Enhancement of mycelial biomass and lignocellulolytic enzymes of milky mushroom *Calocybe indica* through supplementation with organic amendments

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Abstract

Milky mushroom (Calocybe indica) also known as tropical mushroom or summer mushroom has significant attributes of milky white colour with high nutritive value and long shelf life. The yield performance of milky mushrooms again depends on the secretion of lignocellulolytic enzymes that further influence the colonization of mycelium on the substrates. In the present study, organic supplements viz. cakes of cotton, groundnut, sesamum, coconut cake, neem cake, powdered moth bean, red gram, horse gram, green gram, soya bean, lab-lab, maize, rice bran and wheat bran amended at 1% and 5% concentration induced profuse mycelial growth and enhanced ligninolytic enzyme secretion by Calocybe indica under in vitro conditions.

The results revealed that wheat bran and red gram supplements at 5 % concentration in PDA medium were found to influence the mycelial growth on the 7th day (68.67 mm and 62.33 mm respectively). Similarly, both wheat bran and red gram supplements at 5 % in liquid medium supported increase in the biomass production of C. indica on the 15th day (1.83 and 1.40 g) followed by horse gram. The influence of organic supplements on the induction of lignocellulolytic activity showed that the maximum cellulase and xylanase activity (1.7 U/min/g and 0.835 U/min/g respectively) and laccase activity (1.6 U/min/g) were noticed in the substrate supplemented with wheat bran followed by horse gram (1.3 U/min/g) at 5% concentration under solid-state fermentation. However, the lignin peroxidase activity was maximum (0.59 U/min/g) in combination with wheat bran (2.5%) and red gram (2.5%) and manganese peroxidase activity was maximum in wheat bran supplement (0.3 U/min/g) at 5% concentration. It is inferred that amending organic supplements viz. wheat bran, horse gram and red gram to substrates enhance the mycelial growth, biomass production and lignocellulolytic enzyme secretion of C. indica and offers scope for yield improvement.

Keywords: *C. indica*, enzymes, supported, concentration, growth, supplements

Introduction

Calocybe indica is a tropical edible mushroom commonly known as milky mushroom or Dudh Chatta or summer mushroom, belonging to the family Tricholomataceae of the order Agaricales.[9] The appealing nature of milky mushroom with its robust size, long shelf life, delicious nature and nutritive value makes it more profitable for mushroom growers.[10,22] The technology for commercial cultivation of milky mushroom, *C.indica* was first developed by TNAU and released as APK 2.[12] Milky mushroom prefers a temperature range of 30 to 35° C and relative humidity of 85 percent.[12]

Though the appropriate environmental factors such as temperature and relative humidity are essential for the production of milky mushrooms, the substrate nutrients greatly influence the growth and yield.[4] Many researchers have contributed to the studies on the addition of different growth supplements of various C/N ratios, minerals and vitamins to the substrate for better growth and the enhanced yield of milky mushrooms.[16]

Also, Xie et al[25] stated that cellulose enzyme initiates the mycelial colonization and laccase enzymes that help in the induction of sporocarp also rely on substrate nutrition. Degradation of lignocellulose substrates is influenced particularly by the enzymes, cellulase xylanase, lignin peroxidase, manganese peroxidase and laccase which play a major role in the biological efficiency of mushroom.[8] Bhupathi et al[3] reported that the xylanase enzyme activity was significantly found at all the growth stages of *C. indica* besides, the enzymes such as laccase, mannitol dehydrogenase, xylanase, lipoxygenase and tyrosinase activity found to be maximum in the elongation stage of *C. indica*

Likewise, Alam et al[1] reported that the growth supplements such as rice bran, maize powder and wheat bran used at different concentrations revealed that supplementation with maize powder at 30 percent concentration initiated early primordial formation along with increased the biological and economic yield of *C. indica.* In addition, Sardar et al[19] also reported that supplementation of wheat bran to substrates played a significant role in initiating early spawn run, pinhead formation and yield of milky mushroom *C. indica.*

Alternatively, Thiribhuvanamala et al[23] revealed that the addition of organic supplements to substrate enhanced the ligninolytic secretion of white-rot fungi such as *Pleurotus pulmonarius, P. sajor-caju and Schizophyllum commune.*

Based on the above information, the present investigation was taken up with more emphasis on studying the influence of different growth supplements to increase the ligninolytic enzyme secretion and mycelial biomass thereby leading to increased yield.

Material and Methods

Influence of various organic supplements on the mycelial growth and biomass production of *Calocybe indica*: The pure culture of milky mushroom *Calocybe indica* var. APK2 was obtained from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and used for the studies.

To enhance the radial growth and biomass production of *C. indica*, different organic supplements *viz*. cakes of cotton, coconut, sesame, groundnut, neem and powders of moth bean, red gram, green gram, cowpea, horse gram soya bean, lablab, maize, rice bran and wheat bran were supplemented at 1% and 5% concentration separately in potato dextrose agar medium (PDA) in conical flasks and sterilized in an autoclave at 15 lbs (121° C) for 15 min. The sterilized medium was inoculated with a 9 mm mycelial disc of *C. indica* at the center of the Petri dish and incubated at room temperature (28 ± 2°C) and the radial mycelial growth was measured on 7th day.

In a separate study, the above-mentioned supplements were added in potato dextrose (PD) broth at 1% and 5 % concentration and sterilized in an autoclave at 121 ^{0}C for 15 mins. Later, a 9 mm mycelial disc was inoculated into PD broth and incubated at room temperature (28 ± 2°C) and the biomass was recorded on the 15th day. Later, based on the results, the best supplements were selected and tested in combination at 5 % concentration on the mycelial biomass of *C. indica* in PD medium and broth.

Estimation of lignocellulolytic enzyme assay for *C. indica* under Solid-state fermentation: Based on the previous results, wheat bran, horse gram and red gram powders that supported the mycelial growth and biomass of *C. indica* were used for studying the secretion of lignocellulolytic enzymes under solid-state fermentation and liquid state fermentation.

S.N.	Supplements
1	Paddy straw + Wheat bran powder (5%)
2	Paddy straw + Horse gram powder (5%)
3	Paddy straw + Red gram powder (5%)
4	Paddy straw + Wheat bran (2.5%) + Horse gram powder (2.5%)
5	Paddy straw + Wheat bran (2.5%) + Red gram powder (2.5%)
6	Paddy straw + Horse gram powder (2.5%) + Red gram powder (2.5%)
7	Paddy straw + Wheat bran (2%) +Horse gram powder (2%) + Red gram powder (1%)
8	Paddy straw alone (Control)

For solid-state fermentation, about 50 g of chopped paddy straw (50 % moisture content) was supplemented with wheat bran, horse gram and red gram powder added at 5% concentration separately. Treatment without supplements served as control. The bottles were sterilized in an autoclave at 15 lbs for 15 min. A 9 mm mycelial disc of *C. indica* was inoculated and incubated at room temperature (28 ± 2°C) for 15 days. Later, the samples were drawn and tested for enzyme activity.

Assay of lignocellulolytic enzymes

Cellulase enzyme: One gram of paddy straw amended substrate was taken and macerated with 0.01 M phosphate buffer and centrifuged at 12000 rpm for 15 mins. The supernatant was added with 0.5 ml of 1 % carboxymethyl cellulose (CMC) and incubated at 50 ºC for 30 mins in a water bath. Following this, 3ml of dinitro salicylic acid (DNS) was added and boiled at 55 ºC using a water bath for 15 mins. One ml of sodium potassium tartrate (40%) was added. Later, the substrates were cooled. 2 ml distilled water was added. Following this, absorbance was taken at 575 nm in spectrophotometry.[5]

Assay of Xylanase: One gram of paddy straw amended substrate was macerated and extracted with sodium citrate buffer (pH 4.8) and centrifuged at 12000 rpm for 15 min. at 4 ºC and the supernatant was used. as enzyme source. The reaction mixture consisted of 1.8 ml of 1% (w/v) suspension of xylan in 50mM sodium citrate and 0.2ml of enzyme dilution (in 50mM sodium citrate at pH 4.8) was incubated at 50ºC for 5 min. Reducing sugar was determined by the dinitrosalicylic acid reagent (DNS) method, by adding 3ml of DNS solution and then incubating the mixture at 95ºC for 5 min. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme utilizing the release of µmol of xylose equivalent/min.[13]

Assay of Laccase: One gram paddy straw substrate amended with growth supplements was taken separately and macerated using phosphate buffer (0.1 M) at pH 6.0 and centrifuged at 12,000 rpm for 15 min. at 4 ºC. The supernatant was collected and used for the enzyme assay. Later, 0.3 ml of the substrate was taken and add 2.5 ml of 30 µM guaiacol. The absorbance was read at 470 nm in spectrophotometry after incubating the reaction mixture for 30 min at 25ºC against zero-time control. One unit of enzyme activity was calculated as changes in absorbance by 0.001min/ml of enzyme source at 25ºC.[2]

Assay of Lignin peroxidase: One gram of paddy straw amended substrate was taken and macerated using 0.1M of phosphate buffer and centrifuged at 12000 rpm for 15 min. The supernatant served as an enzyme source. Kang et al[11] described lignin peroxidase assay with pyrogallol as substrate. The reaction mixture contained 1 ml of enzyme sample, 0.2 ml of 0.1M pyrogallol in 0.1M phosphate buffer (pH 6.0) and 0.1 ml of 0.1M H_2O_2. The enzyme activity was determined at 30 sec intervals for 5 min at 436 nm.

Assay of Manganese peroxidase: A gram of substrate was macerated using 0.1M phosphate buffer and centrifuged at 12000 rpm for 15 mints and the manganese peroxidase activity was performed as per the method of Wariishi et al[24] with sodium malonate as substrate. The reaction mixture contained 1ml of the enzyme, 0.2 ml of 1mM manganese sulfate in 0.1M sodium malonate and 0.2 ml of 0.1M H_2O_2. The oxidation of sodium malonate was read at 270 nm.

Results and Discussion

Milky mushroom (*C. indica*) is highly suitable for cultivation in hot humid tropical regions of India with sustainable yield.[20] However, the main concern of the farmers is to tap the maximum bio efficiency of milky mushrooms to enhance their income. A perusal of literature shows that organic supplements proved to enhance mycelial growth, biomass production and enzyme activity as observed in both solid and liquid state fermentation in our study.

Among the different organic supplements tested, both wheat bran and red gram powders recorded maximum mycelial growth both at 1% (59.67 mm and 58.67 mm respectively) and 5% concentration (68.67 mm and 62.33 mm respectively). Supplementation of horse gram powder at 1 % and 5% also supported maximum mycelial growth of 54.67 and 57.00 mm respectively compared to other supplements. Also, the wheat bran and red gram supplements supported complete growth (90.00 mm) of *C. indica* in solid medium (PDA) on the 10th day at 1% concentration and 9th day and 9.5 days at 5% concentration respectively. Horse gram supplement at 1% and 5 % supported full growth of *C. indica* on the 11th day and 10th day respectively, other treatments also supported fairly good growth but took longer days for attaining full growth in PDA medium in Petri dish. (Table.1; Plate 1).

The maximum biomass production of the fresh mycelial weight of *C. indica* was obtained in wheat bran (7.19 g and 11.98 g respectively) followed by horse gram (6.01g and 11.25 g respectively) and red gram (5.72 and 13.30 g respectively) at 1% and 5 % concentration in PD broth. Similarly, the maximum mycelial dry weight of *C. indica* was observed in wheat bran (1.26 g and 1.83 g respectively) followed by red gram (1.13 g and 1.40 g respectively) and horse gram (1.13 g and 1.26 g respectively) at 1 and 5 % concentration. Though neem cake also supported fresh mycelial growth at 1% concentration, higher concentration i.e. at 5%, the mycelial growth was inhibited and thereby recorded reduced dry mycelial weight (Table 2).

Combined application of wheat bran, horse gram and red gram supplement was tried for studying the mycelial growth of *C. indica* and recorded maximum radial mycelial growth of 42.3 mm and 41 mm in wheat bran and horse gram respectively on 7th day. However, increased mycelial biomass (fresh weight and dry weight) of *C. indica* was observed in wheat bran (6.24 and 1.41 g respectively) and horse gram alone (6.16 and 1.34 g respectively) supplementation at 5 % concentration (Table 3).

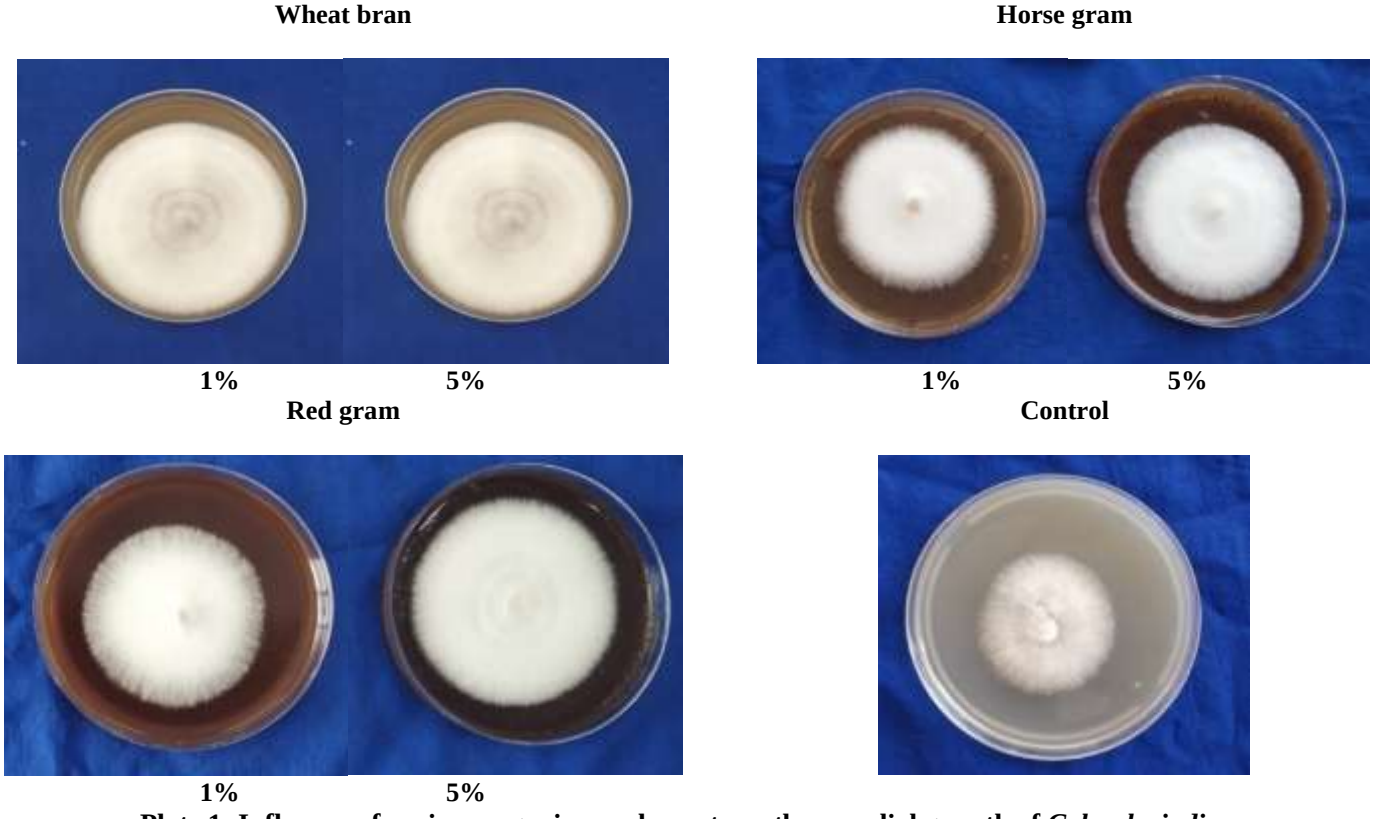

Plate 1: Influence of various organic supplements on the mycelial growth of *Calocybe indica*

Table 1
Influence of various organic supplements on the mycelial growth of *Calocybe indica* var. APK 2

S.N.	Growth supplements	Radial mycelium growth (mm)		Days took to attain full growth	
		1%	5%	1%	5%
1	Cotton cake	24.00^{k} (29.33)	37.33^{gh} (37.66)	15	14
2	Groundnut cake	46.00^{fgh} (42.71)	45.33^{ef} (42.32)	12.5	12.5
3	Sesame cake	42.67^{hi} (40.78)	39.67^{g} (39.04)	13.0	14
4	Coconut cake	39.67^{ij} (39.04)	27.33^{j} (31.52)	13.5	15
5	Neem cake	44.00^{gh} (41.55)	28.33^{ij} (32.16)	13.0	15
6	Moth bean powder	52.00^{cd} (46.15)	57.00^{bc} (49.02)	11.5	10
7	Red gram powder	58.67^{a} (49.99)	62.33^{b} (52.14)	10	9.5
8	Horse gram powder	54.67^{bc} (47.68)	57.00^{bc} (49.02)	11.0	10
9	Cowpea powder	47.33^{efg} (43.47)	49.33^{de} (44.62)	12.50	12.5
10	Green gram powder	51.33^{cde} (45.76)	55.33^{c} (48.06)	11.5	12
11	Soya bean powder	49.33^{def} (44.62)	48.67e (44.24)	12.0	12
12	Lablab powder	42.67^{hi} (40.78)	54.67^{cd} (47.68)	13.0	12
13	Maize powder	44.33^{gh} (41.75)	49.33^{de} (44.62)	13.0	12.5
14	Rice bran	38.00^{j} (38.06)	33.33^{hi} (35.26)	13.5	14
15	Wheat bran	59.67^{a} (50.57)	68.67^{a} (55.96)	10	9
16	Control	40.33^{ij} (39.43)	40.33^{fg} (39.43)	13	13
17	**SED**	**2.03**	**2.69**		
18	**CD (0.05)**	**4.15**	**5.47**		

Mean of three replications. Value in parenthesis indicate arc transformed value

The influence of organic supplements on the induction of lignocellulolytic activity showed that the maximum laccase activity was noticed in the substrate supplemented with wheat bran (1.6 U/min/g) followed by horse gram (1.3 U/min/g). However, the lignin peroxidase activity was maximum in a combination of a wheat gram and red gram (0.59 U/min/g) and manganese peroxidase activity was maximum in wheat bran supplement (0.3 U/min/g). The cellulose and xylanase activity were maximum in wheat bran supplemented substrate (1.7 U/min/g and 0.835 U/min/g respectively) compared to control (Fig. 1).

It is observed that in our study, supplementation of wheat bran, red gram and horse gram has an improved effect on the radial mycelial growth and biomass production of *C. indica*. Probably, the phenolics present in wheat bran would have stimulated the mycelial growth and led to more biomass production. It is found that wheat bran and horse gram supplementation alone at 5% concentration are enough to support maximum mycelial biomass and enzyme secretion of *C. indica* rather than combination treatments.

Moreover, the cost of wheat bran and horse gram is much less compared to other amendments. Control without supplements recorded reduced radial mycelial growth and biomass of *C. indica.* If these supplements are added to, the substrates certainly will enhance the faster and maximum mycelial growth of *C. indica* which will lead to increased secretion of lignocellulolytic enzymes for effective utilization of substrates.

Supporting this study, Sardar et al[19] reported that the substrates supplemented with rice bran and wheat bran enhanced the fruiting body of *C. indica*. Alternatively, Moonmoon et al[14] stated that the sawdust supplemented with 25 % and 40 % wheat bran supported more fruiting body production apart from increasing the quality of *Lentinus edodes* of *the* fruiting body.

Following this, Oseni et al[17] reported that wheat bran supplementation @ 15% with pine sawdust boosted the yield of *Pleurotus osteratus*. Likewise, the substrate supplemented with wheat bran (20 %) initiated the early spawn running, primordial formation and increased the yield in *P. florida*.[6] Wheat bran supplementation also influences the C/N ratio in addition to playing role in increased nutrients *viz.* carbohydrates, amino acids and minerals which might improve the quality and yield of milky mushrooms. Interestingly, lignocellulolytic enzymes play a vital role in biological efficiency as it helps in quick initiation of pinhead formation and expansion of fruiting bodies with an increased yield of *C. indica*.

In our study, the laccase enzyme was found to be maximum in wheat bran supplement both solid and liquid state fermentation followed by horse gram. Besides, enzymes such as cellulase, xylanase and manganese peroxidase were significantly higher in wheat bran supplemented with substrates. Bhupathi et al[3] reported that the xylanase enzyme activity was found to be secreted in all the growth stages of *C. indica* in addition to the enzymes such as laccase, mannitol dehydrogenase, xylanase, lipoxygenase and tyrosinase activity significantly found at the elongation stage.

Table 2
Effect of various organic supplements on the biomass production of *Calocybe indica*

S.N.	Organic supplements	Fresh weight (g)		Dry weight (g)	
		(1%)	(5%)	(1%)	(5%)
1.	Cotton cake	3.53^{i} (10.83)	4.96^{j} 12.86	0.42^{i} (3.70)	0.47^{h} (3.93)
2.	Groundnut cake	3.15^{j} (10.23)	4.14^{l} 11.74	0.62^{g} (4.50)	0.76^{e} (5.00)
3.	Sesame cake	2.52^{l} (9.14)	2.73^{mn} 9.52	0.31^{k} (3.19)	0.31^{i} (3.21)
4.	Coconut cake	4.77^{de} (12.62)	2.88^{m} 9.77	0.53^{h} (4.19)	0.57^{g} (4.33)
5.	Neem cake	4.36^{f} (12.05)	0.07^{o} 1.48	0.81^{e} (5.17)	0.03^{k} (1.05)
6.	Moth bean powder	4.94^{d} (12.84)	7.30^{i} 15.67	0.93^{d} (5.54)	0.78^{e} (5.08
7.	Red gram powder	5.72^{c} (13.83)	13.30^{a} 21.39	1.13^{b} (6.11)	1.40^{b} (6.80)
8.	Green gram powder	4.63^{e} (12.43)	9.44^{f} 17.89	0.64^{g} (4.60)	0.72^{ef} (4.88)
9.	Cowpea powder	5.73c (13.85)	10.77^{d} 19.16	0.91^{d} (5.48)	0.94^{d} (5.56)
10.	Horse gram powder	6.01^{b} (14.19)	11.25^{c} 19.60	1.13^{b} (6.10)	1.26^{c} (6.44)
11.	Soybean powder	4.25^{fg} (11.89)	8.36^{g} 16.80	0.62^{g} (4.53)	0.83^{e} (4.56)
12.	Lablab powder	4.08^{gh} (11.66)	9.81^{e} 18.25	1.03^{c} (5.82)	1.20^{c} (6.29)
13.	Maize kernel powder	3.64^{hj} (11.06)	4.61^{k} 12.40	0.72^{f} (4.88)	0.74e (4.95)
14.	Rice bran	3.23^{j} (10.36)	7.73^{h} 16.15	0.44^{i} (3.82)	0.58^{g} (4.36)
15.	Wheat bran	7.19^{a} (15.55)	11.98^{b} 20.25	1.26^{a} (6.45)	1.83^{a} (7.78)
16.	Control	2.82^{k} (9.66)	2.82^{n} 9.67	0.36^{j} (3.45)	0.37^{j} (3.48)
17.	**SED**	0.1221	0.1362	0.0216	0.0468
18.	**CD (0.05)**	0.2487	0.2775	0.0441	0.0954

Mean of three replications. Value in parenthesis indicate arc transformed value

Table 3
Influence of organic supplements on growth and mycelial biomass of *Calocybe indica* var. APK 2

Organic Supplements	Radial mycelium growth (mm)		Mycelial biomass (g)	
	5th day	7th day	Fresh weight (5%)	Dry weight (5%)
W	22[a] (27.97)	42.3[a] (39.82)	6.24[a] (14.46)	1.41[a] (6.82)
H	23[a] (28.66)	41[a] (40.59)	6.16[b] (14.37)	1.34[a] (6.66)
R	15.3[b] (23.05)	35.3[b] (36.47)	5.45[c] (13.49)	1.04[b] (5.86)
W+H	12.7[c] (19.97)	28[d] (31.95)	3.94[d] (11.45)	0.86[c] (5.31)
W+R	11[c] (19.37)	25.7[d] (30.44)	2.35[e] (8.82)	0.57[e] (4.33)
H+R	15[b] (22.79)	30.7[c] (33.63)	2.23[f] (8.59)	0.59[e] (4.42)
W+H+R	19[ab] (25.84)	38.3[ab] (38.25)	3.03[e] (8.59)	0.72[d] (4.87)
Control	11.7[c] (19.57)	26[d] (30.66)	2.69[e] (9.43)	0.44[f] (3.81)
SED	1.0672	1.9930	0.0166	0.0286
CD (0.05)	2.2624	4.2251	0.0352	0.0614

W- Wheat bran; H- Horse gram; R- Red gram
Mean of three replications. Values in parenthesis indicate arc transformed value

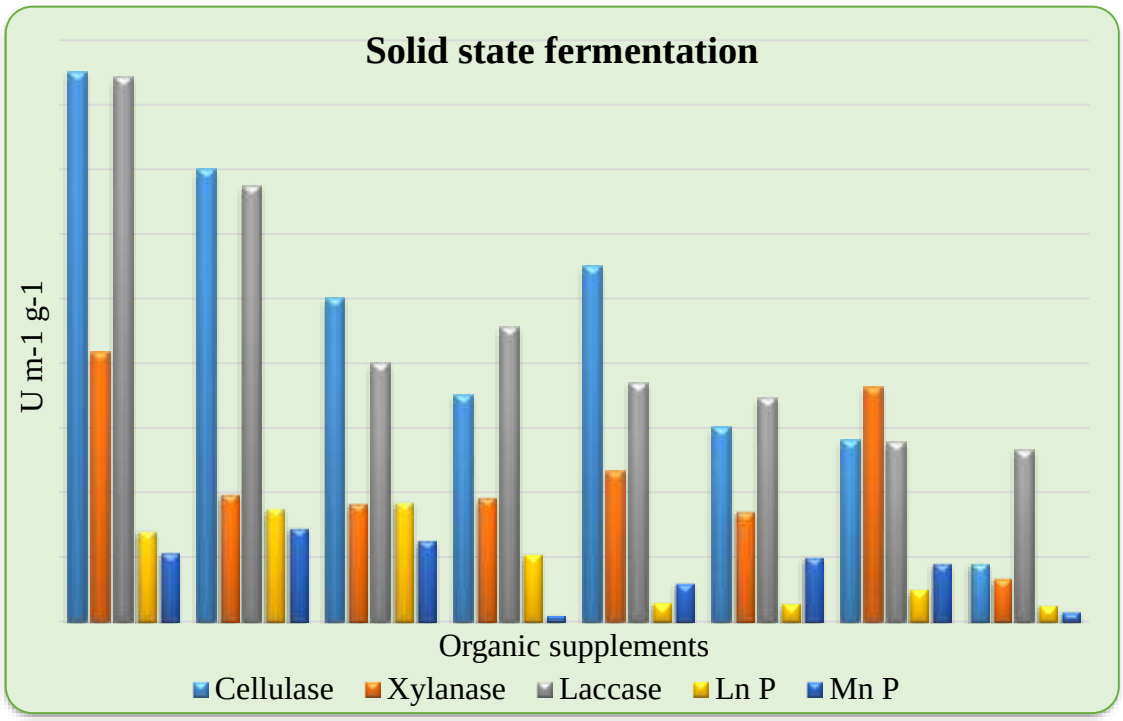

Fig. 1: The lignocellulolytic activity of *C. indica* influenced by organic supplement (Solid-state fermentation)

However, Nannapaneni et al[15] reported that the horse gram and tamarind powder supplemented with paddy straw substrate recorded faster spawn run, pinhead formation and also increased the hydrolytic and proteolytic enzymes produced by *Volveriella volvaceae*. Sugarcane bagasse and wheat bran increased the xylanase and cellulase enzyme production by *Pleurotus* spp on maximum at 25th day under solid-state fermentation[18]. However, the enzyme activity was reduced when sugarcane bagasse was combined with wheat bran in our study where the combination of wheat bran, red gram and horse gram reduced the enzyme activity of *C. indica*. Thus, the ligninolytic enzyme activity is positively correlated with the yield of milky mushrooms.

Conclusion

The study confirms that wheat bran, horse gram and red gram play an important role in promoting the mycelial growth and biomass of *C. indica*. Also, supplementation with wheat bran induces higher secretion of lignocellulolytic enzymes that will facilitate quick degradation of substrates to produce the early primordial formation of milky mushrooms.

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