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RESEARCH ARTICLE

Effect of different additives for the production of lignocellulolytic enzymes (liquid medium) in *Volvariella volvacea*

■ **A. Sudha and M. Rajesh**

SUMMARY

Among the additives liquid culture amended with calcium carbonate was found to be superior for the production of exo β-1,4 glucanase (17.51 µ mol of glucose/ml), endo β-1,4 glucanase (18.39 µ mol of glucose/ml), β-glucosidase (23.21µ mol of p-nitrophenol/ml), xylanase (0.26 µ mol of xylose/ml), laccase (0.17 OD change/min/ml) and polyphenol oxidase (0.24 OD change/min/ml). This was followed by horse gram powder (16.83; 17.54; 20.95; 0.21; 0.14 and 0.21), calcium carbonate + gypsum (16.28; 17.07; 20.66; 0.20; 0.13 and 0.20) as against 13.33; 15.48; 19.05; 0.17; 0.10 and 0.17 in non-amended medium (control).

Key Words : *Volvariella volvacea*, Organic, Inorganic additives, Enzyme production, Liquid medium

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The genus *Volvariella* (paddy straw mushroom) comprised a group of several species, which can be found growing on a variety of substrates in tropical and sub-tropical regions. *V.volvacea* (Bull. ex Fr.) Sing., is probably the best known species, as it has been traditionally cultivated in Southeast Asia since the 18th century (Chang, 1977). At present time, *V. volvacea* is the third most important cultivated mushroom reaching total world production of 287 metric tones (Chang and Miles, 1993). In India, Su and Seth (1940) have first cultivated straw mushroom but the scientific cultivation using spawn was successfully demonstrated by Thomas *et al.* (1943). It is commonly known as Chinese mushroom, the most favourite mushroom in South Asian countries because of its excellent delicacy, high protein, aminoacid, vitamins and minerals contents (Thakur and Vijay, 2006). The climatic conditions prevailing in the Indian plains seems to be quite suitable for large scale

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production of paddy straw mushroom.

It has been cultivated successfully on cotton waste, a relatively crystalline cellulose (Chang, 1974). This implies that the organism may have the potential to produce high exoglucanase activity to degrade crystalline cellulose. If "lignase" activity could be clearly demonstrated, both crystallinity and lignification problems could be overcome simultaneously. A variety of substrates including paddy straw, banana leaf waste, water hyacinth and composted cotton waste have been successfully used for growing *V. volvacea*, (Chang, 1974; Saeed *et al*., 1994 and Krishnamoorthy *et al*., 2005). Practically in India very little improvement in the cultivation technique has been made during the last two decades. Major draw back in the cultivation of *Volvariella* is the very low biological efficiency (BE) compared to other tropical mushrooms. If cultivation technique improves, it can be cultivated widely and cheaply as other common vegetables, which can be consumed regularly by all people. This study was undertaken to provide essential information on quantitative production of cellulase and lignase by *V. volvacea*. For simplicity and economy, it is practical, especially in the developing countnes, to grow microbial cells on lignocellulose waste for human or animal food and produce enzymes and glucose in a unified and continuous process.

Producing mushrooms on lignocellulose waste is a good approach to achieve this goal. By this method, the fruiting body can be harvested as a food commodity and the extracellular enzymes produced on the cellulose material can be used later for the production of sugar through saccharification in the reactor. Since cellulase is strongly absorbed to cellulose at high concentration of substrate (Mandelb, 1975 and Ng *et al*., 1977), the unhydrolyzed residual cellulosic mushroom bed can be put into the reactor without removing the enzyme from the cellulose. Newsprint, because of its high cellulose content, consistent composition, and ready availability was chosen as one source of lignocellulose in this study.

MATERIAL AND METHODS

Czapek's dox broth (Wasite, 1961) was used as a basal medium for lignocellulolytic enzymes assay. The composition of broth for estimation of cellulases were: sodium nitrate 2.0 g; potassium dihydrogen phosphate 1.0 g; magnesium sulphate 0.5 g; sucrose 30 g; carboxy methyl cellulose (CMC) 20 g and distilled water 1000 ml (pH 6.8-7.0). For the estimation of lignases same medium was used, were CMC was substituted with sawdust (10 g). Various organic and inorganic additives, *viz.,* farm yard manure, paddy straw powder, horse gram, calcium carbonate, gypaum and calcium carbonate + gypsum were added, separately to give 2 per cent concentration. Amended media (25 ml) were dispensed in 100 ml Erlenmeyer flasks and sterilized at 15 lbs pressure for 1 h after cooling, flasks were inoculated with a disc (9 mm dia.) taken from the peripheral growth of 7-day-old PDA cultures of the test fungi, separately. In each culture, the concentration of organic and inorganic additives each was replicated thrice. The broth without additives inoculated in a similar manner served as control. They were incubated at room temperature of 25±3ºC for 15 days.

Extraction of enzymes:

The 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). After extracting the supernatants from the individual treatment flasks, the mycelial growth in weighed whatman No.1 filter paper was used for assessing biomass production. The filter paper with mycelial growth was oven dried at 80ºC for 6 h and the dry mycelial weight was determined.

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 solution (one %) 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 toroom temperature (28 to 30ºC). The volume was made upto five ml with distilled water (pH 7.0). The absorbance of the sample was measured at 540 nm in a spectrophotometer (GS5703AT). D-glucose at 20 to 100 µg / 0.5 ml of distilled 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 r-nitro phenyl- ß-D glucopyranosidase and 0.4 ml of 0.1 M acetic acid buffer (pH 5.0), after that the mixture 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 (GS5703AT). The enzyme activity was expressed as µ mol 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 mol of xylose released per 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. 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 in a cuvette and placed in a spectrophotometer set at 495 nm and the absorbance were 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 (GS5703AT). The changes in absorbance for every 30 sec upto three min were recorded. The enzyme activity was expressed as 0.001 OD change / min / ml of culture filtrate.

RESULTS AND DISCUSSION

The effect of various organic and inorganic additives (2 %) on lignocellulolytic enzymes production was studied and results were presented in Table 1.

Liquid culture amended with calcium carbonate was found to be superior for the production of exo β-1,4 glucanase (17.51 µ mol of glucose/ml), endo β-1,4 glucanase (18.39 µ mol of glucose/ml), β-glucosidase (23.21 µ mol of p nitrophenol/ml), xylanase (0.26 µ mol of xylose/ml), laccase (0.17 OD change/min/ml) and polyphenol oxidase (0.24 OD change/min/ml). This was followed by horse gram powder (16.83; 17.54; 20.95; 0.21; 0.14 and 0.21), calcium carbonate + gypsum (16.28; 17.07; 20.66; 0.20; 0.13 and 0.20) as against 13.33; 15.48; 19.05; 0.17; 0.10 0.17 and in non-amended medium (control).

Traditionally *V. volvacea* has been grown on rice straw. That tradition earned it the name of paddy straw mushroom (Chang, 1983). The straw mushroom cultivation in tropical/subtropical areas is still less advanced. The climatic condition prevailing in the Indian plains seems to be quit suitable for large scale production. Practically in India a very little improvement in the cultivation technique has been done during the last two decades. Major draw back in the cultivation of *Volvariella* is the very low biological efficiency (B.E) as compared to other tropical mushrooms. If culture technique improved, it should be cultivated widely and

Table 1: *In vitro* effect of various additives on lignocellulolytic enzymes activity of *V.volvacea*

Sr. No.	Additives (Organic/ inorganic 2 %)	Exo β-1,4 glucanase	Endo β-1,4 glucanase	β glucosidase	Xylanase	Laccase	Polyphenol oxidase
1.	FYM	16.13 ^{d}	17.19 ^{c}	20.18 ^{e}	19.05 ^{g}	0.13 ^{c}	0.20 ^{bc}
2.	Horse gram powder	16.83 ^{b}	17.54 ^{b}	20.95 ^{b}	0.21 ^{b}	0.14 ^{b}	0.21 ^{b}
3.	Paddy straw powder	15.91 ^{e}	17.54 ^{b}	20.39 ^{d}	0.19 ^{bc}	0.13 ^{c}	0.19 ^{cd}
4.	Calcium carbonate	17.51 ^{a}	18.39 ^{a}	23.21 ^{a}	0.26 ^{a}	0.17 ^{a}	0.24 ^{a}
5.	Gypsum	15.20 ^{f}	16.96 ^{e}	19.89 ^{f}	0.19 ^{bc}	0.12 ^{c}	0.19 ^{bcd}
6.	Calcium carbonate + Gypsum	16.28 ^{c}	17.07 ^{d}	20.66 ^{c}	0.20 ^{bc}	0.13 ^{c}	0.20 ^{bc}
7.	Control	13.33 ^{g}	15.48 ^{f}	19.05 ^{g}	0.17 ^{c}	0.10 ^{d}	0.17 ^{d}

Units: 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 / r ml of the culture filtrate
β glucosidase - μ mol of ρ nitrophenol 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
Mean of three replicates
Means followed by a common letter are not significantly different at the 5 per cent level by DMRT

cheaply as other common vegetables which can be consumed regularly by all people.

Efficient utilization of lignocellulolytic substrates by mushroom fungi largely depends on the activity of extracellular enzymes. 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 (Ardon *et al.*, 1996; Munoz *et al.*, 1997; Bommaraju, 2002 and Arunprasad, 2004). As the literature sources on enzymology of *Volvariella* are minimum, the present study was undertaken to probe the influence of various organic and inorganic additives for lignocellulolytic enzyme production. The results of the experiment showed that liquid culture amended with calcium carbonate at 2.0 per cent was found superior (Fig. 1) for production of exo β-1,4 glucanase (17.51 μ mol), endo β-1,4 glucanase (18.39 μ mol), β-glucosidase (23.21 μ mol), xylanase (0.26 μ mol), laccase (0.17 OD change/min/ml) and polyphenol oxidase (0.24 OD change/min/ml), compared to horse gram powder (16.83; 17.54; 20.95 μ mol; 0.21 0.14 and 0.21). High level of enzymes production due to calcium carbonate (0.5 %) in *Lentinus* and *Pleurotus* spp. was reported by several workers (Ardon *et al.*, 1996; Munoz *et al.*, 1997; Bommaraju, 2002 and Arunprasad, 2004). A positive correlation between the biomass production and lignocellulolytic enzyme production in synthetic media was studied by Natarajan and Kaviyarasan (1991). They observed that the fungal biomass production was increased with increase in lignocellulolytic enzymes. The great potentiality of *V. volvacea* 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 per cent concentration was evident from the present study.

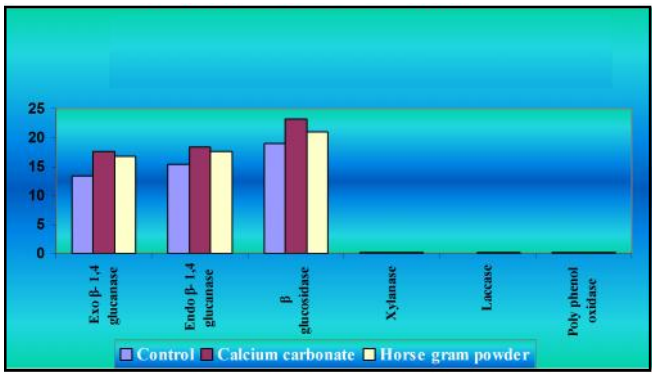

Fig. 1: In vitro effect of various additives on lignocellulolytic enzymes activity of *V. volvacea*

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