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Biomass and Extracellular Lignocellulolytic Enzyme Production by *Calocybe indica* Strains

[1]*Manraj Mangat,* [1]*Pardeep K Khanna,* [1]*Shammi Kapoor and* [2]*Baldev S Sohal*

[1]Department of Microbiology,
[2]Department of Biochemistry, College of Basic Sciences and Humanities,
Punjab Agricultural University, Ludhiana, Punjab, India-141004

Abstract: Three strains of *Calocybe indica,* a tropical basidiomycete fungus, were screened for biomass growth and extra cellular lignocellulolytic enzyme production potentials under *in vitro* stationary conditions at regular intervals for three weeks with the objective of selecting a strain with maximum lignocellulolytic enzyme activity and rapid growth rate. The selected strain could be commercially exploited for mushroom production. Maximum biomass production was obtained with *C. indica* strain Ci-3 after 21 days of incubation in liquid culture medium irrespective of the inducer used in the liquid medium. Strains Ci-1 and Ci-2 produced lower biomass. The strain Ci-3 also showed maximum activity of the three cellulolytic enzymes (namely endoglucanase, β-glucosidase and cellobiohydrolase) quantified. Production of endoglucanase (96.6 U/ml) was maximum followed by cellobiohydrolase (52.2 U/ml) and β-glucosidase (49.2 U/ml). Very low laccase activity was observed in the culture filtrate of all the strains. The enzyme production by the different strains was independent of the biomass production. To corroborate the enzyme production study in liquid medium, solid state fermentation of wheat straw pretreated by different methods *viz.* boiling (45 min), steam under pressure (15 psi for 45 min), steam without pressure (45 min) and untreated wheat straw, was carried out. Strain Ci-3 showed fastest mycelial extension rate (2.54 cm/day) with the concomitant minimum number of days (13 days) for complete impregnation of the straw indicating the potential of this strain to be exploited for commercial mushroom production compared to the other strains evaluated.

Key words: β-glucosidase · Biomass · *Calocybe indica* · Cellobiohydrolase · Endoglucanase · Laccase

INTRODUCTION

Vast quantities of organic residues, particularly lignocellulosic materials are generated annually through the activities of agricultural processes. The Indian peninsula, with diverse agro-climatic zones, alone generates about 5000 million tonnes of agri-residues annually and currently a large proportion of these residues are allowed to degrade *in situ* leading to environmental pollution and loss of valuable resources. Most of the lignocellulosic waste has a pentosan ratio lower than the minimum (0.5) required for the decomposition of organic matter in soil and hence cannot be composted *in situ.* There is thus a need for profitable utilization of this renewable resource in an eco-friendly manner. Mushroom production, a controlled non-axenic solid state fermentation of the lignocellulosic material is currently one of the economically viable techniques for producing protein rich edible mushrooms from the lignocellulosic waste [1]. Mushrooms besides being a food delicacy due to their characteristic texture and flavor are valued for their nutritional [2], high protein conversion efficiency per unit of land and per unit of time [1], medicinal value and various metabolites having antitumor, immunomodulator and hypocholesterolemic effects [3].

The lignocellulosic waste material is composed of three major structural polymers: cellulose (a homopolymer of glucose), hemicellulose (a heteropolymer that includes xylans and mannans), and lignin (a complex polyphenolic polymer). The complexity of the lignocellulosic material makes it recalcitrant to degradation. Fungi are the most potential lignocellulose degraders, since they secrete an array of enzymes including cellulases, xylanases and laccases [4]. The different abilities of an individual mushroom species to colonize and fruit on a particular lignocellulosic waste are determined by both the fungus and substrate associated factors [5]. The repertoire of enzymes (qualitative) that a mushroom possesses and the comparative intensities of the activities

Corresponding Author: Manraj Mangat, Department of Microbiology, College of Basic Sciences and Humanities, Punjab Agricultural University, Ludhiana, Punjab, India-141004

(quantitative) of these enzymes decide its preference for a substrate [6].

Calocybe indica, known as milky mushroom, is a tropical basidiomycete of Indian origin [7]. Owing to its high biological efficiency and good shelf life; it is fast becoming a commercial variety popular not only with the growers but also consumers. However, no work on the lignocellulolytic enzymes of this fungus has been attempted so far. The present study was aimed at evaluating the lignocellulolytic enzyme potential of three strains of *C. indica* in liquid culture. The screening for a hyper producing strain would be important for the selection of a strain showing better colonization of substrate, shorter duration of spawn run and consequently higher yield. Moreover, in addition to commercial mushroom production such strains can also be exploited for biological treatment of lignocellulosics to produce fermentable sugars that could be utilized for bioethanol production.

MATERIALS AND METHODS

Organism: Three strains of *Calocybe indica* (Ci-1, Ci-2 and Ci-3) obtained from the national repository of mushroom cultures, National Research Centre for Mushrooms, Chambaghat, Solan (H.P., India), were used in the study. All cultures were maintained on Malt Extract Agar (MEA) slants at 30±1°C. Inocula of respective strains were prepared on MEA medium (pH 5.5) in 90 mm sterile disposable petri-dishes. Mycelial discs (one cm diameter) obtained from 15-day-old culture plate were used as the inoculum.

Growth and enzyme production: Production of extracellular enzymes was studied in liquid culture medium containing 1.5 g $NaNO_3$, 0.5 g KH_2PO_4, 0.1 g KCl, 0.2 g $MgSO_4.7H_2O$ and 0.2 g $FeSO_4.7H_2O$ per litre, dispensed in 250 ml Erlenmeyer flasks @ 50 ml aliquots before sterilization at 15 pounds per square inch pressure for 20 min. CM-cellulose, cellobiose and crystalline cellulose @ 1% (w/v) were used as the sole carbon source for the induction of endoglucanase (β-1,4-D-glucan-4-glucanohydrolase or C_x; E.C.3.2.1.4), β-glucosidase (β-D-glucoside glucohydrolase, E.C.3.2.1.21) and cellobiohydrolase (1,4-β-D- glucan cellobiohydrolase or C_1, E.C.3.2.1.91), respectively. No inducer was added for laccase enzyme (benzene diol oxygen oxidoreductase, E.C.1.10.3.2) study. Each flask was inoculated with the one cm mycelial disc from a 15-day-old culture plate used for the inoculum preparation and incubated at 30±1°C as stationary culture. Mycelium was harvested periodically on pre-weighed Whatman No.1 filter paper circles (125 mm diameter), dried at 50°C for 48 hrs and re-weighed. Enzyme activities were assayed directly in the filtrate obtained.

Enzyme assays: Activities of the cellulolytic enzymes viz. endoglucanase (C_x), β-glucosidase and cellobiohydrolase (C_1) were estimated by the methods of Mandels *et al.* [8], Toyama and Ogawa [9] and Mandels and Sternberg [10], respectively. Laccase was assayed according to Turner [11] with some modifications as described by Singh *et al.* [12]. The reducing sugars liberated as a result of the assay reaction were estimated as glucose using 3,5-dinitrosalicylic acid (DNS) method [13]. All assays used inactivated enzymes as controls. One unit of cellulolytic enzyme activity was expressed as the activity necessary to release 1 μmol of glucose/h/ml of culture filtrate. One unit of laccase activity was expressed as the activity necessary to increase O.D. by 0.001/min/ml of culture filtrate. All experiments were performed in triplicates and analysed statistically using the computer programme CPCS ver. 1.0 for bi-factorial analysis of variance.

Solid state fermentation (SSF) of lignocellulosic straw: Experiments were conducted to assess the ability of the three strains of *C. indica* to utilize lignocellulosic straw (wheat straw) that had been subjected to different pretreatments. The straw was pre-wetted to 70% moisture level overnight and then filled in graduated corning glass tubes (25 × 70 cm) upto 40 cm and plugged with non absorbent cotton. The tubes were divided into four groups with 10 tubes per group. Tubes of the first group were kept in a water bath at 100°C for 45 minutes, second group was steamed in an autoclave without pressure for 45 minutes, the third group was autoclaved at 15 psi for 45 minutes and the fourth group of tubes served as control. The tubes were then inoculated aseptically with wheat grain spawn (@ 10-12 grains/tube) on the surface of the straw in the tubes. The tubes were incubated at 30±1°C and the linear growth was recorded at regular intervals. Days for complete spawn impregnation for the three strains were recorded to assess the ability of the three strains to utilize the pretreated straw under SSF and the mean mycelial extension rate/day calculated.

RESULTS AND DISCUSSION

Cellulase Activity in Different Strains: Three strains of *Calocybe indica* were evaluated for the production of cellulases namely endoglucanase, β-glucosidase and

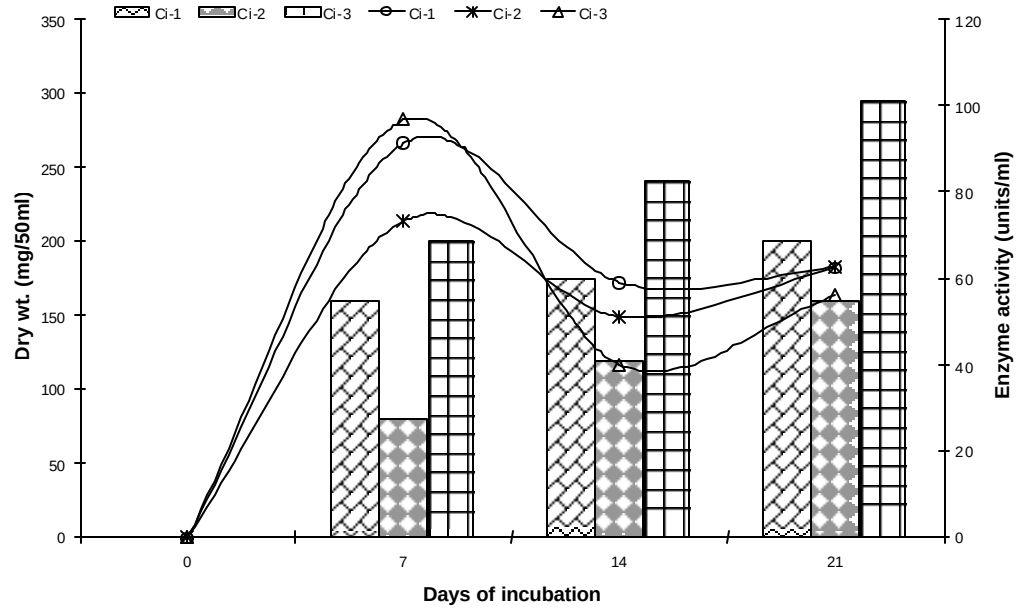

Fig. 1: Biomass production (bars) and endoglucanase activity (line graph) in culture filtrate during the growth of *C. indica* strains with CMC as an inducer

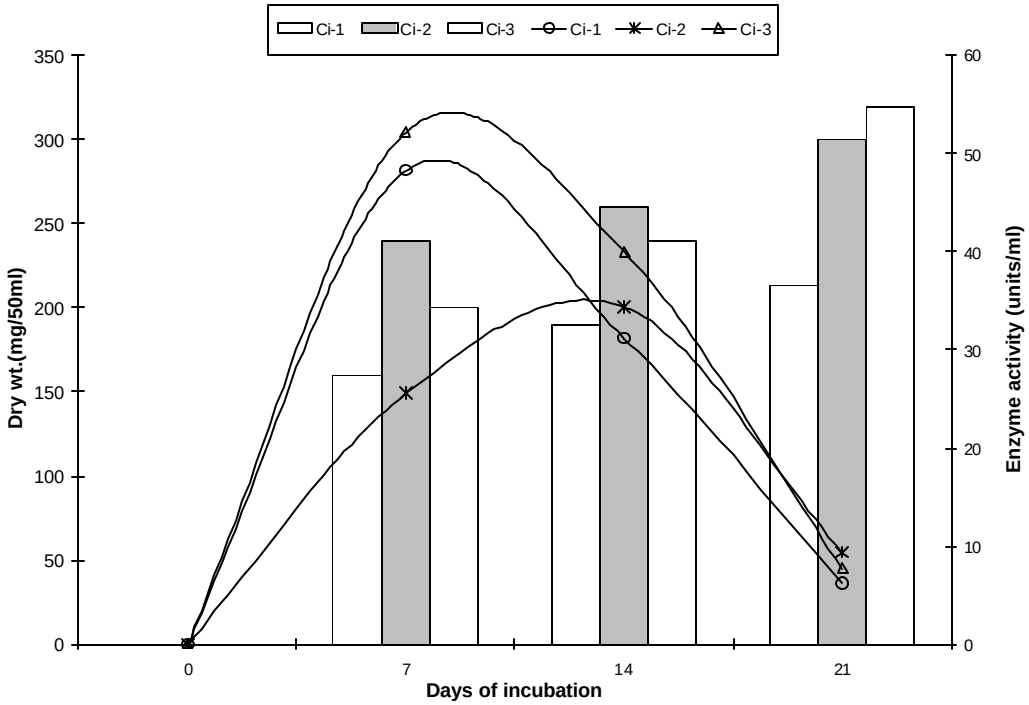

Fig. 2: Biomass production (bar graph) and glucosidase activity (line graph) in culture filtrate during the growth of *C. indica* strains with cellobiose as an inducer

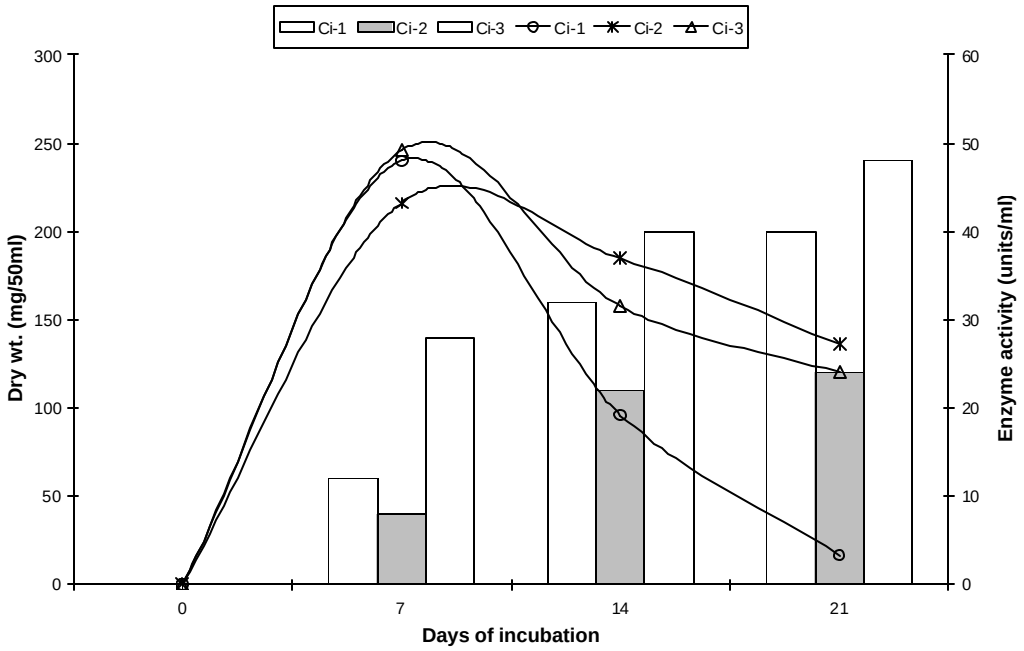

Fig. 3: Biomass production (bar graph) and cellobiohydrolase activity (line graph) in culture filtrate during the growth of *C. indica* strains with crystalline as an inducer

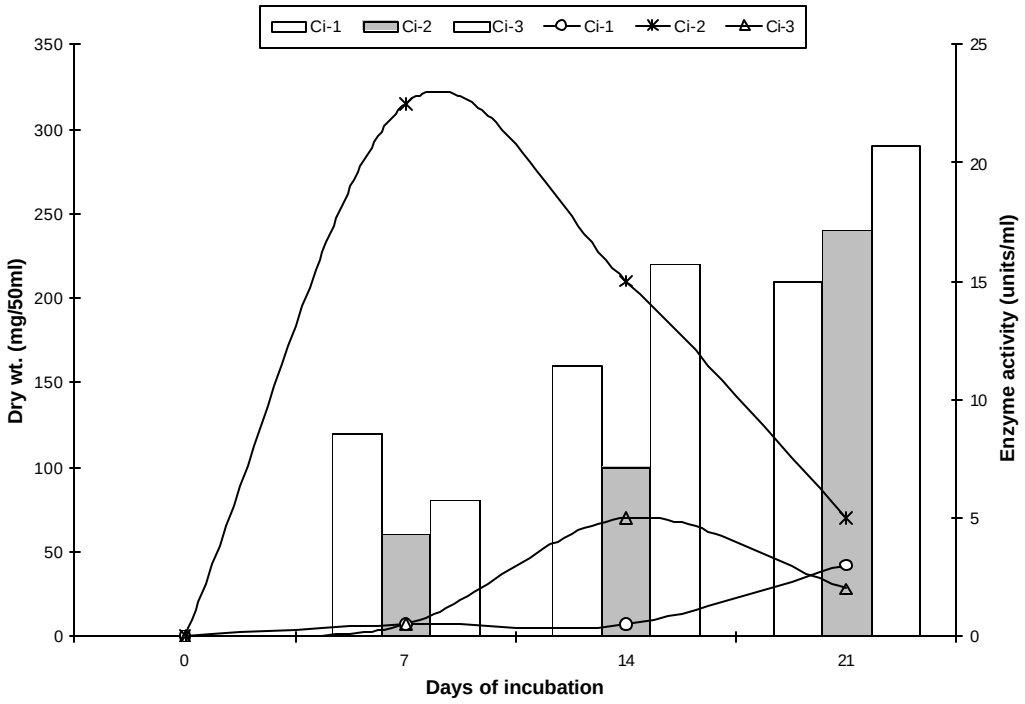

Fig. 4: Biomass prodction (bars) and laccasse activity (line graph) in culture filtrate during the growth of *C. indica* strains

cellobiohydrolase in liquid medium using CM-cellulose, cellobiose and crystalline cellulose, respectively, under static conditions. The three strains showed unique trends in enzyme production and maximum endoglucanase activity (Fig. 1) was obtained with strain Ci-3 (96.6 U/ml filtrate) followed by Ci-1 (91.2 U/ml filtrate) after 7 days of incubation. However, after 21 days, strain Ci-1 showed 62.4 U/ml filtrate of enzyme activity while Ci-3 recorded only 56.0 U/ml filtrate. Strain Ci-2 showed relatively stable endoglucanase activity with a peak value of 73.2 U/ml filtrate on 7th day and 62.4 U/ml filtrate on 21st day. A little decline in the activity of all three strains was observed on 14th day. Biomass production (mg/50ml) with CMC as the inducer continued to increase with incubation period. Maximum value of biomass (295 mg/50 ml) was observed for the strain Ci-3 on 21st day followed by Ci-1 and Ci-2 (200 and 160 mg/50 ml, respectively).

Enzyme β-glucosidase activity (Fig. 2) was also maximal on 7th day of incubation (49.2, 48.0 and 43.2 U/ml filtrate for Ci-3, Ci-1 and Ci-2, respectively) using cellobiose as the inducer in the medium. Strain Ci-1 showed a steep decline in the activity of this cellulase enzyme complex with the final activity being 3.3 U/ml on the 21st day whereas Ci-2 and Ci-3 showed activity of 27.2 U/ml filtrate and 24.0 U/ml filtrate respectively. Biomass obtained with cellobiose showed the same trend as with CM-cellulose. Strain Ci-3 produced a maximum of 240 mg/50 ml followed by Ci-1 with 200 mg/50 ml and Ci-2 with 120 mg/50 ml production.

In cellobiohydrolase production studies (Fig. 3), maximum activity corresponding to 52.2 U/ml filtrate was obtained for Ci-3 on 7th day followed by 48.3 U/ml filtrate for Ci-1 and 25.6 U/ml filtrate for Ci-2. The activity continued to decrease with incubation period except in strain Ci-2 which increased to 34.4 U/ml filtrate on 14th day and decreased thereafter. Biomass obtained with cellobiose was higher than those obtained with CM-cellulose. A maximum biomass of 320 mg/50 ml was obtained for Ci-3 after 21 days of incubation.

Comparison of enzyme pattern revealed production of endoglucanase to be maximum followed by β-glucosidase and cellobiohydrolase. Strain Ci-2 showed most stable system of enzymes while Ci-3 showed maximum cellulolytic activity. High level of endoglucanases may be responsible for extensive fungal colonization of the lignocellulosic growth substrate, an essential prerequisite for high fruit body production [14]. Small quantities of biomass produced were sufficient enough to use the inducers for production of cellulases and release low molecular weight sugars, which were then assimilated for biomass turnover and protein incorporation [15].

Doshi *et al.* [16] reported high cellulase (C_x) activity after 25 days of fungal growth in grain bottles of *Calocybe indica* when compared with the growth at 11 days and had suggested that the cellulase production closely shadows the mycelial biomass production. Anandh and Prakasam [17] in their study on enzymes in *C. indica* during fruiting suggested association of cellulase activity with mushroom flushes.

Laccase Activity in Different Strains: Laccase production studies (Fig. 4) were carried out in liquid medium containing glucose @1% under static conditions, at regular intervals for three weeks. Comparison of laccase activity in the three strains revealed that only Ci-2 strain showed detectable enzyme activity of 22.5 U/ml filtrate after 7 days of incubation, which decreased to 5 U/ml filtrate after 21 days of growth. Biomass production values showed continuous increase in all the three strains with maximum production of 290 mg/50 ml by Ci-3 strains at the end of third week. In strains Ci-3 and Ci-1 very low levels of this enzyme was detectable over the 21 days of study. Studies on *Phanerochaete chrysosporium* showed that detectable ligninase activity was recorded only when the fungus was cultivated on low nitrogen medium [18]. Low laccase activity observed in the cultural filtrate of *C. indica* could be attributed to the nitrogen rich medium used in the present study. Studies suggest that ligninase is produced by several fungi, but is located mainly intracellular, which might explain our failure to detect high levels of activity in the medium [19].

Solid state fermentation of lignocellulosic straw: SSF of wheat straw by the three strains of *C. indica* showed that Ci-3 strain had fastest impregnation rate per day with the concomitant minimum number of days for complete spawn run. Fastest linear mycelial extension was observed in boiled straw by Ci-3 strain (2.54 cm/day) while mycelial impregnation rates for straw pretreated using steam under pressure (2.36 cm/day) were better than steam without pressure (2.20 cm/day). Ci-1 and Ci-2 strains also showed the similar trends but the mycelial extension (2.20 cm/day) was slower than Ci-3 (Table 1). The shorter duration of spawn run for Ci-3 probably points to the better substrate colonization potential of the strain when wheat straw is used as a substrate. All the strains failed to impregnate the straw in the control tubes indicating that heat pretreatment of the wheat straw was an essential prerequisite. The heat pretreatment probably leads to the structural breakdown of the lignocellulosic complex of the straw thereby releasing sugars that are effectively used by the growing *C. indica* strains. Different workers have

Table 1: Effect of pretreatment of wheat straw on average linear mycelial extension rate (cm/day) of *C. indica* strains

Pretreatment	Strain	Days for spawn run	Linear mycelial impregnation/day(cm)
Boiling water	Ci-1	16	2.06
(100°C for 45 mins)	Ci-2	15	2.20
	Ci-3	13	2.54
Steam under pressure	Ci-1	17	1.94
(15 psi for 45 mins)	Ci-2	16	2.06
	Ci-3	14	2.36
Steaming without pressure	Ci-1	18	1.83
(45 mins)	Ci-2	16	2.06
	Ci-3	15	2.20
Control	Ci-1	-	-
(only wetting)	Ci-2	-	-
	Ci-3	-	-

- No mycelial impregnation of the wheat straw

Mean of ten replicates tubes

suggested hot water boiling as the most appropriate method for pretreatment of the substrate for commercial cultivation of this mushroom [20, 21].

The present study on *Calocybe indica* strains indicates the presence of a strong cellulolytic enzyme system in this organism. The quantitative approach of the study helps to compare the enzymatic potentials of the three strains and could provide the basis for further investigations into biochemistry of lignocellulosic bioconversion of agricultural residues by *Calocybe indica*. The strain Ci-3 which possessed higher enzyme activities as compared to the other strains and also faster growth rate could be recommended to the mushroom producers for commercial production of this tropical mushroom.

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