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INCREASED BIOMEDICAL POTENTIAL OF *Pleurotus ostreatus* THROUGH THE USAGE OF EFFECTIVE SUBSTRATE

S.DATTA*, S.SHYAM CHOUDHURI AND A.K.MITRA

Department of Microbiology, St. Xaviers College, Kolkata, India

ABSTRACT

Mushrooms have been used as food supplement from times immemorial not only for their nutritive value, but also for their medicinal properties. This investigation attempts to update the present cultivation status and project the future prospects of the oyster mushroom, *Pleurotus ostreaus* for its biomedical potential. Comparative analysis of the yield and the chemical composition of clusters of *Pleurotus ostreatus* were performed by cultivating them on three different substrates, viz, paddy straw, wheat straw and sugarcane waste (bagasses). Thereafter extrapolating the results to compare their biomedical potential by means of various spectrophotometric assays. The genetic variation among the clusters was studied using RAPD markers, OPAB18, OPAC19, OPAC13 by Polymerase Chain Reaction (PCR) and dendrogram analysis. The yield of *Pleurotus ostreatus* was seen to be comparable in case of paddy and wheat straw while for sugarcane waste it was almost 50% lower. Results correlated with the substrate degrading enzymes, enzymatic and non-enzymatic antioxidants and bioactive compounds.

KEYWORDS: ***Pleurotus ostreatus,*** **lignocellulosic substrates, antioxidant, secondary metabolites, enzymes, RAPD**

S.DATTA
Department of Microbiology, St. Xaviers College, Kolkata, India

INTRODUCTION

Mushroom cultivation is gaining its popularity in India due to its several advantages. With the advent of modern cultivation technology, it is now possible to cultivate mushrooms seasonally throughout the year by employing environmentally controlled conditions. It serves as the most efficient and economically-viable biotechnology for the conversion of ligno-cellulosic waste materials into high-quality protein food#[1]. It can contribute towards goal of conservation of natural resources as well as increased productivity in recycling of agro-wastes including agro-industrial waste. Utilizing these wastes for growing mushrooms can enhance income and impart a higher level of sustainability [2]. Among mushrooms, *Pleurotus* (oyster mushroom) can make use of the largest variety of waste substrates with its rapid mycelial growth and its multilateral enzyme system that can easily biodegrade nearly all types of available wastes#[3]. Oyster mushrooms possess exceptional medicinal potentials and several bioactive substances which have curative and prophylactic properties, especially in diseases such as high blood pressure, asthma, respiratory tract infection, anemia, hepatitis, cancer, tumor, and many more [5, 6]. A wide range of diverse ligno-cellulosic substrates are used for cultivation of *Pleurotus ostreatus in India.* Amongst various cereal straws, paddy straw and cotton waste was reported to be the best substrate for the cultivation of oyster mushroom whereas, next to the paddy straw, wheat straw proved to be the best substrate for the cultivation of *Pleurotus* spp. Other substrates like maize straw, soybean straw, banana waste, sugarcane waste (bagasse) and many agro byproducts are used with variable yield [4, 7]. Several reports indicated that the yield of mushroom is related to the variation in substrate composition. So present study deals with cultivated variety of *P. ostreatus* grown on three commercially used substrates paddy straw, wheat straw and sugarcane waste (bagasse) determining the growth and yield of mature fruiting bodies. This study also attempts to bring to light the hypothesis that whether the difference in substrate composition and growth conditions have any effect on generating variation in biomedicinal parameters and gene expression in cultivated mycelia of *P. ostreatus.* This will help to promote the large scale commercial cultivation and selection of proper growth condition for the production of better quality mushrooms.

MATERIALS AND METHODS

All chemicals were obtained from Merck, India except bovine serum albumin (BSA), nicotinamide adenine dinucleotide phosphate (NADPH), oxidized glutathione, quercetin, Carboxy methyl cellulase (CMC) from Sigma Aldrich (St. Louis, MO, USA).

i) Collection of P. ostreatus

Stock culture of *P.ostreatus* was obtained from Ramkrishna Mission, Narendrapur, West Bengal which carries out cultivation of various mushroom species for commercial purpose.

ii) Maintenance of mycelia culture

Potato Dextrose Agar (PDA) is the medium that contains nutrients in appropriate amounts to favour the optimal growth of mushroom mycelia. The plates and test tubes were incubated at 25ºC for 7 days followed by repeated sub-culturing with fresh PDA media every week for maintenance of culture and prevention of contamination.

Preparation of Spawn# [1]

The substrates were chopped into 3~4 inch lengths. On a dry-weight basis, 0.2% $CaCO_3$ was added to the chopped substrates and mixed thoroughly. Water was added to constitute a moisture level of 65%. Substrate (500 g) was added to polypropylene bags (7 × 10" sizes), and the openings of the bags were plugged with cotton and secured with plastic rings. The bags were autoclaved at 121^{o}C at 15 psi for1 hour, after which they were inoculated with 2 teaspoons of a mother culture of *P. ostreatus*. The spawn packet was kept in a dark room for incubation at 30-32^{o}C for approximately 30 to 35 days.

iii) Cultivation of Pleurotus ostreatus

Paddy straw, wheat straw, and sugarcane waste (bagasse) were used for the cultivation of *P. ostreatus*, following the method proposed

by Bano and Srivastava (1962) with slight modifications. Dried agro wastes were chopped into 1 to 2 cm long bits and soaked in water overnight. Excess water was drained off and these substrates were pasteurized by dipping in hot water at 80 °C. Spawn (20 g/kg) was added and mixed with the wet straw. Polyethylene bags of 30 cm×20 cm with holes were filled with the mixture. One kg of dry substrate was used in each bag. Spawning was done in five layers at the rate of 2% of net substrate. These bags were incubated at room temperature in a room with sufficient light and ventilation for 15 days. Next, the polyethylene bags were cut open on the sides without disturbing the bed and were sprayed with water twice a day using a hand sprayer. The mass of substrate was kept undisturbed for the appearance of fruiting bodies. The spray of water was discontinued a day before the harvest of the fruiting bodies.

Table 1
Types of substrate and their composition used for the cultivation [7]

Name of substrate	Composition
Rice straw	It contains 41% cellulose, 14% lignin, 0.8% total nitrogen, 0.25% P_2O_5, 0.3% K_2O, 6% SiO_2, pH 6.9, Carbon/Nitrogen = 70
Wheat straw	1% protein, 13% lignin 39% hemicellulose, 40% cellulose.
Sugarcane bagasse	Sugar cane rubbish, cane trash, Cellulose 35- 40%, Hemicellulose 20-25%, Lignin 18-24%, Ash 1-4%, Waxes < 1%, 0.7% nitrogen.

iii) Measurement of physical characters

The length and diameter of stalk of the mycelia, the length and diameter of pileus, biological yield and the percentage of moisture content were measured in mycelia.

Spore Print

A drop of nutrient agar was taken on the top of the lid of a sterile petriplate and a piece of intact, fresh fruiting body tissue was placed on it such that the part of the mycelia faced the inside of the petriplate. The lid was closed and incubated at 37ºC overnight following which a spore print was obtained the next morning.

v) Preparation of ethanolic extract for assay of secondary metabolites [11]

The fruiting bodies of the mushroom were cleaned and washed to remove any residual compost by using distilled water. These samples were cut into pieces and stored and they were air-dried to remove the moisture content. After air-drying, the dried samples were ground into fine powder by using a mortar-pestle. 10grams of each powdered sample was extracted with 50 ml of ethanol. The mixture was placed in a conical flask (wrapped with an aluminium foil) and agitated at 200 rpm with orbital shaker for 72 hour at 37°C. The extract was then separated from the residue by filtration through Whatman No. 1 filter paper. The remaining residue was re-extracted once, and then the two extracts were combined. The residual solvent of ethanol extracts was removed by vacuum evaporation to dryness for 3 days. The same method was applied to all the three clusters of *P.ostreatus*. Final 1g/ml concentration was used directly for further analysis.

vi) Preparation of protein lysate

The fruiting bodies of the mushroom were washed and cleaned with distilled water to remove residual compost. They were then taken in a mortar and crushed with a pestle in the extraction buffer, (50mM Tris, 20mM MgSo4, 0.2uM DTT, 0.1% B-Mercaptoethanol and 0.02% PMSF) The extracts were then centrifuged at 10,000rpm at 4ºC for 20 minutes. The supernatant was collected and stored at -20ºC for biochemical analysis. The protein lysate was prepared for the three clusters of mushrooms. The protein in the supernatant was estimated by Bradford assay.

vii) Measurement of substrate degrading enzymes: [8]

Amylase

Amylase activity was measured by preparation of a reaction mixture of 3 ml. For the standard, dextrose (1 mg/ml) containing 500µL of distilled water and 500 µL of dextrose solution and was serially diluted to 0 mg/ml of 500 mg/ml. In three other tubes, reaction mixture of 3 ml was prepared for the mushroom sample containing 500µL of protein extract of a sample, 500µL of starch substrate[1.5 mL of 1% brought to boil and maintained at boiling conditions until

dissolved, cooled at room temperature]. 2 ml of 3,5-Dinitrosalicylic Acid, DNSA was added to each of the above tubes and incubated at 90ºC in a water bath for 10 minutes. Before cooling, 500µL of Rochelle salt [40% Na-K Tartarte] was added to each of the tubes and the colour was measured at 575 nm. One unit of amylase activity was defined as the amount of enzyme that releases one milligram of reducing sugar as glucose per milliliter per minute under the assay conditions.

Cellulase

Cellulase activity was assayed by determination of the reducing sugars released from carboxymethyl cellulase (CMC). A volume of 0.5 mL of the protein extract was incubated with 1 mL of 2% CMC in 0.05 M sodium acetate buffer (pH4.8) at 50 °C for 10 min. The reduced sugar product was assayed using the dinitrosalicylic acid method mentioned above using dextrose as the sugar standard. The activity was expressed in µg/mL of extract.

Protease

Protease enzyme activity was assayed by following a spectrophotometric method. The activity of the reaction mixture, which was prepared by incubating the enzyme extract with casein (1 mg/ml), was terminated by using 110 mM or 2.5% Trichloroacetic acid. Next, the filtrate obtained after filtration through Whatman No. 1 filter paper was added to 2% Sodium carbonate and Folin-Ciocalteu reagent and incubated at 37 °C for 30 min, and its optical density was taken at 660 nm. A standard curve was prepared using tyrosine and expressed as the quantity of the enzyme that releases soluble fragments of Trichloroacetic acid giving a blue color equivalent to 0.5 mg/mL tyrosine under the conditions of the assay.

viii) Measurement of enzymatic and non-enzymatic antioxidants: [9, 10, 11]

Catalase (CAT)

Activity was determined using a reaction mixture containing 200 µL of 40 mM H_2O_2 in a 50mM phosphate buffer (pH 7.0) and 0.1 mL of protein extract prepared for the three clusters in a total volume of 3 ml. The absorbance of H_2O_2 was measured at 240 nm and the activity of the enzyme was expressed in units/mL.

Ascorbate oxidase (AS.ox)

To 3ml of ascorbate solution(18.8mg ascorbic acid dissolved in 300ml of 0.1M phosphate buffer, pH-5.6), 0.1ml of enzyme extract was added and the change in the absorbance at 265nm is measured at an interval of 30sec of a period of 5min. One enzyme unit is equivalent to 0.01 O.D change per min per mg of protein.

Total Antioxidant

An aliquot of each sample (0.05ml) was mixed with 0.5ml of reagent (0.6M H2SO4, 28mM sodium phosphate and 4mM ammonium molybdate) in 1.5ml eppendorf tube. The tubes were capped and boiled in a boiling water bath at 95ºC for 90min and cooled. The absorbance of each sample was measured at 695nm against blank in a spectrophotometer. A typical blank contained 0.5ml of reagent solution and 0.05mlof buffer and treated in the same manner as rest. The antioxidant capacity was expressed as micromoles of ascorbic acid equivalents of antioxidant capacity.

Lipid Peroxidase (Perx)

One volume of protein extract was mixed with 2 volumes of 15% w/v Trichloroacetic acid(TCA) and 0.375% w/v Thiobarbituric Acid(TBA) in 0.25N HCl. Incubated at 100ºC for 30 minutes in a shaking water bath(shaken at 5 min interval). The tubes were cooled and centrifuged at 10,000 rpm for 10 mins.The supernatant was taken and absorbance was measured at 535 nm. Concentration of Thiobarbituric Acid Reactive Substance (TBARS) or Malonaldehyde(MDA) calculated using extinction coefficient(1.56×10^5 M/cm).

Superoxide dismutase (SOD)

The reaction mixture for auto-oxidation consisted of 2 ml Tris-HCl buffer (pH 8.2), 0.5 ml 2 mM pyrrogallol and 2 ml of distilled water. The rate of auto-oxidation was measured at 470 nm every 30 seconds for 5 minutes by a spectrophotometer. The activity of SOD was expressed as unit/mg protein (1 unit was the amount of enzyme that was utilized to inhibit 50% of auto-oxidation of pyrrogallol/min).

Glutathione reductase

GR activity in 0.1 ml of lysate was measured in triplicate in 0.1 M potassium phosphate buffer (pH 7.4) containing 2.3 mM

ethylenediaminetetracetic acid (dipotassium salt) and 0.89 mM oxidised GSSG in a reaction initiated by 80 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a total volume of 3.6 ml. The cuvettes were incubated for 5 min at 37°C before the addition of NADPH, and the decrease in absorbance over 3 min was recorded continuously at 340 nm. The reduction in absorbance reflects the decrease in NADPH.

Glutathione (GSH)

Briefly, 0.5 ml sample from each of three clusters was precipitated with 10% TCA (Trichloro acetate and centrifuged at 1000g. To the aliquot of supernatant, 2 ml of PBS and 0.5 ml of DTNB (5, 5′ -dithio-2-nitro benzoic acid) were added and final volume was made upto 5 ml with distilled water. The yellow colored product's optical density was measured at 412 nm by spectrophotometer. Commercial glutathione was used as a standard. The level of GSH was expressed as µg/mg protein.

ix) Assay of Secondary metabolites and bioactive compounds: [12]

Total Phenolic content

in the sample extracts was measured by Folin-Ciocalteu assay. One milliliter of sample from ethanol extract was mixed with 1 ml of Folin and Ciocalteu's phenol reagent (1:9; Folin-Ciocalteu reagent: distilled water). After 5 min, 1 ml of 13% sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min and its absorbance was read at 725 nm. A calibration curve was constructed with different concentrations of gallic acid as standard. The results were expressed as mg of gallic acid equivalents (GAE) per gram of extract.

Total Flavonoid content

of the sample extracts was estimated by the $AlCl_3$ method with some modifications. The ethanol extracts (1.5 ml) were mixed with 5 ml of deionized distilled water (ddH_2O) and 0.3 ml of 5% NaNO2. After five min incubation at room temperature, 1.5 ml of 2% Aluminium trichloride ($AlCl_3$) solution was added. Two milliliters of 1M NaOH were added after the next 6 min. The mixture was vigorously shaken on orbital shaker for 5 min at 200 rpm and the absorbance was read at 510 nm against a blank. Quercetin with different concentrations was used as a standard.

Carbohydrates content

was quantitated spectrophotometrically using dextrose (stock conc 1 mg/ml) dissolved in 20 mM phosphate buffer as the standard. The varying concentrations of dextrose, ranging from 0-500 mg/ml were prepared by serially diluting in distilled water. 0.2% Anthrone reagent was prepared by dissolving in concentrated H_2SO_4, incubated at 90ºC for 10 minutes and cooled at room temperature. Accordingly, 500µl of each of the three *P.ostreatus* samples was dissolved in 500µl distilled water and 4 ml Anthrone reagent was added and incubated as mentioned above. Absorbance was measured at 620 nm and the unknown concentration was estimated by plotting against the dextrose standard plot.

Qualitative phytochemical tests

The vacuum evaporated ethanolic extract prepared was used for the same purpose. The following qualitative phytochemical tests were performed: Carbohydrate was analysed by Benedict's Test. For Alkaloids crude extract was mixed with 2ml of 1% HCl and heated gently. Wagner's reagents were then added to the mixture. Turbidity of the resulting precipitate is taken as evidence for the presence of alkaloids. Crude extract was mixed with 4 ml of distilled water in a test tube and boiled. Few drops of 0.1% $FeCl_3$ were added. Development of brownish green or blue-black color indicates presence of Flavonoids. Terpenoids were confirmed by Salkowski's test. Presence of cardiac glycosides was analysed by Keller-Kilani test. Lead Acetate test was performed for Phenolic compounds.

x) DNA fingerprinting and Dendrogram analysis: [13]

RAPD analysis helped in examining the genetic variations among the three clusters on the basis of the difference in the substrate used for cultivation. RAPD analysis constitutes the following steps;

DNA Extraction

About 50 mg of mushroom samples were cut into small pieces and grounded with

homogenisation buffer(50 mM Tris, 10 mM EDTA, 50 mM Glucose), extraction buffer(100 mM Tris, 10 mM EDTA, 250 mM NaCl, pH-8.0 and 1% Sodium Dodecyl Sulphate) with Proteinase-K was added and incubated in a dry bath at 60ºC for 1 hr. It was centrifuged at 3000 rpm for 5 mins, supernatant was treated with equal volumes of phenol: chloroform: isoamyl alcohol (25:24:1) and incubated at 37ºC for 30 mins. Centrifugation was carried out at 8000 rpm for 6 mins and phenol:chloroform:isoamyl alcohol(25:24:1) step was repeated and DNA was precipitated using 0.8 volumes of chilled isopropanol. The DNA samples were tested qualitatively on 0.8% agarose gel and quantified using Spectrophotometer.

PCR Reaction

A set of 3 decameric oligonucleotides was used in this investigation as single primers in the Polymerase Chain Reaction [Table 2]. The PCR reaction was carried out in a final volume of 25µL containing 100 ng DNA, 2 U of Taq DNA Polymerase, 2.5 mM $MgCl_2$, 2.5 mM each of the dNTPs and 100 p moles of primers. The DNA amplification was performed in a thermocycler using the following conditions: complete denaturation (94ºC for 5 min), 10 cycles of amplification (94ºC for 45 sec, 32ºC for 1 min, 72ºC for 1.5 min) followed by 30 cycles of amplification (94ºC for 45 sec, 34ºC for 1 min, 72ºC for 1 min) and the final elongation step (72ºC for 5 min).

Agarose Gel Electrophoresis of the PCR products

Total volume of the amplified product (25 µL) of each sample was subjected to electrophoresis on 2.0% agarose gel containing ethidium bromide in 1X TBE buffer at 120V for 1 hr. Finally, the DNA bands were observed in a Gel Doc system and photographs were captured.

Data analysis

The RAPD profiles were analyzed for the presence or absence of individual RAPD bands and a dendrogram analysis was carried out, followed by calculation of percentage polymorphism among the three clusters of *P.ostreatus*.

Table 2
List of primers

Primer	Sequence(5´to 3´)
OPAB 18	CTGGCGTGTC
OPAC 19	AGTCCGCCTG
OPAC 13	GACCCGATTG

RESULTS

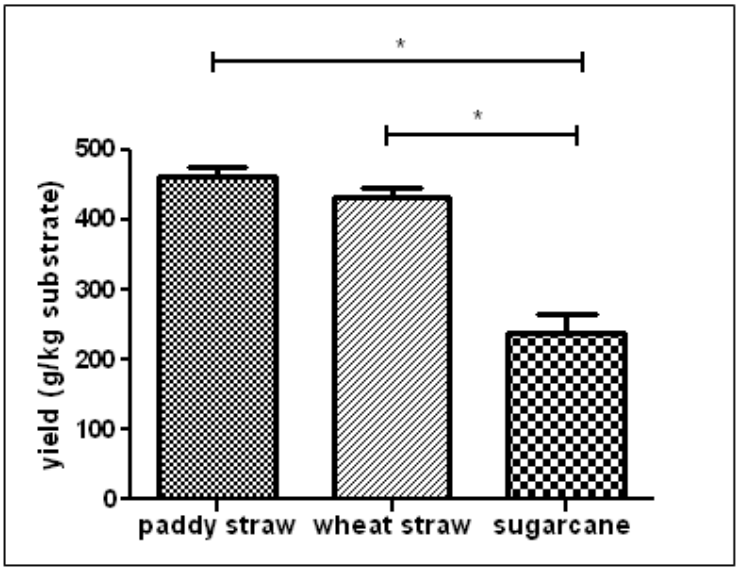
Measurement of yield (gram/kg of substrate)

The biological yield, as measured in gram/kg of substrate utilized was found to be the highest in case of paddy straw, the mean ± SD value obtained from the Graph 1 being 462.60±12.50. This was about 1.03 folds higher than wheat straw and about 1.94 folds higher than in sugarcane waste (bagasses), this difference being significant for sugarcane bagasse.

Table 3
yield (g/kg of substrate used) of paddy straw, wheat straw and sugarcane bagasse.

Paddy	Wheat	Sugarcane
462.60±12.50	433.0±13.11	238.25±25.79

Graph 1
Yield (g/kg substrate) for paddy straw, wheat straw and sugarcane bagasse,



The data is expressed as mean ± SD from the triplicate samples that were tested
**** p<0.05 and indicates a significant difference.***

Measurement of fruit-body head and stipe

The mean value of the length and breadth of the stipe of the fruit bodies were observed to be varying considerably across the three different substrates used (Table 4), with the value of length being 1.94 fold higher and breadth being 1.5 folds higher in paddy as compared to wheat. The difference between paddy and sugarcane was significantly higher, the length and breadth being 3.60 folds and 1.87 folds higher in the former, respectively.

Table 4
length and breadth of head and stipe of fruit-body in paddy straw, wheat straw and sugarcane bagasse.

	Paddy		Wheat		Sugarcane	
	Mean length	**Mean breadth**	**Mean length**	**Mean breadth**	**Mean length**	**Mean breadth**
Measurement of Stipe (cm)	4.04	1.50	2.08	1.00	1.12	0.8
Measurement of head (cm)	7.38	6.40	6.77	5.22	4.82	4.68

Measurement of mean fresh weight, mean dry weight and % moisture

The mean values of fresh weight and dry weight were seen to vary significantly across the three substrates, paddy straw, wheat straw and sugarcane bagasse (Table 5). The mean fresh and dry weights of the fruitbody grown on paddy were 2.8 folds higher than that grown on sugarcane bagasse. However, the percentage of moisture content in the three fruitbodies was found to be comparable.

Table 5
Mean fresh weight and mean dry weight of mushroom in paddy straw, wheat straw and sugarcane bagasse.

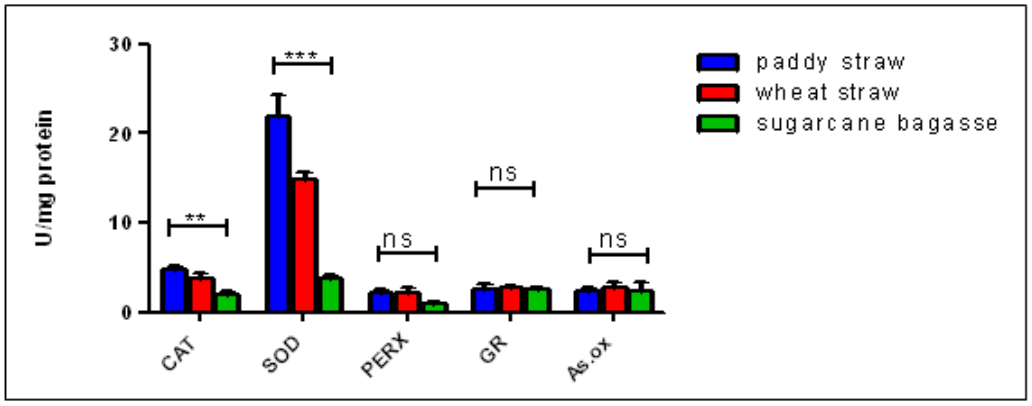
	Paddy	**Wheat**	**Sugarcane**
Mean Fresh weight(gm)	12.32	9.57	4.40
Mean Dry weight(gm)	1.27	0.90	0.44
% moisture	89.69	90.50	90.20

Figure 1
spore print of Pleurotus ostreatus

Levels of enzymatic antioxidants in P.ostreatus fruitbodies

The enzymatic antioxidants like SOD, catalase, peroxidase, Glutathione reductase (GR) and ascorbate oxidase enzyme activities of *Pleurotus ostreatus* cultivated in paddy waste, wheat straw and sugarcane bagasses were tested. As is evident from the graph 2, the SOD activity of the fruitbodies cultivated in paddy was 1.46 fold higher than that cultivated in wheat and 5.98 folds higher than in sugarcane, the mean values of the three being 21.97U/mg, 14.97U/mg, 3.67U/mg respectively. The catalase activity of fruitbodies grown on paddy was 1.28 fold higher than those grown on wheat and 3 folds higher than those grown on sugarcane bagasses. Difference in Peroxidase, glutathione reductase and ascorbic oxidase enzyme activity was found to be not significant (graph 2) as the activity was comparable in all the three substrates. However, in case of GR and Ascorbate oxidase, there was an exception where the enzymatic activity was decreased slightly in the case of paddy as compared to wheat, the mean value for GR being 2.62 U/mg and 2.76 U/mg for paddy and wheat, respectively. In case of Ascorbate oxidase, the mean values were 2.45 U/mg and 2.80 U/mg for paddy and wheat respectively.

Graph 2
Levels of enzymatic antioxidants in U/mg protein.

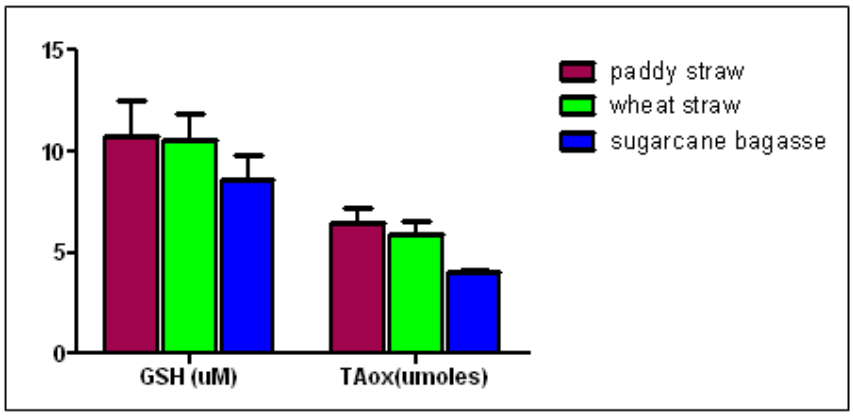


*All values are expressed as Mean ± SD, ** p<0.01 and *** p<0.001, ns = not significant*

Levels of non-enzymatic antioxidants P.ostreatus fruitbodies

Levels of non-enzymatic antioxidants like GSH was also found to be 1.24 folds higher in case of paddy than sugarcane, the mean values being 10.73 µM and 8.63µM respectively. In case of paddy and wheat, the difference was not significant. The antioxidant activity of samples was expressed as equivalents of α-tocopherol (µmoles) which was found to be 6.45 µmoles in paddy and 3.90 µmoles in sugarcane which indicate an increased expression in case of paddy than in sugarcane. The difference in expression levels in paddy and wheat was not significant (Graph 3).

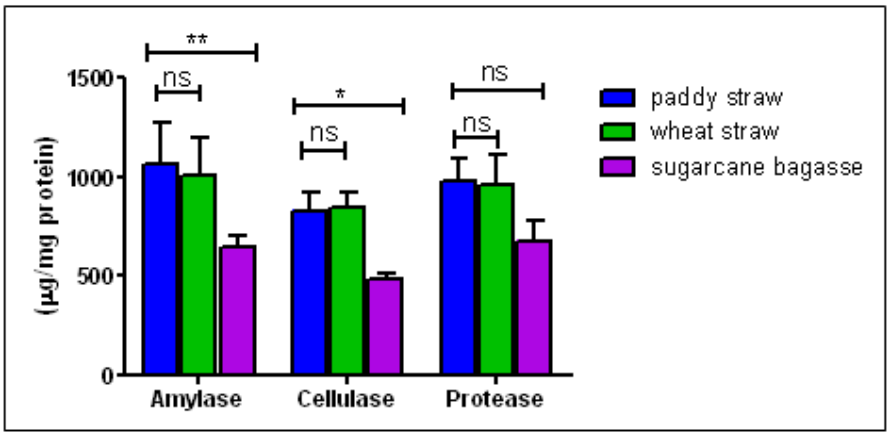
Graph 3
Levels of non-enzymatic antioxidants in paddy straw, wheat straw, sugarcane bagasse



Increased expression of substrate degrading enzymes P.ostreatus fruitbodies

Mushrooms generally grow on lignocellulosic substrates with a suitable nitrogen source. So the enzymes like cellulase, amylase and protease are required for the utilization of substrates and mushroom yield. It was observed from (Graph 4) that the level of these enzymes were significantly higher when paddy was used as a substrate than when sugarcane was used. The difference between paddy and wheat was not significant. Mean value for amylases (in µg/mg protein) were 1063, 1005 and 642.70 in paddy, wheat and sugarcane wastes respectively. Mean value of cellulase (in µg/mg protein) were 832.3, 847.7 and 487.30 for paddy, wheat and sugarcane respectively. Similarly for protease, mean values (in µg/mg protein) were 977, 957, and 679.30 for paddy, wheat and sugarcane respectively.

Graph 4
Expression levels of substrate degrading enzymes for paddy straw, wheat straw and sugarcane bagasse



*All values expressed as mean±SD , ** p<0.005 and * p<0.01, ns = not significant.*

Measurement of bioactive compounds P.ostreatus fruitbodies

When comparison was made between the ethanolic extract of fruitbodies of mushroom grown on paddy straw, wheat straw and sugarcane bagasses as substrates, the phenolic content, in case of sugarcane was observed to be 1.09 folds higher than in case of paddy straw. This difference was 1.10 folds higher in case of wheat than in paddy. Mean value being 140.0 and 154.1 and 153.0 µg/ml extract for paddy, wheat and sugarcane respectively. A similar trend was observed with other important bioactive compounds like

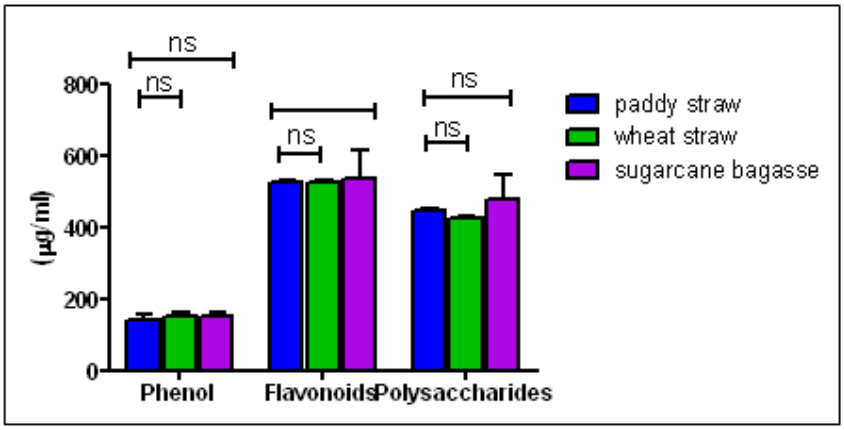
flavonoids and carbohydrates. Mean value being 525.2, 526.8, and 636.7 µg/ml extract in case of paddy, wheat and sugarcane, respectively, for flavonoids and 446.2, 427.2, and 479.0 µg/ml extract in case of paddy, wheat and sugarcane, respectively, for total polysaccharide content (Graph 5).

Table 6
Table showing the results of qualitative analysis of bioactive compounds

Bioactive compounds	Qualitative result
Polysaccharides	+++
Alkaloids	+
Tannin	-
Phenols	++
Flavonoids	+++
Terpenoids	+
Cardiac glycosides	++

(+) indicates positive result and (-) indicates negative result

Graph 5
Expression levels of bioactive compounds for paddy straw, wheat straw, and sugarcane bagasse

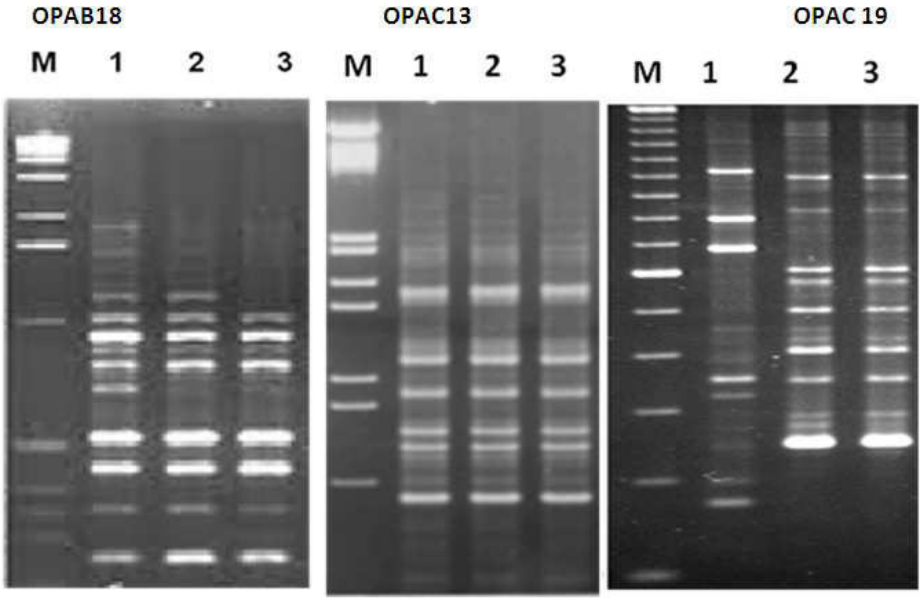


All values expressed as mean±SD , ns indicates not significant

DNA fingerprinting analysis of P.ostreatus

Distinct bands were observed with all the three primers, OPAB18, OPAC19, OPAC13. As is evident from the results, OPAC19 showed the highest percent polymorphism among the three primers and hence, can be used as a marker for differentiating between the fruitbody grown on paddy straw, wheat straw and sugarcane bagasse. Dendogram analysis showed the genetic distance between the clusters of fruitbodies.

Figure 2
DNA fingerprinting profile of P.ostreatus



M- DNA Molecular weight marker, Lane 1- Fruitbody cultivated on paddy straw, Lane 2- Fruitbody cultivated on wheat straw, Lane 3- Fruitbody cultivated on sugarcane bagasse.

Figure 3
Dendrogram tree

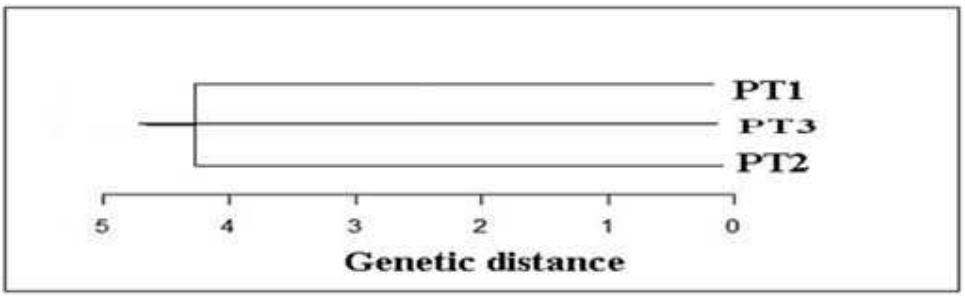


Table 7
Percentage of polymorphism for the three primers and the three substrates, paddy, wheat and sugarcane

	% Polymorphism			
Primers	**Paddy**	**Wheat**	**Sugarcane**	**Total**
OPAB18	34.14	26.82	21.95	12.19
OPAC19	36.0	29.61	32.0	64.0
OPAC13	22.22	22.22	22.22	0

DISCUSSION

In this study the optimum yield and mycelial growth were observed in paddy straw which was statistically similar to that of wheat straw. It was significantly higher than the yield and mycelial growth found in sugarcane bagasse (Table 3). The presence of the right proportion of α-cellulose, hemi-cellulose, pectin, lignin and protein was the probable cause of the highgrowth rate of mycelium in the paddy and wheat straw. A suitable ratio of carbon to nitrogen might have been responsible for the higher mycelial growth. The effect of different substrates on yield contributing characters such as pileus size and stipe length varied significantly. Paddy straw gave the maximum stipe and pileus size, followed by wheat straw

with the least in sugarcane bagasse (Table 4) There appears a definite co-relation between the substrate composition and growth of mushroom. The range of polysaccharide and protein-degrading enzymes formed by oyster mushrooms growing on lignocellulosic substrates like paddy and wheat straw and sugarcane bagasse were monitored. High amount of cellulase, amylase and protease present in case of paddy straw which was comparable to wheat straw and significantly higher than sugarcane bagassse which indicates that higher amount of cellulosic compounds and amount of protein influence the activity of these enzymes. Physiological amounts of reactive oxygen species (ROS) are a mandatory cellular requirement being involved in signaling pathways, regulating a variety of cellular activities including cytokine secretion, growth, differentiation and host defense against invading pathogens. However, ROS can be compared to a double edged sword as it concomitantly has the potential to induce significant biological damage when it counters the cells robust antioxidant system. Reactive oxygen species (ROS) such as super oxide, hydrogen peroxide and hydroxyl radicals are molecules that contain oxygen and have higher reactivity than ground state molecular oxygen. These free radicals can peroxides membrane lipids and attack protein or DNA. In order to avoid these unnecessary reactions there is a requirement of enzymatic and non-enzymatic defense systems that deal with ROS produced as a consequence of aerobic respiration [14]. In this study an increased level of antioxidants like SOD, catalase, GSH, Ascorbic oxidase, peroxidase, glutathione reductase, total antioxidants was present in fruitbodies of *P. ostreatus* cultivated on paddy straw and wheat straw. It was significantly lower in sugarcane bagasse. It is evident from the result that if grown on proper substrate mushroom can be considered as a potential organism for antioxidant therapy. Oyster mushrooms are considered as the most potent edible mushroom with high therapeutic values. Secondary metabolites are compounds that are used as food and medicine to protect against illness and to maintain human health. Oyster mushrooms accumulate a variety of secondary metabolites, including phenolic compounds, polysaccharides, polyketides, terpenes, steroids. Present study indicated presence of all these above mentioned bioactive compounds with significantly high amount of phenols, flavonoids and polysaccharides. Comparative study between three different substrate showed no significant differences which showed substrate composition does not influence the secondary metabolite production. New improved samples or improved characteristics of mushrooms are needed to make mushroom cultivation more sustainable and highly productive. In this study DNA fingerprinting technique with RAPD primers were used to assess the genetic variation in *P. ostreatus* caused by substrate composition correlating with morphological and biochemical variations. The RAPD patterns of genomic DNA of *P.ostreatus* by primers OPAB19, OPAC13 and OPAC 14 were analyzed for polymorphism and OPAC13 gave the distinctive band pattern with highest % polymorphism.

CONCLUSION

An overall study indicated that variable substrate composition were responsible for differential yield of fruitbodies and expression of substrate degrading enzymes, antioxidant system, bioactive compounds. Different growth conditions lead to generation of genetic polymorphism in fruitbodies. Thus, it can be concluded that if grown under optimum growth conditions oyster mushroom can prove to be and potential therapeutic agent with increased biomedical properties.

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