LIGNOLYTIC ENZYMES OF *CALOCYBE INDICA*

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ABSTRACT

Calocybe indica which is gaining popularity in the tropical parts of India and cultivated round the year while in subtropical parts it is preferred during summer seasons. The fruiting temperature requirement is from 28 ^{0}C to 35 ^{0}C. It is cultivated on wheat or paddy straw after chemical sterilization, hot water treatment or on pasteurized straw. The mycelial growth and fruiting takes place at higher temperature than the other cultivated mushroom like white button mushroom, shiitake mushroom or oyster mushroom. The lignocellulolytic enzymes responsible for growth on substrate and secretion of enzymes at higher temperatures of *C. indica* have not been thoroughly studied. So studies were undertaken to estimate influence of temperature on biomass production, changes in pH, total protein production, laccase activity, tyrosinase activity, aryl alcohol oxidase (AAO), manganese peroxidase (MnP), lignin peroxidase (LiP) and versatile peroxidase (VP) on wheat straw medium, saborauds medium and modified Czapek dox medium (10g/l glucose) after 6, 12, 18, 24 days. It was interesting to record that *C. indica* produced Versatile peroxidase on all three culture medium and maximum quantity was recorded on wheat straw media after 12 days at 35 ^{0}C. Maximum protein was recorded on straw medium and least in Czapek dox medium. Wheat straw medium produced maximum laccase after 24 days at 30 ^{0}C (187.38 u/ml) followed by 35 ^{0}C and 25 ^{0}C. Maximum biomass was recorded on saborauds medium at 300 ^{0}C and 350 ^{0}C after 24 days followed by wheat straw and Czapek dox medium. This is the first report of versatile peroxidase in *C. indica* in addition to *Pleurotus eryngii*, *P. pulmonarius, P .ostreatus, Bjerkandera* adusta and *Bjerkandera* sp. In all *Pleurotus* spp and Bjerkandera the production of VP is stimulated under peptone containing media. In the present case the wheat straw produced maximum VP followed by Sabourauds medium (peptone containing medium) and least in Czapeck dox medium.

Keywords: *Calocybe indica*, ligninolytic enzymes, oxidases, peroxidases, laccase, manganese peroxidase, lignin peroxidase, versatile peroxidase

INTRODUCTION

Calocybe indica (milky mushroom) is an indigenous edible mushroom belonging to Lyophyllae of the family Trichlomataceae. This mushroom is identified on the bases of cyanophilic basidiospores and siderophyllous granules in basidia. It is found during rainy season in the indogangetic plains of the West Bengal (India) and in Bangladesh [1]. This mushroom becoming popular in tropical and subtropical parts of India due to its robust basidiomes, fibrous texture, attractive white colour, suitable for cultivation during warmer seasons at higher temperature, consistent yield and better keeping quality are the major reason for its popularity. Nutritionally also it has higher amounts of $B_{2,}$ niacin and rich in minerals like Na, K, P [2]. The fruit bodies contains eighteen fatty acids including essential fatty acids like eicosapentaenoic and docasohexaenoic acids [3]. The substrate requirement for *Calocybe indica* is also like *Pleurotus* spp. and various kinds of agricultural wastes including wheat straw, paddy straw, ragi maize, bajra, cotton stalks and leaves. Sugar cane bagasse, cotton and jute wastes, tea and coffee waste, dehulled maize cobs and coconut coir substrate can be utilized for cultivation of *Calocybe indica* [4, 5]. There is no need to compost the substrate for cultivation of *Calocybe indica* as the mycelium can degrade cellulose, hemicellulose and lignin by secretion of various extracellular enzymes. Doshi *et al.* [6] have reported pectinolytic and cellulolytic enzyme production from the fruit bodies of the *Calocybe indica*. However, no information is available on the ligninolytic oxidases and peroxidases enzyme, change in protein, biomass and role of temperature during incubation for enzyme secretion. Therefore studies were undertaken to estimate various ligninolytic enzymes of *Calocybe indica* in liquid culture medium inoculated at three different temperature conditions.

MATERIALS AND METHODS

Culture: Culture of *C. indica* (DMRO- 38) was obtained from the "Gene Bank" of ICAR - Directorate of Mushroom Research, Solan.

Liquid broth medium for growth and enzyme studies:- Three culture media namely, wheat straw extract (50 g/l), Saborauds medium and Czapek dox medium (modified- 10g/l glucose instead of 30 g/l sucrose.) were prepared and 25 ml medium was transferred in 150 ml conical flasks. Each medium has 48 flasks and 16 flasks were incubated at 25, 30, 35 ^{0}C in BOD under dark. Four samples were taken after 6, 12, 18 and 24 days for enzyme analysis.

Biomass, pH and protein estimation: Mycelial biomass was quantified on pre weighed Whatmann filter paper (No.1) by taking out culture filtrate from mycelium and washing twice with sterilized distilled water. The filter papers were oven dried (50 ^{0}C) to a constant weight. The culture filtrate was used to estimate protein and change in pH and enzyme activity from each medium under different incubation temperature. Protein was estimated by following the method of Bradford [7] using bovine serum albumin (BSA) as protein standard. The enzyme samples were drawn using sterile pipette tips in 2ml sterilized micro centrifuge tubes. The enzymes samples were stored at ±4 ^{0}C for enzyme estimation.

Enzyme estimation:- All the enzymes and protein samples were estimated using Perkin Elmer (Lambda-12) UV-VIS. Double beam spectrophotometer. One International Unit (U) of enzyme activity was defined as the activity that would change 1uMol of substrate or synthesize 1μM of product per minute. Enzyme activity divided by per mg protein gave enzyme activity per mg of protein. Laccase(p-diphenol oxidase, EC1.10.3.2): Laccase was estimated by following the method of Prilliger modified by Haars *et al*. [8]. Tyrosinase: (o-diphenol oxidase, EC1.14.18.1): The tyrosinase activity was measured with L-DOPA (3,4- dihydroxyphenylalanine) as the substrate [9]. Aryl alcohol oxidase: (AAO, EC1.1.3.7): AAO activity oxidizes primary aromatic alcohols like benzyl, cinnamyle, naphthyl and aliphatic unsaturated alcohols to corresponding aldehydes by transfer of two hydrogen from alcohol to molecular oxygen with the release of hydrogen peroxide. 4-methoxy benzyl alcohol (anis alcohol) was used as substrate. The enzyme activity was measured as per the method of Muheim *et al*. [10]. Lignin peroxidise: (LiP, EC1.11.1.14): The method of Tien and Kirk (11) was followed for the lignin peroxidase activity. Veratryl alcohol (3,4- dimethoxy benzyl alcohol) was used as substrate.Versatile peroxidise: (VP, EC 1.11.1.16): The VP activity was measured by decrease in absorbance of reactive Black-5 (Sigma) at acidic pH in sodium tartarat buffer. This enzyme has the capacity to oxidize Mn^{2+} and different aromatic compounds and dyes, including compounds that are not efficiently oxidized by LiP and MnP.

RESULTS AND DISCUSSION

The effect of temperature and pH on mycelial biomass with different culture media is shown in Table-1. There was less reduction in pH in Czapek dox medium and the pH remained almost neutral to slightly alkaline, while in Saborauds medium the medium pH becomes acidic in all the three temperature conditions. In wheat straw medium the pH slowly decreased upto 12 days and then again increased to neutral.

Maximum mycelial biomass was recorded in Saborauds medium at 30 and 35 ^{0}C (17.36 and 17.03 mg/ml) followed by 25 ^{0}C after 24 days. Least mycelial biomass (2.75 mg/ml) was recorded after 6 days in Czapek Dox medium. The effect of temperature on extra cellular protein is shown in Table 2. Wheat straw medium supported maximum protein (16.34 μg/ml) after 24 days at 30 ^{0}C followed by 25 ^{0}C and 35 ^{0}C(14.50 and 14.43 mg/ml).. Saborauds medium gave 8.86 μg/ml protein after 24 Days. Least protein was found in Czapek dox medium.

The laccase activity in different culture medium and at different temperatures is shown in Table 3. There was vast difference in laccase activity in all three culture medium. Maximum laccase activity (183.38U/ml) was observed in wheat straw medium after 24 days at 30 ^{0}C. While maximum activity per unit protein was in Czapek dox at all the three temperatures after 24 days. Although Saborauds medium has maximum biomass and reasonable amount of protein /ml. but it gave poorest laccase/unit ml and per unit protein concentration.

Table 1. Change in pH and mycelial biomass (mg/ml) in different culture media

		pH			Biomass(mg/ml)		
Day Interval	**Temp.**	**Wheat st.**	**Sab.**	**Cza. Dox**	**Wheat st.**	**Sab.**	**Cza. Dox**
6 Days	25ºC	5.65	5.35	6.74	3.4	5.7	2.76
	30ºC	6.47	5.68	6.87	4.5	6.9	2.46
	35ºC	6.54	5.8	6.97	5.8	6.06	2.66
	25ºC	7.1	5.6	7.7	3.76	6.96	3.5
12 Days	30ºC	7.0	4.4	7.8	5.2	13.16	3.6
	35ºC	6.4	4.5	7.5	6.16	14.26	3.43
	25ºC	7.3	5.1	7.4	4.06	14.86	3.3
18 Days	30ºC	7.1	3.5	7.45	4.26	13.5	3.5
	35ºC	6.0	3.3	7.3	6.23	16.46	3.93
	25ºC	7.09	3.8	7.7	4.76	14.53	5.2
24 Days	30ºC	6.9	3.4	7.7	9.1	17.36	4.96
	35ºC	6.2	3.1	7.1	7.76	17.03	5.46

Table 2. Extracellular proteins in different culture media

		Protein(ug/ml)		
Day Interval	**Temperature**	**Wheat st µg/ml**	**Sab. µg/ml**	**Cza. Dox µg/ml**
6 days	25ºC	10.31	0.132	0.331
	30ºC	11.2	0.761	0.143
	35ºC	8.84	0.397	0.165
	25ºC	12.70	2.20	0.48
12 Days	30ºC	13.86	2.69	1.92
	35ºC	12.39	3.36	1.26
	25ºC	13.60	3.14	0.40
18 Days	30ºC	15.19	4.92	0.187
	35ºC	15.36	6.14	0.264
	25ºC	14.50	5.13	0.23
24 Days	30ºC	16.34	6.31	0.34
	35ºC	14.43	8.86	0.35

Table 3. Laccase activity of *C.indica* in different culture media and temperatures

		Laccase								
Day Interval		**Wheat st.**			**Sab.**			**Cza. Dox**		
	Temp.	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**
6 days	25ºC	6.023	0.58	1.77	1.22	9.24	0.21	5.76	17.40	2.1
	30ºC	12.73	1.13	2.82	2.4	3.15	0.34	8.8	61.53	3.6
	35ºC	3.23	0.36	0.55	1.11	2.79	0.18	9.8	59.39	3.76
	25ºC	4.54	0.35	1.20	2.3	1.04	0.33	5.69	11.85	1.62
12 Days	30ºC	5.51	0.39	1.05	3.33	1.23	0.25	10.38	5.40	2.88
	35ºC	6.73	0.54	1.09	2.23	0.66	0.15	13.93	11.05	4.06
	25ºC	25.91	1.90	6.38	19.54	6.22	1.31	11.85	29.62	3.59
18 Days	30ºC	48.33	3.18	11.34	1.41	0.28	0.10	19.86	106.2	5.6
	35ºC	71.3	4.64	11.44	0.65	0.10	0.039	29.13	110.3	7.41
	25ºC	97.49	6.72	20.48	12.74	2.48	0.87	29.12	126.6	5.6
24 Days	30ºC	187.38	11.46	20.59	3.96	0.62	0.22	39.09	114.9	7.88
	35ºC	113.07	7.83	14.57	4.77	0.53	0.28	44.38	126.8	8.12

The Tyrosinase activity in different culture media and temperature is shown in Table 4. Tyrosinase activity was maximum in wheat straw followed by Czapek dox medium, while Sabourauds medium produced least activity. Czapek Dox medium gave more tyrosinase per unit mg of protein after 18 and 24 days at all the three temperature. Incubation temperature of 25 and 30 ^{0} cel gave more Tyrosinase then at 35 ^{0} cel.

The results of aryl alcohol peroxidase (AAO) are shown in Table 5. AAO is the main hydrogen peroxidase generating enzyme for the activity of lignin peroxidase (LiP), Manganese peroxoidase (MnP) and versatile peroxidase (VP). The AAO activity started after 6 days in all the three culture media. Maximum activity was observed in Sabourauds medium and it continuously increased from 6th day to 24th day. It indicates that production of AAO requires more nutrient. Czapek dox medium gave more laccase per unit mg of protein than other media.

Table 4. Tyrosinase activity in *C.indica* in different culture media and temperatures

Tyrosinase activity										
Day Interval	**Wheat st.**				**Sab.**			**Cza. Dox**		
	Temp.	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**
6 days	25ºC	9.69	0.93	2.85	3.8	28.78	0.66	2.36	7.12	0.85
	30ºC	0.0	0.0	0.0	0.0	0.0	0.0	4.26	29.79	1.73
	35ºC	6.47	0.73	1.11	4.9	12.34	0.80	2.83	17.15	1.06
	25ºC	49.88	3.92	13.26	3.91	1.77	0.56	2.45	5.10	0.7
12 Days	30ºC	38.18	2.75	7.34	1.42	0.52	0.10	4.66	2.42	1.29
	35ºC	16.84	1.35	2.73	4.68	1.39	0.32	4.22	3.34	1.23
	25ºC	66.07	4.85	16.27	3.96	1.26	0.26	1.88	4.7	0.56
18 Days	30ºC	63.70	4.19	14.95	5.97	1.21	0.44	2.84	15.18	0.81
	35ºC	23.06	1.50	3.70	1.70	0.27	0.10	4.40	16.66	1.11
	25ºC	15.63	1.07	3.28	2.93	0.57	0.20	5.62	24.43	1.08
24 Days	30ºC	39.81	2.43	4.37	2.59	0.41	0.15	7.10	20.88	1.43
	35ºC	26.63	1.84	3.43	1.89	0.21	0.11	6.43	18.37	1.17

Table 5. Aryl alcohol peroxidase (AAO) activity of *C. indica* in different culture media and temperature

AAO										
Day Interval	**Wheat st.**				**Sab.**			**Cza. Dox**		
	Temp.	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**
6 days	25ºC	9.95	0.96	2.92	2.91	22.04	0.51	0.48	1.45	0.17
	30ºC	7.76	0.69	1.72	1.35	1.77	0.19	0.88	6.15	0.35
	35ºC	9.56	1.08	1.64	0.91	2.29	0.15	1.41	8.54	0.53
	25ºC	0.92	0.072	0.24	3.94	1.79	0.56	1.44	3.0	0.41
12 Days	30ºC	2.35	0.16	0.45	5.79	2.15	0.43	1.69	0.88	0.46
	35ºC	1.76	0.14	0.28	5.69	1.69	0.39	1.29	1.02	0.37
	25ºC	8.2	0.60	2.01	10.54	3.35	0.70	2.66	6.65	0.80
18 Days	30ºC	4.0	0.26	0.62	15.33	3.11	0.13	2.0	10.69	0.57
	35ºC	7.03	0.45	1.08	6.2	1.00	0.37	3.42	12.95	0.87
	25ºC	12.02	0.82	2.52	21.51	4.19	1.48	3.95	17.17	0.75
24 Days	30ºC	2.99	0.18	0.32	23.55	3.73	1.35	3.18	9.35	0.64
	35ºC	6.68	0.46	0.86	19.29	2.17	1.13	4.10	11.71	0.75

The versatile peroxidase activity (U/ ml, U/mg protein and U/mg biomass) in different culture media is shown in Table 6. VP was the main peroxidase activity which started just after 6th day in all the culture media. The VP activity increased from 12th day to 24th day in wheat straw medium and maximum activity was recorded at 35 ^{0} cel on 12th day. Temperature of 35 ^{0}C is more suitable for VP. Activity of VP was much more in Czapek dox medium per unit of protein .

The effect of culture media on manganese peroxidase activity is shown in Table 7. The MnP activity was detected in all the three culture media after 6th day and recorded till 24th day. In wheat straw medium on 6th day at 30 ^{0}C max MnP activity was recorded followed by at 35 ^{0}C on 12th day. Similar to other enzymes Czapek dox medium gave highest enzyme /unit mg of protein.

Table 6. Versatile peroxidase (VP) activity of *C. indica* in different culture media and temperature

Versatile peroxidase										
Day Interval	**Wheat st.**				**Sab.**			**Cza. Dox**		
	Temp.	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**
6 days	25ºC	135.3	13.12	39.79	40.46	306.5	7.09	12.38	37.40	4.48
	30ºC	109.6	9.79	24.35	42.80	56.24	6.20	21.16	147.9	8.60
	35ºC	52.9	5.98	9.12	30.38	76.52	5.01	57.18	346.5	21.49
	25ºC	265.0	20.86	70.47	31.27	14.21	4.49	14.45	30.10	4.12
12 Days	30ºC	213.6	15.41	41.07	56.30	20.92	4.27	41.98	21.86	11.6
	35ºC	396.0	31.96	64.28	54.76	16.29	3.84	73.72	58.50	21.49
	25ºC	295.85	21.75	72.86	32.43	10.32	2.18	106.34	265.85	32.2
18 days	30ºC	161.66	10.64	37.94	85.53	17.38	6.33	56.36	301.39	16.10
	35ºC	266.57	17.35	42.78	93.2	15.17	5.66	110.0	416.66	27.88
	25ºC	137.74	9.49	28.93	165.24	32.21	11.37	9.66	42.0	1.85
24 Days	30ºC	140.39	8.59	15.42	68.12	10.79	3.92	106.10	312.0	21.39
	35ºC	193.25	13.3	24.90	91.07	10.27	5.34	101.48	289.9	18.58

Table 7. Manganese peroxidase (MnP) activity of *C. indica* in different culture media and temperature

MnP										
Day Interval	**Wheat st.**				**Sab.**			**Cza. Dox**		
	Temp.	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**
6 days	25ºC	62.59	6.07	18.4	19.33	146.4	3.39	20.77	62.74	7.52
	30ºC	124.55	11.13	27.6	15.15	19.9	2.19	2.76	19.30	1.12
	35ºC	39.95	4.51	6.88	20.29	51.1	3.34	10.4	63.03	3.90
	25ºC	23.2	1.82	6.17	45.65	20.75	6.55	5.27	10.97	1.50
12 Days	30ºC	37.2	2.68	7.15	12.12	4.50	0.92	27.9	14.53	7.75
	35ºC	118.6	9.57	19.25	18.14	5.39	1.27	10.5	8.33	3.06
	25ºC	39.1	2.875	9.63	9.66	3.07	0.65	2.38	5.95	0.72
18 days	30ºC	29.6	1.948	6.94	15.04	3.05	1.11	2.53	13.52	0.72
	35ºC	35.4	2.30	5.68	27.24	4.43	1.65	3.43	12.99	0.87
	25ºC	52.57	3.62	11.04	28.78	5.61	1.98	9.45	41.08	1.81
24 Days	30ºC	52.21	3.19	5.73	26.80	4.24	1.54	7.09	20.85	1.42
	35ºC	35.68	2.47	4.59	18.27	2.06	1.07	6.26	17.88	1.14

The results of LiP activity in different culture media and temperature is shown in table 8. LiP activity started after 6th day in all the three culture media. In wheat straw medium max LiP activity was recorded on 18th day followed by 24th day. Czapek dox medium gave maximum enzyme per unit mg of protein.

This is the 1st report of VP peroxidase in *Calocybe indica* after *Bjerkandera adust* and *Pleurotus* spp. We can use Czapek dox medium for enzyme production and purification, however sraw medium can be used for bulk production of VP for industrial uses.

Table 8. Lignin peroxidase (LiP) activity of *C. indica* in different culture media and temperature

	LiP									
Day Interval	**Wheat st.**				**Sab.**			**Cza. Dox**		
	Temp.	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**	**U/ml**	**U/mg protein**	**U/mg Biomass**
6 days	25ºC	3.36	0.32	0.98	1.93	14.62	0.33	2.40	7.25	0.86
	30ºC	4.80	0.42	1.06	1.75	2.29	0.25	1.63	11.39	0.66
	35ºC	2.90	0.32	0.50	1.80	4.53	0.29	3.30	20.0	1.24
	25ºC	0.0	0.0	0.0	5.06	2.30	0.72	4.83	10.06	1.38
12 Days	30ºC	6.73	0.48	1.29	5.76	2.14	0.43	2.10	1.09	0.58
	35ºC	3.28	0.26	0.53	2.13	0.63	0.15	0.88	0.69	0.25
	25ºC	36.6	2.69	7.68	5.93	1.88	0.40	5.06	12.65	1.53
18 Days	30ºC	12.53	0.82	1.37	7.56	1.53	0.56	5.43	29.03	1.55
	35ºC	18.46	1.20	2.37	11.2	1.82	0.68	3.83	14.50	0.97
	25ºC	13.36	0.92	2.80	10.26	2.0	0.70	8.97	39.0	1.72
24 Days	30ºC	15.26	0.93	1.67	5.52	0.87	0.31	9.33	27.4	1.88
	35ºC	14.89	1.03	1.91	14.19	1.60	0.83	9.63	27.5	1.76

REFERENCES

[1] Chakravarty DK *et al.* (1981). *Calocybe indica*, a tropical edible mushroom. *Current Science* 50: 550.

[2] Rajesh Babu D *et al.* (2012). Proximate, vitamins and mineral elemental analysis of cultivated edible mushrooms: *Calocybe indica* and *Hypsizygus ulmarius*. *Mushroom Research* 21(2): 129-135.

[3] Usha S. (2007). Fatty acids analysis of *Calocybe indica* cultivated on various bio wastes. *Mushroom Research* 16(1): 27-29.

[4] Amin R *et al.* (2010). Effect of the different substrate and casing materials on the growth and yield of *Calocybe indica*. *Mycobiology* 38(2): 97-101.

[5] Upadhyay RC. (2010). Milky mushroom (*Calocybe indica*) cultivation. In Advances in Mushroom Biology and Biotechnology. (Sharma, Satish Kumar and Wackchaure eds). 133-136, DMR, Solan.

[6] Doshi A *et al.* (1987). Pectinolytic and cellulolytic enzyme production at various stages of spawn growth of *Calocybe indica*. *Mush. J. Tropics* 7: 83-85.

[7] Bradford MM (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilising the principle of protein dye binding. *Analyst Biochem*. 72: 248-254.

[8] Haars A. and Huttermann. (1981). Effect of phenolic compounds and tannin on growth and laccase activity of *Fomes annosus*. *Eur. J. For. Pathol.* 11: 67-76.