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STRAIN IMPROVEMENT OF SPECIALTY MUSHROOM, *Calocybe indica*, THROUGH MUTAGENESIS

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ABSTRACT

***Calocybe indica*, a specialty mushroom, is the third most popular mushroom in India. During present investigation 9 strains of *C. indica* were cultivated on wheat straw using the recommended technology. Maximum biological efficiency was obtained in strain Ci-3 (81.28%). The improvement in Ci-3, a high yielding strain, was attempted using mutagenic treatment on protoplasts. Four mutagenic treatments (one irradiation and three chemical) yielded 30 putative mutants. Growth studies of putative mutants indicated maximum growth rate of CMB-4 on wheat straw while CMN-11 gave maximum biomass on complete yeast extract medium. Mutant CMN-9 gave maximum endoglucanase (0.345 µg min^{-1} ml^{-1}) and xylanase (0.54 µg min^{-1} ml^{-1}) activity. Seven mutants were identified on the basis of growth and enzymes studies. Cultivation of mutants was carried out on wheat straw following hot water treatment. Four mutants (CMN-3, CMN-9, CMN-11 and CMB-4) gave significantly higher yield than the parent.**

Keywords: *Calocybe indica*, enzyme activity, mushroom, mutagenesis, putative mutants, yield

INTRODUCTION

Specialty mushrooms are the wild edible mushrooms found in natural habitat which are locally available to regional consumers. Tremendous revolution in mushroom technology with respect to types and strains of cultivated mushrooms has been made in recent years. *Calocybe indica*, a specialty mushroom, is the third most popular and commercially grown mushroom in India after button and oyster mushrooms. This mushroom, commonly called "Dudh Chatta" due to the shape of milky umbrella, is known by diverse names in different parts of India. It was first reported in India from West Bengal (Purkaystha and Chandra, 1974) and its edibility confirmed later (Purkaystha and Chandra, 1976). Major part of India is endowed with tropical and subtropical climates suitable for the cultivation of milky mushroom throughout the year. The approximate annual production of this mushroom in India estimated to be 10,000 tonnes. The cultivation of milky mushroom has gained momentum in Andhra Pradesh, Goa, Karnataka, Kerala, Maharastra, Tamil Nadu and Uttar Pradesh. The climate of Punjab is somehow favourable for the cultivation of *C. indica*, as the optimum temperature required for this mushroom (28-35°C) prevails at least for six months (March-May and August-October). Productivity and quality of widely cultivated mushrooms mainly depend on strain make up, therefore many strains have constantly been produced with an aim of achieving higher yields and improved quality attributes such as accumulation of nutrients of interest, disease resistance, adaptability to wide temperature range and sporelessness. Genetic manipulation of mushrooms can be achieved in number of ways which include cross-breeding, protoplast fusion or protoplast mutagenesis. Mutagenesis has been attempted

using a number of approaches. Chemical mutagenesis may be performed using agents such as NTG (N-methyl-N'-nitro-N-nitrosoguanidine), sodium nitrite, BU (5'-bromouracil), etc. and this usually results in a single nucleotide substitution in the target genome. Ultraviolet light (UV) induces errors in DNA replication, while ionizing radiation, as a mutagen, induces deletions and translocations. Mutagenesis of protoplasts from a *Volvariella volvacea* monokaryon had given both auxotropic and morphological mutants (Mukherjee and Sengupta, 1986). Auxotrophic mutants have also been obtained via protoplasts from *Pleurotus* spp. (Toyomasu *et al.,* 1986, Toyomasu and Mori, 1987) and from *Oudemansiella musida* (Homolka *et al.,* 1988). Irradiations by gamma rays lead to production of lignocellulolytic mutants in *P. ostreatus* (Lee *et al.,* 2000) while in *P. florida,* three putative mutants were developed by Djajnegara and Harsoyo (2008) using gamma rays. A lot of work has been done with respect to the cultivation technology of *C. indica* (Kaur, 2003, Mangat, 2005, Kaur, 2010, Redhu, 2010) but no systematic work has yet been undertaken for its genetic improvement. The present investigation was carried out to improve the productivity of *C. indica* through protoplast mutagenesis.

MATERIALS AND METHODS

***Strains used*:** Nine strains of *Calocybe indica*, Ci-1, Ci-2, Ci-3, Ci-4, Ci-5, Ci-6, Ci-7, Ci-9 and APK-2 were maintained on potato dextrose agar (PDA) medium and subcultured routinely after every 6 week.

Cultivation of Calocybe indica strains: Cultivation trials were conducted using the above strains of *C. indica* during summer season (May-September). The cultivation was done indoor under natural climatic conditions with temperature ranging from 25 to 40°C and relative humidity 70-90% at University Mushroom Research Complex using the recommended technology. Proper ventilation and diffused light in growing rooms was maintained.

Mutagenic treatment of high yielding strain: The mycelium of high yielding strain was subject to protoplast mutagenesis. For protoplast liberation, 4-5 days old mycelium was used. The content of inoculated flask was shifted to sterilized centrifuge tubes and centrifuged at 8000 rpm for 15 min. The supernatant was discarded and mycelium washed thrice with sterile distilled water. The next two washings were given with 0.2 M phosphate buffer (pH 5.7). To each centrifuge tube 2 ml enzyme solution was added and incubated at 30±2°C for 18 h. The mycelial remnants were removed by filtration through cotton wool. The protoplasts were again freed from mycelial fragments by centrifugation at 2,000 X g for 10 min. The pelleted protoplasts were re-suspended in osmotic stabilizer. The preparation was checked under microscope to ensure the absence of any mycelial fragment. The protoplasts obtained were subject to irradiation or mutagenic treatments. UV light was used for irradiation. The protoplast suspension was subjected to UV (250 nm) irradiation for 3 to 12 min. The exposure was carried out at distance of 5-25 cm from the center of germicidal lamp (UV light source). The protoplasts were then kept for 30 min in dark to allow full expression of damage induced by UV light. For chemical mutagenesis, three mutagenic agents viz., alkylating (NTG), base analog (BU) and intercalating mutagen (ethidium bromide, $EtBr_2$) were used. Protoplasts were exposed to these chemical mutagens for different periods from 30 to 120 sec. and used at five concentrations 5, 10, 15, 20 and 25 µg ml^{-1} of osmotic stabilizer solution. The mutagen treated protoplasts were washed with osmotic stabilizer and plated on regeneration medium containing 1% glucose and 0.5% yeast extract in osmotic stabilizer solution. For regeneration on solid medium, 0.1 ml of mutagen-treated protoplast suspension, containing 65 to 70 protoplasts was spread out on regenerating medium containing 2% (w/v) agar and overlaid with the same soft agar medium containing 0.5% (w/v) agar. The inoculated plates were incubated at 30±2°C for 4-5 days. The purity of protoplast suspension was checked by using a control plate, on which a suspension of protoplasts initially lyzed with distilled water, was plated in similar way. The absence of mycelial colonies on control plate was taken as a measure of the purity of protoplast preparation. The colonies were picked and shifted to potato dextrose agar slants.

Evaluation of mutants: *Calocybe indica* mutants were assayed for linear growth and biomass. For linear growth, 10 mm mycelial bits were placed in the centre of petriplates containing boiled wheat straw. The inoculated petriplates were incubated at 30±2°C for 6 days. The linear growth of mutants was recorded in terms of colony diameter at 2 days interval. Complete yeast extract medium (CYM) [Raper *et al.,* 1972] was used for biomass study. *C. indica* mutants were tested for their ability to produce extracellular enzymes on CYM broth. Estimation of endo-β-D-1,4-glucanase was made using the method of Miller (1959). Test tubes containing a mixture of 0.5 ml CMC solution and 0.5 ml culture filtrate were incubated at 50°C for 30 min. in water bath. Controls devoid of enzyme extract were also run simultaneously. Reducing sugars produced were measured using DNS method. 3 ml DNS was added to each tube and kept in boiling water bath for 15 min. While still hot 1 ml Na-K-tartarate solution was added and the contents cooled to room temperature followed by the addition of 2 ml distilled water to each test tube. Enzyme extract was added to controls after adding DNS. The light absorbance by the resulting solutions was measured at 575 nm in Spectronic 20. The corresponding enzyme activity was calculated from standard curve. Xylanase was estimated as per Bucht and Erikson (1968). One ml sample of approximately diluted culture was mixed with 1 ml 1% xylan solution and incubated for 30 min. at 50°C. Reducing sugar was measured as xylose equivalents by DNS method. The unit of enzyme activity was one unit of endoxylanase that catalyzed the formation of 1 µmol of xylose min^{-1} ml^{-1} culture filtrate.

Cultivation of developed mutants: On the basis of growth studies and enzyme activity seven mutants were selected for cultivation on wheat straw using recommended technology. Least significant difference (LSD) was used for statistical analysis of data obtained.

RESULTS AND DISCUSSION

Cultivation of selected strains: Nine strains of *Calocybe indica* Ci-1 to Ci-7, Ci-9 and APK-II were grown on wheat straw following the standard adapted cultivation technology. Observations on days of spawn run, number of fruit bodies, yield and average weight of fruit body were recorded along with stipe length and pileus diameter of the random sample of each strain. Spawn run period ranged from 31 to 40 days. The number of fruit bodies harvested was maximum for Ci-7 (1890) followed by Ci-4 and lowest in Ci-9 (890). The biological efficiency, estimated from the harvested yield (kg q^{-1} dry straw), was maximum in strain Ci-3 (81.28%). Five strains Ci-4, Ci-5, Ci-6, Ci-7 and APK-II gave yields at par

Table 1: Yield performance of *Calocybe indica* strains on wheat straw

Strains	Spawn run (days)	NFB* (No. q^{-1} dry straw)	Yield (kg q^{-1} dry straw, %BE)	Av. fruit body wt (g)	Av. fruit body dimension (cm)			
					PD	TL	SL	SD
Ci-1	40	1105	50.70	46	7.5	15.3	13.1	1.9
Ci-2	33	1167	49.56	42	6.9	14.8	10.9	2.6
Ci-3	35	1605	81.28	51	9.4	14.2	10.5	1.4
Ci-4	35	1834	71.13	39	8.1	15.7	13.6	2.3
Ci-5	31	1447	70.80	49	6.9	13.9	10.0	2.2
Ci-6	37	1334	70.31	53	7.7	16.1	13.9	1.8
Ci-7	33	1890	71.73	38	6.2	15.9	12.7	2.2
Ci-9	40	890	47.17	53	8.3	15.3	13.6	1.6
APK-2	33	1447	71.82	50	9.3	16.8	14.2	3.1
$LSD_{0.05}$		30.64	2.49	-	-	-	-	-

No. of replicates: 8 (4 + 4);

Substrate used: wheat straw boiled for 45 minutes; Substrate/bag: 1 kg wet straw/bag; Spawn rate: 10% on dry wt. basis; Casing: FYM + sandy loam soil (4:1); Date of sowing: 3rd June 2009, 12th July 2009; Date of casing: 6th July 2009, 14th August 2009; Date of termination: 23rd August 2009, 4th October 2009; NFB: Number of fruit bodies; PD = Pileus diameter; TL = Total length of fruit body; SL = Stipe length; ST = Stipe thickness

with each other whereas Ci-1, Ci-2 and Ci-9 showed low biological efficiency (47.82-51.28%). Average weight of fruit body ranged from 38 to 53 g with maximum in Ci-3, Ci-6 and Ci-9 strains and least in Ci-7 strain. The pileus diameter of strains ranged between 6.2 and 9.4 cm and stipe length between 10 and 14.2 cm (Table 1). Considering the yield potential of *C. indica* strains, only one strain Ci-3 had maximum biological efficiency. Similar results for *C. indica* strain Ci-3 under natural climatic condition of Punjab have been reported by Mangat (2005) and Kaur (2010).

Mutagenic treatment of high yielding strain: High yielding strain Ci-3 was subjected to strain improvement via protoplast mutagenesis. *C. indica* protoplasts (Fig. 1) were exposed to one physical (UV exposure) and three chemical (alkylating, base analog and intercalating) mutagenic treatments. UV treatment was given onto the protoplasts spread on regeneration medium containing 1% glucose and 0.5% yeast extract in osmotic stabilizer solution (v/v) upto 12 min. at a distance between 5 and 25 cm. After UV treatment, regeneration was allowed in dark. Seven regenerants obtained were assigned CMU-1 to CMU-7 as UV mutants (Table 2). NTG was used as an alkylating mutagen and protoplasts exposed to 5-25 µg ml^{-1} NTG for 30 to 120 sec. The treated protoplasts were spread on regeneration medium which yielded 12 regenerants. The base analog selected for the mutagenesis of *C. indica*, strain Ci-3 protoplasts was bromouracil (BU). The protoplasts were treated for 30 to 120 seconds with varying

Table 2: Effect of mutagenic treatments on *C. indica* strain Ci-3 on the protoplasts generated

A) Physical treatment	**Exposure time (min.)**	**Distance of spread plated protoplasts from UV source (cm)**					**Total**
		5	10	15	20	25	
UV	3	-	1 (CMU-1)	-	-	-	1
	6	2 (CMU-2, CMU-3)	-	2 (CMU-4, CMU-5)	-	-	4
	9	-	1 (CMU-6)	-	1 (CMU-7)	-	2
	12	-	-	-	-	-	-
Sub-total		2	2	2	1	-	7
B) Chemical treatment	**contact time (sec.)**	**Concentration used for treatment (µg ml^{-1})**					**Total**
		5	**10**	**15**	**20**	**25**	
Base analog: 5'- Bromouracil (BU)	30	2 (CMB-1, CMB-2)	-	1 (CMB-3)	-	-	3
	60	-	-	1 (CMB-4)	-	-	1
	90	1 (CMB-5)	-	-	-	-	1
	120	-	-	-	-	-	
Intercalating agent: N-methyl-N'-nitro-N-nitrosoguanidine (NTG)	30	2 (CMN-1, CMN-2)	-	2 (CMN-3, CMN-4)	1 (CMN-5)	1(CMN-6)	6
	60	-	1 (CMN-7)	1 (CMN-8)	-	-	2
	90	1 (CMN-9)	-	1 (CMN-10)	-	-	2
	120	-	2 (CMN-11, CMN-12)	-	-	-	2
Intercalating agent: Ethidium bromide ($EtBr_2$)	30	-	2 (CME-1, CME-2)	-	-	-	2
	60	-	-	2 (CME-3, CME-4)	-	-	2
	90	1 (CME-6)	-	-	-	-	1
	120	-	-	1 (CME-7)	-	-	1
Sub-total		7	5	9	1	1	23

Numerical figures indicate number of protoplasts regenerated;
Regeneration medium: 1% glucose and 0.5% yeast extract in osmotium; Incubation temperature: 30±2°C; Incubation period: 10 days

concentration of BU (5-25 µg ml^{-1}) and the putative mutants named as CMB-1 to CMB-5. Intercalating mutagenesis was done by $EtBr_2$ using 5-25 µg ml^{-1} concentration for 30 to 120 sec. Four mutagenic treatments gave a total of 30 protoplast regenerants which were treated as putative mutants evolved from the parent strain Ci-3. UV and NTG at 15 and 30 µg ml^{-1} concentration have been used by Mukherjee and Sengupta (1986) for mutagenesis of protoplasts in *Volvariella volvacea*. Radiation using gamma ray (60 CO) at 0.75 KGray with doze velocity of 1.149 KGray hr^{-1} on white oyster mushroom (*Pleurotus florida*) mycelia yielded several mutants. Based on isozyme analysis using two enzyme markers, esterase (EST) and acid phosphatase (ACP) indicated 3 putative mutants (PO-3, PO-4 and P-5) had been identified (Djajanegara and Harsoyo, 2008).

Evaluation of mutants: In comparison to parent *C. indica* Ci-3 no mutant showed improved growth rate, except CMB-4. Only two mutants CMN-4 and CMN-7 showed slow growth (Table 3). The study on biomass production in complete yeast extract broth indicated maximum biomass for six mutants, CMU-2, CMN-4, CMN-9, CMN-10, CMN-11 and CMN-12 which were better than that of parent Ci-3

Table 3: Growth performance and endoglucanase and xylanase enzyme activities of mutants

Mutants	Linear growth (mm day^{-1})	Biomass (g L^{-1})	Endoglucanase activity (µg min^{-1} ml^{-1})	Xylanase activity (µg min^{-1} ml^{-1})
CMU-1	5.83	13.6	0.210	0.085
CMU-2	4.58	15.4	0.033	0.105
CMU-3	7.67	13.7	0.142	0.094
CMU-4	7.50	13.9	0.129	0.226
CMU-5	6.88	13.6	0.046	0.070
CMU-6	6.83	14.5	0.256	0.134
CMU-7	7.17	12.8	0.158	0.129
CMN-1	6.13	15.1	0.213	0.112
CMN-2	5.33	14.6	0.098	0.134
CMN-3	7.80	13.8	0.205	0.379
CMN-4	4.08	15.4	0.114	0.078
CMN-5	7.42	12.9	0.234	0.094
CMN-6	6.37	13.9	0.312	0.105
CMN-7	5.08	14.0	0.254	0.131
CMN-8	7.55	14.8	0.074	0.232
CMN-9	6.22	15.7	0.345	0.540
CMN-10	7.75	15.3	0.305	0.173
CMN-11	7.22	16.0	0.299	0.196
CMN-12	6.30	15.9	0.088	0.124
CMB-1	5.56	14.3	0.236	0.143
CMB-2	7.00	14.8	0.351	0.254
CMB-3	6.13	13.6	0.302	0.135
CMB-4	9.83	14.2	0.120	0.028
CMB-5	6.59	14.8	0.024	0.196
CME-1	6.83	13.9	0.017	0.057
CME-2	6.72	13.0	0.013	0.365
CME-3	5.98	14.0	0.142	0.153
CME-4	5.80	13.8	0.245	0.128
CME-5	7.00	13.0	0.156	0.207
CME-6	6.07	14.6	0.236	0.211
Ci-3	6.75	14.2	0.226	0.091
$LSD_{0.05}$	0.72	0.93		

Medium used for linear growth: Wheat straw boiled for 45 min;
Medium used for biomass study: Complete yeast extract medium;
Incubation temperature: 30±2°C; Incubation period: 6 days; Drying temperature/period: 60°C for 8 h;
Enzyme assay: Incubation period: 6 days; Incubation temperature: 30±2°C

whereas one mutant CMU-7 showed poor biomass (12.8 g L^{-1} (Table 3). Kaur (2003) has reported best growth on malt extract agar and PDA with a colony diameter of 59 and 58 mm, respectively followed by wheat extract agar (54 mm) after 12 days of incubation. Growth of *Calocybe indica* in 11 different liquid media has also been studied by Pani (2010). Maximum mycelial biomass was harvested from wheat seed extract medium followed by malt extract medium and potato dextrose medium, although the difference in fungal biomass was statistically insignificant. The endoglucanase production was maximum in CMN-9 and CMB-2 putative mutants of *C. indica*. Seven mutants showed enzyme activity at par with that of parent Ci-3. Xylanase activity of putative mutants revealed maximum activity for CMN-9 mutant (0.54 µg min^{-1} ml^{-1}). Four mutants viz., CMN-4, CMU-5, CMB-4 and CME-1 showed xylanase activity less than the parent (0.091 µg min^{-1} ml^{-1}) while others showed either higher activity or were at par with the parent. On the basis of characteristics of *C. indica* mutants, 7 mutants (CMU-2, CMU-5, CMN-3, CMN-9, CMN- 11, CME-2 and CMB-4) were selected for further study. Krishnamoorthy *et al.* (2002) in a study on enzyme-related biodegradative potential of *C. indica* found direct relationship between production of cellulases and yield. Mangat (2005) reported laccase activity in only one strain (Ci-2) of *C. indica.*

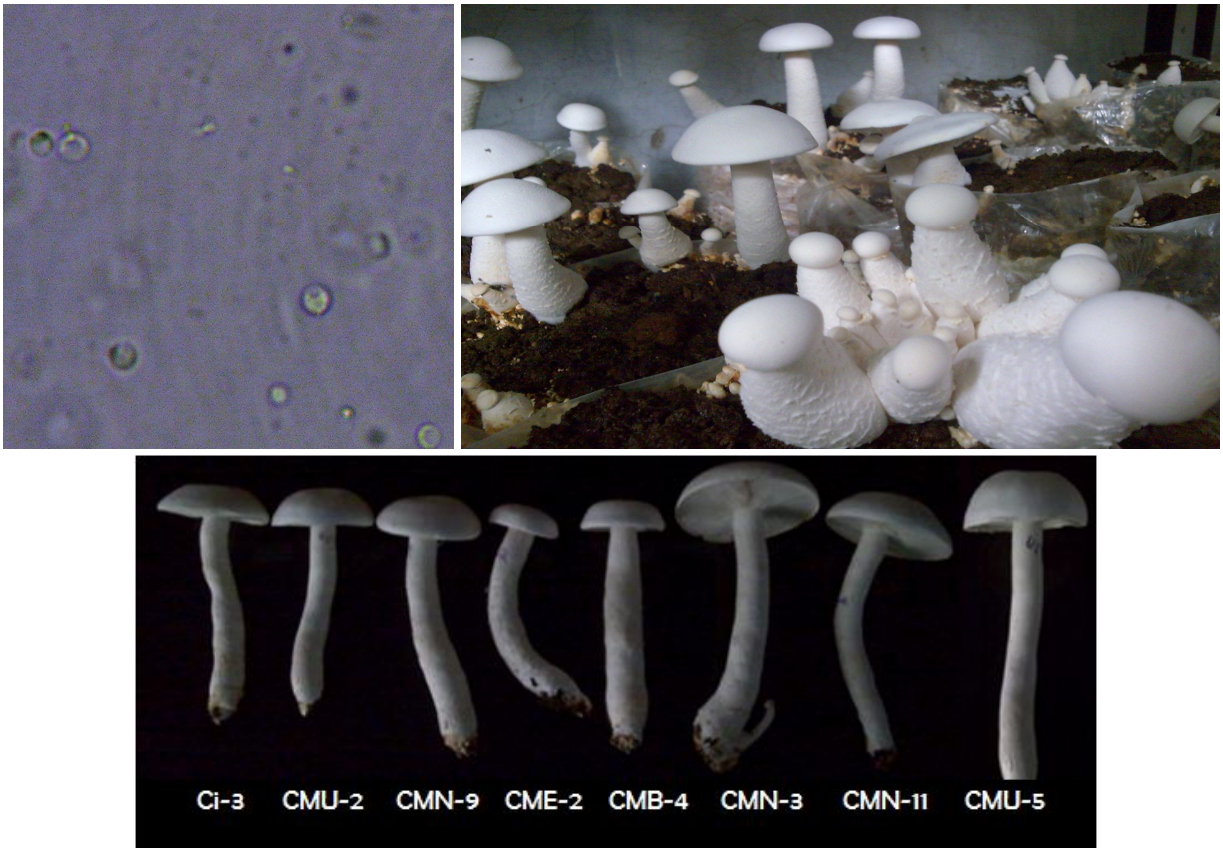

Fig 1: a) (upper left) Protoplasts of *Calocybe indica*, Ci-3 strain at 45X; b) (upper right) Cultivation of mutants on wheat straw; c) (bottom) Individual fruit bodies of *C. indica* mutants

Cultivation of developed mutants: Cultivation of seven mutagenically developed strains namely, CMU-2, CMU-5, CME-2, CMN-3, CMN-9, CMN-11 and CMB-4 along with parent strain (Ci-3) was carried out on wheat straw following hot water treatment (Fig. 1b). Spawn run period for these mutants varied from 38 to 49 days (Table 4). The fruit bodies harvested were maximum for CMN-11 (1466) followed by CME-2 (1334). Average weight of fruit bodies ranged from 47.75 g (CME-2) to 98.97 g (Ci-3).

Table 4: Yield performance of *Calocybe indica* mutants on wheat straw

Mutant strain	Spawn run (days)	NFB (No. q^{-1} dry straw)	Yield (kg q^{1} dry straw; %biological efficiency)	Av. fruit body weight (g)
CMU-2	42	666	47.28	70.99
CMU-5	49	934	54.52	58.37
CME-2	49	1334	63.70	47.75
CMN-3	42	1066	74.46	69.85
CMN-9	38	1200	97.90	81.58
CMN-11	49	1466	86.58	59.05
CMB-4	42	1200	73.94	61.61
Ci-3	42	666	65.92	98.97
$LSD_{0.05}$		53.2	3.05	

No. of replicates: 5;
Substrate used: Wheat straw boiled for 45 minutes; Substrate/bag: 1kg wet straw/bag; Spawn rate: 10% on dry wt. basis; Casing: FYM + Sandy loam soil (4:1); Date of sowing: 3rd April 2010; Date of casing: 28th May 2010; Date of termination: 18th July 2010; NFB: Number of fruit bodies

Four mutants, CMN-9, CMN-11, CMN-3 and CMB-4, were found to give significantly higher yield (97.90, 86.58, 74.46 and 73.94%, respectively) than the parent strain (32.96%). Yield potential of mutants CMU-5 and CME-2 was at par with Ci-3 while CMU-2 (23.64%) was found to be least yielding mutant (23.64%). The individual fruit bodies of all the seven mutants are shown in Fig. 1c. Mangat (2005) reported that 45- 50 min boiling of wheat straw was the best pre-treatment for obtaining maximum biological efficiency (41.6-62.9%) for 3 strains of *C. indica.*

Conclusion: Nine strains of *C. indica* were subjected to cultivation trial using the recommended technology out of which Ci-3 strain out-yielded the other eight strains. Therefore, Ci-3 strain was selected for further improvement. Protoplasts from Ci-3 strain were obtained and subjected to irradiation and chemical treatments which resulted in the regeneration of 30 putative mutants. Seven putative mutants, namely CMN-3, CMN-9, CMN-11, CMB-4, CME-2, CMU-2 and CMU-5, were selected on basis of their growth and enzyme activities used for evaluation of putative mutants. Of these seven mutants, four mutants viz., CMN-3, CMN-9, CMN-11 and CMB-4 out-yielded the parent when grown on wheat straw. Hence mutagenesis of protoplasts is a preferred and reliable method for obtaining high yielding strains of mushrooms which can revolutionize the mushroom industry.

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