SOLID STATE FERMENTATION OF LACCASE FROM NEW PULSE HUSKS: PROCESS OPTIMIZATION AND BIOPROCESS STUDY

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ABSTRACT

A comparative study was done for feasibility of pulse husk waste (Green gram husk (GgH), Black gram husk (BgH), Bengal gram husk (BegH) and Red gram husk (RgH)) as substrates for laccase production by Pleurotus ostreatus 1804 under solid state fermentation. Various process parameters like incubation time, pH of the culture, initial moisture content, particle size, inoculum size, substrate loading, and effect of inducer (2,5-xylidene) on laccase production were investigated. To characterize the solid substrate (pulse husks) before and after fermentation FT-IR, substrate components (cellulose, total carbohydrates, and lignin) and CHNS analysis were done. Inducer enhanced 2-fold of the laccase yield at optimized culture conditions. GgH was proven to be the best source for laccase production with maximum yield of 2200 U compared to all other substrate studied [1350 U (BgH), 1010 U (BegH) and 810U (RgH)].

Key words: Laccase, Pleurotus ostreatus, solid state fermentation, optimization, husk.

INTROCUTION

Lignin degrading enzymes (Laccases) have been the subject of continuous study since the end of the 19th century [1]. Lignin is either directly degraded to CO_2 and H_2O or converted to humus, which is very resistant to biological deterioration. Complete degradation of lignin is believed to be a result of cooperative action of various fungi and bacteria [2]. Species of the genus *Pleurotus* were among the most efficient natural species in lignin degradation. Nowadays the mechanism of lignin depolymerisation by white-rot fungi or by isolated enzymes is under intensive study in connection with a wide range of biotechnical applications such as biopulping [3], biobleaching [4], treatments of hazardous chemicals and wastes [5] and improvement of digestibility of lignocellulosic substrates for animal feeding [6].

Solid state fermentation (SSF) is environmental friendly as it resolves the problem of solid wastes disposal. It has been generally claimed that product yields are mostly higher in SSF when compared to submerged fermentation (SmF). Production of these biocatalysts using agro-biotech substrates under solid-state fermentation conditions provide several advantages in productivity, cost-effectiveness in labour, time and medium components in addition to environmental advantages like less effluent production, waste minimization, etc. [7]. There are several reports describing use of agro-industrial residues for the production of laccase e.g. Banana skin by *Trametes pubescens* [1], Coconut flesh, groundnut shells and groundnut Seeds by *T. hirsuta* [2, 8], hampas, rubberwood sawdust, and OPFPt. Sagohampas by *Pycnoporus sanguineus* [9] grape seeds [10]. However, these production characteristics would have to offer a competitive advantage over existing products. In general, each microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties. This warrants thorough characterization of isolated individual microbial species to evaluate its potential at commercial level.

Furthermore, most of these wastes contain lignin or/and cellulose and hemicellulose, which act as inducers of the ligninolytic activities. Moreover, most of them are rich in sugars, which make the whole process much more economical. All these make them very suitable as raw materials for the production of secondary metabolites of industrial significance by microorganisms. In particular, the present investigation aimed to exploit the locally available, inexpensive four common pulse husks from agro-processing units

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viz. red gram, black gram, green gram and bengal gram husks were evaluated as potential solid state substrates for the laccase production first time. The influence of substrate on the enzyme yield by *P. ostreatus* 1804 strain was investigated with respect to substrate composition.

MATERIAL and METHODS

Microorganism

The white-rot fungus *P. ostreatus* 1804, a hyperlaccase producing strain was used in the present study to examine the production of laccase during SSF. *Pleurotus ostreatus*-1804 was procured from Microbial Testing and Collection Center (MTCC), Institute of Microbial Technology (IMTech), Chandigarh, India and fungi was maintained on potato dextrose agar (PDA) plates and stored at 4 ^{o}C.

Inoculum preparation

Inoculum of *P. ostreatus* on wheat grains was prepared as per the procedure recommended by Kumar and Chandra [11]. 200 g of wheat grains were taken in 1000 ml of Erlenmeyer conical flask, to that two volumes of distilled water was added and boiled for 30 min. Boiled wheat grains were supplemented with 0.2% calcium carbonate and 1.2% of calcium sulphate and sterilized at 1 kgf/cm2 for 2 h. After sterilization, grains were cooled to room temperature and were inoculated with mycelial plugs grown on PDA agar plate and incubated at 25 ^{o}C. After 15 days of incubation the mycelial cultivated wheat grains were colleted and used as the initial inoculum for the SSF studies.

Solid-state substrates and their characterization

Four commonly available (in India) agro-pulse husks viz. Cajanus cajan (red gram, local name) [RgH], Vigna mungo (black gram, local name) [BgH], Phaseolus aureus (green gram, local name) [GgH] and Cicer artinum (bengal gram, local name) [BegH] husks were evaluated as potential solid-state substrates for the laccase production. Pulse husks were collected from a pulse-processing mill where it is generated as a primary waste. The four husks were characterized for total carbohydrates, cellulose, lignin according to [12, 13, 14] respectively Crude protein content (it was calculated by multiplying total Kjeldahl nitrogen content with a factor N x 6.25) and total ash content as mentioned in [15] and were depicted in percentage proximate composition of husk on dry weight basis (Table 1). Carbon and nitrogen content of the husks were also determined using Elemental analyzer (Elementer Vario-EL, Germany).

Table 1. Composition of agro-pulse husks

Sub-strate	Protein (%)	Total carbohy-drates (%)	Cellu-lose (%)	Lignin (%)	Ash content (%)
GgH	4.05	64.5	35.4	4.2	4.54
BgH	3.32	54.1	31.5	3.2	4.93
BegH	2.31	50.1	26.2	2.8	3.98
RgH	1.45	46.9	21.8	2.4	2.92

Solid state fermentation experiments

Two grams of each husk was taken in 250 ml Erlenmeyer flasks separately and was moisturized with salt solution (KH_2PO_4- 2.0 g, $MgSO_4.7H_2O$- 0.5 g, $CaCl_2$- 0.1 g, KCl- 0.5 g, Urea- 1 g and $CuSO_4.5H_2O$- 0.25 g dissolved in 100 ml of distilled water) to set the desired moisture level. Then the media were sterilized at 121 ^{o}C for 1 h to provide proper cooking of the substrate and to increase its susceptibility to microbial attack. Media were cooled after autoclaving to room temperature and inoculated with 3 g of the inocula of *P. ostreatus* grown on wheat grain and cultures were incubated in static conditions at 25 ^{o}C in an incubator. The contents were mixed thoroughly four times daily during the fermentation period by gently hitting the flask bottom on the palm of the hand. Two experiments for each substrate were conducted in parallel and triplicate samples were analyzed. The values in the figures corresponded to mean values with a standard deviation.

Optimization of solid-state fermentation conditions

To elucidate the relative efficiency of the selected agro pulse husks as solid substrate a series of fermentation experiments were designed and conducted. The strategy adopted for optimization of various process parameters influencing laccase yield included consecutive evaluation of parameters. Initially one parameter was evaluated and it was then incorporated at its optimized level in the subsequent optimization experiments. Details of optimization experiments for various process parameters were discussed below.

Fig 1 Effect of initial moisture content on laccase production (a) and biomass growth (b) during SSF

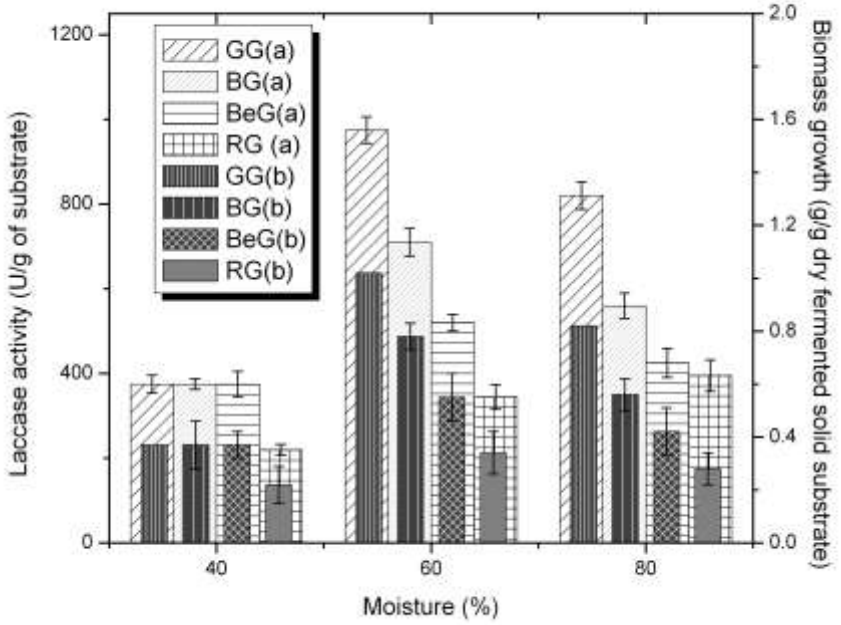

Fig. 2. Effect of substrate particle size on laccase production (a) and biomass growth (b) during SSF

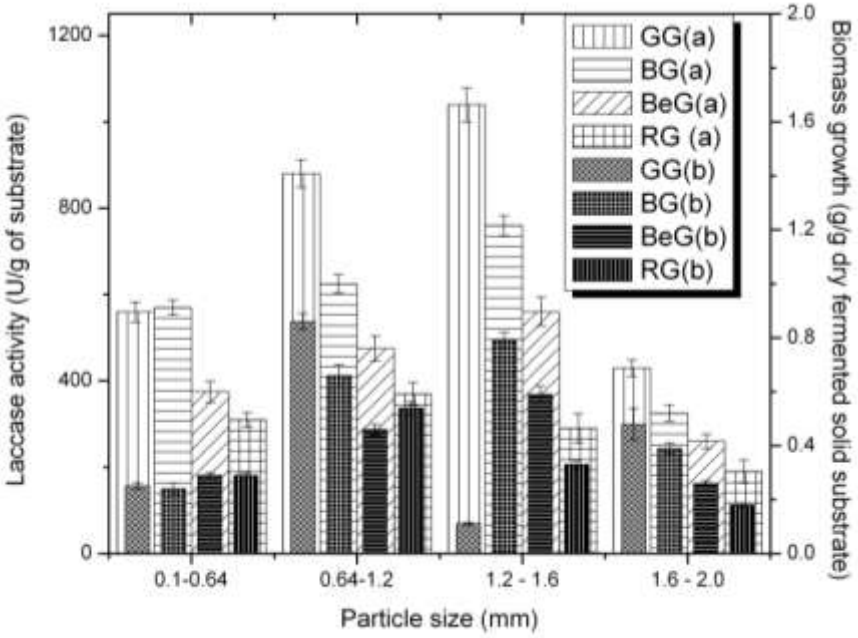

Fig. 3. Effect of inoculum size on laccase production (a) and biomass growth (b) during SSF

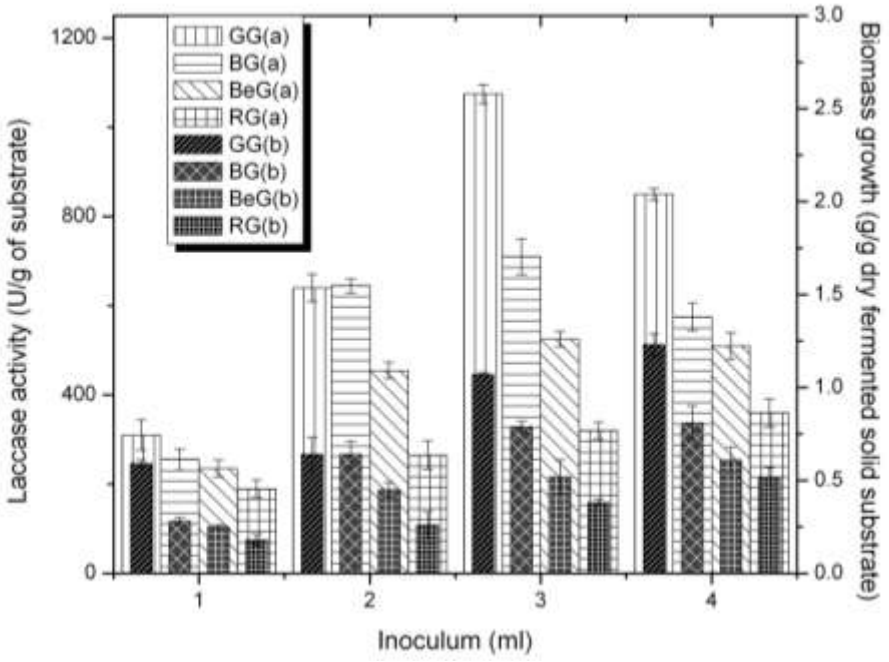

Fig. 4. SDS-PAGE showing the laccase expression during SSF at optimized conditions. Lane 'M' indicates standard molecular weight marker, lanes 1-4 are enzyme samples extracted from fermented solid substrates (1. Black gram husk, 2. Green gram husk, 3. Bengal gram husk, 4. Red gram husk)

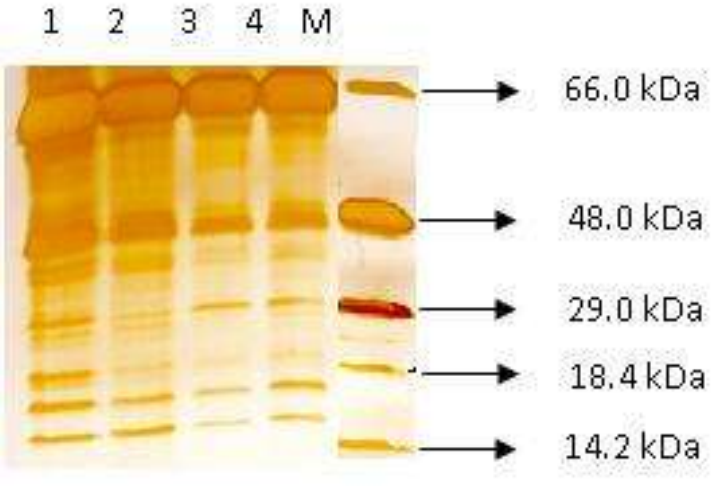

Fig. 5 [a-d]. FT-IR spectra of husk before and after SSF by P. ostratus 1804

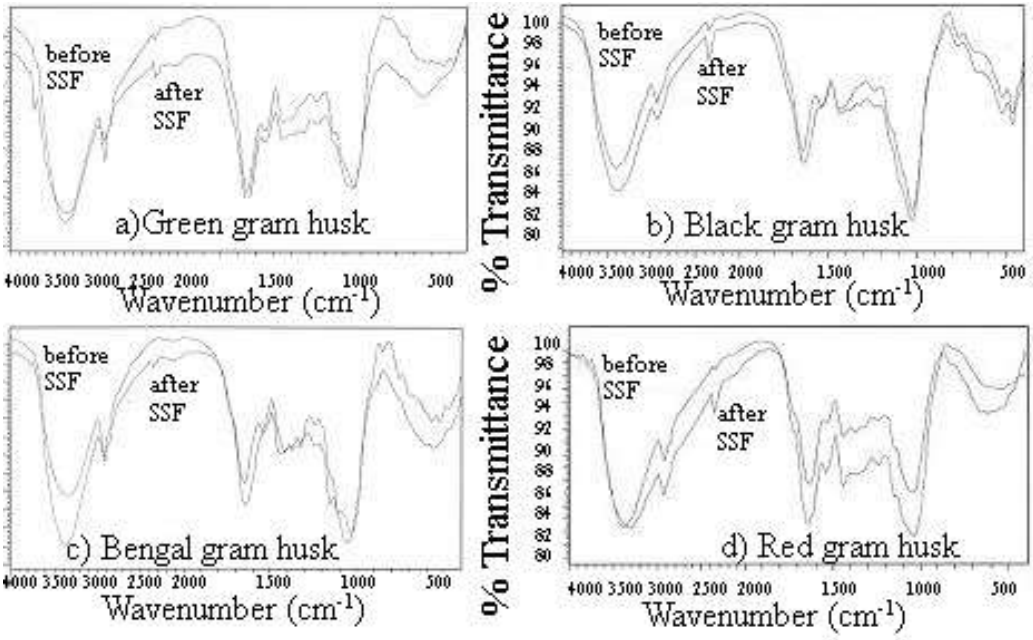

Fig. 6. Photograph showing the colonization of P. ostreatus mycelia to husk particle

Incubation time

To determine the optimum incubation time experiments were carried out for laccase production with 10 sets of duplicate flasks for each substrate. The inoculum concentration (3 g) and moisture content (60%) was maintained constant in all the flasks studied. During the fermentation period, the samples were analyzed for laccase activity at every 3 days interval until the 30th day of incubation time.

Effect of pH

To find out the optimum pH for laccase production by *P. ostreatus* during SSF, the initial pH of the solid substrate was adjusted to pH 4.0 to 7.0 (with variation of 0.5) and experiments were conducted. The required pH of the substrate was maintained by adding pre-adjusted salt solution. The desired pH of the salt solution was obtained by using 3N HCl or NaOH.

Effect of particle size

In order to find out the effect of particle size on laccase production four fractions of different particle sizes viz. less than 0.64 mm, between 0.64 and 1.2 mm, 1.2 and 1.6 mm and 1.6 and 2.0 mm were used. The desired particle size of studied substrates was obtained by sieving (using USA standard sieves) the air dried substrates.

Effect of initial moisture content

Samples containing 3 moisture levels (40%, 60% and 80%) were prepared by moisturing 2 g of studied substrates with salt solution. During this period the nutrient levels were maintained the same.

Effect of inoculum size

The effect of inoculum size on laccase production was studied using an inoculum size of 1, 2, 3 and 4 g of wheat grain per 2 g of substrate.

Effect of weight of the solid substrates

Each substrate in three separate sets of flasks containing 1, 2 and 3 g were incubated in order to find out the optimum substrate concentration for maximum laccase yield. After carrying out the fermentation for optimum time, the contents of the flasks were extracted for laccase and its activity was measured.

Effect of inducer

In order to assess the enhancement of laccase induction during solid state fermentation, the putative laccase inducer 2,5-xylidine was investigated in the concentration range of 0.1, 0.2 and 0.3 mg/g of substrate. Stock solution of xylidine was prepared by dissolving in ethanol and required concentration of sterilized (passing through 0.2 μ filter) inducer solution was added to the actively growing fungal cultures on the 10th day of incubation. Control flask without aromatic inducer (with ethanol) was also incubated along with inducer supplemented flasks in order to understand the effect of aromatics on the SSF of laccase by *P. ostreatus*-1804.

Laccase Extraction

After the given period of incubation, the fermented substrate cultures (2 g) were extracted twice for laccase by mixing with 50 ml of chilled phosphate buffer (50 mM, pH 6.0). Each time extraction was performed with 25 ml of chilled buffer solution under rotary shaking (100 rpm) at 25 ^{o}C for 1 h. The homogenate was filtered through nylon cloth (200 mesh size) and the filtrate was centrifuged at 10,000 x g at 40 ^{o}C for 15 min. The clear supernatant was used for estimating the laccase activity.

Analytical Assay

Laccase activity

Laccase activity was measured by ABTS oxidation procedure [16]. Enzyme activity was expressed as units/g of cultured substrate and was defined as the amount of enzyme producing 1 µM of product per min per gram of substrate extracted.

Fourier transform infra red spectroscopy (FT-IR)

In the present study, FT-IR spectroscopy was used to detect the changes occurring in solid-state substrates due to the action of *P. ostreatus*. Solid substrate samples were analysed before and after SSF. The pellets were prepared by mixing 2 mg of grounded homogenous sample with 300 mg KBr and later compressing the mixture under vacuum for 10 min. In order to limit moisture interference, both composting samples and KBr were dried at 105 ^{o}C for 72 h before making pellets. The IR spectra in 4000-500 cm^{-1} regions for four agro waste husks were illustrated using FT-IR model Thermo Nicolet NEXQS, 670.

Sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE)

Clear enzyme extracts after SSF were subjected to SDS-PAGE, in order to know the laccase expression along with its molecular weight. SDS-PAGE was carried out on a 10 % resolving gel and a 5 % stacking gel. Samples (10 μg of protein) were treated prior to loading onto the gel with 0.5% SDS and 5% β-mercaptoethanol, and were boiled at 95 ^{o}C for 5 min. SDS-PAGE was performed using a Torson Mini-Vertical Electrophoresis system (Apex scientific instruments, India) and proteins were separated at 60 mA per gel. Proteins were visualized by staining for 3 h with silver nitrate and it was compared with low range molecular markers (Sigma).

Scanning electron microscopy (SEM)

P. ostreatus growth and its mycelial formation around the solid-state substrate particles during SSF were visualized by SEM according to Bozzola and Russell [17].

Estimation of fungal biomass in SSF

Estimation of fungal biomass in SSF was done calorimetrically by chitin estimation procedure on the basis of N-acetyglucosamine content present in the fungal biomass [18].

RESULTS AND DISCUSSION

One of the effective approaches to reduce the cost of enzyme production was to replace pure carbohydrates as substrates with relatively cheaper materials namely lignocellulosics. Majority of organic materials available in nature like polysaccharides, proteins and lignin were polymeric in structure. In general, microorganisms could use all these as substrates (carbon source). Solid substrates used in SSF were insoluble in water. Filamentous fungi had other features, which gave them advantages for SSF processes over unicellular organisms [19]. The mycelial growth was ideally suited to rapid colonization of the whole of a solid surface, which could later be followed by an increase in density. The hyphal growth mode also allowed fungi to penetrate into substrate particles, which might play an important role in degrading the particle structure and making nutrients available [20]. The utilization of solid substrates by the microorganisms was affected by several physical and chemical factors. Among the physical factors, accessibility of substrate to microbes, film effects and mass effects were important [21]. The physical morphology, especially porosity and particle size of the substrate, governed the accessible surface area to the organism. The chemical nature of the substrate was also an important criterion [20, 22]. The conditions for growth and cultivation played an important role in the laccase production by fungi, but the composition of media was equally important [23].

Composition of solid substrates

CHNS analysis was performed on the solid substrates (Table 2) and the data revealed higher percentage of C, H, N and S composition in GgH followed by BgH, BeH and RgH respectively. The composition of the substrates with respect to protein, ash, cellulose, lignin and total carbohydrates was determined to acquire detailed information about the solid substrates (Table 1). GgH showed higher percentage of cellulosic composition.

Table 2. CHNS analysis of agro-pulse husks

Substrate	Carbon (%)	Nitro-gen (%)	Hydro-gen (%)	Sulphur (%)
GgH	47.81	1.98	6.02	ND
BgH	40.31	1.16	5.69	ND
BegH	30.81	0.47	5.99	ND
RgH	29.10	0.24	5.89	ND

ND: not detected

Incubation time for laccase production

Incubation (fermentation) time required for the maximum laccase yield varied from substrate to substrate and it was strongly associated with the composition of the substrate (Table 1 and 2). With GgH, maximum laccase activity (1100 U) was observed on the 18th day. In the case of BgH, BegH and RgH, the maximum laccase activity of 760 U, 525 U and 375 U was observed on 24th day, 27th day and 30th day of incubation respectively. Where as it were only 7.6 U/g and 7.5 U/g for sago hampas and oil palm frond parenchyma tissue respectively by *Pycnoporus sanguineus* in 11 days of fermentation [9]. Thus the incubation time required was found to be governed by the characteristics of the solid substrate and organism used.

Effect of substrate pH

Continuing consecutive optimization of laccase production by *P. ostreatus*, the effects of initial pH of the substrate was investigated. Production of laccase did not vary appreciably in the initial pH range of 4.0 to 4.5. At pH of 5.0, maximum laccase activity of 1005 U, 740 U, 480 U and 350 U were obtained with GgH, BgH, BegH and RgH respectively. However increase in substrate pH from 5.0 to 7.0 showed reduction in the laccase yield. Although the fungus grew well over a pH range of 4 to 8, the pH of the culture might change in response to metabolic activities and also it was obvious that the culture pH had a striking effect on secretion of laccase. Stability of enzymes and secondary metabolites was pH dependent. Even though the rate of production might not be affected by change in pH, the overall process productivity declined because of destruction of the product [24]. The pH variation during fermentation depended highly on the nature of microorganism used. With *Aspergillus* sp., *Penicillium* sp., and *Rhizopus* sp. a rapid drop in pH below 3.0 was reported due to the possibility of secretion of organic acids. In the case of *Trichoderma*, *Sporotrichum* and *Pleurotus* sp. the pH was more stable between 4 and 5 during fermentation [25].

Effect of initial moisture content (IMC) of solid culture

The moisture content in SSF had significant influence on the final outcome. Variations in laccase yield and biomass growth at different initial moisture contents presented in Fig 1. Experimental data showed significant increase in the biomass growth at 60% of IMC for all the studied solid substrates along with higher laccase yield (Fig 1). With the other two IMC studied, comparatively lower biomass growth was observed. The highest levels of enzyme yield were attained in the media containing 60% of IMC, while the lowest laccase expression was observed in the media with 40% of IMC. At 80% of IMC a significant suppression in the enzyme expression was observed. Optimum moisture content of the solid substrate media facilitated better access to the microorganism to nutrients, which led to effective growth in SSF. Media contained a reasonable amount of oxygen in the beginning, which was indispensable for the growth and thus the microorganisms profited from the availability of both the nutrients and oxygen. In the later phase of the fermentation process, the oxygen available initially was depleted and further oxygen supply to the media was difficult as excessive/higher level of moisture content was present in the solid substrate due to the presence of relatively smaller number of channels through which air could more easily circulate. Considering the two variables, nutrients and oxygen, it might be concluded that the medium with a 60% IMC offered more favorable conditions for the growth of this fungus in a solid state process with the studied substrates.

Optimum IMC for growth also depended on the water holding capacity of the solid medium. The inert support system allowed usage of a highest IMC around 70%. *Achrimonium chrysogenium* produced the greatest amount of Cephalosporin C on rice bran at an IMC of 49-51% [26]. In some SSF systems, bacteria (*Actinomycetes* and *Bacillus*) required higher water content than fungi. In wheat bran SSF, optimum IMC for production of giberrelic acid by *Gibberella fujikuroi* was 60%, whereas the optimum value for itruin production by *Bacillus* was 68%. Optimum IMC for Cephalosporin production in a barley system by *Streptomyces claviligerus* and the fungus Achromonium were reported to be 39.5% and 35%, respectively [26].

Effect of particle size of substrate

Influence of particle size on laccase yield was presented in Fig 2. The higher biomass growth and highest laccase expression was observed in the cultures with large particle size (0.64 to 1.2 mm - RgH and 1.2 to 1.6 mm- other substrates studied). A decrease in both the biomass growth and enzyme expression was observed with small particle size of the substrate (<0.64 mm). Such behavior of the fungus could be attributed to the non-availability of oxygen in the medium. Larger particle sizes provided larger inter particle spaces and therefore, a better passage of air and better penetration of the mycelia. Sarikaya and Ladisch [27] reported the effective growth of *P. ostreatus* NRRL 2366 in the media with larger particles than in those with smaller ones on the solid media composed of three different particle sizes of a mixture of stems, pods and leaves of rapeseed plant. While Prakasham et al., [22] reported 1.4-1.0 mm of GgH was optimum for the protease production.

Effect of inoculum size

In the present study, an attempt was made to investigate the influence of four different inoculum

sizes on the fermentation efficiency. Inoculation size in terms of wheat grain based immobilized fungi of 1 g, 2 g, 3 g and 4 g per 2 g of substrate was studied. The fungal growth was evidently observed more in the media inoculated with higher inoculum size (Fig 3). However, in the case of enzyme activity, higher yield was noticed at 3 g of inoculum size (Fig 3). On the contrary, in the case of RgH as the solid substrate higher laccase yield was evident at an inoculum size of 4 g. In SSF process, the inoculum density is of great importance. Too low a density may give insufficient biomass and permit the growth of undesirable contaminants, while for some processes high inoculum densities may produce too much biomass and deplete the carbohydrates and nutrients necessary for product formation.

Effect of substrate loading

Surface-to-mass ratio of solid substrate was one of the important factors in SSF, as it was directly related to the surface area available for the growth of cells [26]. In this direction three variations in solid substrate loading (1 g, 2 g and 3 g of solid substrate) were studied to enumerate its role on the laccase yield in SSF. It was found from the results that 2 g of solid substrate yielded effective laccase expression when GgH, BgH and BegH (970 U, 700 U and 540 U respectively) were used as solid substrates compared to the 1 g (580 U, 625 U and 350 U respectively) and 3 g solid substrate loading (725 U, 560 U and 600 U respectively). However, in the case of RgH, 3 g of solid substrate loading expressed maximum laccase yield (440 U) compared to other two substrate loading rates. This phenomenon might be attributed to the presence of relatively higher availability of the less total carbohydrate and nitrogen content in the RgH. Cellulose concentration rich substrate enhanced the laccase yield as per literature report [28] and the same phenomenon was observed in GgH substrate used in this study. However, in the case of other solid substrates, the presence/availability of higher concentrations of carbon and nitrogen source might cause the repression of laccase expression at the higher substrate loading condition.

Effect of inducer

Laccases were generally produced in low concentrations by white-rot fungi [29], but higher concentrations are obtainable with the addition of various supplements to media [23]. No specific studies on the role of inducers especially on SSF were performed in laccase fermentation. However, the effect of different inducers on laccase production in submerged cultures was studied using various white-rot fungi [30, 31].

Hence in the present study, effect of 2,5-xylidene as inducer on the production of laccase was investigated in SSF. The selection of 2,5-xylidene as inducer was done based on the earlier studies by the author in submerged fermentation with *P. ostreatus* for laccase expression [16]. 2,5-xylidene resembles phenolic structure of lignin molecule and it helped to increase the rate of biosynthesis of laccase during the fermentation of white rot fungi. After 10 days of cultivation, xylidene was supplemented to the fermented substrate and this manifested enhanced yield of laccase expression compared to the control. The resemblance in phenolic structure of lignin molecule might trigger the preferential synthesis of one of the metabolites of the ligninolytic enzymes i.e. laccase. It is evident from experimental data that 0.2 mg xylidine/g of substrate had shown to be optimum concentration for the maximum laccase yield [2140 U (GgH), 1125 U (BgH), 1025 U (BegH) and 790 U (RgH)] when compared to control [1020 U (GgH), 710 U (BgH), 510 U (BegH) and 355 U (RgH)]. At 0.3 mg xylidine/g of substrate concentration, the laccase expression was found to be suppressed (1525 U (GgH), 925 U (BgH), 825 U (BegH) and 705 U (RgH)) compared to the control.

SSF at optimized culture conditions

The selection of an ideal substrate for enzyme production in a solid-state fermentation process is one of the critical factors to be considered. In order to find out a suitable substrate among the

Table 3. Optimum culture conditions for studied substrates

Parameters	GgH	BgH	BegH	RgH
Fermentation time (days)	18	21	27	30
Substrate initial pH	5.0	5.0	5.0	5.0
Initial moisture content (%)	60	60	60	60
Particle size (mm)	1.2-1.6	1.2-1.6	1.2-1.6	0.64-1.2
Inoculum g/g of substrate	1.5	1.5	1.5	2.0
Substrate load (g/g of substrate)	2.0	2.0	3.0	3.0
Inducer (mg/g of substrate)	0.2	0.2	0.2	0.2

studied agro wastes for maximum laccase yield, SSF experiments were conducted for all the substrates at their corresponding optimum levels already established (Table 3). After conducting the SSF at optimized conditions, the solid substrates were analyzed for laccase activity, biomass growth, FT-IR analysis, elemental analysis (for carbon and nitrogen), substrate component analysis (for cellulose, total carbohydrates and lignin content) and SEM imaging in order to monitor the on going biological process.

Laccase production

Expression of laccase was monitored using SDS-PAGE and it was found that various proteins were expressed and the molecular weight of laccase was approximately 66 kDa (Fig 4). Also it was evident from the experiments conducted at optimized culture conditions resulted in effective yield of laccase. GgH showed highest laccase expression of 2200 U (GgH) followed by 1350 U (BgH), 1010 U (BegH) and 810U (RgH).

Biomass growth

One of the approaches to determine the biomass was estimation of chitin, a polymer of N-acetylglucosamine, which formed the cell wall of the fungal organisms [18]. Quantification of N-acetylglucosamine represented the fungal biomass. This was done by analyzing a known mass of fungal culture and correlating it to a standard graph of N-acetylglucosamine. In the present work, 1 g of dry mycelium contained 52.2 mg of N-acetylglucosamine. The correlation for fungal organisms was reported to vary from 67 to 126 mg glucosamine/g dry mycelium [26]. Effective growth of mycelia was observed in the case of GgH fermentation compared to other substrates studied BgH, BegH and RgH (1.23, 1.0, 0.76, and 0.61 g/g of dry fermented solid substrate respectively). This data was well correlated with the laccase expression.

FT-IR analysis

During SSF, white-rot fungi *P. ostreatus* 1804 decay structural cell wall constituents and also the decay differ from substrate to substrate. A White-rot fungus generally removes lignin and structural carbohydrates at a similar rate, resulting in homogeneous cell wall decay. The influence of white-rot decay on wood chemistry was investigated by various methods [32, 33]. FT-IR was one of the useful techniques for studying wood decay chemistry, since minimal sample preparation is required and very small quantities of sample can be analysed (a few milligrams) when compared to conventional gravimetric techniques where several grams are required. Fungal decay of wood was reported using FT-IR [33, 34]. FT-IR spectra were taken on the solid substrate before and after

Table 4. Details of FT-IR bands observed in solid substrates before and after SSF

Assignment	Frequency changes of aromatic skeletal vibration reported Wave number (cm^{-1})	Frequency changes of aromatic skeletal vibration during SSF Wave number (cm^{-1})
A strong hydrogen bonded (O–H) stretching	3400	3373 [GgH]; 3380 [BgH]; 3362 [BegH]; 3379 [RgH]
Prominent C–H stretching	2997	2925 [GgH]; 2922 [BgH]; 2928 [BegH]; 2928 [RgH]
Absorbed O–H and conjugated C–O	1650	1647 [GgH]; 1644 [BgH]; 1644 [BegH]; 1640 [RgH]
Aromatic skeletal in lignin	1596 and 1505-1511	1511 [GgH]; 1541 [BgH]; 1543 [BegH]; 1539 [RgH]
C–H deformation in lignin and carbohydrates	1462 and 1425	1445 [GgH]; 1440 [BgH]; 1419 [BegH]; 1445 [RgH]
C–H deformation in cellulose and hemicellulose	1375	1324 [BegH]
syringyl ring and C–O stretch in lignin and xylan	1244	1246 [GgH] 1244 [RgH]
C–O stretch in cellulose and hemicellulose	1048	1038 [GgH]; 1034 [BgH]; 1029 [BegH]; 1038 [RgH]
C–H deformation in cellulose	898	856 [BegH]

fermentation by direct transmittance using the KBr pellet technique and the spectral changes in the study of solid state substrates (husks) were monitored in the range 4000-500 cm^{-1}. The resulted FT-IR spectra of GgH, BgH, BegH and RgH are shown in (Fig 5a-d) respectively. The band assignments of spectra were made according to Pandey and Pitman [33] and compared assignments of bands are given in Table 4.

Substrate components analysis

The components of the solid substrates before and after fermentation were analysed to enumerate the relative consumption of individual substrate in fermentation process. Agro wastes are mainly composed of polysaccharides (cellulose and hemicellulose) and lignin which significantly effect the growth of the fungus and production of lignocellulosic enzymes. After collection of fermentation substrates, mycelium was removed from their surfaces and was oven-dried to constant weight. Then the structural components of the substrates in terms of cellulose, total carbohydrates and lignin were analysed for each sample in order to know the utilization of substrates during SSF. The mean percentage of components lost (XSub) from individual samples was calculated using XSub (%) = [(Wi -Wf)/Wi] x 100. The data was depicted in Table 5.

Table 5. Percentage of solid substrate components loss during SSF

Component	GgH (%)	BgH (%)	BegH (%)	RgH (%)
Total carbohydrates	24.3	18.4	18.2	10.9
Cellulose	14.4	11.2	10.9	4.8
Lignin	25.3	19.3	18.7	11.9
Carbon	14.7	11.7	8.1	5.3
Nitrogen	51.2	39.2	23.7	12.7

Where, Wi represents initial components concentration of the solid substrate and Wf denotes concentration of components of the solid substrate after SSF. During SSF of laccase, significant loss in the solid substrate composition was evident from the experimental data (Table 5). GgH showed maximum consumption of each of the solid substrate components analysed among the studied solid substrates. Subsequently, BgH and BegH showed next higher component consumption, while RgH showed comparatively low consumption of components during the fermentation process. The component consumption data of solid substrates correlated well with the laccase yield and it was knew that, secondary metabolite production was directly related to components of substrates (i.e. carbon and nitrogen source) consumed by fungi during its growth.

SEM analysis during SSF

During SSF, the growth patterns of mycelia on the solid substrate was examined under SEM and the scanning electron images before and after fermentation of GgH husk are shown in Fig 6. Solid substrate before fermentation was also examined in order to know the surface characteristics. It was evident from SEM images that solid particles possessed rough surface and contained internal porous network. Colonization of *P. ostreatus* was observed in the form of mycelia branching on the solid substrate after SSF. Lengthened and sub-parallel hyphae with smooth surface were observed. The growth of hyphae led to the formation of densely formed aggregation around the solid particle resulting in consistent and profuse growth of mycelia. In fungi mycelia, hyphae extend by a highly polarized process called as cell extension or tip extension [35]. From the images, it was clearly observed that as the tip extended, periodic branches also appeared in a polarized manner forming into new tips. The processes of tip extension and branching permitted the fungi to colonize and penetrate into particle and efficiently utilize the substrate, which could be directly related to the process performance.

CONCLUSION

From the results obtained, it can be concluded that *P.ostreatus* 1804 is capable of utilizing all the pulse husk studied and produce the laccase enzyme under SSF conditions. But the laccase enzyme production pattern was not similar with all the husks studied. Because the composition of the husk were not similar and it was a key parameter to get more yield. In optimizing SSF process the factors such as the carbon source and the levels of the nitrogen and trace metals, could influence the growth and production of the metabolites. It followed the trend of the husk which has more amount of total carbohydrates and nitrogen content gave the more yield of laccase by the *P. ostreatus* 1804*.* GgH was most suitable material of

all the studied husks. After optimization of the fermentation conditions like pH, IMC, inoculum size, incubation time and the induction of an inducer 100% improvement was noticed. These promising results suggest the application of the system to industrial-scale operation in order to produce laccase enzyme economically challenging.

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