculture, Indira Gandhi Krishi Vishwavidyalaya, Raipur (Chhattisgarh). The changes in biochemical constituents with the activity of different species of *Ganoderma* was studied. The degradation of cellulose, lignin and total sugars was found to be less after 20 days of inoculation but, as the fungal growth in the substrate advanced, the rate of degradation was faster and reached to maximum, when the fungus attained fruiting stage. No particular trend was found in phenol content as it showed marked variation at different stages of growth.

Keywords: *Ganoderma applanatum*, *G. Lucidum*, cellulose, hemicullulose, lignin, ligocellulosic waste

Introduction
Medicinal mushrooms are known to have effective biochemical substances responsible for antifungal, anti-inflammatory, antitumor, antiviral, antibacterial, hepatoprotective, antidiabetic, hypolipedemic, antithrombotic and hypotensive activities (Wasser and Weis, 1999). The medicinal mushroom genus *Ganoderma* is established by Karsten (1881) with *Ganoderma lucidum* (Curtis) P. Karst. As the type species (Moncalvo and Ryvarden, 1997). *Ganoderma* species are distributed worldwide and the fruiting bodies of *Ganoderma* grow from living, or more commonly, from dead trunks or branches of trees. *Ganoderma* species are white rot fungi and have ecological importance in the breakdown of woody plants for nutrient mobilization. They hold effective mechanisms of lignocellulose-decomposing enzymes useful for bioenergy production and bioremediation (Adaskaveg *et al*. 1991, Coetzee *et al*. 2015, Kües *et al*. 2015, Hapuarachchi *et al.* 2018) [1, 3, 16, 12]. Root and stem rots caused by *Ganoderma* species result in loss of forestry yields (Singh 1991, Kinge and Mih, 2015 and Monkai *et al*., 2016) [22, 15, 18] worldwide. Turner (1981) [25] reported 15 species of *Ganoderma* from various parts of the world, such as Africa, India, Indonesia, Malaysia, North America, Papua New Guinea and Thailand as being associated with basal stem rot of oil palm, including *G. applanatum*, *G. boninense*, *G. tornatum*, *G. tropicum* and *G. zonatum*. Most of the basidiomycetous fungi have the capacity to degrade lignin. White rot fungi utilize cellulose, hemicellulose and lignin component with the help of cellulose and ligninolytic enzymes. Brown rot fungi could only utilize cellulose and hemicellulose and lignin part remain unutilized as brownish tissue. Several ligninolytic enzymes like lignin peroxidase, mangamese peroxidase, Laccase and aryl alcohol oxidase have been reported from white rot fungi.

White-rot fungi (Basidiomycetes) produce various extracellular enzymes, such as laccase (Lac), manganese peroxidase (MnP) and lignin peroxidase (LiP), which are involved in the degradation of lignin and their natural lignocellulosic materials. The degradation of complex lignocellulosic waste is brought about by the activity of several enzymes like β-glucosidase, cellulase, xylanase, lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase among others. It also largely depends upon the synergistic action of extracellular enzymes like ligninases, cellulases and hemicellulases

These enzymes are characteristically produced by higher fungi particularly the wood degrading basidiomycetes. It is well established that mushroom yield depends largely on the nature of substrates used. The enzymatic degradation of lignocellulosics is primarily governed by their structural features, since 1. Cellulose present in the biomass possess a highly resistant crystalline structure. 2. Lignin surrounding the cellulose forms a physical barrier 3. The sites available for enzymatic attack limited. Any means that increase the amorphons content of cellulose enhance the hydrolysis rate (Fan *et al.* 1980 a, 1980 b) [10, 11]. Pre-treatment, therefore is an essential pre requisite to enhance the susceptibility of lignocellulosics to enzyme action. Several studies on the cultivation of *Agaricus bisporus, Pleurotus flabellatus, P. sajor-caju, P. cornuicopiae* var. *citrinopileatus, P. djamor* and *P. ostreatus* have suggested that changes in extracellular activities of cellulase and laccase are directly correlated with growth and fruit body formation (Manning and Wood, 1983; Claydon and Wood, 1988, Eriksson, 1988, Rai and Saxena, 1990, Kaviyarasan and Natarajan, 1997 and Sharma *et al.* 1999) [17, 2, 9, 21, 9, 21, 14, 24]. The enzymes involved are laccase, phenol oxidase, cellulose, amylase and pectinase. Dejong *et al.* (1992) [5] also found manganese peroxidase activity in all fungi which were able to decolourize poly R-478 but these fungi did not produce any ligninases except *P. chrysoporium.* The ability of a lignin degrading basiodiomycetes PM-1 (CET-2971) to decolorize Kraft lignin and poly R-478 dye had very high laccase activity (Coll *et al.*, 1993) [4]. An attempt for therefore made to study the changes in biochemical constituents of the fruiting substrates used for cultivation of *Ganoderma* spp., a magic mushroom.

Materials and Methods

Changes in biochemical constituents of fruiting substrates: From seven fruiting substrates, samples were derived at five stages *viz*. initial stage, 20 days after inoculation (DAI), 40 DAI, 60 DAI and 80 DAI. The samples were then processed for degradation of cellulose, phenol, total sugars and lignin component. Each treatment had three replications.

Determination of Cellulose

Materials used were acetic/nitric reagent, 150ml of 80% acetic acid and 15ml of concentrated nitric acid. Anthrone reagent (dissolved 200 mg anthrone in 100ml concentrated sulphuric acid) was prepared fresh and chilled for 2 hrs. Before use. Added acetic/nitric reagent (3ml) to a known amount (0.5g) of the sample in a test tube and mixed in a vortex. Placed the tube in a water bath at 100 °C for 30 min. Cooled and then centrifuged the contents for 15-20 min. Discarded the supernatant. Washed the residue with distilled water and again centrifuged it and discarded the supernatant from tube. Added 10 ml of 67% sulphuric acid and allowed it to stand for 1 hr. Diluted 1 ml of the above solution of 100 ml of distilled water. To 1 ml of this diluted solution, added 10 ml of anthrone reagent and mixed well. Heated the tubes in a boiling water bath for 10 min. Cooled and measured the colour at 630nm. Set a blank with anthrone reagent and distilled water. Cellulose content (mg g^{-1}) was determined as under.

= 344.83 x OD x 100/1 x 10/1 x 1/0.5 x 100/1000 x 1/1000

Determination of Phenol

Borate buffer (0.2 M, pH 7.6), 80% alcoholic borate buffer, saturated Na_2CO_3 and folin-cioculteau reagent were used for determination of phenol. Sample of 0.5g was weighed and grind it with a pestle and mortar in 7 ml of 80% alcoholic borate buffer. Centrifuged the homogenate at 10000 rpm for 20 min. and saved the supernatant. Pipetted out different aliquots to make up the volume 3 ml into test tube. Sample extract-100 ml, distilled water 800 ml, folin-cioculteau reagent-100 ml and saturated Na_2CO_3-2000 ml were taken and mixed thoroughly. Placed the tubes in boiling water for exactly one min, cooled and recorded the observation at 660 nm against a blank. Set a blank with distilled water + folin-cioculteau reagent + saturated Na_2CO_3. Phenol content (mg g^{-1}) was calculated as under:

= 363.63 x 2 x 7000/100 x 10/0.5 x 1/1000 x OD

Estimation of Lignin

For estimation of lignin content, the collected samples (0.5 g in weight) were digested in hot concentrated sulphuric acid and the insoluble residue obtained by filtration were oven dried and weighted (Effland, 1977) [8].

Estimation of total sugars

Total sugars were estimated by using sodium phosphate buffer (0.05 M 7.2 pH) as per methods by Dubios (1956). The samples were dried and powered (200 mesh) using Willy mill. IEC centrifuge was used at 3000 rpm. Micropipettes were used for serial dilution by Milli Q super distilled water. Constant temperature water bath was used for colour development. Fresh Anthrone reagent (0.2%) was prepared in concentrated sulphuric acid which was chilled for 2 hours prior to use. 0.1 g sample was taken in 3 ml phosphate buffer (0.05 M, 7.2 pH) for extraction of total sugars. Thereafter, it was centrifuged at 3000 rpm for 15 minutes. The supernatant was collected in test tube and 10 µl supernatant was taken with the help of the micropipette from each sample then 208 ml anthrone reagent was mixed and final volume (4 ml) was prepared by adding distilled water. These tubes were placed in boiling water for 10 minute. Glass balls were kept on each test tube to avoid loss of water by evaporation. All samples were allowed to cool at room temperature. A bluish green colour appeared. A blank was used. The optical density of each sample was recorded at wavelength 625 nm with the use of blank. It was calculated as under:

Total sugars (%) = 82.98755 x reading x 5000/10 x 10/0.1 x 1/1000 x 1/1000

Results and Discussion

Change in biochemical composition of substrates with the activity of *Ganoderma* spp. during different period of incubation Natural plant wastes are the basic substrates for growth and yield of a mushroom. Chemically, these inedible plant wastes are lignocellulosics, which are difficult to degrade or by far the most abundant renewable organic materials available for biological and chemical conversion into usable products. Lignocellulose is the building block of the stem and are not easily degradable. A wide variety of microorganisms including mushroom fungi produce enzymes which can degrade cellulose, hemicelluloses and lignin. Mushroom fungi are efficient and effective examples of the microbial world gifted with the unique ability of ligninolytic activity, to degrade lignin besides cellulose and hemicellulose, in turn producing fruiting bodies which are highly nutritious and have varieties of health benefits. The change in biochemical constituents of different substrates by the activity of *Ganoderma* spp. are as under:

Degradation of cellulose

The degradation of cellulose in different substrates with the activity of *Ganoderma* spp. was studied and the results are presented in Table 1. The initial content of the cellulose in different substrates differed significantly. The initial cellulose content was significantly higher in saw dust (14.54 mg g^{-1}), while, it was significantly less (7.94 mg g^{-1}) in sugarcane baggase. The degradation of cellulose after 20 days of inoculation was less but the rate of degradation increased rapidly as the *G. applanatum* reached to its fruiting stage i.e. 80 days after inoculation. Similar trend was also observed in case of local strain of *G. lucidum.* Maximum degradation of cellulose was observed in a local strain of *G. lucidum* colonised paddy straw + wheat straw substrate (76.03%) followed by wheat straw (75.96%) and paddy straw alone (75.53%) whereas it was least (68.77%) in saw dust. On the other hand, degradation of cellulose was comparatively lesser in all the substrates (67.57-74.79%) colonized by *G. applanatum* after 80 days of inoculation. Degradation of cellulose in paddy straw+ wheat straw, wheat straw and paddy straw substrates might be due to average bigger size of the capillaries in the biomass facilitating more enzymatic activities and thus fast degradation compared to that of saw dust substrate.

Table 1: Degradation in cellulose content of different substrates by the activity of *Ganoderma* spp. during different period of incubation

Substrates	Initial stage (Before inoculation)	Days after inoculation							
		20		40		60		80	
		Ganoderma appIanatum	*G.a lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain
Saw-dust	14.54	12.22 (16.75)*	12.63 (13.13)	10.20 (30.51)	10.41 (28.40)	7.58 (48.36)	7.32 (49.65)	4.45 (69.68)	4.54 68.77)
Wheat straw	8.78	7.32 (13.78)	7.03 (19.93)	5.88 (30.74)	6.10 (30.52)	4.06 (52.17)	4.10 (53.30)	2.14 (74.79)	2.11 75.96)
Paddy straw	9.89	9.31 (4.61)	8.34 (15.67)	17.87 (19.36)	6.90 (30.23)	5.23 (46.41)	4.38 (55.71)	3.03 (68.95)	2.42 (75.53)
Paddy straw+wheat straw	9.64	8.90 (8.53)	8.24 (14.52)	6.97 (28.36)	6.84 (29.04)	5.46 (43.88)	4.25 (55.91)	3.29 (66.18)	2.31 (76.03)
Sugarcane baggase	7.94	6.58 (12.38)	6.88 (13.35)	5.78 (23.03)	5.41 (31.86)	4.07 (45.80)	4.12 (48.11)	2.02 (73.10)	2.02 (74.55)
Paddy straw+saw-dust	10.24	8.99 (13.33)	9.08 (11.32)	6.13 (40.88)	7.19 (29.78)	5.57 (55.93)	5.19 (49.31)	2.62 (74.73)	2.95 (71.19)
Wheat straw+saw-dust	11.36	10.48 (7.66)	10.14 (10.73)	8.67 (23.61)	8.23 (27.55)	6.87 (39.47)	5.94 (47.71)	3.68 (67.57)	3.14 72.35)
F value	S	S	S	S	S	S	S	S	S
SEm±	0.193	0.86	0.211	0.751	0.684	0.72	0.149	0.0771	0.095
CD (0.05)	0.59	2.63	0.64	2.28	2.08	2.20	0.45	0.23	0.29

*: Values in parenthesis denotes per cent degradation in cellulose

Degradation in total sugar

The initial sugar content was significantly higher in sugarcane baggase (50.05 mg g^{-1}), while it was significantly lower (12.75 mg g^{-1}) in saw dust substrate (Table 2). The rate of degradation was higher in paddy straw + wheat straw (63.88%) which was closely followed by wheat straw + saw dust (59.49%). The maximum sugar was removed by local strain of *G. lucidum* (GR/L/04/1) at 80 DAI in sugarcane baggase (64.75%) closely followed by paddy straw+wheat straw substrates, whereas, maximum sugar degradation in case of *G. applanatum* was observed in wheat straw (56.78%). The minimum sugar was removed by *G. applanatum* in wheat straw + saw dust (42.68%), whereas, local strain of *Ganoderma* (GR/L/04/1) showed minimum degradation (53.41%) of sugar in saw dust alone after 80 days of inoculation. It is obvious that sugarcane baggase with more total sugars supports better vegetative as well reproductive growth of *Ganoderma* spp. and was also reported by Sharma and Thakur (2010) [23].

Table 2: Degradation in total sugar content of different substrates by the activity of *Ganoderma* spp.

Treatments	Initial stage (Before inoculation)	Days after inoculation							
		20		40		60		80	
		Ganoderma appIanatum	*Ganoderma lucidum* local strain GR/L/04/I	*Ganoderma appIanatum*	*Ganoderma lucidum* local strain GR/L/04/I	*Ganoderma appIanatum*	*Ganoderma lucidum* local strain GR/L/04/I	*Ganoderma appIanatum*	*Ganoderma lucidum* local strain GR/L/04/I
Saw-dust	12.75	10.04(21.25)*	10.09(20.86)	9.22(27.68)	9.12(28.47)	8.32(34.74)	7.35(42.35)	6.81(46.58)	5.94 53.41)
Wheat straw	24.25	22.91(5.52)	22.84(5.81)	17.37(28.37)	17.35(28.45)	14.15(41.64)	14.12(41.77)	10.48(56.78)	9.91 59.13)
Paddy straw	26.07	25.11(3.68)	24.41(6.36)	20.38(21.82)	19.25(26.16)	15.34(41.15)	14.13(45.79)	11.52(55.81)	11.45(56.07)
Paddy straw+wheat straw	27.91	24.96(10.56)	24.23(13.18)	18.35(34.25)	17.22(38.30)	16.55(40.70)	15.32(45.10)	14.41(48.36)	10.08(63.88)
Sugarcane baggase	50.05	44.68(10.72)	45.41(9.27)	38.85(22.37)	37.82(24.43)	30.34(39.38)	29.32(41.41)	22.94(54.10)	17.64(64.75)
Paddy straw+sawdust	19.56	17.91(8.43)	17.24(11.86)	14.88(23.92)	14.42(26.27)	10.37(46.98)	10.54(46.11)	8.88(54.60)	8.47(56.69)
Wheat straw+saw dust	17.01	16.04(5.70)	16.15(5.05)	14.38(15.46)	14.82(12.87)	11.50(32.39)	11.32(33.45)	9.75(42.68)	6.89(59.49)
F-Value	S	S	S	S	S	S	S	S	S
SEm±	0.26	0.15	0.28	0.19	0.13	0.19	0.21	0.21	0.18
CD(0.05)	0.82	0.46	0.63	0.58	0.42	0.59	0.65	0.64	0.55

*: Values in parenthesis denotes per cent degradation in sugar content

Degradation in lignin content

The initial lignin content was significantly higher in saw dust (63.58 mg/g) whereas, it was less (51.65 mg g^{-1}) in sugarcane baggase (Table 3). After 20 days of inoculation, the degradation of lignin started in all the substrates and the degradation increased rapidly as the fungus reached to its fruiting stage. Maximum degradation in *G. applanatum* (60.89%) was observed in paddy straw + saw dust substrates whereas, local strain of *Ganoderma* spp. had maximum degradation (61.47%) of lignin in paddy straw + wheat straw substrate.

Table 3: Degradation in lignin content of different substrates by the activity of *Ganoderma* spp.

Treatments	Initial stage (Before inoculation)	Days after inoculation							
		20		40		60		80	
		Ganoderma appIanatum	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain
Saw-dust	63.58	45.89(27.82)*	46.37(27.06)	35.66(43.91)	36.46(42.66)	30.84(51.49)	30.96(51.30)	24.86(60.89)	26.03(59.05)
Wheat straw	58.02	39.58(31.78)	37.91(34.66)	35.91(38.10)	36.07(37.83)	31.01(46.55)	30.43(47.55)	26.91(53.61)	26.15(54.92)
Paddy straw	54.20	44.81(17.32)	40.79(24.74)	35.43(34.63)	35.53(34.44)	29.23(46.07)	28.72(47.01)	23.50(56.64)	23.43(56.77)
Paddy straw+wheat straw	57.94	34.80(39.93)	34.08(41.18)	33.16(42.76)	32.81(43.37)	28.75(50.37)	27.04(53.33)	22.89(60.49)	22.32(61.47)
Sugarcane baggase	51.65	33.46(35.21)	34.60(37.82)	32.16(37.73)	33.46(39.87)	27.53(46.69)	28.23(49.27)	25.78(50.00)	24.03(56.81)
Paddy straw+saw-dust	59.93	40.12(33.05)	40.24(32.85)	84.00(40.00)	34.55(42.35)	35.98(39.96)	32.32(52.74)	24.99(58.30)	23.70(60.45)
Wheat straw+saw-dust	56.36	43.88(22.62)	43.34(23.10)	37.64(33.28)	35.50(37.01)	32.55(42.24)	28.00(50.31)	25.16(55.35)	24.60(56.35)
F-Value		S	S	S	S	S	S	S	S
SEM±	1.417	1.663	1.500	NS	NS	NS	NS	NS	NS
CD(0.05)	4.030	5.050	4.560	1.884	1.665	1.731	1.629	1.810	1.660

*: Values in parenthesis denotes per cent degradation in lignin content

Change in phenol content

The initial phenol content was significantly higher in saw dust (38.19 mg g^{-1}) whereas, it was significantly lower (15.37 mg g^{-1}) on sugarcane baggase (Table 4). The phenol content showed marked variation in different fruiting substrates but no trend was obtained at different stages of incubation. The declining trend was observed in wheat straw + saw dust up to 40 DAI and thereafter, the increasing trend was noticed. In paddy straw, an increase was observed up to 40 days after inoculation and then decreased. In paddy straw + saw dust, the phenol content decreased at 20 and 40 DAI but increased at 60 DAI and again decreased at 80 DAI. Increase in phenol at 60 DAI may be associated with requirement of more phenol at ripening stage of fruiting body.

Table 4: Change in phenol content in different substrates by the activity of *Ganoderma* spp

Treatments	Initial stage (Before inoculation)	Days after inoculation							
		20		40		60		80	
		Ganoderma appIanatum	*G.a lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain	*Ganoderma appIanatum*	*G. lucidum* local strain
Saw-dust	38.19	43.50(13.90)*	43.46(13.79)	44.44(16.36)	43.63(14.24)	45.48(19.08)	44.65(16.91)	36.41(-4.55)	33.27(-12.88)
Wheat straw	20.73	28.91(39.45)	29.04(40.08)	31.08(49.92)	33.41(61.16)	30.23(45.82)	31.28(50.89)	33.80(63.04)	34.91(68.40)
Paddy straw	21.86	24.92(14.27)	22.08(1.00)	32.30(47.75)	31.02(41.90)	19.84(-9.24)	28.91(-13.49)	15.22(-30.37)	14.21(-34.08)
Paddy straw+wheat straw	21.62	25.29(16.97)	24.91(15.21)	29.74(37.55)	29.91(38.34)	20.22(-6.47)	17.81(-17.62)	32.19(48.88)	30.48(40.98)
Sugarcane baggase	15.37	22.66(47.43)	24.46(59.14)	23.39(52.17)	22.92(49.12)	24.41(58.81)	25.24(64.21)	27.74(78.26)	28.44(85.03)
Paddy straw+saw dust	30.48	24.35(-20.11)	23.29(-23.58)	20.57(-32.51)	21.74(-28.67)	34.41(12.89)	35.93(17.88)	23.04(-24.40)	24.47(-19.71)
Wheat straw+saw dust	28.93	21.63(-25.23)	23.46(-18.90)	25.79(-10.85)	27.21(-5.94)	34.44(19.04)	37.21(28.62)	41.21(42.44)	44.74(54.64)
F value	S	S	S	S	S	S	S	S	S
SEm±	0.46	0.11	0.28	0.12	0.10	0.09	0.17	0.06	0.10
CD(0.05)	1.42	0.35	0.92	0.36	0.30	0.28	0.57	0.20	0.32

*: Values in parenthesis denotes per cent degradation in phenol content

Degradation of cellulose, total sugars and lignin was found by the activity of *G. applanatum* and local strain of *G. lucidum* whereas no trend was found in case of phenol content. The degradation of cellulose, lignin and total sugars was less after 20 days of inoculation but the rate of degradation increased rapidly when both the fungus reached to their fruiting stages. Changes in phenol content was also observed but no particular trend was found as it showed marked variation in different fruiting substrates. Thus, changes in different biochemical constituents and the speed at which they changed is an indication of good growth and development in the substrates. Dewangan (2006) [6] also reported the similar changes in lignin, cellulose and phenol content in different substrates colonised by *Lentinula edodes*. Similar results were also reported by Kaviyarasan and Natrajan (1997) [14] and Sharma *et al.* (1999) [24] in paddy straw due to the activity of *Pleurotus cornucopiae* var. *citrinopleatus* at growth and fruiting stages. Rai (2003) [20] also found that *G. lucidum* produced full assay of enzymes namely ligninases, manganese peroxidase, laccase, polyphenol oxidase for lignin degradation, exglucanase, endoglucanase and β glucosidase for degradation of cellulose. *Ganoderma* species also play an important role in breakdown of woody plants for nutrient mobilization, production of bioenergy and in bioremediation, as it contains, the mechanism of lignocellulose decomposing enzyme (Adaskaveg *et al*. 1991, Coetzee *et al*. 2015) [1, 3].

Conclusions

Mushroom species grow and yield on a wide spectrum of plant waste consisting of cellulose, hemicellulose and lignin. Growing medicinal or other mushrooms on lignocellulosic wastes represent the most successful example of solid state fermentation to generate a quick separation of valid and valued form of biomass represented by the mushrooms. Biodegradation and biotransformation of lignocellulosic wastes by different strains and species of *Ganoderma* through production of varying extracellular enzymes serves to return carbon to the atmosphere in its most natural form. Mushrooms are, thus, the potential degradors of lignocellulosic wastes obtained from agriculture, forestry and industries.

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