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In vitro effect of organic and inorganic additives from the production of radial mycelial growth and lignocellulolytic enzyme in *Lentinus edodes* (Berk.) Sing.

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Abstract: Shiitake mushroom globally a well known cultivated species is yet to find a place in India. The success of commercial cultivation of this mushroom is largely depending on improved cultivation technique on locally available substrate. For isolation of *Lentinus edodes* (Berk.) Sing., inner tissue bits from the basidiocarp, after sterilization with 10% sodium hypochloride recorded maximum tissue growth with least microbial contamination on potato dextrose agar medium. In culture method organic additive calcium carbonate significantly increased the biomass and lignocellulolytic enzyme production of *L. edodes*. For spawn production illfilled paddy + calcium carbonate (20 g/ Kg) significantly enhanced the mycelial growth and basidiocarp formation. In polybag method, ill-filled paddy with calcium carbonate recorded highest yield of 360.70 Kg of substrates in a separate experiment. It has been concluded that in Tamil Nadu shiitake can be cultivated commercially using ill-filled substrate with additives calcium carbonate using poly bag method.

Key words: *Lentinus edodes*, sodium hypochlorite, mycelial growth, lignocellulolytic enzymes, ill-filled paddy, calcium carbonate

تأثير الإضافات العضوية وغير العضوية المستخلصة علي النمو الفطري الشعاعي وأنزيمات السليلوز من بذور لنتس ادودس

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الملخص: يعتبر فطر الشيتاكي المعروف عالميا من مصادر الغذاء والتجارة التي أدخلت حديثا إلى الهند ، ونجاح زراعته على مستوى تجارى يعتمد على تطوير تلك التقنية لتتوافق مع معطيات البيئة المحلية . وتدل النتائج أن اعلي معدل نمو سجل بعد التطهير باستخدام محلول 10% من هيبوكلورين الصوديوم والتي تزامنت مع اقل معدل تلوث ميكروبي عند استخدام بيئة دكستوروز البطاطس والأجار . كما سجلت ارتفاعات ذات مغذى إحصائي لكتلة الحياة وأنزيمات البيجلوسليلوز من البذور عند إضافة كربونات الكالسيوم. أما بالنسبة للتكاثر الفطري فزاد المعدل الإحصائي عند إضافة 20 جرام/ كجم من خليط البادي وكربونات الكالسيوم وكذلك أدت هذه الإضافات إلى بيئة النمو إلى زيادة الإنتاجية الكلية إلى 360.7 كجم عند استخدام تقنية الأكياس . ويوصى البحث باستخدام هذه التقنية لرفع الإنتاجية على المستوى التجاري في منطقة التاميل بالهند

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Introduction

The black oak mushroom [*Lentinus edodes* (Berk.) Sing. = *Lentinula edodes* (Berk.) Peg.] commonly known as shiitake, is the second most important edible mushroom in the world. This mushroom has been cultivated in Japan and China for about 2000 years (Royse, 2005). In addition, shiitake has also been cultivated in Thailand, Korea and Brazil. Among the countries Japan is the main world producer of this mushroom, reaching production of 7.5 million tones (Smith et al., 2002). The great interest in shiitake's commercialization is due to its unique flavour / taste, nutritive value and medicinal properties (Sugui et al., 2003; Silva et al., 2007). Its fungal mycelia has high content of proteins, fibres, vitamins, minerals and low content of lipid specifically cholesterol (Yang et al., 2002).

The production of shiitake mushroom has increased at an accelerated rate during last five years (Royse, 2005). Shiitake is globally a well known cultivated species, yet to find a place in Indian markets. Lack of cultivation technology on locally available substrates and suitable high temperature strains are the reason for its non-availability in India. Despite sporadic research efforts to standardize its cultivation technology in India (Sharma et al., 2006), it could not reach the commercial level, as available technology was not viable. A few growers in the state of Manipur and Mizoram initiated the cultivation of shiitake mushroom based on Japanese log system with limited success. However, the scenario was rapidly changing now. The two major research centres *viz.,* National Research Centre for Mushroom (Solan) and Indian Institute of Horticultural Research (Bangalore) are trying to develop cultivation technology based on locally available substrates (Sharma et al., 2006).

Materials and Methods

Isolation Techniques

Shiitake mushroom fungus was isolated from freshly harvested basidiocarp on PDA (potato-dextrose agar) medium, by adopting various techniques *viz.,* Swabbing of basidiocarp with ethyl alcohol + surface sterilization inner tissue bits without surface sterilization, swabbing with ethyl alcohol + surface sterilization of inner tissue bits with Sodium hypochloride 5%, sodium hypochlordte 10%, sodium hypochloride 20%, carbendazim 0.05%, carbendazim 0.1%, carbendazim 0.2% and mercuric chloride 0.1% (Control). After surface sterilization, tissue bits were washed with three changes of sterile distilled water and placed on PDA medium. They were incubated in laboratory conditions (25 ± 3ºC). For each treatment ten replicated plates were maintained @ 3 tissue bits / plate.

Suitable media for the growth

Based on the results of above *in vitro* experiment, the basidiocarp was swabbed with 80% ethyl alcohol using absorbent cotton. Then, the inner tissue bits collected using a sterile scalpel were surface sterilized with 10% sodium hypochloride for 5 min. After surface sterilization the tissue bits were placed separately (@3 bits / plate) on Petri dishes containing Czapek dox agar, malt extract agar, malt extract peptone agar, malt yeast extract agar, potato malt agar and potato dextrose agar. For each treatment ten replicated plates were maintained in the laboratory (25 ± 3ºC).

Radial mycelial growth

Poisoned food technique (Nene and Thapliyal, 1979) was followed to study the effect of various organic additives (sawdust, rice bran, corn flour, sawdust + rice bran and sawdust + corn flour) and inorganic additives ($CaCO_3$, gypsum, lime and $CaCO_3$ + gypsum) at 1.0, 2.0 and 3.0% concentrations on radial mycelial growth. The additives were added separately in 100 ml PDA medium in a 250 ml conical flask, to obtain 1.0, 2.0 and 3.0% concentrations. They were sterilized at 15 lbs pressure for 30 min. Twenty ml of medium was poured into each petri plate. After solidification, a

9-mm mycelial disc of *L. edodes* was placed on the centre of the medium separately and incubated at laboratory temperature (25 ± 3°C). Ten replications were maintained for each treatment. Non-amended medium inoculated in a similar manner served as control. Observation on radial mycelial growth of *L. edodes* was recorded after 7 days of incubation.

Lignocellulolytic enzymes production

Czapek's Dox broth was used as a basal medium for lignocellulolytic enzymes assay. Various organic and inorganic additives were added separately to give 2% concentration. In this activity was evaluated by following the method described by Wasite (1961).

Extraction of Enzymes

Broth containing mycelial mat from the individual treatments of each culture was filtered through Buchner funnel, using Whatman No. 1 filter paper, separately. The filtrates were centrifuged at 2000 rpm for 10 min. at 6ºC. The supernatants collected were used for enzymes assay (Bateman, 1964).

Exo-β-1, 4 glucanase

Exo-β-1, 4 glucanase was assayed in terms of filter paper activity was assayed following the method described by Miller (1972).

Endo- β-1, 4 glucanase

The activity of Endo- β-1, 4 glucanase (µM of glucose released/ml) was determined by the method of (Miller, 1972) in the method involving dinitrosalicylic acid was followed for estimating endo- β-1, 4 glucanase activities by measuring the production of reducing sugar, namely, glucose.

β-glucosidase

The β-glucosidase activity was expressed as µM of p-nitrophenol released/mg. In these activities was evaluated by following the method described by Miller (1972).

Xylanase

Mean Xylanase activity (µM of xylose released/mg) of the reaction mixture was measured according to the method described by Miller (1972).

Laccase

Laccase activity was determined by the oxidation of guaiacol. The enzyme activity was expressed as 0.001 OD (optical density) change/min./ml, assay was determined according to the method described by Frochner and Eriksson (1974).

Polyphenol Oxidase

The mean activity of Polyphenol Oxidase (0.001 OD change/min./ml), phosphate buffer and catechol solution was estimated by a spectrophotometric assay, based on procedures described by Sadasivam and Manickam (1992).

Spawn production

Various substrates *viz.,* sorghum grains, ill-filled paddy and sawdust were used for spawn production. Ill-filled paddy spawn was prepared as per the method followed by (Lakshmanan, 2004). Ill-filled paddy spawn preparation procedure was followed for the preparation of sawdust spawn. However, sorghum grain spawn was prepared as per the method of Kalaiselvan (2007) for each treatment ten replicated bottles were maintained. Observation on fungal growth and basidiocarp formation was recorded daily.

Growth and yield

Shiitake mushroom was cultivated using cylindrical polybag method. The substrates *viz.,* silver oak, sawdust, ill-filled paddy, paddy straw and sugarcane trash were mixed separately with additives corn flour and calcium carbonate (20 g/ Kg of substrate), the bed preparation method was described by Royse (2005).

Statistical analysis

All experiments were done in three replicates except first experiment (Ten replicates), and mean values are presented.

Statistical analysis was performed on the data by Duncan's Multiple Range Test (DMRT) with means followed by a common letter are not significantly different at the 5% level by DMRT.

Results and Discussion

The success of the commercial mushroom cultivation is largely depending on the quality of culture used. The commercial mushroom seed (spawn) growers of Tamil Nadu usually adopt the crude method of isolation for culture preparation. They simply split the basidiocarp after surface sterilization with 80% ethyl alcohol and transfer the inner tissue bits directly on the PDA medium in slants without any surface sterilization. This method is very simple, but the chance of contamination is very high (Lakshmanan, 2004). The present study also clearly indicated that by adopting crude method of isolation about 95.05% of tissue was germinated with maximum contamination of 97.09%. Among the scientific method of isolation, maximum percentage of tissue germination (99.22%) with least tissue contamination (0.79%) was recorded in inner tissue bits with surface sterilization with 10% sodium hypochlorite (Figure 1). However, the existing scientific method *viz.,* surface sterilization with 0.1% mercuric chloride drastically inhibited the tissue germination, though the contamination was very less. The beneficial effect of sodium hypochlorite (10%) as surface steriliant on mushroom tissues has been reported by several workers (Lakshmanan, 2004). The drastic inhibition of tissue growth of *L. edodes* due to mercuric chloride 0.1% might be due to high residual toxicity of the chemical which killed the soft tissues (Lakshmanan, 2004).

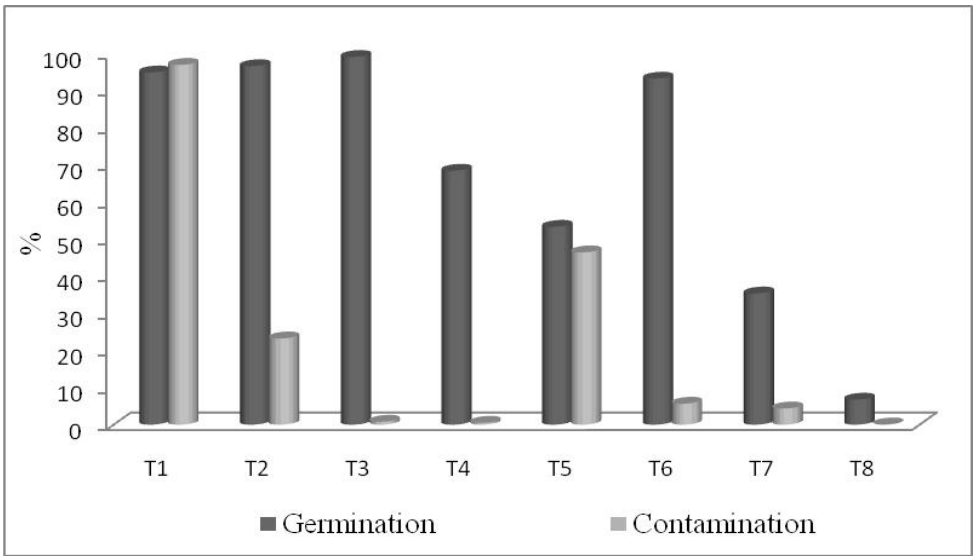
Among the various media tested, potato-dextrose agar encouraged the radial mycelial growth of the fungus and covered the plate within 8.6 days (Table 1). There was no influence of media on cultural character of the fungus. There were several controversial reports on growth of *L. edodes* in different media (Rangaswami, 1956) reported that corn meal and oat meal agar were found to be highly satisfactory for growing of various edible mushroom fungi. Jodon and Royse (1976 recorded maximum growth of *L. edodes* in PDA medium. Sharma et al. (2006) observed a good growth of Malaysian strain of shiitake mushroom in Brown's medium and Czapek dox liquid medium. The different concentrations of organic and inorganic additives, almost all additives at 2% concentration enhanced the fungal growth. Among the additives, calcium carbonate found superior in enhancing the radial mycelial growth (5.7 days) of the fungus (Table 2).

Table 1. Selection of suitable medium.

Media	Days to cover the Petri plates (90 mm dia)	Cultural character (mycelia)
Czapek dox agar	19.60 [f]	Pure white
malt extract agar	18.80 [e]	Pure white
malt extract peptone agar	12.63 [c]	Pure white
malt yeast extract agar	15.80 [d]	Pure white
potato malt agar	11.21 [b]	Pure white
potato dextrose agar	8.60 [a]	Pure white

Mean of ten replicates, Means followed by a common letter are not significantly different at the 5% level by DMRT.

Figure 1. Effect of various isolation techniques on percent tissue germination and contamination.



T1- Swabbing with ethyl alcohol + surface sterilization inner tissue bits without surface sterilization, T2- Swabbing with ethyl alcohol + surface sterilization of inner tissue bits with: Sodium hypochlorite 5%, T3- Sodium hypochlorite 10%, T4- Sodium hypochlorite 20%, T5- Carbendazim 0.05%, T6- Carbendazim 0.1%, T7- Carbendazim 0.2%, T8- Mercuric chloride 0.1% (Control).

Table 2. In vitro effect of organic and inorganic additives on radial mycelial growth.

Additives	Days to cover the Petri plates (90 mm dia)		
	1 %	2 %	3 %
Organic			
Sawdust	9.50 ^{f}	8.60 ^{e}	9.80 ^{e}
Rice bran	10.30 ^{g}	9.80 ^{g}	10.66 ^{g}
Corn flour	9.40 ^{f}	9.20 ^{f}	10.42 ^{f}
Sawdust + rice bran	7.80 ^{c}	7.70 ^{c}	8.40 ^{bc}
Sawdust + corn flour	7.20 ^{b}	6.60 ^{b}	8.30 ^{b}
Inorganic			
$CaCo_3$	6.30 ^{a}	5.70 ^{a}	7.50 ^{a}
Gypsum	8.80 ^{e}	7.80 ^{d}	9.30 ^{d}
Lime	0.0 *	0.0 *	0.0 *
$CaCo_3$+ gypsum	11.80 ^{h}	10.60 ^{h}	12.76 ^{h}
Control	8.50 ^{dec}		

Mean of ten replicates, Means followed by a common letter are not significantly different at the 5% level by DMRT. * No fungal growth.

The increasing expansion of agro-industrial activity over last 40 years has lead to the accumulation of a large quantity of lignocelluloses residues all over the world (Villas-Boas et al., 2002). The utilization of lignocellulosic residues to cultivate mushroom fungus species is environmental importance. The fungi convert the residues into mushroom for human consumption, and the spent substrate can be employed in animal feeding (Zhang et al., 1995). The use of a lignocelluloses solid waste supplement can improve the nutrient content in substrate composition substantially to benefit the commercial production of mushrooms (Omoanghe et al., 2009). An efficient utilization of a lignocellulosic substrate by

fungi is directly related to their capacity to metabolize the wood components (Buswell et al., 1995). The use of lignocellulose as carbon source depends on the capacity of the fungus to produce lignocellulolytic enzymes and to excrete them to the extracellular medium (Mata and Savoie, 1998). *L. edodes* is a white rot fungus that produces a set of lignocellulolytic enzymes, which allow it to grow on lignocellulosic substrates rich in lignin (Leatham, 1986). Several lignocellulolytic enzymes that are released play a major role in the biodegradation process. Production of these enzymes is mostly influenced by various organic and inorganic additives (Munoz et al., 1997). The present study was undertaken to probe the influence of additives on lignocellulolytic enzymes production. The result of the experiment showed that $CaCo_3$ (2%) amended medium recorded high level of lignocellulolytic enzymes production *viz*., exo-β-1,4-glucanase (2.31), endo β-1,4-glucanase (1.59), β-glucosidase (1.79), xylanase (1.94), laccase (1.85) and polyphenol oxidase (0.82) compared to other additives (Table 3). High level of lignocellulolytic enzymes production due to $CaCo_3$ in *Lentinus* and *Pleurotus* has been reported by several workers (Munoz et al., 1997). A positive correlation between the biomass production and lignocellulolytic enzymes production in synthetic medium was studied by Natarajan and Kaviyarasan (1991). They reported that fungal biomass production was increased with increase in lignocellulolytic enzymes. The great potentiality of *L. edodes* by virtue of its fast mycelial growth, greater biomass production activity to produce high level of lignocellulolytic enzymes due to calcium carbonate additive at 2% concentration was evident from the present study.

Table 3. *In vitro* effect of various additives on lignocellulolytic enzymes activity.

Additives (2 %)	Exo β -1,4 Glucanase[1]	Endo β -1,4 glucanase[2]	β – glucosidase[3]	Xylanase[4]	Laccase[5]	Polyphenol oxidase[6]
			Organic			
Sawdust	2.05 [d]	1.12 [d]	1.43 [c]	1.60 [d]	1.10 [e]	0.71 [b]
Rice bran	1.19 [h]	0.65 [h]	0.89 [h]	1.32 [h]	0.53 [h]	0.21 [h]
Corn flour	1.32 [g]	0.80 [g]	1.03 [f]	1.39 [g]	0.84 [f]	0.24 [g]
Sawdust + rice bran	2.11 [c]	1.25 [c]	1.31 [d]	1.69 [c]	1.58 [c]	0.52 [d]
Sawdust + corn flour	2.20 [b]	1.33 [b]	1.62 [b]	1.79 [b]	1.62 [b]	0.63 [c]
			Inorganic			
$CaCo_3$	2.31 [a]	1.59 [a]	1.79 [a]	1.94 [a]	1.85 [a]	0.82 [a]
Gypsum	1.69 [e]	0.96 [e]	1.23 [e]	1.53 [e]	1.31 [d]	0.31 [f]
$CaCo_3$+ gypsum	1.53 [f]	0.86 [f]	0.96 [g]	1.41 [f]	0.66 [g]	0.41 [e]
Control	0.76 [i]	0.13 i	0.85 [i]	0.45 [i]	0.35 [i]	0.18 [i]

Mean of three replicates. Means followed by a common letter are not significantly different at the 5% level by DMRT.

Units:

1 Exo β 1, 4 glucanase- µ mol of glucose released / ml of the culture filtrate; 2Endo β 1, 4 glucanase - µ mol of glucose released / ml of the culture filtrate

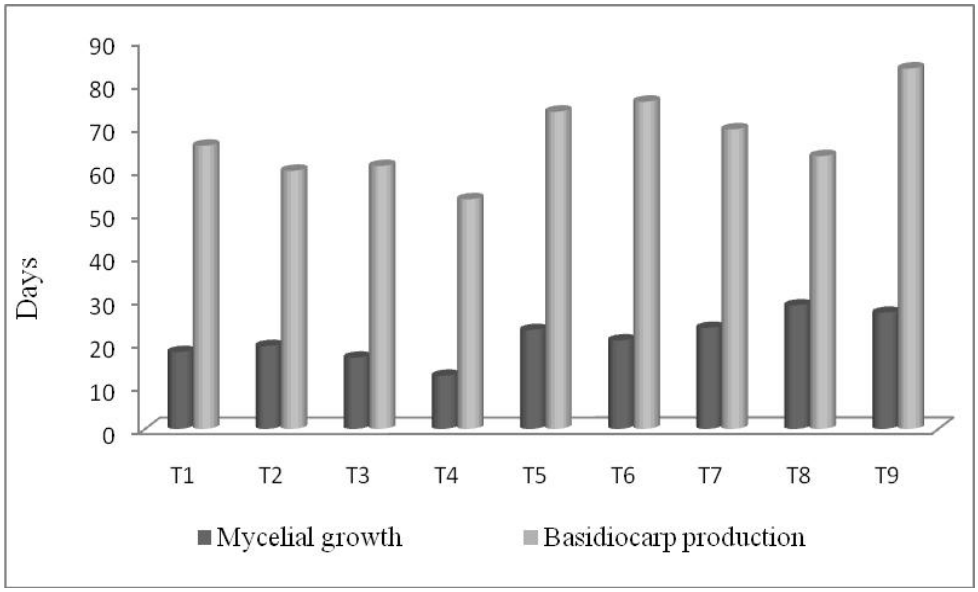
3β glucosidase - µ mol of ρ-nitro phenol released / ml of the culture filtrate; 4Xylanase - µ mol of xylose released / ml of the culture filtrate

5Laccase – 0.001 OD change / min. / ml of the culture filtrate; 6Polyphenol oxidase – 0.001 OD change / min / ml of the culture filtrate

At present, for spawn production, grains like wheat, barley, sorghum and maize are commonly employed for the commercial cultivation of a variety of edible mushroom (Sharma et al., 2006). The use of cereals for the production of spawn is not ideal because the grains often favour the development of saprophytic moulds and bacteria in the beds (Mathew et al., 1996). Since, these substrates have various industrial and domestic uses and the cost is very high. Uses of such a costly substrate for spawn production increase the cost of production. Moreover beds prepared from grain spawn were prone to rat damage (Lakshmanan, 2004). Hence in the present study, agro based industrial wastes *viz.,* ill-filled paddy which is cheaply available in plenty was used as substrate for spawn production. The efficiency of ill-filled paddy with various pre treatments was compared with cereals and sawdust spawns.

The results of the present experiment clearly indicated that ill-filled paddy overnight soaking in carbendazim 0.1% and $CaCo_3$ (20 g/Kg) significantly increased the mycelial growth and covered the container within 12 days. Basidiocarp formation inside the container was also early (53 days) by this treatment (Figure 2). This was followed by ill-filled paddy + sawdust + corn flour (17 and 61 days), sorghum grain + sawdust + corn flour (18 and 66 days), sorghum grain + $CaCo_3$ (19 and 60 days), sawdust + $CaCo_3$ (21 and 76 days) and sawdust + corn flour (23 and 74 days). The suitability of ill-filled paddy + $CaCo_3$ for spawn production has also been reported by (Kalaiselvan, 2007).

Figure 2. Influence of various substrates with additives on spawn production.



Additives (2%) T1- Sorghum grain + sawdust + corn flour, T2- Sorghum grain + CaCo3,T3- Ill- filled paddy + sawdust + corn flour, T4- Ill- filled paddy + CaCo3, T5- Sawdust + corn flour, T6- Sawdust + CaCo3, T7- Sorghum grain alone, T8- Ill -filled paddy alone, T9- Sawdust alone.

Among the substrates, ill-filled paddy substrate with additive calcium carbonate significantly enhanced the browning of mycelial (< 36 days), basidiocarp formation (< 52 days) and yield (360.90 g/Kg). This was followed by silver oak sawdust (< 45 days; < 71 days; 310.8 g/Kg), ill filled paddy with corn flour (< 39 days; < 56 days; 290.5 g/Kg) and silver oak sawdust with corn flour (Table 4) (< 46 days; < 74 days; 10.8 g/Kg).

Table 4. Effect of various substrates with additive on growth and yield.

Substrates/ additives	Browning (days)	Basidiocarp formation (days)	Yield (g/Kg)
Silver oak sawdust+ corn flour	45.60 [g]	73.60 [e]	280.45 [d]
Silver oak sawdust+ $CaCo_3$	41.40 [d]	70.60 [d]	310.84 [b]
Illfilled paddy+ corn flour	39.40 [c]	56.20 [b]	260.50 [c]
Illfilled paddy+ $CaCo_3$	35.70 [a]	51.80 [a]	360.90 [a]
Paddy straw+ corn flour	42.20 [e]	88.20 [h]	60.20 [h]
Paddy straw + $CaCo_3$	43.50 [f]	84.70 [g]	90.10 [g]
Sugarcane thrash+ corn flour	61.20 [j]	99.40 [k]	25.05 [k]
Sugarcane thrash+ $CaCo_3$	60.90 [i]	95.30 [j]	35.20 [j]
Silver oak sawdust	48.80 [h]	78.60 [f]	200.49 [f]
Illfilled paddy	38.70 [b]	59.40 [c]	220.15 [e]
Paddy straw	41.30 [d]	89.60 [i]	40.75 [i]
Sugarcane thrash	62.80 [k]	94.60 [j]	15.10 [l]

Mean of ten replicates. Means followed by a common letter are not significantly different at the 5% level by DMRT. * Additive $CaCo_3$ (20g / Kg).

The fast mycelial growth and basidiocarp formation due to incorporation of additive $CaCo_3$ might be due to the neutral pH of the substrate and prevention of substrate aggregation. Moreover, this additive provides sulphur and calcium which are essential mineral nutrients for the growth of mushroom (Curvetto et al., 2002). Good growth of the fungus without contamination might be due to incorporation of fungicide carbendazim 0.1%. Ill-filled paddy spawn has been reported to prevent rat damage in mushroom production. Non preference of ill-filled paddy to rat might be due poor grain filling and throat irritation (Lakshmanan, 2004).

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