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RESEARCH ARTICLE

EFFICACY OF VARIOUS ORGANIC ADDITIVES ON LIGNOCELLULOLYTIC ENZYMES ACTIVITY, SPAWN AND SPOROPHORE PRODUCTION OF *Hypsizygus ulmarius* (BLUE OYSTER MUSHROOM)

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ABSTRACT

Studies were conducted to assess the effect of different organic additives on the production of lignocellulolytic enzymes and their influence on the spawn and sporophore production. Horsegram flour influenced the maximum production of Exo β-1,4 glucanase, Endo β-1,4 glucanase, β-glucosidase, Laccase, Xylanase and Polyphenol oxidase in *Hypsizygus ulmarius.* Among the different spawn substrate paddy grain recorded the fastest mycelia growth covering the spawn bottle in 12.05 days. Mushroom beds inoculated with Paddy grain spawn recorded maximum number of sporophores (118.00), maximum weight of sporophores (492.88 g bed^{-1}) and the best biological efficiency (98.57 %). Among the different additives *viz.,* Blackgram flour, Cotton seed Powder, Finger millet flour, Groundnut cake, Horsegram flour, linseed cake, Redgram flour and sorghum flour. Horsegram flour supplemented paddy grain recorded minimum spawn run days (10.1 days) and subsequently inoculation of spawn supplemented with horsegram provided highest biological efficiency (103.01%) of *Hypsizygus ulmarius.*

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INTRODUCTION

Oyster mushrooms, are widely cultivated edible mushrooms. They are valued as a luxury food because they are a source of good quality protein, dietary fiber, vitamins, minerals, and flavor, and they have a variety of medicinal and tonic qualities (Wasser, 2002, Lindequist *et al*., 2005). They are highly adaptable to grow and fruit on a wide variety of forest and agro-industrial lignocellulosic wastes because of their ability to synthesize the relevant hydrolytic and oxidative enzymes that convert the individual components of the substrate (cellulose, hemicellulose, lignin) into low-molecular weight compounds, which can be assimilated for fungi nutrition. 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). Production of these enzymes are mostly influenced by various organic and inorganic additives (Kalaiselvan, 2007), which results in severe degradation and reduction in the cellulose, hemicelluloses and lignin content of the substrate in which the mushroom fungus ramifies. The success of mushroom cultivation and its yield solely depends to a large extent on the purity and quality of spawn and suitable substrates.

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At present, spawn on cereal grains are commonly employed for the commercial cultivation of variety of edible mushrooms. Lack of cultivation technology and lack of easily available, accessible and low cast substrates are the major constraints in spawn production in India. With this background, this research has been carried out to identify the suitable substrate and supplements for the cultivation of *Hypsizygus ulmarius* mushrooms.

MATERIALS AND METHODS

Extraction of enzymes: The broth containing mycelial mat from the individual treatments 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 thus collected were used for the assay of enzymes (Bateman, 1964).

ASSAY OF ENZYMES

Exo-ß -1, 4 glucanase

Exo-ß -1, 4 glucanase was assayed based on its activity on filter paper discs. Whatman No.1 filter paper was cut into four mm dia. discs to ensure uniform surface area of the substrate in each tube. The enzyme source (0.5 ml) in 0.1 M sodium citrate buffer (pH 5.8) was added to 32 mg of the filter paper. The

mixture was incubated at 50ºC for one h and the amount of reducing sugar was determined. The enzyme activity was measured following the procedure of Miller (1972) and expressed as µg of sugar released per ml. of the culture filtrate.

Endo- ß -1, 4 glucanase

Dinitrosalicylic acid method suggested by Miller (1972) was followed for the estimation of endo- ß -1, 4 glucanase activity. Production of reducing sugars in µg per ml of the culture filtrate was determined. Carboxy Methyl Cellulose (CMC) solution (1 %) at 0.45 ml was mixed with 0.05 ml of enzyme source. The mixture was incubated at 5ºC for 15 min., immediately followed by placing the enzyme substrate mixture in warm water bath (55ºC) for five min. While the tubes were still immersed in warm water bath, one ml of 40 per cent potassium sodium tartarate solution was added and cooled to room temp (28± 30ºC). The volume was made up to five ml with distilled water (pH 7.0). The absorbance of the sample was measured at 540 nm in a spectrophotometer. D-glucose at 20 to 100 µg / 0.5 ml of dist. water (pH 7.0) was prepared and used to plot the standard graph. Comparing the standard graph, actual amount of glucose released in the enzyme substrate mixture was calculated and the enzyme activity was expressed as µ mol of glucose released / ml of the culture filtrate.

ß –glucosidase

The ß –glucosidase activity was estimated by following the method described by Miller (1972). In this case, the assay mixture containing 0.1 ml of enzyme, 0.5 ml of ρ-nitro phenyl-ß-D glucopyranosidase and 0.4 ml of 0.1 M acetic acid buffer (pH 5.0), was incubated at 45ºC for 20 min. The reaction was stopped by adding two ml of sodium carbonate. The absorbance was measured at 420 nm in spectrophotometer. The enzyme activity was expressed as µmol of ρ-nitro phenol released / ml of the culture filtrate.

Xylanase

Xylanase activity was assayed following the procedure suggested by Miller (1972). The reaction mixture contained 0.5 ml of xylan, 0.25 ml of acetate buffer and 0.25 ml of enzyme. The mixture was incubated at 50ºC for 20 min. The amount of reducing sugar was determined by calorimetric method and the absorbance was measured at 325 nm. The enzyme activity was expressed as µmol of xylose released / ml of the culture filtrate. Laccase: Assay of laccase was carried out as per the method suggested by Frochner and Eriksson (1974). Assay mixture consisting of five ml of 10 M guaiacol in 0.1 M sodium phosphate buffer (pH 6.0) was pipetted out into test tubes and equilibrated at 25ºC. Then, 0.1 ml of the enzyme source was added to the mixture and incubated for five min. and the absorbance was determined at 412 nm. Boiled enzyme served as control. Activity of laccase was expressed in terms of enzyme units (one unit is equivalent to the change in absorbance of 0.01 per min.).

Polyphenol oxidase

Polyphenol oxidase activity was assayed by the method described by Sadasivam and Manickam (1992). Assay mixture consisting of 2.5 ml of 0.1 M phosphate buffer (pH 6.0) and 0.3 ml of catechol solution (0.01 M) was taken in a cuvette and placed in a spectrophotometer set at 495 nm and the absorbance was adjusted to zero. The cuvette was removed and 0.2 ml of enzyme source was added. After shaking, the cuvette was placed immediately in to spectrophotometer. The changes in absorbance for every 30 sec., up to three min. were recorded. The enzyme activity was expressed as 0.001 OD change / min. / ml of culture filtrate.

Spawn production

Ten different commonly available spawn substrates *viz.,* blackgram, cumbu, ill-filled paddy grain, maize grain, paddy grain, panivaragu grain, sorghum grain, thenai, and wheat grains were tested for their efficacy in supporting the mycelial growth of *Hypsizygus ulmarius*. The spawn substrates were presoaked overnight. The solution was drained completely and dried under shade. At 50 per cent moisture level the substrate was filled in wide mouth glass bottles or heat resistant polypropylene bags to their 3/4th capacity. Then, the mouth was tightly plugged with non absorbent cotton and sterilized at 15 psi pressure for two hours. The substrates were allowed to cool after sterilization and inoculated with a nine mm mycelial disc obtained from the actively growing region of eleven days old culture aseptically and incubated at 25±2^{0}C. for complete coverage of mycelium. After the incubation period, the observations like spawn run (days), yield and biological efficiency were assessed and recorded (Sivaprakasam, 1980).

Various spawn supplements

The paddy grain spawn was prepared as detailed earlier. Different supplements *viz.,* blackgram flour , cotton seed powder, fingermilllet flour, groundnut cake, horse gram flour, linseed cake, red gram flour and sorghum flour were added separately at two per cent level. The supplements were thoroughly mixed with the grains and sterilized. Paddy grain spawn without any supplement served as control. The spawn bottles were incubate and observations were recorded.

Bed preparation

Cultivation of *H. ulmarius* was carried out in transparent polythene bags of 60 x 30 cm size with a thickness of 100 gauge and cyclindrical beds were prepared using 0.5 kg of paddy straw on dry weight basis described by Sivaprakasm (1980). The biological efficiency of *H. ulmarius* was calculated by the following formula

$$BE = \left[\frac{\text{Fresh weight of the mushroom / bed}}{\text{Dry weight of the substrate / bed}} \times 100 \right]$$

Statistical analysis

Statistical evaluation was done using analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The statistical significance was expressed at $p < 0.05$.

RESULT AND DISCUSSION

The results showed that (Table 1) horse gram flour amended liquid medium was found to be most supportive to the production of exo-β-1, 4-glucanase (2.58 µ mol), endo-β-1,4-

Table 1. *In vitro* evaluation of various organic additives on lignocellulolytic enzymes activity of *Hypsizygus ulmarius*

Tr. No.	Additives (2 %)	Exo β -1,4 glucanase	Endo β -1,4 glucanase	β-glucosidase	Xylanase	Laccase	Polyphenol oxidase
1	Corn flour	2.26^{c}	1.69^{e}	1.43^{c}	1.65^{c}	1.38^{c}	0.66^{c}
2	Black gram flour	1.32^{h}	0.86^{g}	0.86^{g}	1.18^{f}	0.53^{h}	0.28^{f}
3	Horse gram flour	2.58^{a}	2.10^{a}	1.84^{a}	1.95^{a}	1.74^{a}	0.79^{a}
4	Red gram flour	2.31^{b}	1.82^{b}	1.69^{b}	1.78^{b}	1.61^{b}	0.72^{b}
5	Rice flour	1.47^{f}	1.10^{e}	0.90^{f}	1.31^{e}	0.87^{g}	0.40^{e}
6	Sorghum flour	2.10d	1.57^{d}	1.28^{d}	1.61^{c}	1.41^{d}	0.61^{c}
7	Tapioca flour	1.86e	1.28^{f}	1.02^{e}	1.49^{d}	1.26^{e}	0.52^{d}
8	Wheat flour	1.64^{g}	0.98^{g}	0.96^{f}	1.36^{e}	1.10^{f}	0.43^{e}
9	Control	0.81^{i}	0.17^{h}	0.75^{h}	0.59^{g}	0.38^{i}	0.18^{g}

Mean of three replicates: Means followed by a common letter are not significantly different at the 5% level by DMRT: Exo β 1,4 glucanase- µ mol of glucose released / ml of the culture filtrate : Endo β 1,4 glucanase - µ mol of glucose released / ml of the culture filtrate : β glucosidase - µ mol of ρ-nitro phenol released / ml of the culture filtrate : Xylanase - µ mol of xylose released / ml of the culture filtrate : Laccase – 0.001 OD change / min / ml of the culture filtrate : Polyphenol oxidase – 0.001 OD change / min / ml of the culture filtrate

Table 2. Efficacy of different spawn substrates on sporophore production of *Hypsizygus ulmarius*

Tr.no.	Spawn substrate(grain)	Spawn run(days)	No of sporophore /bed	Sporophore yield (g)	Biological efficiency (%)
1	Black gram	16.33^{f}	79.33^{f}	321.38^{f}	64.27^{f}
2	Cumbu	15.06^{e}	84.16^{f}	390.51^{e}	78.10^{e}
3	Ill-filled paddy	12.98^{b}	116.42^{b}	478.72^{b}	95.74^{b}
4	Maize	14.74^{d}	98.65^{e}	402.02^{e}	80.40^{e}
5	Paddy	12.05^{a}	118.00^{a}	492.88^{a}	98.57^{a}
6	Panivaragu	17.46^{g}	71.85^{g}	246.14^{g}	49.28^{g}
7	Sorghum	13.62^{c}	109.58^{c}	460.63^{c}	92.00^{c}
8	Thenai	18.35^{g}	68.55^{g}	200.79^{h}	40.16^{h}
9	Wheat	14.10^{d}	101.26^{d}	451. 90^{d}	90.38^{d}

Values not sharing a common superscript differ significantly at P < 0.05 (DMRT)

Table 3. Effect of various supplements of spawn base on the weight of sporophores and biological efficiency of *Hypsizygus ulmarius*

Tr.No.	Supplements (@ 2%)	Spawn run (days)	Weight of sporophore (g/bed)	Biological efficiency (%)
1	Black gram flour	12.9^{e}	330.59^{h}	66.11^{h}
2	Cotton seed powder	11.6^{c}	415.70^{f}	83.14^{f}
3	Finger millet flour	13.4^{f}	295.61^{i}	59.10^{i}
4	Groundnut cake	12.3^{d}	461.00^{d}	92.20^{d}
5	Horse gram flour	10.1^{a}	515.07^{a}	103.01^{a}
6	Linseed cake	12.4^{d}	392.61^{g}	78.52^{g}
7	Red gram flour	10.9^{b}	501.25^{b}	100.25^{b}
8	Sorghum flour	11.5^{c}	485.64^{c}	97.12^{c}
9	Control	12.8^{e}	454.38^{e}	90.88^{e}

Values not sharing a common superscript differ significantly at P < 0.05 (DMRT)

glucanase (2.10 µ mol), β-glucosidase (1.84 µ mol), xylanase (1.95 µ mol), laccase (1.74– 0.001 OD change) and polyphenol oxidase (0.79– 0.001 OD change), followed by red gram flour and corn flour. The utilization of insoluble lignocelulosic substrates by edible mushroom depends upon the production of enzymes (cellulases, hemicellulases and lignases) that bring about hydrolysis of the macro molecules of cellulose, hemicelluloses and lignin components (Buswell *et al*., 1993). 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).

Efficacy of different spawn substrates and additives on sporophore production of *Hypsizygus ulmarius*

The experimental results (Table 2) revealed that paddy grain is the most suitable substrate for early spawn run which took only 12.05 days to complete mycelia growth. Further, it was observed that the formation and the density of mycelia in spawn were found to be less in minor millets when compared to cereals and pulses. Inoculation of paddy spawn in bed substrates recorded maximum number of sporophores (118.00), maximum weight of sporophores (492.88 g bed $^{-1}$) and the best biological efficiency (98.57 %). followed by ill-filled paddy grains. From the table 3, it is discernable that paddy grain supplemented with 2% horsegram flour recorded the least no of spawn run days (10.01) followed by redgram flour. Among the various additives used horse gram flour was found to influence the maximum sporophore production (515.07g) and biological efficiency (103.01%). Spawn, the vegetative seed material plays an important role in mushroom cultivation. The substrate, on which the spawn is prepared, affects the mushroom cultivation. (Mathew *et. al.,* 1996). Hence, several authors have tried a variety of grains and agricultural waste because of availability, accessibility and low cost as substrates for the preparation of spawn of different edible mushrooms (Sharma and puttoo, 2004; Prabakara, 2006). The poor utilization panivaragu and thenai seed as spawn substrate in the present study could be due to less moisture absorbance, lack of aeration and spaces for the mycelia spread and colonization, the favorable result in paddy grain could be due to increased surface area. It is comparatively cheaper and gives more number of spawn bottle per unit weigh as observed by Eswaran

et al., (1998). The stimulation activity of horse gram flour supplements might be due to the increase in the activity of beneficial saprophytic bacteria which can help in bio-degradation of organic substances and thereby increasing in nutrient availability in the substrates (Krishnamoorthy and Narasimhan 1994; Sangeetha, 2006). The study has brought out a useful finding that the paddy grains can be used successfully as a substrate for spawn preparation with supplementation of horsegram powder at two per cent level for enhanced production of *Hypsizygus ulmarius.*

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