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Formulation of liquid spawn base of paddy straw mushroom, *Volvariella volvacea* (Bull. Ex Fr.) sing

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Abstract
Mushroom spawn in liquid form could be more handily employed and its dispersion would be greatly superior to solid spawn. Underpinning this, an isotonic liquid spawn base is formulated with gum acacia, trehalose, carboxymethyl cellulose (CMC), glycerol, polyethylene glycol (PEG) and aloe gel, based on their water activity for uniform suspension and preservation of chlamydospores and basidiospores. The spores were suspended in the formulation and viability counts were recorded at 15 days interval. The outcome perspicuously indicated that the chlamydospores suspended in PEG were found to be the best even after 45 days (1.5×10^4 spores ml^{-1}) followed by aloe gel based formulation (1.2×10^4 spores ml^{-1}). In the case of basidiospores, the isotonic formulation with glycerol as preservative unveiled more number of viable spore counts (8.7×10^4 spores ml^{-1}) followed by aloe gel (7.5×10^4 spores ml^{-1}). The efficiency of liquid formulation tested in terms of laccase activity indicated that the substrate seeded with chlamydospore based formulation had exhibited comparatively more laccase activity than that of the basidiospore based formulation.

Keywords: Basidiospore, chlamydospore, mycelium, spore, *Volvariella volvacea*, water activity

Introduction
Species of *Volvariella* are perishable and tend to lose their appearance due to short shelf life, an impediment to the distribution and marketing of fresh mushrooms. Extension of the quality and shelf life is therefore, a scientific, technical and economical challenge. Despite of multifarious approaches for extending the shelf life of mushrooms, an alternative safe, cheaper and benign advent is of utmost importance (Fernandes *et al.*, 2013) [8]. The production of liquid spawn is much more rapid than with a solid base material. Liquid spawn is widely used in the cultivation of many mushrooms, including button mushroom, *Agaricus bisporus* (Friel and McLoughlin, 2000) [9]; *Pleurotus ostreatus* (Laniece, 1966 and Silveira *et al.*, 2008) [13, 23]; *Lentinus edodes* (Kirchhoff and Lelley, 1991 and Pellinen *et al.*, 1987) [12, 19]; Brazilian oyster mushroom, *Pleurotus ostreatus* (Rosado *et al.*, 2002) [21]; Paris mushroom (*A. bisporus* var. *hortensis*), wood blewit (*Tricholoma nudum*), garden morel (*Morchella hortensis*), yellow morel (*Morchella esculenta*) and Chanterelle, *Cantharellus cibarius* (Laniece, 1966) [13].
Monosporous and vegetative hyphae of *Volvariella volvacea* (Bull.Ex Fr.) Sing. is known to produce two kinds of spores namely, brown, thick walled and multinucleate asexual chlamydospores and brown, sexual basidiospores (Chang, 1969) [3]. Chlamydospores represent enlarged, highly refractile, thick-walled vegetative cells with varied forms and condensed cytoplasm that form within hyphae or at hyphal tips (Odds, 1988) [17]. They are not formed by direct transformation of hyphal cells but are borne on specialized spore-bearing branches consisting several swollen cells and at maturity, these spores are detached. Commonly, chlamydospores are spherical with an average diameter of 58.8µ. Chlamydospore is thickened uniformly; the germ tube or tubes can emerge out of the spore at any point by means of rupturing the wall (Chang and Yau, 1971) [4]. Mycelium borne chlamydospores rapidly lose their viability in water when subjected to stress, suggesting that they are unlikely to act as long-term storage structures (Teixido *et al.*, 1998) [25]. Basidiospores tend to be in egg shape with an average length of 7-9µ; the widest part 5-6µ and the narrowest part 3-4µ across. The spore is generally uninucleate, but occasionally multinucleated. Spore wall is relatively thick and brown in color when spores shed. Protrusion of the germ tube is always at the hilum region. Germination of the basidiospore commences with the appearance of a small clear round vesicle, which emerges from a minute pore on the cell wall.

The temperature of 20 ºC and above is imperative for spore discharge by the fruiting body and the process continues for 18 hours at 25 ºC (Chang, 1969) [3].

The spores may be collected and formulated in a liquid suspension for more uniform distribution of inoculum during the process of seeding the substrate (Eyal, 1991) [7]. Highly concentrated biomass in a liquid formulation is a worthwhile prerequisite in order to curtail the cost of transit (Abadias *et al.,* 2003) [1]. In the course of developing spore based liquid spawn, the viability of the spores needs to be given sheer priority. Lowering water activity (a_w) was an efficient way to control bacterial growth in liquid formulations (Mugnier and Jung, 1985) [16]. The fermentation process was scaled up using a low-cost culture medium for the mass production of yeast, *Rhodotorula minuta* (10^9 CFU ml^{-1}) by adding glycerol (20%) and xanthan (5g l^{-1}) to avert contamination and sedimentation (Patino-Vera *et al.,* 2005) [18].

The liquid spawn of oyster mushroom (*P. ostreatus*) was immobilized by using a mixture of cottonseed hull, corn core and wheat bran with a ratio of 4.5:4.5:1 by weight to prolong the storage time and also to provide accessible transportation of liquid spawn (Wang *et al.,* 2011) [27]. Compared to solid spawn, there are ample advantages of adapting the liquid spawn which entail improved automation, more uniform distribution of inocula in substrate, quick and year-round production of large mycelial chunks, more homogeneous fungal growth, lower cost, early fruiting and elevated yield (Eyal, 1991; Kirchhoff and Lelley, 1991; Kawai *et al.,* 1995 and Rosado *et al.,* 2002) [7, 12, 10, 21]. Besides, it is strenuous to store and transport liquid spawn. Sometimes the residual nutrients may cause contamination. In addition, liquid spawn of several mushrooms does not colonize on all substrates (Friel and McLoughlin, 2000 and Leatham and Griffin, 1984) [9, 14]. The liquid spawn without nourishment, could not survive in pasteurized compost and that the biomass levels were significantly lower than that of conventional grain spawn with the entrapment of both mycelium and nutrients (Friel and McLoughlin, 2000) [9]. Consistent, more homogenous spawn production of *V. volvacea* is a laborious and more sensitive process and the requirement of mushrooms is proliferating constantly. Alternate spawn base to replace the widely used straw spawn assents. Hence, the present inquest has been fascinatingly conducted to identify the ideal liquid formulation for maintaining the spore viability.

Materials and Methods

Volvariella volvacea strain CBE TNAU 1505, which is found to show superior characteristics in terms of growth and chlamydospore production in our previous work (Kumar *et al.,* 2016) [11] was used in this study. This was maintained on Potato Dextrose Agar (PDA) medium by incubating at 32+2ºC. Chlamydospore production is proportional to vigor, in order to maintain vigor; fresh isolations were made from the fruiting bodies every time after 2 to 3 subcultures. For this purpose, the strains were propagated in straw spawn and grown on paddy straw following the method suggested by Thomas *et al.* (1943) [26]. Freshly harvested sporophores were swabbed with 70 percent ethanol. At the junction of the pileus and stipe, tissue bits were removed aseptically, surface sterilized with 70% ethanol for 30 seconds and repeatedly washed in sterile water and placed on PDA medium taken in sterile Petri dishes. The dishes were incubated at 30 to 35 ºC for seven days. Following single hyphal tip method (Rangasamy, 1972) [20] pure culture was made and stored in PDA slants to carry out further studies. Micrometric observations were recorded with the help of image analyzer (N-400T, Optika, Italy).

Collection and Purification of Chlamydospores and Basidiospores

A Nine mm mycelial disc of seven days old actively growing culture was inoculated into potato dextrose broth. Flasks were incubated at 30-35ºC for 3 days without shaking and were kept in an orbital shaker at 150 rpm with incubation at 30-35ºC for 6 days in the stationary condition. After 15 days formation of red to pinkish colored dots (chlamydospores) on the lower surface of the mycelial mat was observed. The mycelial mat was then collected and macerated in a pestle and mortar for 5 minutes to ensure the detachment of spores from the hyphae as suggested by Chang (1969) [3]. The final macerated product was filtered through glass wool in the funnel where most of the hyphae were barred by the glass wool while chlamydospores passed through it easily. The filtrate was kept in sonicator at 25 kHz for 10 min for complete separation of chlamydospores, following the method suggested by Citiulo *et al.* (2009) [6] from hyphae. The filtrate was once again filtered through glass wool kept in the funnel to harvest the chlamydospores (completely separated from hyphae). Fruiting bodies were produced by following the method suggested by Sangeetha, 2002 [22]. Basidiospores were collected by placing the freshly harvested pileus at elongation stage on a white sheet and keeping it undisturbed for 7 hours in aseptic condition. The basidiospores were further collected by scrapping the spore print on the white sheet using the sterile blade. The collected basidiospores were suspended in 10 ml sterile water and serially diluted to get 1:10 dilution, which is further used for formulation. The chlamydospores and basidiospores obtained were counted by using hemocytometer.

Standardization of Buffer for the Storage of Chlamydospores and Basidiospores

Viable chlamydospores were stored at 30-35 ºC in an isotonic buffer prepared based on the water activity of the spores as suggested by Patino-Vera *et al.,* 2005 [18]. The buffer preservative solution contained NaCl, 8.0 g; KH_2PO_4, 2.0 g; KCl, 2.0 g and Na_2HPO_4, 2.9 g l^{-1} (pH 6.9). The water activity of chlamydospores and basidiospores was measured by water activity meter at 30 ºC (a_w = 0.89 and 0.60, respectively) (Aqua LAB Model series TE8056). The water activity of phosphate buffer was adjusted to a_w = 0.89 and 0.60 by adding several protective agents like gum arabica, trehalose, carboxymethyl cellulose (CMC), glycerol, polyethylene glycol (PEG) and *Aloe vera* extract. Each preservation medium was added with L-ascorbic acid (0.02 g w/v) as an antioxidant. The formulations to preserve chlamydospores were named as VVCF1 to VVCF6 and those used to preserve basidiospores were named as VVBF1 to VVBF6. Each formulation contained 100 ml of isotonic buffer with 0.02 g of L- Ascorbic acid. In addition, protective agents *viz.*, 1.78 g of gum Arabica (VVCF1), 1.33 g of trehalose (VVCF2), 1.22 g of CMC (VVCF3), 1.44 ml of glycerol (VVCF4), 1.78 g of PEG (VVCF5), 1.78 g of *Aloe vera* extract (VVCF6), 1.09 g of gum Arabica (VVBF1), 0.81 g of trehalose (VVBF2), 1.75 g of CMC (VVBF3), 0.88 ml of glycerol (VVBF4), 1.09 g of PEG (VVBF5), 1.09 g of *Aloe vera* extract (VVBF6) were added. The spores suspended in the buffer solution with L-ascorbic acid (VVCF7 and VVBF7) and in sterile water (VVCF8 and VVBF8) served as controls. All liquid formulation media were inoculated with 10 ml of chlamydospore and basidiospore suspensions. The initial

concentration of viable cells was determined by counting the spores (ml^{-1}) by using hemocytometer. The samples were stored at 30ºC in order to maintain the water activity and shelf life. The observations were recorded at 15 days interval. The spore viability was confirmed by spraying 1ml of spore suspension on 1-3 cm pounded paddy straw bits.

Assay of Laccase (EC 1.10.3.2)

As laccase is essential for colonization of substrate its activity is determined. The conventional, paddy straw based spawn (Sangeetha, 2002) [22] and spore suspension inoculated fresh straw bits of 1 g were grounded in one ml of 0.1 M sodium phosphate buffer (pH 6.8). The homogenate was centrifuged at 15,000 rpm for 15 min at 4ºC and the supernatant was used as the enzyme source. Laccase was assayed by adding 0.3 mL enzyme source to 2.5 mL of 30 µM Guaiacol in phosphate buffer (0.1 M) at pH 6.0. Absorbance was read at 470 nm after incubating the reaction mixture for 30 min at 25ºC against zero time control. One unit of laccase activity was calculated as the change in absorbance by 0.001 min^{-1} mL^{-1} of enzyme source at 25ºC.

Statistical Analysis

In order to avoid errors, the experiment has been carried out in 4 replicas and the results presented are mean values. Statistical software AGRES was used for the data analysis. In the case of zero values, the data was square-foot transformed (X+0.5) before statistical analysis.

Results

Viability of basidiospores in isotonic formulation

Chlamydospores collected were tested for their total count and viability by using different substances in the isotonic buffer designed based on the water activity (0.89 a_w at 30ºC) and the viability counts were taken at 15 days interval for 45 days by using haemocytometer, with initial count of 7.5×10^4 spores ml^{-1} at 1:10 dilution. The results are presented in table 1. Among the substances tested the spores diffused in PEG performed significantly better in maintaining spore viability at 15, 30 and 45 DAI (5.7×10^4, 2×10^4 and 1.5×10^4 spores ml^{-1}). This was followed by aloe gel (5.1×10^4, 1.7×10^4 and 1.2×10^4 spores ml^{-1}), gum acacia on 15 DAI (4.7×10^4 spores ml^{-1}) and glycerol at 30 and 45 DAI (1×10^4, 8×10^3 spores ml^{-1}), respectively. Among the substances evaluated, gum acacia suspension had registered the minimum number of spores at 45 DAI (3.9×10^3) ml^{-1}. Very less number of viable spores were observed in water at 45 DAI (1.7×10^2 spores ml^{-1}) when used as a control.

Table 1: Sustainability of chlamydospores in isotonic solution

Formulation	15 days*	30 days*	45 days*
VVCF1	$4.7 \times 10^{4\,c}$ (4.67)	$7.6 \times 10^{3\,e}$ (3.88)	$3.9 \times 10^{3\,e}$ (3.59)
VVCF2	$3.3 \times 10^{4\,e}$ (4.52)	$8.7 \times 10^{3\,d}$ (3.94)	$5.5 \times 10^{3\,d}$ (3.74)
VVCF3	$2.9 \times 10^{4\,f}$ (4.46)	$6.2 \times 10^{3\,f}$ (3.79)	$2.7 \times 10^{3\,f}$ (3.43)
VVCF4	$3.6 \times 10^{4\,d}$ (4.56)	$1 \times 10^{4\,c}$ (4.00)	$8 \times 10^{3\,c}$ (3.90)
VVCF5	$5.7 \times 10^{4\,a}$ (4.76)	$2 \times 10^{4\,a}$ (4.30)	$1.5 \times 10^{4\,a}$ (4.18)
VVCF6	$5.1 \times 10^{4\,b}$ (4.71)	$1.7 \times 10^{4\,b}$ (4.23)	$1.2 \times 10^{4\,b}$ (4.08)
VVCF7	$2.5 \times 10^{4\,g}$ (4.40)	$6 \times 10^{3\,f}$ (3.78)	$2.3 \times 10^{3\,g}$ (3.36)
VVCF8	$2 \times 10^{3\,h}$ (3.30)	$5.9 \times 10^{2\,g}$ (2.78)	$1.7 \times 10^{2\,h}$ (2.23)
CD (P = 0.05)	0.030	0.034	0.031

VVCF1 - gum acacia, VVCF2 - trehalose, VVCF3 - carboxy methyl cellulose, VVCF4 - glycerol, VVCF5 - poly ethylene glycol, VVCF6 - aloe gel, VVCF7 - buffer and VVCF8 - water. Viability counts were taken at 15 days interval for 45 days by using haemocytometer, with initial count of 7.5×10^4 spore's ml^{-1} at 1:10 dilution. *The data presented are mean of 4 replications and the data in parenthesis are square root transformed values. Means in a column followed by the same letter are not significantly different at P = 0.05 by one way ANOVA.

Viability of basidiospores in isotonic formulation

Basidiospores were collected and diluted to 1:10 dilution with the isotonic buffer designed based on the water activity (0.60 a_w at 30ºC). The spores were diffused in various substances and the viable spore counts were taken by using hemocytometer up to 45 days at 15 days interval, with the initial count of 8.2×10^6 spores ml^{-1}. The results obtained are presented in table 2. The basidiospores diffused in glycerol were significantly different in terms of spore viability at 15, 30 and 45 DAI (1.4×10^6, 1.02×10^6 and 8.7×10^4 spores ml^{-1}). This was followed by aloe gel (1.35×10^6, 9.25×10^5 and 7.5×10^4 spores ml^{-1}) and gum acacia (1.1×10^6, 7.4×10^5 and 5.3×10^4 spores ml^{-1}), respectively. Comparatively less number of viable spores was recorded in PEG at 15, 30 and 45 DAI (9.7×10^5, 7.3×10^5 and 4.6×10^4 spores ml^{-1}). Less number of spores was observed in water i.e., 8.6×10^5, 4×10^5 and 3.5×10^4 spores ml^{-1} at 15, 30 and 45 DAI, respectively.

Table 2: Sustainability of basidiospores in isotonic formulation

Formulation	15 days*	30 days*	40 days*
VVBF1	$1.1 \times 10^{6\,c}$ (6.04)	$7.4 \times 10^{5\,c}$ (5.86)	$5.3 \times 10^{4\,c}$ (4.72)
VVBF2	$1.02 \times 10^{6\,d}$ (6.01)	$6.65 \times 10^{5\,d}$ (5.82)	$5.1 \times 10^{4\,c}$ (4.70)
VVBF3	$1.25 \times 10^{6\,b}$ (6.10)	$8.8 \times 10^{5\,b}$ (5.94)	$7.3 \times 10^{4\,b}$ (4.86)
VVBF4	$1.4 \times 10^{6\,a}$ (6.15)	$1.02 \times 10^{6\,a}$ (6.00)	$8.7 \times 10^{4\,a}$ (4.93)
VVBF5	$9.7 \times 10^{5\,de}$ (5.99)	$7.3 \times 10^{5\,c}$ (5.86)	$4.6 \times 10^{4\,e}$ (4.66)
VVBF6	$1.35 \times 10^{6\,a}$ (6.13)	$9.25 \times 10^{5\,b}$ (5.96)	$7.5 \times 10^{4\,b}$ (4.87)
VVBF7	$9.5 \times 10^{5\,e}$ (5.98)	$5.8 \times 10^{5\,e}$ (5.76)	$5.08 \times 10^{4\,c}$ (4.54)
VVBF8	$8.6 \times 10^{5\,f}$ (5.93)	$4 \times 10^{5\,f}$ (5.60)	$3.5 \times 10^{4\,f}$ (4.54)
CD (P = 0.05)	0.030	0.037	0.037

VVBF1 - gum acacia, VVBF2 - trehalose, VVBF3 - carboxy methyl cellulose, VVBF4 - glycerol, VVBF5 - poly ethylene glycol, VVBF6 - aloe gel, VVBF7 - buffer and VVBF8 - water. Viable spore counts were taken by using haemocytometer up to 45 days at 15 days interval, with the initial count of 8.2×10^6 spore's ml^{-1} at 1:10 dilution. *The data presented are mean of 4 replications and the data in parenthesis are square root transformed values. Means in a column followed by the same letter are not significantly different at P = 0.05 by one way ANOVA.

Laccase activity in colonized substrate

The chlamydospores and basidiospores formulated in the isotonic buffers containing glycerol and PEG were sprayed on steam sterilized paddy straw bits keeping conventional straw spawn as a control. The activity of laccase was determined and expressed as change in absorbance min^{-1} ml^{-1} of enzyme source in the colonized paddy straw bits after 6 days of inoculation. Laccase activity was found to be more in conventional spawn colonized substrate prepared from tissue culture (0.4). This was followed by chlamydospores colonized substrate (0.19). Basidiospores colonized substrate recorded the minimum laccase activity of 0.12 as shown in figure 1.

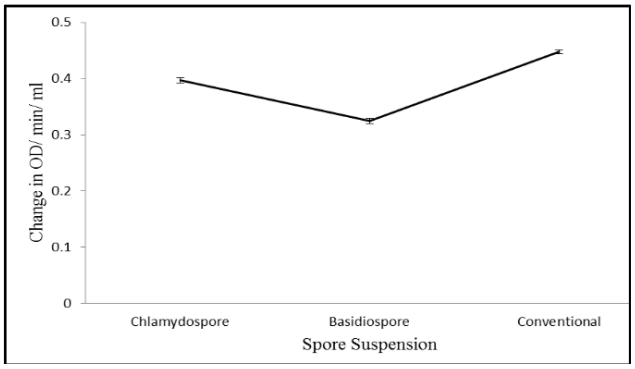

Fig 1: Laccase Activity in Spore Suspension Colonized Substrate

Discussion

The addition of chemical amendments to the formulation is capable of enhancing cell tolerance to desiccation, osmotic pressure and temperature stress (Streeter, 1985). In the present study, chlamydospores and basidiospores were collected and formulated in an isotonic buffer designed with disparate preservatives for perpetuating the viability of spores. It was observed that chlamydospores suspended in PEG and basidiospores suspended in glycerol significantly performed better over a period of 45 days when stored at 30 to 35°C. The outcome obtained by chlamydospores based isotonic suspension pertained to PEG, a polymer commercially used in the manufacturing of medicines, in commensuration to immobilization by entrapment in polymer gels, allows for a greater level of microenvironmental control (McLoughlin, 1994). The results were cognate to other immobilization techniques. Patino-Vera *et al.* (2005) [18] developed a liquid formulation of yeast, *Rhodotorula minuta* by adding glycerol (20%) which helped to uphold the viability of cells @ 10^7 cfu ml^{-1} up to 6 months at 4°C. During the current investigation, oval shaped basidiospores and three types of chlamydospores were observed *viz.*, spores with full, partial and no protoplasm (Figure 2 and 3). In consonance with Chang (1969) [3], the spores with full protoplasm are considered as viable spores. The breaking down of chlamydospores might be due to osmotic imbalance and change in the environment around spore walls. Accordingly, it is difficult to store and transport liquid spawn and sometimes, residual nutrients may cause contamination (Friel and Mcloughlin, 2000) [9]. During the course of the investigation, bacterial contamination of chlamydospore and basidiospore based isotonic suspension was observed. This is surmounted by the addition of 0.3 ml of 500 ppm streptomycin sulfate to the suspensions. For avoiding damage during storage and transit, the spores may be immobilized by entrapment in alginate beads or polymer gel matrix to protect the cells from fluctuations in the macro environments.

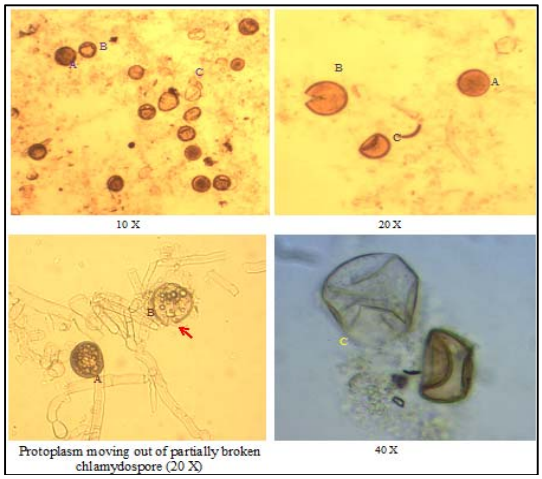

Fig 2: Chlamydospores suspended in isotonic buffer
A - Viable chlamydospore, B - Partially broken chlamydospore, C - Fully broken chlamydospore (empty spore).

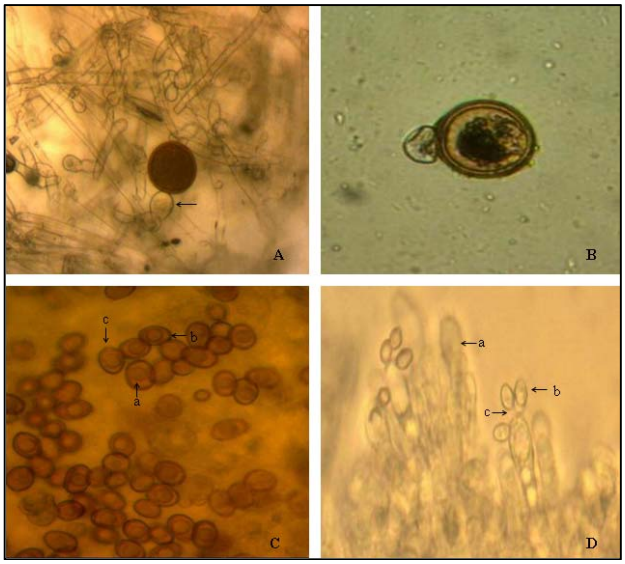

Fig 3: Chlamydospores and Basidiospores of *Volvariella volvacea*
A - Thick double walled chlamydospore on swollen hyphal cells (20 X)
B - Double walled chlamydospore with half-filled protoplasm (20 X)
C - Oval shaped Basidiospores (plasmamembrane (a), outer wall (b), hilum (c)
D - Basidium (a) with basidospores (b) on sterigmata (c)

In mushroom fungi, laccases play a dynamic role in lignin degradation and sporophore development (Ardon *et al.*, 1998 and Wood, 1980). According to Chen *et al.* (2003), laccase is imperative in the mushroom developmental cycle involving fruit body morphogenesis and also indicated that low levels of laccase are detected all during the vegetative growth phase but enzyme activity increases sharply during sporophore development. Laccase activity was determined in paddy straw

substrates colonized by conventional straw spawn, and isotonic suspensions of chlamydospore and basidiospore on the sixth day after inoculation. It is divulged that, conventional straw spawn colonized substrate had shown more laccase activity, followed by chlamydospore based liquid formulation. The upshot also highlighted the discrepancy in adoption period of both the inocula on sterilized and partially fermented paddy straw. Friel and McLoughlin (2000) [9] tested mycelia based liquid spawn of *A. bisporus* for laccase activity and disclosed its superiority. In conformity with Chang (1969) [3], basidiospores desire 40ºC for 48 hours for effective germination. The basidiospores suspended in liquid formulation might not have experienced 40ºC at bed surface for effective colonization. Henceforth, it is propounded that the basidiospore inoculated substrate should be incubated at 40ºC at least for 48 hours to promote spore germination and subsequent colonization. Future research in this context is warranted by augmenting the concentration of spores in suspension for effective colonization of substrate and also by including more benign protective agents for maintaining the spore viability.

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