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Effect of Different Agricultural Waste Substrates on the Biological Efficiency and Therapeutic Value of *Calocybe indica* (P and C)

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Abstract

Purpose: Agro-wastes generated from agricultural industry needs to be recycled from ecosystem by an eco-friendly way. One of the most sustainable method is cultivation of mushroom which follows all the principles of solid waste management. **Methods*:*** The effect of five different agricultural waste substrates on cultivation of *Calocybe indica* (milky mushroom) were investigated. Morphological characters, total yield and biological efficiency were evaluated. Total protein, carbohydrate, phenolic compounds and total β -glucan content was estimated as known to be functional constituents of mushroom. Total genomic DNA expression were studied by gel analsyis. Fruit bodies were screened for enzymatic and non-enzymatic antioxidants by spectro-photometric method, antimicrobial activity by agar disc diffusion method. **Results:** Maximum yield and biological efficiency were obtained for paddy straw (PS) + sugarcane bagasse (SB, 1:1) followed by paddy straw (PS) + cotton waste (CW, 1:1) combination with garden soil (GS): vermicompost (VC): sand (1:1:0.5) casing. Substrate degrading enzymes were significantly variable with substrate composition. DPPH radical scavenging activity was significantly present in methanolic crude extract (MCE) of PS+SB fruit-bodies (IC_{50} = 3.57±0.75 mg/ml). The maximum zone of inhibition (ZOI) was obtained for minimum inhibitory concentration (MIC) 10 mg/ ml of methanolic extract of selected fruiting bodies of *C.indica* against pathogenic bacteria. **Conclusion:** Proper utilization of ligno-cellulosic waste products as substrates will upgrade the production and therapeutic value of milky mushroom which will generate a high impact on our society and environment.

Keywords
Milky mushroom, ligno-cellulosic substrates, biological efficiency, therapeutic value

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INTRODUCTION

Cultivation of edible mushroom is gaining its popularity due to its ability to convert a wide variety of ligno-cellulosic substrates by a low-cost biotechnological process (Tiwari et al. 2017). Large scale ligno-cellulosic mass is generated from agro-wastes or food industries which needs to be managed by an eco-friendly way (Mercy et al. 2011). One of the most sustainable method is the cultivation of mushroom using agro-wastes as substrate and increased utilization of nutrients.

Several ligno-cellulosic substrates majorly wheat straw, paddy straw, rice straw, sugarcane leaves and bagasse, coffee straw, banana leaves, tea leaves, cotton straw and wastes, saw dust are used for cultivation (Vijaykumar et al. 2013) of a profitable product like mushroom. *Calocybe indica* P and C also known as milky mushroom belongs to the phylum basidiomycotina and are generally grown in eastern Indian region (Venkatesh B et al. 2015). P and C indicates that it was first identified by Purkayastha and Chandra (P and C), Department of Botany, University of Calcutta (Purkayastha et al .1976). They are a promising source of biologically active compounds like phenolic compounds, protein and carbohydrates that are associated with cell wall (Gopal et al, 2012). Due to its high protein content they are considered as a good alternative of animal protein (Pani.2012). So, the demand for this mushroom is highly increasing in food industry for consumption but the rate of production is not sufficient (Kotgire et al. 2014). To fulfil the demand for production alternative cultivation methods, need to be standardized. Awareness should be created among common people about its high nutritional and medicinal value which will in turn create balance in the environment and improve human health as a huge quantity of animal meat is getting consumed unnecessarily.

For cultivation of mushroom paddy straw is used as a conventional method (Ramji S et al. 2012). Several reports (Amin et al. 2010) have showed better yield and production of fruit-bodies along with a change in neutraceutical value when supplemented with several ligno-cellulosic waste substrates (Ganesh et al.2012, Sharma et al. 2013).

Accordingly, the objective was optimization of substrate for the growth and productivity of edible milky mushroom *Calocybe indica* P&C. Several ligno-cellulosic wastes like sugarcane bagasse, cotton wastes, coconut coir and rice husk were selected for supplementation with paddy straw and different casing combinations were used to evaluate the variation in morphology and yield. The most potent cultivation method was finalised by screening of *Calocybe indica* P&C fruit-bodies for biochemical and bioactive potentials with respect to nutritional, antioxidant and antimicrobial activity (Sahoo et al.2014, Gimmay et al.2016).

MATERIAL AND METHODS:

All chemicals were purchased from Himedia and ß-glucan standard was purchased from Sigma-aldrich. Substrates were collected from different local agro-processing unit

Collection of fruit body: Fruit bodies of mushroom were collected from the farms of Ramkrishna Mission Narendrapur, West Bengal, a place which does routine cultivation of mushroom of various species for commercial purposes.

Preparation of pure culture: Potato Dextrose Agar (PDA) was used as a basic culture media for the growth and maintenance of mycelia. Mushroom culture media (MCM) was used for submerged liquid culture of mycelia.

Preparation of spawn: Whole wheat grain was used for the spawn preparation. Dry wheat grain was boiled in water for 30-45 minutes. Excess water was drained off; the spawn substrate was solidified using 2% $CaSO_4$ and 4% $CaCO_3$. The polypropelene bags were filled with 200g of solidified substrate, cotton plugged, and the bags were autoclaved at 121℃ and 15 psi for 2 hr. Spawn bags were inoculated with mycelial suspension using a 10ml sterile syringe. The spawn packets were kept in dark at 28-30℃ for approximately 20 to 30 days till the spawns were ready for fruiting.

Preparation of substrate: Five different ligno-cellulosic substrates were used for cultivation. Chopped Paddy straw (PS) was supplemented with four other substrates like sugarcane bagasse (SB), cotton waste (CW), coconut coir (CC) and rice husk (RH) in1:1 ratio. All the substrates were autoclaved at 121℃ and 15 psi for 2 hr and sundried. Polypropelene bags (24-inch X 12 inch) were filled with the mixture of 2kg of dry chopped substrates according to Figure 1. Each combination was replicated three times. Spawn was added in different layers and mixed with the substrate. Cultivation bags were incubated in a room with temperature 20-25°c and relative humidity of 80% for 15-20 days. After complete colonization of mycelial masses different casing combinations (Fig 1, 2) were applied with routine spraying of water until pinheads appeared. The data on period of spawn running, pinhead formation first harvest, yield and biological efficiency of mushroom were recorded during the study. Yield performance of different treatment combinations was calculated by BE and expressed in percentage. Substrate samples were dried by an oven at 40℃ to a constant weight and ground to powder samples. Total carbon (C) content and total nitrogen (N) content was carried out by standard method. Then the C/N ratio of each substrate was calculated.

Estimation of total protein: Fresh fruit-bodies were extracted with 50mM Tris-HCl buffer, pH-7.4 extraction buffer. The extracts were then centrifuged at 10,000rpm at 4ºC for 20 minutes. The supernatant was collected and stored at -20°C for biochemical analysis. The protein in the supernatant was estimated by the Bradford assay.

Evaluation of substrate degrading enzymes: Amylase was evaluated by DNSA (Di nitrosalicylic acid) method, Cellulase was measured by DNSA method using carboxymethylcellulose (CMC) as substrate, Protease was quantified by Folin-Ciocalteu method using caesin as substrate by spectrophotometry. (Mitra et al. 2013)

Evaluation for enzymatic antioxidants: Protein lysates obtained from fresh fruiting bodies as above mentioned were evaluated for enzymatic antioxidants superoxide dismutase (SOD) catalase (CAT), peroxidase (PERX): glutathione reductase (GR), Ascorbate oxidase (As. Ox) by standard spectrophotometric method (Surekha Ch et al. 2011).

Estimation of DNA content: 100mg of mycelia tissues from each set of fruit-bodies were harvested from 10 days old culture flask under laboratory conditions. They were immediately flask freezed with liquid nitrogen and genomic DNA was extracted with the CTAB method. The quality and quantity of extracted DNA was examined through spectrophotometer at A260 /A280. The quality of DNA was further tested by agarose gel electrophoresis, on a 1% agarose gel with 1X TAE buffer. The DNA was compared on a gel with a 1 kb gene ladder (Himedia).

Procedure for solvent extraction: 1 kg of air-dried *Calocybe indica* fruiting bodies were extracted with 2L methanol and kept for 72hrs and filtered with Whatman filter paper no.1 the supernatants were collected, and vacuum evaporated to obtain crude methanolic extract (MCE). The residue was dried in an oven at 50°C and subsequently boiled with double distilled water for 3hrs. Supernatants were filtered and lyophilized to obtain crude water extract (WCE).

Estimation of total carbohydrate and lipid: Total carbohydrate content was measured spectrophotometrically by anthrone method. Total lipid was determined by slight modified method of Folch et al. 1957.

Estimation of β-glucan: Total β- glucan content was measured in C.indica by Semedo et al., 2015. Briefly 0.7ml of crude polysaccharide solution was placed into a test tube with 1.3ml of water by an auto sampler. 1.4ml of congo red solution (140ppm congo red into 0.66M tris buffer, pH 8.0) was added into it. Absorbance was measured at 545nm by a spectrophotometer. 0-1mg/ml of β -glucan (sigma) was used as standard.

Study for secondary metabolites: Total phenol, flavonoids and glycosides were found qualitatively (data not shown) to be predominant bioactive compounds present in methanolic extract. They were quantified by spectrophotometric method (Asmah et al. 2013).

Evaluation of DPPH radical scavenging activity: Antiradical activity was measured in methanolic extract by a decrease in absorbance at 517 nm of DPPH (2,2-Diphenyl-1-Picrylhydrazyl) solution.

***In vitro* Antimicrobial activity:** The antimicrobial activity of methanolic extract was determined on opportunistic human pathogens E. *coli* MTCC 443, *P.aeruginosa* MTCC 741, *Staphylococcus aureus* MTCC 3160, *Bacillus subtilis* MTCC 9041. After 24 hrs of incubation 50µl of active inoculum was added in 5ml nutrient broth to make a final inoculum of 5×10^5 CFU/mL with increasing concentration of 0.5-15mg/ml of the methanolic extracts of selected fruit-bodies. The tubes were incubated at 37°C for overnight. The lowest concentration of the compound that inhibits bacterial growth compared to control was regarded as MIC. Methanol alone did not affect the growth of the bacteria during the experiments (data not shown).

For further confirmation 100 µl of inoculum was spread on Mueller Hinton *Agar* (*HiMedia*) with MIC dose of selected extracts in different wells and their zone of inhibitions were measured after 24 hours by agar disc diffusion method. Antibiotics were used as the positive control.

Statistical methods:

All the graphs and statistical analysis was done in Graph pad prism software version 5

RESULTS:

***Effect of different agro waste supplement and casing mixture*:**

Several agro-wastes supplemented to the paddy straw increased the growth and total yield of *Calocybe indica*. Minimum time 18.33±1.50 days and 18.25±2.10 days were recorded for spawn run with paddy straw and PS+SB (1:1) respectively. Minimum average time of pinhead formation and first harvest were observed for PS+SB combination with 24.00 ±2.09days and 33.20±2.55 days respectively (Table 1). Among the tested casing mixtures, the combination of garden soil: VC: sand (1:1:0.5) was proved to be maximum effective for reducing the average duration for pinhead formation and first

harvest as well as enhancing the total yield and biological efficiency (Table 1, 2). Maximum yield of 950.50±70.80 g/ 2kg substrate and BE of 47.50±0.50% were obtained for PS+SB combination with GS:VC:sand (1:1:0.5) casing followed by PS and PS+CW (Fig 3, Table 2). PS+RH combination showed minimum yield and BE. Maximum average weight per fruit-body was recorded for PS+SB with GS:VC:sand casing mean ± SD being 80.72±7.50g. C/N ratio obtained for selected substrates had positive correlation (r=0.8) with C/N ratio of fruit-bodies cultivated on different substrates (Fig 4A).

Table 1 Effect of different substrates and casing mixtures on mycelial development

Substrate	Avg Time of spawn running (in days)	Casing combination	Avg Time of pinhead formation	Avg Time of first Harvest (in days)	Avg Time of second Harvest (in days)
PS	18.33±1.50	Soil:VC:Sand	28.15 ± 2.50	40.35 ±1.59	48.50±2.10
		VC	28.25 ± 2.85	40.15 ±2.01	48.25±1.96
		Soil	32.56 ±1.82	45.56 ±1.80	50.42±2.53
PS+SB	18.25±2.10	Soil:VC:Sand	24.00 ±2.09	33.20 ±2.55	40.55±2.00
		VC	28.15 ±1.50	40.25 ±1.81	45.87±2.88
		Soil	33.75 ±1.28	42.50 ±2.65	45.50±2.53
PS+CC	21.20 ±1.02	Soil:VC:Sand	28.60 ±1.85	40.50 ±2.68	48.20±1.80
		VC	30.15 ±2.50	45.70 ±1.95	52.54±1.56
		Soil	32.45 ±2.91	48.50 ±2.81	52.51±2.05
PS+CW	24.50 ±2.06	Soil:VC:Sand	30.52 ±1.98	42.25 ± 1.67	50.59±2.17
		VC	30.45 ±3.22	42.50 ± 2.07	50.54±1.66
		Soil	38.70 ±2.59	45.50 ±1.50	55.12±2.40
PS+RH	28.75 ±1.80	Soil:VC:Sand	40.75 ±1.70	48.50 ±2.09	-
		VC	-	-	-
		Soil	-	-	-

Table 2 Effect of different substrates and casing mixtures on morphological variation, total yield and biological efficiency (BE) of *C.indica*

Substrate	Casing combination	Mean pileus diameter (cm)	Mean stalk length (cm)	Mean weight of a fruitbody(g)	Total Yield (g/ 2kg) substrate	Biological efficiency %
PS	Soil:VC:Sand (1:1:0.5)	5.50 ±0.90	6.50 ±0.9	50.25 ±5.50	800.20 ±65.56	40.50±1.00
	VC	4.50 ±0.60	6.50 ±0.6	48.5 9±3.80	425.50 ±46.55	20.10±0.60
	Soil	4.20 ±0.70	6.00 ±0.7	35.57 ±5.60	320.80 ±30.2 1	16.50±0.05
PS+SB	**Soil:VC:Sand (1:1:0.5)**	**6.50 ±0.6 0**	**9.5 0±0.5**	**80.72 ±7.50**	**950.50 ±70.80**	**47.50±0.50**
	VC	6.00 ±0.50	8.5 0±0.7	78.85 ±6.80	660.30 ±85.50	33.10±0.90
	Soil	5.50 ±1.0 0	7.00 ±0.8	70.10 ±9.50	480.1 ±50.50	24.20±0.60
PS+CW	Soil:VC:Sand (1:1:0.5)	6.50 ±0.80	10.0 ±1.2	78.54 ±6.20	700.19 ±95.50	35.20±0.60
	VC	5.7 0±0.50	9.20 ±0.7	75.00 ±5.40	450.50 ±50.15	22.50±0.07
	Soil	4.50 ±0.7 0	8.50 ±0.9	68.72 ±6.30	380.09 ±68.94	19.20±0.25
PS+CC	Soil:VC:Sand (1:1:0.5)	3.50 ±0.50	4.50 ±0.4	35.86 ±3.20	650.54 ±87.52	32.30±0.55
	VC	3.00 ±0.30	4.02 ±0.3	30.0 0±2.50	350.18 ±25.50	17.10±0.06
	Soil	2.50 ±0.60	3.00 ±0.2	25.60 ±3.20	200.52 ±30.12	10.70±0.64
PS+RH	Soil:VC:Sand (1:1:0.5)	2.50 ±0.60	3.50 ±0.6	25.46 ±2.90	25.00 ±5.50	0.012±0.16

VC	-	-	-	-
Soil	-	-	-	-

a) All values expressed as mean± SD

Nutritional Analysis: The result of % moisture content, protein, carbohydrate, lipid, DNA content, total phenolic compounds and total beta glucan content are given in Table 3 (a and b). The moisture content of fresh mushroom fruit bodies grown on various substrates ranged from 87.84±1.50 to 90.50±1.00%. The protein content of mushroom fruit bodies ranged from 1.10±0.33 to 1.60±0.30 mg/100mg of fruit bodies. The mushroom fruit bodies produced on PS+SB and PS+CW showed more protein content than paddy straw alone. The yield of total DNA was ranged from 0.50 ±0.08 and 0.85±0.10mg/100mg from mycelial tissue cultured from each fruiting bodies obtained from different substrate combinations. Quality of total DNA was further confirmed on 1% agarose gel. (Fig 4B) Total sugar content recorded in the mushroom fruit bodies varied from 3.56 ±0.90 to 6.50±1.50 mg/100mg of dried mushroom. Maximum total sugar content was observed in the mushroom fruit bodies produced on paddy straw and sugarcane bagasse followed by paddy straw and cotton waste combination. Total phenol and flavonoid content were found to be maximum in PS and PS+SB with 3.54. ±0.57 and 5.26± 1.20 mg/100mg methanolic extract (Table 3b)

Table 3 a) Effect of different substrates on nutritional properties of *C.indica*

	Moisture content %	Total Polysaccharide content mg/100mg	Total Protein content mg/100mg	Total lipid content mg/100mg	DNA Content mg/100mg
PS	90.50 ±1.00	5.15 ± 0.95	1.57 ± 0.60	0.74±0.09	0.80±0.09
PS+SB	90.00 ± 0.80	6.50± 1.50	1.50 ±0.50	0.67±1.00	0.85±0.10
PS+CW	89.55 ±1.65	6.20±0.71	1.60 ±0.15	1.09±0.82	0.72±0.08
PS+CC	90.50 ±1.15	4.95 ±0.80	1.60 ±0.30	0.54±0.40	0.50 ±0.08
PS+RH	87.84 ±1.50	3.56 ±0.90	1.10±0.33	0.66±0.23	0.55 ±0.05

a) All values expressed as mean± SD

Substrates:

Table 3 b) Effect of different substrates on secondary metabolites of *C.indica*

Substrates	Phenol Content mg/100mg	Flavonoid content mg/100mg	β-glucan content mg/100mg
PS	3.54 ±0.57	4.26 ±0.95	20.68±1.10
PS+SB	3.12±0.25	5.26± 1.20	24.30±1.50
PS+CW	2.18±0.60	4.15±0.58	22.5± 0.90
PS+CC	2.50 ±0.50	3.50 ±0.3 3	14.7 ±1.80
PS+RH	1.55 ±0.89	2.25 ±0.56	10.00±1.20

All values expressed as mean± SD

Analysis of substrate degrading enzymes: Mushrooms generally grow on ligno-cellulosic substrates with a suitable nitrogen source. So, enzymes like cellulase, amylase and protease are required for the utilization of substrate and mushroom yield. It was observed from Fig 5 that the level of these enzymes was significantly higher for PS+SB and PS+CW fruiting bodies than PS alone. Mean value for amylase ranges from 965.00 ±50.60-1141.75±135.10 U/mg protein. Mean value for cellulase ranges from 681.70 ± 80.25 to 784.33±75.65 U/mg protein and similarly for protease mean value was 752.80 ±98.50 to 1077.20 ± 145.75 U/mg protein as evaluated for each set of fruit-bodies (Fig 5).

Estimation of antioxidants: The enzymatic antioxidants like SOD, catalase, peroxidase, Glutathione reductase (GR) and ascorbate oxidase of *Calocybe indica* grown on different substrate combinations were tested. As shown in the (table 4) the catalase activity in PS+SB grown fruiting bodies was found to be 2.2-fold higher than PS variety, mean value being 4.75± 1.00 U/mg and 2.50± 0.85 U/mg respectively. No significant differences were found in peroxidase, glutathione reductase and ascorbic oxidase enzyme activity in all the fruiting bodies. In case of SOD enzymatic activity were significant in PS, PS+SB and PS+CW mean value being 21.58 ±2.21 and 20.80±3.20 U/mg and 18.57±2.99 U/mg respectively. Significant amount of GSH was present in PS+SB and PS+CW, mean value being 17.56±2.95 and 18.51±1.05 µM respectively. DPPH radical scavenging was significant ranging from 3.57±0.75-14.75 ±1.10 mg/ml of extract assessed in methanolic crude extract of different set of fruit-bodies. (Fig 6A)

Evaluation of antimicrobial activity: The antimicrobial activity of MCE of selected fruiting bodies of *C.indica* was determined preliminarily by performing MIC study. The positive control for Gram +ve bacteria was Tetracyclin which showed 21-25mm zone of inhibition and streptomycin for Gram –ve bacteria with ZOI of 22-24 mm which is taken as a standard (Table 5). No significant difference was found in antibacterial activity with variation in substrate Most significant inhibitory activity was observed with *B.subtilis S.aureus and E.coli* when treated with MIC dose of 10mg/ml of MCE on MH-agar (Fig 6B).

Table 4 Effect of substrate variation on enzymatic and non enzymatic anti-oxidants in C.indica fruit-bodies

Substrate	SOD (U/mg protein)	Catalase (U/mg protein)	Peroxidase (U/mg protein)	Ascorbate oxidase (U/mg protein)	Glutathione reductase (U/mg protein)	GSH (µM)
PS	21.58 ±2.21	2.50 ± 0.85	5.50 ± 1.40	3.56 ± 0.60	3.21 ± 0.89	15.50±2.05
PS+SB	20.80±3.20	4.75. ± 1.00	2.50±1.50	5.50±2.10	3.55±0.80	17.56±2.95
PS+CW	18.57±2.99	2.66 ±1.85	2.04±1.10	4.12±1.45	2.50±0.95	18.51±1.05
PS+CC	15.66±2.15	1.20±2.01	1.95±2.46	2.25±1.0 8	2.82±0.97	8.50±1.56
PS+RH	15.31±2.60	0.58±4.25	1.88±2.19	2.58±0.95	3.21±1.20	5.95±0.59

All values expressed as mean± SD

Table 5 Agar disc diffusion method showing ZOI (mm)

	E.coli	***P.aeruginosa***	***S.aureus***	***B.subtilis***
	ZOI (mm)			
1.PS	**23.5 ± 1.1**	**18.5±0.9**	**21.5 ±0.6**	**20.1±0.4**
2.PS+SB	**22.5±0.4**	**15.5±0.5**	**20.5±0.9**	**24.5±0.5**
3.PS+CW	**20.5±0.5**	**13.2±0.5**	**19.8±0.6**	**20.1±0.5**
4.PS+CC	19.5±0.4	15.7±0.8	20.1±0.5	21.5±0.4
5. PS+RH	15.5±0.3	16.1±0.6	13.2±0.6	14.5±0.6
Tet			23.2±0.8	25.1±0.8
Strep	22.5±0.6	24.1±0.9		

a) All values expressed as mean± SD

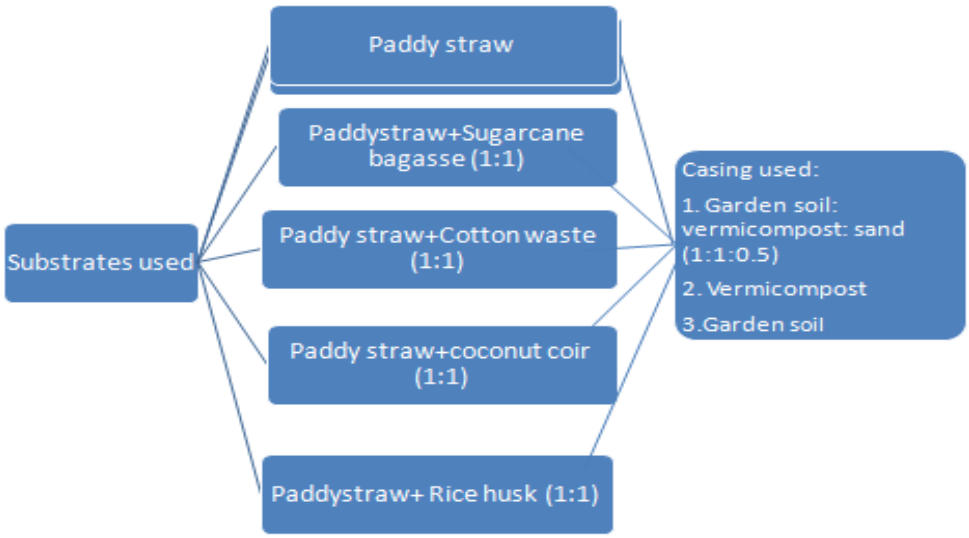

Fig 1 Scheme of substrate preparation and addition of casing

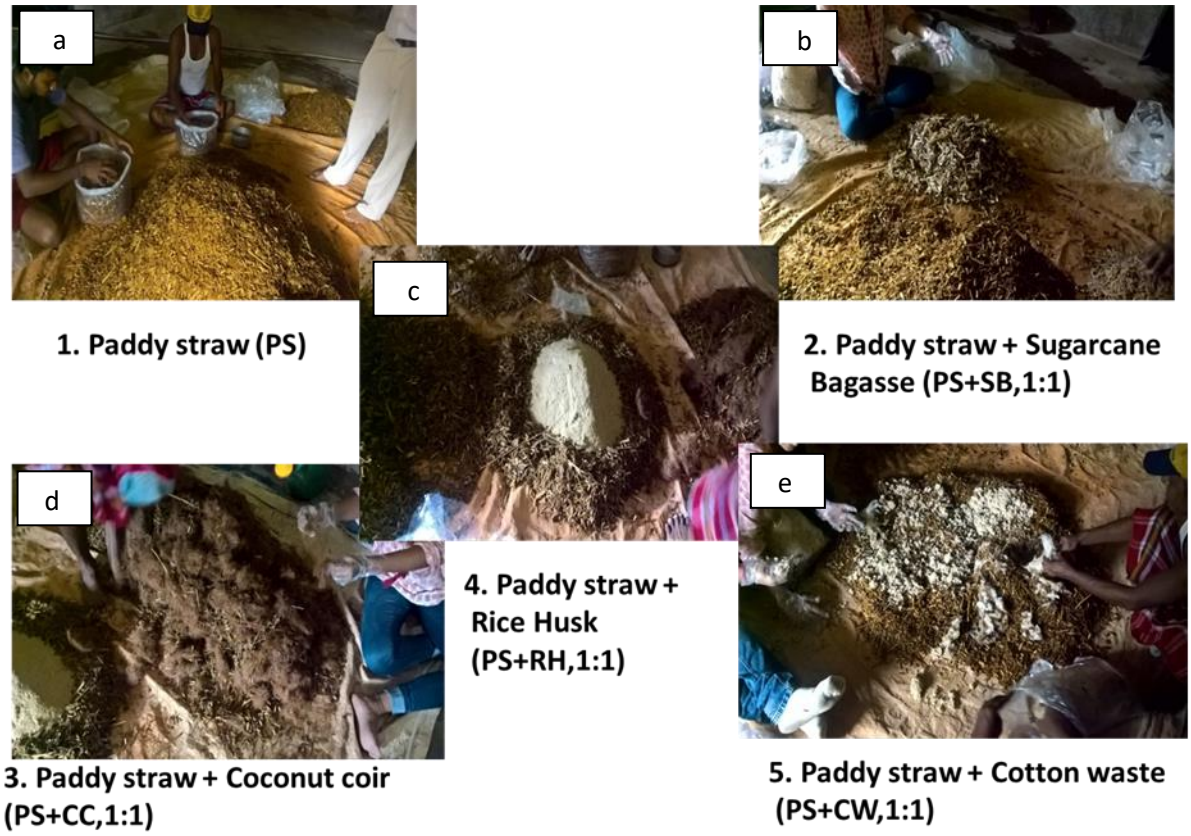

Fig 2 Pictures showing processing and preparation of substrates a) preparation of paddy straw, b) preparation and mixing of Paddy straw +sugarcane bagasse (1:1), c) preparation and mixing of paddy straw + cotton waste (1:1), d) preparation and mixing of paddy straw + coconut coir (1:1), e) preparation and mixing of paddy straw + rice husk (1:1)

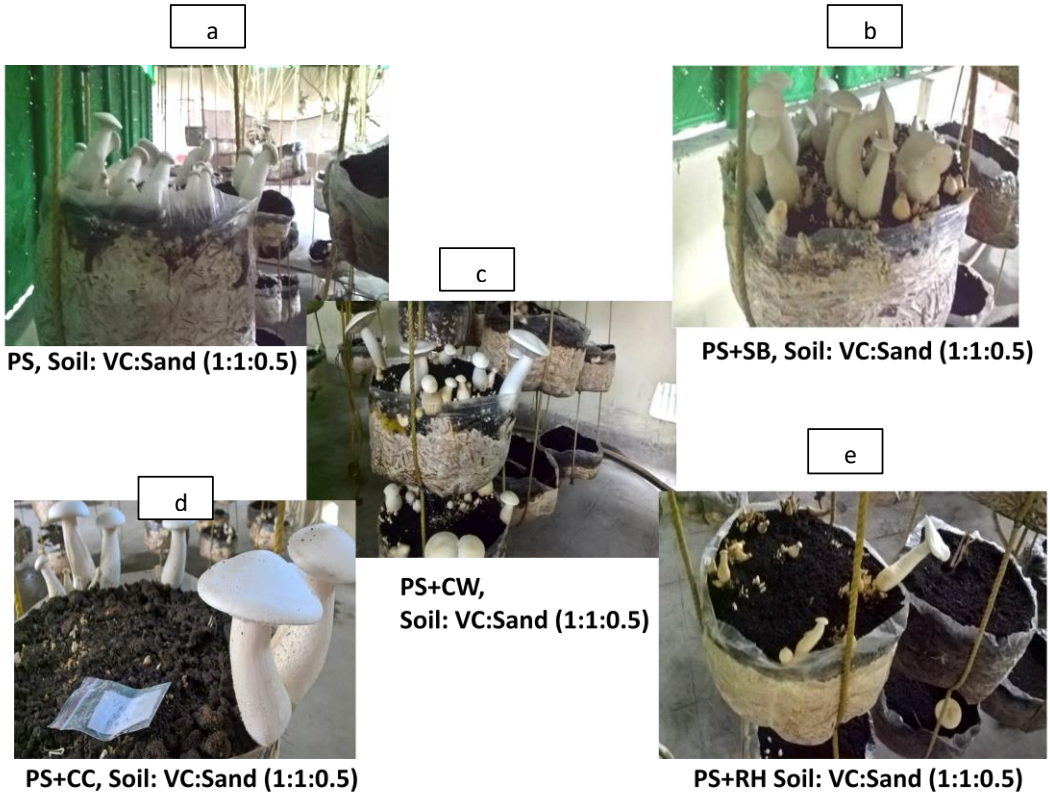

Fig 3 Effect of different substrates and casing mixtures on total yield of *C.indica* fruit-bodies a) yield of *C.indica* fruiting bodies cultivated on paddy straw with soil:VC:sand (1:1:0.5) casing mixture, b) yield of *C.indica* fruiting bodies cultivated on paddy straw + sugarcane bagasse (1:1) with soil:VC:sand (1:1:0.5) casing mixture, c) yield of *C.indica* fruiting bodies cultivated on paddy straw + cotton waste(1:1) with soil:VC:sand (1:1:0.5) casing mixture, d) yield of *C.indica* fruiting bodies cultivated on paddy straw + coconut coir (1:1) with soil:VC:sand (1:1:0.5) casing mixture, e) yield of *C.indica* fruiting bodies cultivated on paddy straw + rice husk (1:1) with soil:VC:sand (1:1:0.5) casing mixture.

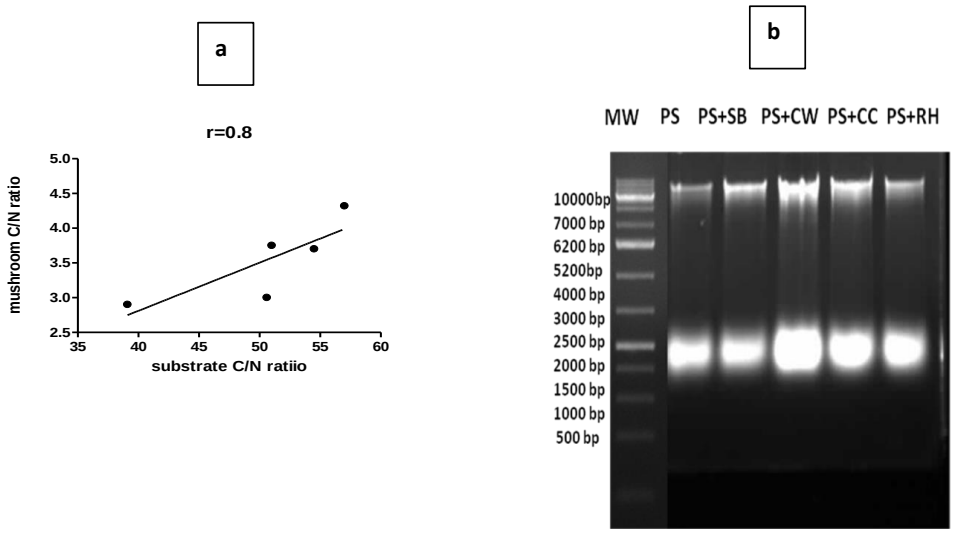

Fig 4 a) Correlation of substrate C/N ratio with mushroom C/N content b) Evaluation of total genomic DNA expression by agarose gel analysis.

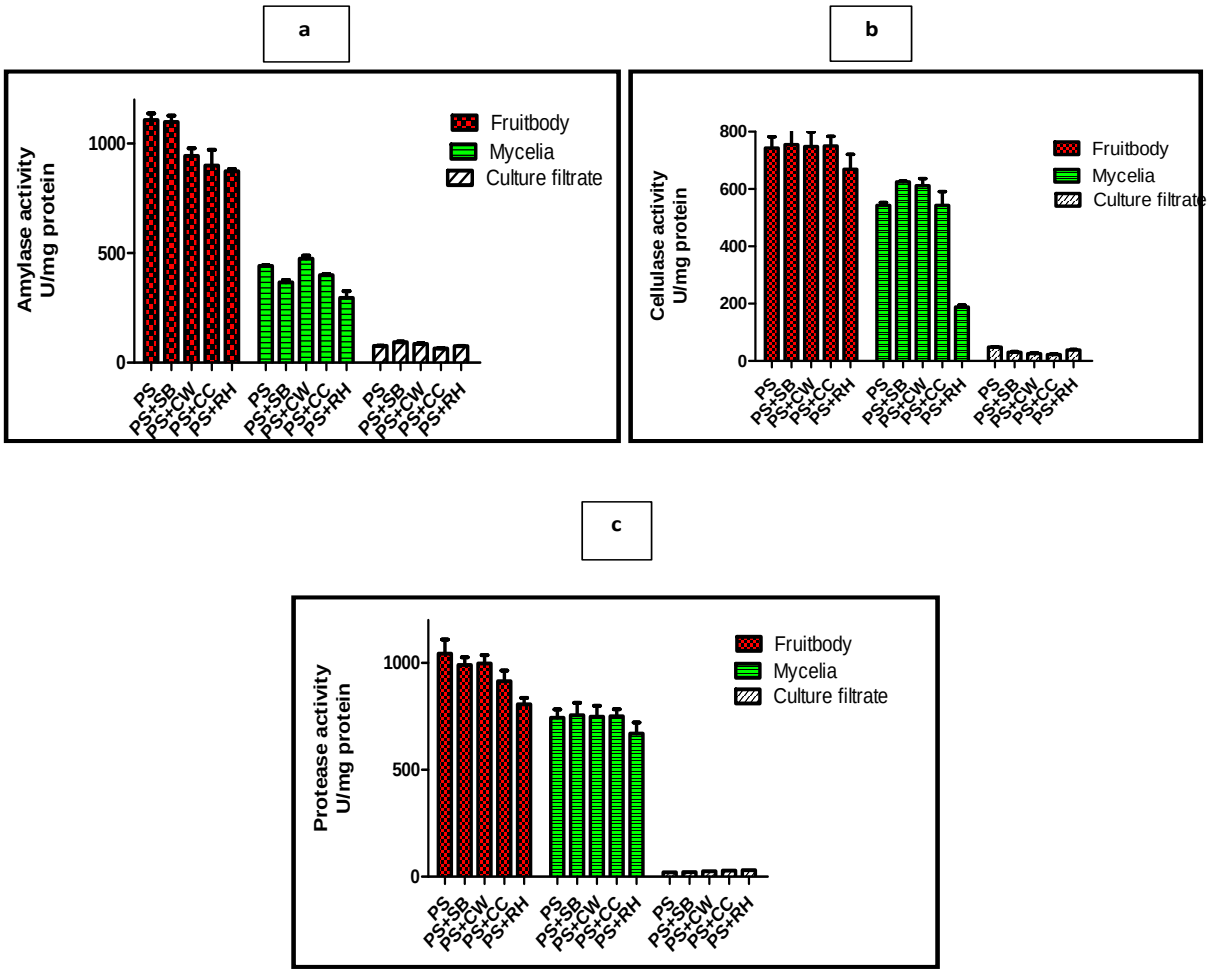

Fig 5 Evaluation of substrate degrading enzymes in fresh fruitbody lysate, mycelial lysate and culture filtrate of *C.indica* fruiting bodies cultivated on different substrates a) estimation of amylase, b) cellulase, c) protease.

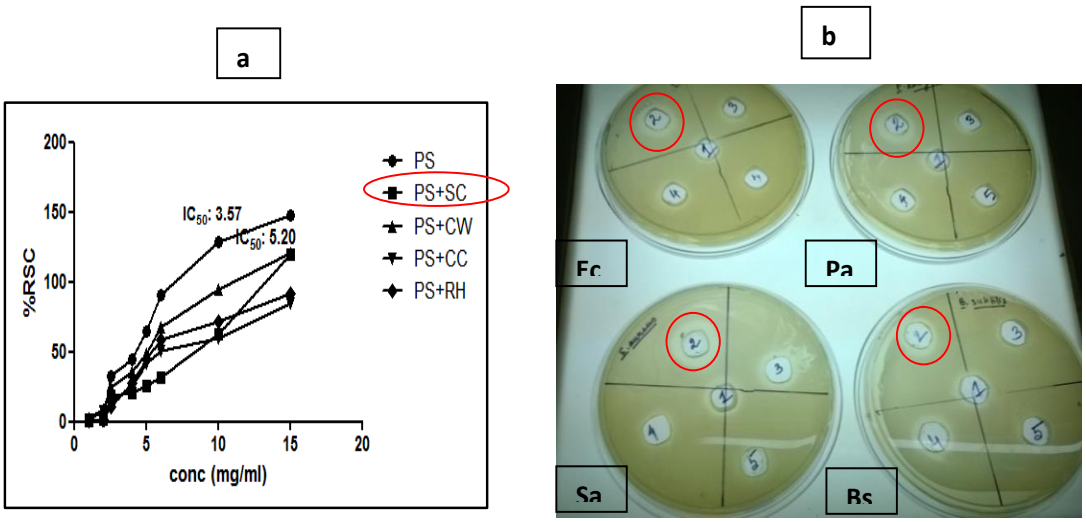

Fig 6 a) DPPH radical scavenging assay of *C. indica* fruiting bodies cultivated on different substrates b) Evaluation of antimicrobial activity by agar well diffusion method. Plates showing ZOI after treatment with 10mg/ml of methanolic extract of 1) PS 2) PS+SB (red circle) 3) PS+CW, 4)PS+CC, 5)PS+RH (EC: *E.coli*, Pa: *P. aeruginosa*, Sa: *S. aureus*, Bs: *B. subtilis*)

DISCUSSION:

The effectiveness of substrate in enhancement of mushroom growth and production lies in the presence of a right amount of cellulose, hemicellulose, lignin and protein (Kumar et al.2012, 2015). Paddy straw is composed of mainly 40-45% cellulose, 14-16% lignin, 35-40% hemicelluloses, 0.8-1% total nitrogen and sugarcane bagasse mainly contains Cellulose 36-40%, Hemicellulose 20-25%, Lignin 18-25% Nitrogen 0.7% (Tiwari et al. 2017). In our study *C.indica* fruit-bodies showed maximum yield and nutritional properties when cultivated on PS+SB (1:1) combination with Garden soil: VC: sand (1:1:0.5) casing which may due to their respective chemical composition (Table 1, 2, Nagaratna et al.2007). Current study showed a positive correlation was present (r=0.8) between C/N ratio of the substrates with C/N ratio of the different cultivated mushroom fruit-bodies (Figure 4A). GS: VC: sand was selected as the best casing combination as they are promoting the pinhead formation and early growth of fruiting bodies. Composition of substrate also affect the substrate degrading enzymes like amylase, cellulase, protease which are required for the degradation of substrates and increased utilization of nutrients for the conversion of waste material into a commercially viable environmentally safe edible product like the mushroom (Ganesh et al.2013). Several reports (Perumal et al.2014) showed cellulose and lignin ratio is directly proportional with the higher expression of these enzymes. Current study also corroborated with this finding as a significant amount of substrate degrading enzymes are present in cultivated mushroom fruiting bodies with highest in PS+SB combination (Figure 5). It can be justified as both substrates are rich in lignin and cellulose content as previously mentioned.

Coming to the antioxidant study reactive oxygen species (ROS) when accumulated can induce significant biological damage of healthy cells. In order to avoid this unnecessary reaction, there is a requirement of enzymatic and non-enzymatic defence mechanism (Shweta S et al. 2014). In this study increased amount of antioxidant enzymes like SOD and catalase and non-enzymatic antioxidant like GSH (Table 4) and DPPH radical scavenging (Solak et al.2010, Ferreira et al.2008) activity (Figure 6A) were detected in fruiting bodies cultivated on PS+SB followed by PS+CW and PS alone. It is evident from the result that if grown under proper growth conditions *C.indica* can be considered as a potential organism for antioxidant therapy (Subramanium et al. 2013)

Secondary metabolites are compounds that are used as food and medicine to protect against illness and to maintain human health. They are considered being generated in organisms under stressed condition (Wang et al.2015). The present study indicated presence of high phenolic compounds, glycosides, steroids, alkaloids and low amount of terpenoids. Comparative study between different substrate showed significant presence of phenolic compounds which indicated that substrate composition may influence secondary metabolite production in milky mushroom (Table 3)

The bioactive compounds present in methanolic crude extract of *Calocybe indica* were effective against Gram-positive bacteria like *S.aureus, B.subtilis* and Gram-positive *E.coli* and *P.aeruginosa* which can be considered as an opportunistic pathogens (Sirikhwan 2015, Appiah et al. 2017). High ZOI of 20-28mm were observed with MIC dose of 10mg/ml of MCE. Substrate variation doesnot significantly increase the antibacterial activity (Table 5, Figure 6B).

CONCLUSION:

Hence the novelty of this work can be explained by the recycling of huge amount of agricultural waste or by-products for the production of high protein product like milky mushroom in an eco-friendly way. Since it is morphologically similar to button mushroom, the demand for this mushroom is slowly increasing in food industry, but its potentiality is still under explored. Currently, with the growing concern about environmental degradation and our effort to make sustainable development as a necessity for a better life popularization of commercial cultivation of *C.indica* using several agro-wastes will generate a high impact on our society and environment. Proper utilization of ligno-cellulosic waste products as substrates will produce value-added product like mushroom. It will be beneficial for human health due to the presence of various bioactive compounds of massive therapeutic importance which was not previously studied.

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