Calocybe indica STRAIN EVALUATION FOR LIGNOCELLULOLYTIC ENZYMES IN RELATION TO MUSHROOM YIELD

Thesis

Submitted to Punjab Agricultural University
in partial fulfilment of the requirements
for the degree of

MASTER OF SCIENCE
in
MICROBIOLOGY
(Minor Subject: Biochemistry)

By

Karanpreet Singh
(L-2017-BS-286-M)

Department of Microbiology
College of Basic Sciences and Humanities

LUDHIANA-141004

2019

CERTIFICATE I

This is to certify that this thesis entitled, **"*Calocybe indica* strain evaluation for lignocellulolytic enzymes in relation to mushroom yield"** submitted for the degree of **Master of Science,** in the subject of **Microbiology** (Minor subject: **Biochemistry**) of Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Mr. Karanpreet Singh (L-2017-BS-286-M)** under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

_____

(Dr. Harpreet Singh Sodhi)
Major advisor
Senior Mycologist
Department of Microbiology
Punjab Agricultural University
Ludhiana- 141004

CERTIFICATE II

This is to certify that the thesis entitled, **"*Calocybe indica* strain evaluation for lignocellulolytic enzymes in relation to mushroom yield"** submitted by **Mr. Karanpreet Singh (L-2017-BS-286-M)** to the Punjab Agricultural University, Ludhiana, in partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Microbiology** (Minor subject: **Biochemistry**) has been approved by the Student's Advisory Committee along with Head of the Department after an oral examination of the same.

_____
(Dr. Harpreet Singh Sodhi)
Major Advisor

_____
(Dr. S.K. Soni)
External Examiner
Chairperson
Department of Microbiology
Panjab University, Chandigarh

_____
(Dr. Shammi Kapoor)
Dean-cum-Head of the Department

_____
(Dr. Gurinder Kaur Sangha)
Dean, Post graduate studies

ACKNOWLEDGEMENT

At the very onset, I bow with all humility and reverence to the Almighty God, creator of the universe for his blessing hand bestowing a creative and healthy environment throughout my academic and research period.

No one can ever make any achievement alone. It is the debt that could only be paid through gratitude. I bow with gratitude to all the teachers who moulded me and enabled me to add a new dimension in my life.

I wish to record my deep admiration and heartfelt gratitude to my Major Advisor ***Dr. Harpreet Singh Sodhi,*** *Senior Mycologist, Department of Microbiology, Punjab Agricultural University, Ludhiana for his selfless attitude, able guidance, keen interest, constant motivation and encouragement and professional and creative suggestions which helped me in completing the degree and this study in its wholeness.*

I am highly grateful to the members of my advisory committee viz. ***Dr. Shivani Sharma****, Assistant Mycologist, Department of Microbiology,* ***Dr. Rachana D. Bhardwaj,*** *Assistant Biochemist, Department of Biochemistry and* ***Dr. G.S. Kocher****, Senior Microbiologist (Dean PGS Nominee), Department of Microbiology for their constant support and valuable suggestions during the course of investigations and preparation of this manuscript.*

I am indebted to my family members specially my respected parents, ***S. Gurpreet Singh*** *and* ***Smt. Narinder Kaur*** *for their constant support, deep affection and their words of encouragement. I feel privileged to applaud my grandfather* ***Late S. Manohar Singh,*** *grandmother* ***Late Smt. Kuldeep Kaur*** *for their blessings and affection are not to be reciprocated in words but are to be felt in the deepest corner of my heart. I am thankful to my brother* ***Dr. Jaspreet Singh*** *and* ***Dr. Kanwalpreet Singh*** *for their co-operation and love. I express my heartfelt sentiments for their moral and emotional support for enabling me to carry on my academic pursuits.*

I express my thanks to my friends ***Suhani, Harshdeep Singh, Tanjot Kaur, Preetiman, Ravneet Kaur , Aashish, Alka, Balwinder Singh*** *and* ***Mani Kaler*** *for their support, memorable company, ever willing help, and for providing me few moments of refreshment in between the exhausting hours to shed the workload which kept me energetic during this period and made my stay at the university memorable.*

Mr. Deepak Kumar *and* ***Mr. Beer Bahadur*** *(Ekta Computer Centre) deserve special thanks for bringing out this manuscript in its presentable form.*

My sincere thanks are due to the faculty and staff of the Department of Microbiology for their encouragement and cooperation.

Memory and words may fail to acknowledge somebody but the heart can't. Heart-felt thanks to all for providing me even a minuscule of help during this period of investigation.

Place: _____

Date: _____ **(Karanpreet Singh)**

Title of the Thesis	:	*Calocybe indica* strain evaluation for lignocellulolytic enzymes in relation to mushroom yield
Name of the Student and Admission No.	:	Karanpreet Singh (L-2017-BS-286-M)
Major Subject	:	Microbiology
Minor Subject	:	Biochemistry
Name and Designation of Major Advisor	:	Dr. Harpreet Singh Sodhi Senior Mycologist
Degree to be awarded	:	M.Sc.
Year of award of Degree	:	2019
Total Pages in Thesis	:	62 + Appendix (vii) + Vita
Name of University	:	Punjab Agricultural University, Ludhiana – 141 004

ABSTRACT

Calocybe indica, milky mushroom is a tropical mushroom of Indian origin that grows well at 28-35°C. *C. indica* strains were evaluated for their mycelial growth, extracellular enzymes producing capability and their yield potential. Different characteristic of nine strains of *Calocybe indica* Ci-01 to Ci-09 and a *Macrocybe* were grown on Potato Dextrose (PDA), Complete Yeast Extract (CYM), Mushroom Minimal Media (MMM) and their broth at 30±2°C. After 10 days, maximum radial growth was observed on PDA in Ci-06 (87.6 mm) and Ci-09 (87 mm) strain of *C. indica*. The maximum biomass was recorded in CYM in Ci-06 (16.1g/l) and Ci-07 (15.5 g/l). The maximum linear growth on wheat straw after 10 days was observed in Ci-07 (63mm) and Ci-09 (66mm). In wheat straw, the maximum biological efficiency estimated from the harvested yield (kg/q dry straw) was observed in the strain Ci-06 (57.3%). Two strains Ci-07 (48.5%) and Ci-09 (45.7%) were found to give yield at par with each other. Maximum number of fruiting bodies were recorded in the strain Ci-06 (1552 no./q dry straw). In culture filtrate endoglucanase enzyme activity range between 1.91-4.42 U/mg, endoxylanase activity ranged between 2.73-4.76 U/mg, laccase activity ranged between 6.94-10.2 U/mg. During spawn run, endoglucanase and endoxylanase enzyme activity ranged between 0.58-1.03 U/mg and 0.74 -2.11 U/mg, laccase activity was ranged between 1.51-4.54 U/mg. During pinhead, endoglucanase and endoxylanase activity ranged between 0.61-1.59 U/mg and 1.57-2.96 U/mg, whereas laccase activity range between 6.53-10.6 U/mg. In fruiting bodies, endoglucanase and endoxylanase activity of the strains ranged between 0.94-1.88 U/mg and 0.93-2.36 U/mg, laccase activity ranged between 5.43-9.21 U/mg. A positive correlation of yield with biomass and endoglucanase has been observed in *C. indica* strains Ci-03, Ci-06, Ci-07 and Ci-09.

Keywords – Biomass, *Calocybe indica* strains, enzyme, yield

_____ _____

Signature of Major Advisor **Signature of the student**

ਖੋਜ ਦਾ ਸਿਰਲੇਖ	:	ਖੁੰਭਾਂ ਦੇ ਝਾੜ ਦੇ ਸਬੰਧ ਵਿੱਚ ਲਿਗਨੋਸੈਲੂਲੋਲਟਿਕ ਇੰਜ਼ਾਇਮ ਲਈ *ਕੈਲੋਸਾਇਬ ਇੰਡੀਕਾ* ਸਟਰੇਨ ਦਾ ਨਿਰੀਖਣ
ਵਿਦਿਆਰਥੀ ਦਾ ਨਾਂ ਅਤੇ ਦਾਖ਼ਲਾ ਨੰਬਰ	:	ਕਰਨਪ੍ਰੀਤ ਸਿੰਘ (ਐੱਲ-2017-ਬੀ.ਐੱਸ-286-ਐੱਮ)
ਪ੍ਰਮੁੱਖ ਵਿਸ਼ਾ	:	ਸੂਖਮ ਜੀਵ ਵਿਗਿਆਨ
ਸਹਿਯੋਗੀ ਵਿਸ਼ਾ	:	ਜੀਵ ਰਸਾਇਣ
ਮੁੱਖ ਸਲਾਹਕਾਰ ਦਾ ਨਾਂ ਅਤੇ ਅਹੁੱਦਾ	:	ਡਾ. ਹਰਪ੍ਰੀਤ ਸਿੰਘ ਸੋਢੀ ਸੀਨਿਅਰ ਮਾਈਕੋਲੋਜਿਸਟ
ਡਿਗਰੀ	:	ਐੱਮ.ਐੱਸ-ਸੀ.
ਡਿਗਰੀ ਨਾਲ ਸਨਮਾਨਿਤ ਕਰਨ ਦਾ ਸਾਲ	:	2019
ਖੋਜ ਪੱਤਰ ਵਿੱਚ ਕੁੱਲ ਪੰਨੇ	:	62 + ਅੰਤਿਕਾ (vii)
ਯੂਨੀਵਰਸਿਟੀ ਦਾ ਨਾਮ	:	ਪੰਜਾਬ ਖੇਤੀਬਾੜੀ ਯੂਨੀਵਰਸਿਟੀ, ਲੁਧਿਆਣਾ-141 004 ਪੰਜਾਬ, ਭਾਰਤ ।

ਸਾਰ-ਅੰਸ਼

ਕੈਲੋਸਾਇਬ ਇੰਡੀਕਾ ਭਾਰਤੀ ਮੂਲ ਦੀ ਇੱਕ ਟਰੋਪੀਕਲ ਖੁੰਭ ਹੈ ਜੋ 28-35°C ਤਾਪਮਾਨ ਵਿਚਕਾਰ ਬਹੁਤ ਵਧੀਆ ਉੱਗਦੀ ਹੈ। *ਕੈਲੋਸਾਇਬ ਇੰਡੀਕਾ* ਸਟਰੇਨ ਨੂੰ ਮਾਈਸੀਲੀਅਲ ਵਾਧੇ, ਐਕਸਟਰਾਸੋਲੂਲਰ ਇੰਜ਼ਾਇਮ ਉਤਪਾਦਨ ਸਮਰੱਥਾ ਅਤੇ ਝਾੜ ਸਮਰੱਥਾ ਲਈ ਪਰਖੇ ਗਏ। *ਕੈਲੋਸਾਇਬ ਇੰਡੀਕਾ* Ci-01 ਤੋਂ Ci-09 ਦੀ 9 ਸਟਰੇਨ ਅਤੇ ਮੈਕਰੋਸਾਇਬ PDA, CYM, MMM ਉੱਪਰ ਅਤੇ ਇਹਨਾਂ ਦੀ ਝੱਗ ਉੱਪਰ 30±2°C ਤੇ ਉਗਾਏ ਗਏ। 10 ਦਿਨਾਂ ਬਾਅਦ ਸਭ ਤੋਂ ਵੱਧ ਰੇਡੀਅਲ ਵਾਧ PDA Ci-06 (87.6 mm) ਅਤੇ Ci-09 (87 mm) ਸਟਰੇਨ ਵਿੱਚ ਪਾਇਆ ਗਿਆ। ਸਭ ਤੋਂ ਵੱਧ ਬਾਇਓਮਾਸ CYM ਵਿੱਚ Ci-06 (16.1 g/l) ਅਤੇ Ci-07 (15.5 g/l) ਵਿੱਚ ਪਾਇਆ ਗਿਆ। 10 ਦਿਨਾਂ ਬਾਅਦ ਕਣਕ ਦੀ ਤੂੜੀ ਵਿੱਚ ਸਭ ਤੋਂ ਵੱਧ ਲੀਨੀਅਰ ਵਾਧਾ Ci-07 (63 mm) ਅਤੇ Ci-09 (66 mm) ਵਿੱਚ ਪਾਇਆ ਗਿਆ। ਕਣਕ ਦੀ ਤੂੜੀ ਵਿੱਚ ਸਭ ਤੋਂ ਵੱਧ ਬਾਇਓਲਾਜੀਕਲ ਕੁਸ਼ਲਤਾ Ci-06 (57.3%) ਵਿੱਚ ਪਾਈ ਗਈ। ਦੋ ਸਟਰੇਨ Ci-07 (48.5%) ਅਤੇ Ci-09 (45.7%) ਨੇ ਇੱਕ ਦੂਸਰੇ ਦੇ ਬਰਾਬਰ ਝਾੜ ਦਿੱਤਾ। ਸਭ ਤੋਂ ਵੱਧ ਫਰੂਟਿੰਗ ਬਾਡੀ ਸਟਰੇਨ Ci-06 (1552 ਪ੍ਰਤੀ ਕੁਇੰਟਲ ਸੁੱਕੀ ਤੂੜੀ) ਵਿੱਚ ਪਾਈ ਗਈ। ਕਲਚਰ ਫਿਲਟਰੇਟ ਵਿੱਚ ਐਂਡੋਗਲੂਕਾਨੇਜ ਇੰਜ਼ਾਇਮ ਕਿਰਿਆ 1.91-4.42 U/mg, ਐਂਡੋਜ਼ਾਇਲਾਨੇਜ ਇੰਜ਼ਾਇਮ ਕਿਰਿਆ 2.73-4.76 U/mg, ਲੇਕੇਸ ਕਿਰਿਆ 6.94-10.2 U/mg ਦੇ ਵਿਚਕਾਰ ਪਾਈ ਗਈ। ਰੇਸ਼ਾ ਫੈਲਣ ਦੌਰਾਨ ਐਂਡੋਗਲੂਕਾਨੇਜ ਅਤੇ ਐਂਡੋਜਾਇਲਾਨੇਜ ਕਿਰਿਆ 0.58-1.03 U/mg ਅਤੇ 0.74 ਤੋਂ 2.11 U/mg ਦੇ ਵਿਚਕਾਰ ਸੀ। ਜਦਕਿ ਲੇਕੇਸ ਕਿਰਿਆ 1.51-4.54 U/mg ਪਿਨਹੈਡ ਦੌਰਾਨ ਐਂਡੋਗਲੂਕਾਨੇਜ ਅਤੇ ਐਂਡੋਜਾਇਲਾਨੇਜ਼ ਕਿਰਿਆ 0.61-1.59 U/mg ਅਤੇ 1.57-2.96 U/mg ਦੇ ਵਿਚਕਾਰ ਸੀ ਜਦਕਿ ਲੇਕੇਸ ਕਿਰਿਆ 6.53-10.6 U/mg ਦੇ ਵਿਚਕਾਰ ਸੀ। ਫਰੂਟਿੰਗ ਹਿੱਸਿਆਂ ਵਿੱਚ ਸਟਰੇਨ ਦੀ ਐਂਡੋਗਲੂਕਾਨੇਜ ਅਤੇ ਐਂਡੋਜਾਇਲਾਨੇਜ ਕਿਰਿਆ 0.94-1.88 U/mg ਅਤੇ 0.93-2.36 U/mg ਦੇ ਵਿਚਕਾਰ ਅਤੇ ਲੇਕੇਸ ਕਿਰਿਆ 5.43-9.21 U/mg ਦੇ ਵਿਚਕਾਰ ਸੀ। *ਕੈਲੋਸਾਇਬ ਇੰਡੀਕਾ* ਦੀ ਸਟਰੇਨ Ci-03, Ci-06, Ci-07 ਅਤੇ Ci-09 ਵਿੱਚ ਝਾੜ ਅਤੇ ਬਾਇਓਮਾਸ ਅਤੇ ਐਂਡੋਗਲੂਕਾਨੇਜ ਵਿੱਚ ਸਾਕਾਰਾਤਮਕ ਸਹਿਸਬੰਧ ਪਾਇਆ ਗਿਆ।

ਮੁੱਖ ਸ਼ਬਦ: ਬਾਇਓਮਾਸ, *ਕੈਲੋਸਾਇਬ ਇੰਡੀਕਾ* ਸਟਰੇਨਸ, ਇੰਜ਼ਾਇਮ, ਝਾੜ, ।

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿੱਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

CHAPTER I

INTRODUCTION

Mushrooms are reproductive structures of fungi belonging to Basidiomycota. They are comprised of various shapes, size, colour, appearance and edibility. Mushrooms derive their nutrients from organic materials referred to as substrate which may be a byproduct from industry, household and agriculture. Mushrooms have been recognized as an alternative good quality food source due to their high nutritional and medicinal values. Edible mushroom production has been developed industrially in more than 80 countries and in the last 20 years production has increased rapidly to 37.3 billion kg with annual growth rate of above (4%) (Sharma 2019 pers. comm.). About 14000 different varieties of mushroom are available in nature, but only 100 varieties of mushroom have been domesticated and about 60 varieties of mushroom are grown commercially which are produced on industrial scale including *Agaricus bisporus* (Button mushroom), *Pleurotus Spp*. (Oyster mushroom), *Lentinus edodes* (Shiitake mushroom), *Calocybe indica* (Milky mushroom), *Volvariella volvacea* (Paddy straw mushroom) and *Auricularia auricula* (Wood ear mushroom). Predominantly in Punjab five different varieties of mushroom are cultivated *Agaricus bisporus* (Button mushroom), *Calocybe indica* (Milky mushroom), *Lentinula edodes* (Shiitake mushroom), *Pleurotus spp.* (Oyster mushroom), *Volvariella spp.* (Paddy straw mushroom).

Calocybe indica commonly known as milky mushroom belongs to Kingdom: Fungi, Phylum:Basidiomycota, class-Basidiomycetes, order:Agaricales and family: Tricholomataceae (Krick *et al* 2001). It is an edible fungal species belonging to India. *Calocybe* genus have about 40 species of mushroom. *Calocybe indica* can grow in grasslands, road verges and farm fields. Purkayastha and Chandra (1974) reported this mushroom in India for the first time and studied the morphology of *Calocybe indica*. The milky mushroom has a large sized fruiting bodies and very attractive white sporophore with delicious flavor and consumer's acceptability. During summer months when other established varieties cannot grow, cultivation of milky mushroom was suitable due to its ability to grow at a temperature above 30^{o}C. The pileus was having diameter of 10.0-14.0 cm. Cap showed convexity at initial stage and later it expanded and became flat, stipe was centric but sometimes it could be eccentric. The length of stipe was about 10 cm and its width varied from top to base. At the top it was 1.8 cm thick, 3.5 cm in the middle and 2.4 cm at the base. Basidiospores were white, hyaline, broadly ellipsoidal, thin walled, without ornamentation, with prominent apiculus, non-amyloid, 5.9-6.8 x 4.2-5.1 µm (Purkayastha and Chandra 1976).

Calocybe indica generally grows on substrate rich in organic material in tropical region. This Mushroom grow well in between the month of May and September**.** It is primarily cultivated in India as it is a heat loving mushroom require a temperature of about

25-35°C and relative humidity of 80-90%. As the fungus has effective enzyme system for the lignocellulosic substrate degradation, it can be cultivated on wide range of agro wastes and byproduct. It can be grown on cereal straws like paddy, wheat, ragi, maize/cotton stalks and leaves, sugarcane bagasse, jute and cotton wastes, tea coffee waste etc (Tewari 2004). It has been studied that fungus could colonize paddy straw, maize stalks and sorghum stalks with high levels of exo and endo-cellulases. For its commercial cultivation we need cylindrical polythene bag beds 15*30 cm size. The best casing soil for *Calocybe indica* is clay loam soil pH 8.4 (Bokaria *et al* 2014). As the optimum temperature for the growth of this mushroom is (25-35°C), the climate of Punjab provides good scope for its cultivation during (May-September). Extract of milky white mushroom have anti lipid peroxidation and anti-hyperglycemic effect. Because of its high productivity and high shelf life as compared to other mushrooms, milky mushroom could play an important role in fulfilling the growing market demands in future. Presently it is being cultivated on large scale in Tamil Nadu, Andhra Pradesh and Karnataka (Jalali *et al* 2003).

Nutritive value of *C. indica* is comparable with other edible mushrooms. Due to high fiber content, low concentration of fats and carbohydrates; nutritionist recommend this mushroom as a good diet for people with hyperacidity, cardiac and diabetes problem. Due to its good rehydration qualities; the dried produce attains 80-90 per cent original weight in 10-15 minutes of soaking. Therefore *C. indica* has a high prospect in processing industries. (17.69 %) protein, (4.1 %) fat, (3.4 %) crude fiber and (64.26 %) of carbohydrates and eleven amino acids has been recorded in *C. indica* (Doshi *et al* 1988). *C. indica* is rich in protein, lipids, mineral, fiber, carbohydrate and essential amino acids (Alam *et al* 2008, Mallavadhani *et al* 2006). It is a good source of thiamine, riboflavin, nicotinic acid, pyridoxine, biotin, and ascorbic acid (Breene 1990). On dry weight basis, mushrooms have high protein contents compared with other common food (Crisan and Sands 1987, Chang 1980) and they also have medicinal values.

Lignocellulose is composed of cellulose, hemicellulose and lignin. Cellulose is a linear homopolymer of glucose units linked with β-1,4-glucosidic bonds whereas hemicelluloses are heteropolysaccharides consisting of short branched chains of hexoses, e.g. mannose and pentoses such as xylose. Lignin contain three pioneer aromatic alcohols; coniferyl, sinapyl and p-coumaryl alcohols which by linking to hemicellulose and cellulose act as barrier. Fruiting and growth depend on the ability of mushroom to attack these constituent as nutrient sources. Laccase is a copper containing blue oxidase; they are polyphenol oxidase contain in its active site four copper ions. They are produced by many fungi and contain cooper in their sites (Hofrichter and Ullrich 2010).Fungal laccases contribute to several processes such as lignin degradation, sporulation, pigment production, fruiting body formation and plant pathogenesis (Thurston 1994, Mayer and Staples 2002).

Lignocellulolytic enzymes contain protein which are extracellular containing hydrolytic enzymes (cellulases, hemicellulases, proteases, chitinases, amylases) and ligninolytic enzymes (oxidases and peroxidases). It has been observed that loss of cellulose, hemicelluloses and lignin from the biodegradation of waste showed positive correlation with cellulases such as endo-â-1,4-glucanase (E.C.3.2.1.91.), exo-1,4-â-glucanase (E.C. 3.2.1.4), xylanase (E.C.3.2.1.8) and laccase (E.C.1.10.3.2) activity of fungus. Lignocellulolytic enzymes have great future prospect in research towards physiological regulation of enzymes, use of different fungal strains, use of inducers to increase production, use of different fungal strain. In the past years there is advancement in the lignocellulolytic enzymes research and development from cellular to molecular and micro to nano levels (Mtui 2012). For the cultivation of *Calocybe indica* various kinds of substrate have been used such as wheat straw, paddy straw, bajra, sugarcane bagasse, cotton and jute waste. Mushroom Strains can be grown on potato dextrose agar (PDA), complete yeast extract (CYM) and mushroom minimal media (MMM) at 30^{o}C for 10 days.

The investigation related to molecular basis for mating provides access to the use of mating type genes in order to facilitate breeding. The genes of mating type control formation of the dikaryon from two haploid strains. Under the optimum environment condition only dikaryon is fertile and able to form mushroom. The gene present in the A loci lead to the formation of transcription factors. Heterodimerization of two proteins of different alleles result in formation of active transcription factors. The B gene form a pheromone and receptor system that helps the fungi to initiate nuclear migration (Ohm *et al* 2010). Interspecies and interstrain hybridization has led to development of a number of hybrids (Fritsche 1983, Yu and Chang 1987, Eichlerova and Homolka 1999, Kapoor *et al* 1996, Kumar 2010).

The strains of mushroom play important role in the productivity and quality of mushroom. Different strains have steadily been produced for the purpose of higher yield and improved variety attributes such as pathogen resistance and adaptability to wide range of temperature (Kumara and Edirimanna 2009). For the growth of mushroom mycelium, growth media is one of the most important factors. Different media such as yeast extract agar, potato dextrose agar, malt extract agar is used for the growth of Mycelium. Under uniform environmental condition a degree of radial symmetry was observed in fungal colonies. Under different physiological condition the rate and the extent of growth has been considered a useful scale for the growth of fungus. As far its cultivation technology is concerned, a lot of work has been done (Kaur 2003, Mangat 2005, Kaur 2010 and Redhu 2010) but no efficient work has yet been undertaken for its genetic improvement. Smaller size is desirable for the development of new strain. Wheat straw is the best substrate for the cultivation of *C. indica*, but it is uneconomical due to its high cost. The cultivation technology still needs to be improved with the preferred use of paddy straw as a substrate to make the process profitable

and economical.

The proposed objectives for the present investigation are given below:

i. Evaluation of *Calocybe indica* strains for cellulase, xylanase and laccase enzyme producing capability.

ii. Observe a possible correlation of the enzyme production from different strains with their yield potential.

CHAPTER II

REVIEW OF LITERATURE

The literature pertaining to the present study has been reviewed under the following headings:

2.1 INTRODUCTION

Mushrooms are known for their high quality/quantity of protein per unit time and area along with to nutritive, medicinal attributes besides unique flavour and texture. The mushroom production and consumption are growing very fast throughout the World. World production of mushrooms is estimated about 37.3 billion kg and the annual growth rate is still above 4% (Sharma pers.comm.). The total mushroom production in India at present is approximately 0.13 billion kg. The average growth rate of 4.3% per annum has been recorded from 2010-2017. Out of total production, the production white button mushroom is maximum with 73% followed by oyster mushroom (16%), paddy straw mushroom (7%) and milky mushroom (3%) (Sharma *et al* 2017).

Fungi have been known from the fossil records as far back in time. Probably the mushroom was used for medicinal purpose by the prehistoric humans. Some of the early civilization like Romans, Chinese, Greeks and Mexicans admire mushroom as a delicacy. In 1600's biggest advancement in mushroom cultivation came in France when *Agaricus bisporus* was cultivated upon a composted substrate. Milky mushroom belongs to Kingdom: Fungi, Phylum: Basidiomycota class- Basidiomycetes order: Agaricales and family: Tricholomataceae (Krick *et al* 2001). *Calocybe indica* now become the third commercially

grown mushroom in India after oyster and button mushroom (Beelman *et al*1989). This mushroom contains higher amounts of B2, niacin and rich in minerals like Na, P, K. Any kind of agricultural waste is used as a substrate for the cultivation of milky mushroom like wheat straw, paddy straw, ragi maize, bajra, cotton stalks and leaves. Doshi *et al* (1987) presented the pectinolytic and cellulolytic enzyme production from the fruit bodies of the *Calocybe indica.* Milky mushroom grows well in artificial indoor condition on uncomposted substrate. Due to suitable environment and availability of substrate, milky mushroom grows well in the tropical regions. The Milky mushroom is easy to cultivate, need less investment and result in very attractive fruiting body. Some small-scale growers prefer this mushroom to grow because, it can be stored upto (5-7) days without refrigerator at room temperature. Purkayastha and Chandra (1976) reported that when culture of *Calocybe indica* kept in dark, gave no fruiting bodies but when the culture was exposed to diffused light, considerable elongation of stipe occurred. Low light intensity of about 800 lux favoured spawn run. But increased light intensity of about 1600 lux favoured increased yield of mushroom (Purkayastha *et al* 1976).

2.2 BIOMASS PRODUCTION

For the growth of fungal mycelium growth, media is one the most important factor because it supplies necessary nutrients for the growth. Different media are used for the growth of fungal mycelium such a yeast extract media, potato dextrose agar, malt extract etc. Under uniform environmental condition a degree of radial symmetry has been observed in fungal colonies (Edelstein and Segel 1983). Under different physiological condition the rate and extent of growth (diameter) has been considered a useful scale for growth of fungus. In another experiment to see the radial growth of milky mushroom, 10 media were prepared. For preparation of different media, 200 grams of substrate were washed in water 2-3 times and then boiled in 500 ml of distilled water for 45 min, allowed to pass the liquid suspension through a muslin cloth. Dextrose and agar each of 20 gm were added to substrate. Standard method was used to prepare the media for PDA and MEA. When the colony covered the entire plate than the observation for the radial growth were measured by Kumar *et al* (2011).

For the growth of fungus under different physiological conditions, the rate and the extent of growth (diameter) has been considered a useful criterion (Lonergen *et al* 1993). Culture media permits acceleration of mycelial growth, ensure quality and production (Chang 2001). *Calocybe indica, Pleurotus florida and Agaricus bisporus* were grown on potato dextrose agar, malt extract agar and yeast potato dextrose extract agar medium containing indole acetic acid (IAA) and naphthalene acetic acid NAA @ 0, 1, 5, 10 and 20 ppm. The best mycelium growth (9.35cm) was observed in the YPDA (yeast potato dextrose agar medium) (Uddin *et al* 2012). The growth of *Calocybe indica* has also been studied on different broth media (potato dextrose, basal maltova, basal malt extract and czapeck's dox) and maximum

biomass (0.22g/100ml) recorded on potato dextrose broth (Phutela and Phutela 2012). It was observed that the highest linear growth (87mm) was observed in response to the tissue culture obtained from the mushroom which consisted of stipe with well differentiate pileus (Pani 2016)

2.3 OCCURRENCE AND MORPHOLOGY

The milky mushroom *Calocybe* belongs to family *Ticholomataceae* of the order *Agaricales.* The three species of *Calocybe* namely, *C. ionides, C. carnea* and *C. gambosa* had been collected from forests but there was no report as yet of their domestication (Dickinson and Lucas 1979). *Calocybe indica* is a tropical milky white mushroom (P&C) that was first identified in India in West Bengal. It can be grown at high temperature range of about (30-38)^{0}C and was not commercially grown until 1998. Krishnamoorthy and Muthuswamy (1997) rediscovered the fungus from Tamil Nadu and commercially produced this mushroom for the first time. This mushroom was morphologically similar to *Agaricus bisporus*, therefore it was quite popular in southern states of India and now gained popularity in other countries too. It is one of the best edible mushrooms.

Purkayastha and Chandra (1974) studied the morphology of *Calocybe indica*. The pileus was having diameter of 10.0-14.0 cm. Cap showed convexity at initial stage and later it expanded and became flat, stipe was centric but sometimes it could be eccentric. The length of stipe was about 10 cm and its width varied from top to base. At the top it was 1.8 cm thick, 3.5 cm in the middle and 2.4 cm at the base. Basidiospores were white, hyaline, broadly ellipsoidal, thin walled, without ornamentation, with prominent apiculus, non-amyloid, 5.9-6.8 x 4.2-5.1 µm (Purkayastha and Chandra 1976). Pandey and Tiwari (2003) reported the sporophore to show large milky white pileus, thick fibrous and cylindrical stipe and no annulus. The gills were giving white spore print. The sporophore often occurred in clusters.

2.4 SUBSTRATE PRE-TREATMENT

Besides disinfection, pre-treatment of substrate helped reduce C:N ratio, change nature of cellulosic complex and phenol content, thereby, enhanced the suitability of the substrate for cultivation of milky mushroom. To avoid contamination of moulds pasteurization of straw was an important step. For good mycelium run of milky mushroom, sun drying was done to attain moisture content upto 70-75%. For the mushroom growers in North East India, sun drying was a difficult task during May to July. So, sterilization techniques and boiling of straw as a pasteurization to avoid contamination of moulds had been adapted (Upamanya and Rathaiah 2000, Pani and Das 1998 and Saxena and Rai 1992).

Soaking of substrate in hot water (60-80°C) for 1-3 hrs had been found to be the best pre-treatment for better yield (Purkayastha 1984, Krishnamoorthy and Muthusamy 1997, Theradimani *et al* 2001). Doshi *et al* (1989) cultivated mushroom on paddy or wheat straw sterilized by either chemical treatment or steam or hot water. Using formalin @ 500 ppm and

bavistin @ 75 ppm, 700g fresh mushroom/kg on chemically sterilized wheat/paddy straw was obtained by Doshi and Sharma (1995). According to Sharma and Upadhyay (1998) this mushroom was reported to grow on both composted or freshly chopped paddy straw soaked in water and subsequently dipped in hot water (65-80ºC) for 2-3 hrs.

Among steam, hot water and chemical sterilization, the best growth was seen in steam (15 lbs for 1 hr) and also observed higher yields with 6 days old compost (Pandey 1998). Kaur (2003) found that help of sterilized straw obtained a maximum yield of 478.0 g/2 kg substrate. It has also been reported that autoclaving method of sterilization favored higher rate of degradation than steaming and fungicide soaking (Anandh and Prakasam 2002). While Bhavana and Thomas (2002) were able to produce an average mushroom yield of 486.8 g/bag with a B. E. (81.2 %) and individual mushroom weight of 99.5g on coirpith. Retted, non-retted and composted coirpith were used as substrates for *C. indica*. The highest yield and sporophore production recorded with non-retted coirpith in combination with 75% rice straw. Retted coirpith showed a biological efficiency of 60% and composted coirpith proved to be inefficient as a substrate for mushroom production (Salam *et al* 2004). It has been 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* (Mangat 2005). For the cultivation of milky mushroom, pasteurization method was found to be the best followed by hot water treatment and autoclaving among the various methods of substrate treatment. Chemical sterilization didn't result in spawn run (Sharma *et al* 2006).

2.5 CULTIVATION STUDY

Calocybe indica, being new to the mushroom industry was cultivated on different substrate to check the ability of different substrate such as paddy straw, wheat straw, soybean straw, cotton waste and sugarcane bagasse for the cultivation. The minimum days of spawn run were recorded on wheat straw for about 15 days, pinhead formation in 28 days, for first harvest 33 days with highest number of fruit bodies 24 (Vijaykumar *et al* 2014). Wheat straw was employed for evaluating yield of composted and non-composted substrate using sterilized and unsterilized casing soil. It was observed that the compost gave highest yield in sterilized casing material. Uncomposted substrates with sterilized casing material gave adequate yield than unsterilized casing material (Chakravarty *et al* 1981). Some basal substrates like wheat, maize and sorghum straws, maize, rice and sorghum meal and wheat bran were evaluated for the cultivation of *C. indica*. They reported wheat straw as a suitable substrate for the cultivation of this mushroom (Doshi *et al* 1989). About 700g fresh mushroom per kg was obtained on chemically sterilized wheat/paddy straw on dry weight basis (Doshi and Sharma 1995). It was reported that cultivation of *C. indica* on wheat straw, using sorghum spawn and sandy loam soil (40-50% sand) as a casing medium, with biological efficiency (B.E.) of 60-100% (Pandey and Tiwari 1993). On the other hand, Kochuthresiamma (1998) suggested

rubber wood saw dust as an ideal substrate for *C. indica*. Kaur (2003) and Mangat (2005) reported the suitability of wheat straw for cultivation of *C. indica* under Punjab conditions. Tandon and Sharma (2006) reported cultivation of *C. indica* on four different substrates: wheat straw, paddy straw, coconut coir and saw dust. Mycelium did not spread at all on saw dust. Though the spawn run was fast in coconut coir substrate but it could not result in higher mushroom yield (296 g) whereas paddy straw as a substrate gave yield of 480g. The highest yield was obtained in bags where wheat straw was used as a substrate. The possibility of growing milky mushroom on rubber wood saw dust was done by Joseph *et al* (1998). The mushroom grew well and produced a fresh weight of 533g per kilogram of saw dust. Bhavana and Thomas (2002) used fermented coir pith as a substrate for the cultivation of *Calocybe indica* which resulted in biological efficiency of (81.16%). Kochuthresiamma (1998) suggested rubber wood saw dust as an ideal substrate for *C. indica*. Availability of substrate generally depends upon the season of cultivation.

Rice straw supplemented with wheat bran or maize meal was found to be the most suitable substrate for production of *C. indica* (Purkayastha and Roy 1982, Purkayastha 1984). Sivaprakasam and Shanmugam (1984) suggested a polythene wrapped bed method using paddy straw for the cultivation and improved yield of milky mushroom. Higher yields have been reported from paddy straw and maize stalks (356.5g and 354.3g respectively per bed containing 250g of substrate on dry weight basis) among ten different substrates. Krishnamoorthy *et al* (2000) evaluated nine substrates viz. paddy straw, sorghum stalks, maize stalks, sugarcane bagasse, vativer grass, groundnut, palmrosa grass, soyabean hay and black gram hay for cultivation of *C. indica* (strain APK_2). The mycelium of this mushroom was reported to grow both on composted or freshly chopped paddy straw soaked in water and subsequently dipped in hot water (65-80°C) for 2-3 hrs (Sharma and Upadhyay 1998). Substrates like sorghum sticks, sugarcane bagasse, groundnut hulls, soybean hay and black gram hay can also be used at 65-75 per cent moisture level. Singh *et al* (2009) reported higher yield from paddy straw followed by wheat straw, sugarcane bagasse and gram straw. Amin *et al* (2010) reported rice straw as the best substrate for cultivating milky white mushroom. Pani (2011) prepared spawn with the help of wheat grain and stored the spawn for many days (14-60) days, found that the best yield of milky mushroom was obtained using 21 days old spawn. He proposed that prolonged storage of spawn reduced the yield and productivity.

Growth regulator also played important role in the development of milky mushroom. Several growth regulators were tested for their effect on sporophore size and the yield of mushroom. It was clearly indicated in the result that gibberllic acid at 40 ppm increase the yield of mushroom. Spraying of kinetin and GA result in high weight of the mushroom (Pani 2011b). It has been reported that the composition of mushroom (carbohydrate, protein, fats, fibres, moisture content) depends upon the substrate and the supplement used for the

cultivation of mushroom. It was found that the composition of fats, carbohydrates and ash contents were high in milky white mushroom, results in increasing its total calorific value (50.03 kcal/100 grams). It has been studied that substrate like coconut coir pith compost, paddy straw compost and saw dust did not favour the growth of milky mushroom. Protein (20.2%) was recorded from the caps of milky white mushroom on the basis of dry weight (Doshi *et al* 1988). In a medium sized milky mushroom, it was reported it was reported 32.2% protein (Krishnamoorthy and Muthuswamy 1997).

Lignocellulosic materials do require different supplements or additives with sufficient amounts of nitrogen, phosphate, potassium, and vitamins for better growth and yield of mushrooms (Mangat *et al* 2008). Organic and inorganic supplement added to the substrate during cultivation are known to influence the yield of various species of mushroom (Marimuthu and Krishnamoorthy 1991). Various experiments conducted by Schisler and Sinden (1962) have shown that organic supplements enhanced mushroom yield. Nitrogen supplementation was also an important factor for developing fruiting bodies on mushrooms (Naraian *et al* 2009). Rice bran is one of the most popular organic substrate supplements for growing a number of edible mushrooms in Asia (Peng *et al* 2000). Supplementing oil seed cakes and rice straw with cereal bran increases the yield and quality of oyster mushrooms (Shashirekha *et al* 2002). Milky mushroom was successfully cultivated under *in vitro* conditions on various substrates. Earlier fruitification was recorded in Basal soil/sand medium supplemented with wheat bran and maize meal were found the best than other media tried by Purkayastha and Chandra (1976). Purkayastha and Nayak (1977) studied the effect of supplementation of cereals, millets, pulses, and oilseed to the soil sand maize meal compost on the yield and protein content of sporocarp. Maximum production of fruit bodies was found from maize meal added at 5 per cent concentration to the medium. The fruiting bodies had 13 per cent protein. They were of the opinion that the mushroom preferred P&K but not N. These workers also observed that the yield of the mushroom was reduced at higher N levels. Doshi *et al* (1989) also reported significantly higher weight of sporophores obtained from maize meal substrate supplemented with NPK (1:1:1) followed by P and K.

Increased (B. E.) with wheat straw supplemented with rice husk, maize meal and coconut husk at the time of spawning was recorded. However, wheat bran, cotton seed, banana pseudo stem, dehydrated Lucerne, cotton linter and termitorium soil performed well when added at the time of casing (Sharma *et al* 1994). Investigations showed an increase in yield by (44.8 %) with 10 per cent rice bran, 37.9 per cent with 5 per cent oat meal, 33.2 per cent with 10 per cent crushed sorghum grains, 22.4 per cent with 15 per cent boiled sorghum grains and 25.9 per cent with 5 per cent boiled maize grains supplementation done at the time of spawning (Pandey 1998) . According to report by Mangat (2005) supplementation of rice bran, wheat bran and cotton seed meal @ 1-4 per cent at the time of spawning, had no effect

on the improvement in yield. Actually, increased incidence of competitor moulds was recorded with addition of supplementation. The highest biological and economic yield was obtained when 30% maize powder was used as a supplement for rice straw substrate (Alam *et al* 2010).

Spent mushroom substrate were currently being implemented internationally for its beneficial use. It has been studied that spent mushroom substrate was rich in organic matter which added nutrient to the soil and promote plant growth (Rinker 2002). It also improved the nutrient poor soil by improving its texture and water holding capacity (Beyer 1996). The experiments were conducted to find the role of media, temperature and pH level for the growth of different strain of milky mushroom. Strains CI-3, CI-4, CI-5, CI-10 showed fluffy growth during experiment while CI-6 exhibited cottony appearance. Creamy white appearance was observed in the colonies of CI-3, CI-10. In CI-3, CI-4, CI-6, CI-10 circular colonies were observed. Irregular shaped colony was observed in CI-5 strain. Maximum stipe length and pileus weight was observed in the strain CI-6 (25g/fruit body FB) followed by CI-4 (23g/FB), CI-10 (22g/FB). It has been observed that at the temperature of 28 C and 7.5 pH on 8th day after inoculation, all strain showed more mycelium growth (Kumar *et al* 2011). Strain improvement can also be achieved by selection and hybridization.

2.6 CASING

For the cultivation of *Calocybe indica,* casing is an important cultural practice. Workers have suggested the use of different soils like clay loam soil, garden soil, sand, peat moss, biogas slurry, farm yard manure and coir pith in different combination with a pH range of 7.0-8.4 for casing (Chakravarty *et al* 1981, Purkayastha 1984, Krishnamoorthy and Muthuswamy 1997, Krishnamoorthy *et al* 2002). Clay loamy soil with the pH of 8.4 gave the maximum yield of 288 g of mushroom per bed with higher number of fruit bodies. The second-best soil for the production of mushroom was Peat soil with the (pH 6.0). It has been reported that two-year-old cow dung patties with casing thickness of 1" gave higher yield. They also suggested that pH of 7.0 for casing soil prevent the attack of moulds. (Trivedi *et al* 1991 and Doshi *et al* 1993). Sharma et (1997) reported that two-year-old cow dung patties and biogas slurry gave 98.7% and 100% biological efficiency. Purkayastha *et al* (1976) did casing of *C. indica* beds for the first time with mixture of soil and sand (1:1) and calcium carbonate (12%) @ 2 kg of casing soil per tray during production of *Calocybe indica*. Sharma and Upadhyay (1998) studied that garden soil or dried loam soil and sand (1:1; v/v) and addition of calcium carbonate @ 12 per cent of the soil: sand mixture proved to be the best casing mixture. It had been reported that one-year old farm yard manure as the best casing mixture for the commercial cultivation of *C. indica* (Kaur 2003). Tandon and Sharma (2006) recorded the casing materials, FYM +loam soil (3:1; v/v), spent compost and loam soil gave higher mushroom yield of 540, 489.6 and 438.3 g per 0.5 kg dry substrate, followed by

farmyard manure (329.3 g) and the lowest yield was recorded in vermicompost gave (270g).

2.7 LIGNOCELLULYTIC ENZYME

Lignocellulosic material of plant consists of three main components, namely cellulose, hemicellulose and lignin. The capacity of the mushroom to grow on a particular substrate, thus lies in its ability to degrade it, which in turn is decided by the repertoire of enzymes that a mushroom species possess. The comparative intensity of the activities of different enzymes, therefore, decide its preference for a particular substrate (Rai and Saxena 1990, Cavazzoni and Manzoni 1994). Lignocellulolytic enzymes are biocatalysts which are responsible for Breakdown of lignin and cellulosic materials (Mtui 2012). Different *C. indica* strains are presented to show much more diversity in their adaptation to the wide range of the temperature and pH levels. Krishnamoorthy *et al* (1997) found a potential strain of *Calocybe indica* present in a sugarcane field near Coimbatore. Later, it was discovered as a new variety called APK-2 from Tamil Nadu Agriculture University and cultivation of this variety was started under artificial condition. According to previous research workers lignocellulose degrading mushroom species are used in solid state fermentation applications for biodegradation and bioremediation of hazardous compounds (Perez *et al* 2007). Use of crop residues depends on the capability of the fungus to produce a lignocellulolytic enzyme complex (Buswell *et al* 1996).

Cellulose is a linear homopolymer of glucose units linked with β-1,4-glucosidic bonds. The hydrolytic breakdown of cellulose in nature is catalysed by extracellular enzymes: cellobiohydrolases, endoglucanases and β-glucosidases produced by microorganisms (Kuhad *et al* 1997, Teeri 1997). The ability to degrade cellulose complex and insoluble carbohydrates can be determined by the level of endoglucanase enzyme which facilitate the degradation of cellulose into soluble carbon compounds required as nutrient for the fungal mycelium growth (Manning and Wood 1983). High cellulase activity of fungal growth has been recorded in spawn bottles after 25 days when compared with growth at 11 days in case of *C. indica* Doshi *et al* (1987). In a study on the enzyme related biodegradative potential of *C. indica*, it has been found that there is a direct relationship between the productions of cellulases and yield. They observed that fungus colonized paddy straw, sorghum stalks and maize stalks showed high level of exocellulases and endocelllulases (Krishnamoorthy *et al* 2002). Enzymatic studies by Anandh and Prakasam (2002) unveil that cellulase activity increased upto second harvest in the cased beds and is associated with the initiation of mushroom flushes.

Hemicelluloses are heteropolysaccharides consisting of short branched chains of hexoses, e.g. mannose and pentoses such as xylose (Kuhad *et al* 1997). Endoxylanases and Endomannanases are the major hemicellulose degrading enzymes. Xylanases are typical endoenzymes and attack the xylan chain in a random manner, releasing short oligomers, xylobiose and even xylose. Akhmedova (1994) studied composition and accumulation of

individual extracellular enzymes of the cellulase, hemicellulase and laccase in *Pleurotus osteratus*. It has been observed that all the three high quantity enzymes produced by fungus led to better substrate utilization and high yield potential. Activity changes has been observed in several extracellular enzymes in different growth periods of *P. citrinopileatus* cultivated on fermented cotton seed hull compost (Kalmis *et al* 2008). Activity peaks of endoglucanase, filter paper activity and hemicellulase activity appeared in periods between primordial formation and fruit body maturity.

The capacity of fungus to produce lignocellulolytic enzyme complex depends upon the use of crop residues (Buswell *et al* 1996). The complex includes the oxidative enzymes laccase and manganese peroxide. It also contain hydrolytic enzymes xylanase and cellulase involved in degradation of hemicellulose and cellulose degradation. Lignocellulolytic enzymes act as biocatalyst responsible for the degradation of lignin and cellulosic material. Municipal solid waste and solid waste have been used as substrate to isolate two cellulase producing fungi (*Aspergillus niger*and *Trichoderma sp.*). It has been observed that cellulase activity increased during primordial formation and fruiting (Ohga *et al* 1999). It has been observed that in *Pleurotus sojor-caju* cellulase and xylanase were elaborate (Madan and Bisaria 1983). It has been observed that the cellulase activity was high for *Pleurotus ostreatus* as compared to *Calocybe indica*. The maximum cellulase production observed in *C. indica* is (0.652 U/ml) at 70 C (Karthikeyan 2015). Cellulase activity in *C. indica* (0.49 U/ml) and *Pleurotus ostreatus* (0.60U/ml) was observed maximum at 70°C (Karthikeyan 2015). The maximum Xylanase activity was observed in the strain VvS-4 (147 U/100ml) (Choudhary *et al* 2009). Maximum activity of endoglucanase and endoxylanase was recorded for the strain Ci-6 (2.72,2.29 U/mg) and Ci-2 (1.70,1.88) (Redhu 2010).

Laccase is a copper containing blue oxidase; they are polyphenol oxidase contain in its active site four copper ions. They are produced by many fungi and contain cooper in their sites (Hofrichter and Ullrich 2010). The laccase activity was found to increase with the mycelial growth in *Pleurotus sajor-caju* (Tan and Wahab 1997). It is proved from the reports of (Palmieri *et al* 1997) that laccase has been the most studied enzyme. In the presence of a mediator such as 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonate) (ABTS) or 1-hydroxybenzotriazole, laccases are capable of oxidation of nonphenolic compounds (Eggert *et al* 1996). Laccases were first isolated from plants but are also present in fungi and some bacteria (Thurston 1994). In plants laccases helps in formation of lignin by polymerization reactions. Fungal laccases contribute to several processes such as lignin degradation, sporulation, pigment production, fruiting body formation and plant pathogenesis (Thurston 1994, Mayer and Staples 2002). It has been reported that wheat straw produced maximum laccase (187.38 u/ml) after 24 days at 30°C (Upadhyay and Tripathi 2014). Laccases are usually the first ligninolytic enzymes secreted to the surrounding media by the fungus

(Choudhary *et al* 2009). It has been observed that absence of laccase and cellobiase activity was much higher than than CMCase and FPase in cultural filtrates of six strains of *Volvariella*. Of the three strains of *C. indica*, Ci-1, Ci-2 and Ci-3, only Ci-2 showed detectable laccase activity (Mangat 2005). Krishnamoorthy *et al* (2002) reported high laccase production of *C. indica* when grown on black gram hay, soybean hay, paddy straw, groundnut haulms.

2.8 POSTHARVEST STUDIES

2.8.1 Nutritional status

Nutritive value of *C. indica* is comparable with other edible mushrooms. Due to its high fiber content, low concentration of fats and carbohydrates; nutritionist recommend this mushroom as a good diet for people with hyperacidity, cardiac and diabetes problem. Due to its good rehydration qualities; the dried produce attains 80-90 per cent original weight in 10-15 minutes of soaking. Therefore *C. indica* has a high prospect in processing industries (Doshi *et al* 1988).

Calocybe indica is rich in protein, lipids, mineral, fiber, carbohydrate and essential amino acids (Alam *et al* 2008, Mallavadhani *et al* 2006). It is a good source of thiamine, riboflavin, nicotinic acid, pyridoxine, biotin, and ascorbic acid (Breene 1990). On dry weight basis, mushrooms have high protein content compared with other common food (Crisan and Sand 1978, Chang 1980) and they also have medicinal values. It has been estimated by Purkayastha and Chandra (1975a) that artificially produced fruit body of *C. indica* developed in 10-week old culture as well as 12-day-old mycelial grown in a culture medium contained 23±0.38 and 19±1.52 % crude protein on dry weight basis.

Five edible fungi have been studied including *Calocybe indica* that contained (19.81%) protein and thirteen amino acids by Purkayastha and Chandra (1976). The protein content of sporocarps cultivated on 28 different substrates has been estimated to show 32.3% of protein content and 41% of crude fibre (Purkayastha and Roy 1982, Krishnamoorthy 2003).The soluble sugar in *C. indica* constitute 22.4 per cent of the total carbohydrate while it was just 12.1 per cent in case of *Pleurotus florida* indicating easy digestibility of *C. indica* (Sivaprakasam *et al* 1986). Calcium and phosphorus content were also high in *C. indica* as compare to other mushrooms (Sivaprakasam and Shanmugam 1984). Doshi *et al* (1988) studied that the dried sporocarp of *C. indica* contains protein (17.69%) and eleven amino acids i.e. alanine, aspartic acid, glutamine, glycine, hydroxyproline, tyrosine, proline, histidine, lysine, threonine and valine. It also contained 4.1 percent fat, 3.4 per cent of crude fiber and 64.26 per cent carbohydrate. Selvi *et al* (2007) studied the amount of vitamin A, C, D, E and reduced glutathione in fresh and dried fruit bodies of *C. indica*. It has been recorded in fresh samples, vitamin A, C, D, E and glutathione were found to be 0.324mg/l, 1.033mg/l, 2.93 mg/l and 0.156 nmoles/g respectively, while in dried form it was 0.215mg/l, 0.4 mg/l,

0.80mg/l and 0.123 nmoles/g respectively, indicating *C. indica* to be rich in non-enzymatic antioxidant property in both fresh and dried forms

Calocybe indica was cultivated on four different agro-residues *viz* paddy straw, coir pith, wood shaving and banana trash. The energy value of substrates based on cellulose, hemicellulose and lignin content was observed maximum in coirpith (466 Kcal per 100 gm) substrate followed by banana trash (435 Kcal per 100 gm), paddy straw (391 Kcal per 100 gm) and wood shavings (380 Kcal per 10 gm values) (Usha 2007).

2.8.2 Postharvest treatments

Like all fleshy fruits and vegetables, Mushrooms are delicate food material in which deterioration sets in within hours. Storage conditions greatly affect the freshness of mushroom including temperature and relative humidity. Fresh mushrooms (*Agaricus bisporus*) lack the protective cuticle and carbohydrate reserves that allow mushrooms to stay fresh during storage, which causes rapid deterioration of mushroom after harvest. Hence, treatments are given to the mushroom to increase their shelf life.

2.8.2.1 Washing

Washing is one of the preliminary processing measures of mushrooms. The washing of pilei in water considerably decreases the quality of stored mushroom products. The delicate cell membrane that separated the enzyme phenol oxidase from the substrata were damaged, causing darkening of pilei (Burton and Noble 1993). Hence mushrooms were washed in solutions of such compounds as metabisulfite, which had a beneficial effect on the whiteness of pilei by inhibiting undesirable changes in colour in order to maintain proper quality of product (Czapski 2002).

The good quality of mushroom products can be obtained by eliminating adverse changes in the colour and texture of pilei (Czapski and Szudyga 2000). Particular attention should be given to minimize weight loss during preliminary processing and canning procedures (Devece *et al* 1999). Washing (Sapers *et al* 2001); blanching (Coskuner and Ozdemir 2000); soaking or vacuum moistening in solutions of compounds preventing the darkening of pilei (Jaworska *et al* 2003) were included in preliminary processing. Among these compounds are table salt, citric acid, L-ascorbic acid, hydrogen peroxide, versenic acid (EDTA), sodium erythorbate, cysteine hydrochloride (Czapski 2002).

2.8.2.2 Blanching

Blanching is another treatment used in processing of mushrooms. This treatment is used to inactivate enzymes by least heat severe treatment that cause discoloration, change in flavor, aroma and removes certain harsh flavor commonly found in mushrooms (Girdhari *et al* 1998). It has been studied that blanching and cooling also brings about many undesirable changes in mushrooms due to leaching of valuable nutrients such as total solid losses, flavor and textural change (Khurdiya 1995). It has been studied that losses may be upto 40% for

vitamins (Vitamin C and thiamine) and mineral, 35% sugar, 20% of protein and amino acids (Selman 1998).

It has been observed by Czapski (1995), 20 sec blanching in water can be applied to preserve good color and minimize the hardening of frozen pilei of *Agaricus bisporus* (Lange) Sing. which followed full blanching (5-8 minutes), however, in this case blanching must be proceeded by washing the raw material in a solution of sodium metabisulfite. Rodrigo *et al* (1990) suggested that the replacement of citric acid by glucono-δ-lactone also enabled canned mushrooms to maintain good texture, color and yield but unlike citric acid, glucono-δ-lactone did not leave a acidic or strange taste and smell in the pilei. Unlike blanching in EDTA solution, which decreases the content of iron and copper. It has been reported that blanching of mushroom in the solutions of citric acid at different concentrations does not significantly affect the level of iron, copper, manganese or zinc in pilei (Coskuner and Ozdemir 2000).

Jaworska *et al* (2003) claimed that blanching in water is sufficient in the production of frozen mushroom stuffing stored for 4 weeks. It has been recommended to precede blanching with soaking or vacuum moistening with water in order to minimize weight loss in mushrooms and increase their yield (Czapski 1994).

Sandhu and Aggarwal (2001) recorded that blanching time was also an important parameter to control color, flavor and textural parameters of mushrooms. It has been observed that the mushrooms blanched for 3 minutes have better color retention than for 2.5 mins. Das and Kalita (2006) used 22 to 25% solution of common salt as steeping solution. According to their report these steeped mushrooms could be utilized for preparation of various products with acceptable organoleptic quality.

2.8.2.3 Drying

Mushroom start deteriorating immediately after the harvesting, cause difficulty in marketing (Tano *et al* 1999). The shelf life of mushroom varies from 2-6 days depending upon variety. Canning, freezing and drying are some of the methods for preservation of mushrooms. Drying seems to be an effective approach among the various techniques employed for preservation of mushrooms to extend shelf life and ensure distribution. The moisture content of fresh mushrooms varies in the range of 70-90 per cent depending upon the harvest time and environmental conditions while that of dried mushroom is about 10-13 per cent (Bhupinder and Ibitwar 2007).

Drying can be defined as a process of moisture removal as there is a process of heat and mass transfer between the product and the drying air by mean of evaporation. One of the simplest methods of drying is conventional hot air drying. It is the oldest technique for preservation of food known to mankind (Lewicki and Jakubczyk 2004). It is simple, economical and efficient method to extend the shelf life of mushrooms. It involves chemical or thermal treatment prior to drying and operational temperature ranged between 50 to 80°C.

2.9 SPENT MUSHROOM SUBSTRATE

When one full crop of mushroom has been taken and further extension became unprofitable that mushroom substrate was considered as ‘spent substrate’ (Wuest and Fahy 1991). Recently spent mushroom substrate has been replaced by ‘post mushroom substrate’ because it is further to be attacked by new set of micro-organisms (Ahlawat *et al* 2009). Million of tonnes of used mushroom substrate become available for other uses after the mushroom crops were harvested (Rinker 2002). It has been observed that more than 50 million tonnes of used mushroom substrate each year needed to be disposed off by the mushroom industry (Fox and Chorover 1999). Improper disposal of spent mushroom substrate (SMS) can cause problem to the environment. Contaminated SMS can cause recontamination in the mushroom house without proper treatment (Lopez *et al* 2008).

CHAPTER III

MATERIALS AND METHODS

Present investigation on Strain evaluation of *Calocybe indica* in relation to mushroom yield was carried out at Dr. HS Garcha Mushroom Laboratories, Department of Microbiology, Punjab Agricultural University, Ludhiana. The materials and methods used during the study have been presented under the following headings and sub-headings:

3.1 Culture procurement and maintenance
- 3.1.1 Source of culture
- 3.1.2 Culture medium
- 3.1.3 Maintenance of culture

3.2 Mycelial growth
- 3.2.1 Preparation of standard inoculum on agar plates
- 3.2.2 Linear growth
- 3.2.3 Radial growth
- 3.2.4 Biomass production

3.3 Cultivation of *Calocybe indica*strains
- 3.3.1 Spawn preparation
- 3.3.2 Cultivation
  - 3.3.2.1 Substrate, pretreatment and spawning
  - 3.3.2.2 Casing
  - 3.3.2.3 Fruiting and harvesting

3.4 Post harvest observation
- 3.4.1 Yield
- 3.4.2 Quality parameter

3.5 Enzyme assay
- 3.5.1 Endoglucanase
- 3.5.2 Endoxylanase
- 3.5.3 Laccase

3.6 Estimation of total protein content

3.7 Statistical analysis

3.1 CULTURE PROCUREMENT AND MAINTENANCE

3.1.1 Source of culture

Calocybe indica strains and a *Macrocybe* strain were obtained from the Culture Collection Bank, Dr. HS Garcha Mushroom Laboratories, PAU, Ludhiana. The strains were originally procured from the Directorate of Mushroom Research (DMR), Chambaghat, Solan, H.P.

3.1.2 Culture Medium

Potato Dextrose Agar (PDA) medium of following composition:

Ingredients	Concentration (g l^{-1})
Potatoes	250
Dextrose	18.0
Agar-Agar	20.0
pH	6.5

About 250 g of good quality potatoes were washed in tap water, peeled off, cut into small pieces and boiled in 600-700 ml of distilled water for about 15-20 minutes or till they become tender. The extract was collected after filtration through a muslin cloth. Dextrose was added in the extract and the volume was made to 1000 ml with the help of distilled water. The pH was adjusted to 6.5. Agar-agar was used as solidifying agent to dissolved in the filtrate by heating till the formation of gel. The hot gel medium was poured into clean test tubes to 1/4th of their capacity, plugged with cotton and wrapped tightly with paper. These tubes were then sterilized by autoclaving at 121°C for 20 minutes at 15 psi and were thereafter tilted on some support so as to get slants. For two days the slants were kept at room temperature. Only the contamination free slants were used for sub-culturing.

Complete yeast extract agar media (CYM) of the following composition

Ingredients	**(g l^{-1})**
Peptone	2.0
Yeast extract	2.0
Glucose	20.0
$MgSO_4$	0.5
KH_2PO_4	0.46
K_2HPO_4	1.0
Agar	20.0
pH	6.5

Mushroom minimal media (MMM) of following composition was used.

Ingredients	**(g l^{-1})**
Glucose	20.0
$MgSO_4$	0.5
KH_2PO_4	0.5
K_2HPO_4	1.0
Agar	20.0
pH	6.5

3.1.3 Maintenance of culture

The culture of *Calocybe indica* and a *Macrocybe* species were maintained on PDA slants at 30±2°C by sub-culturing them regularly after 6 weeks. The mycelial agar bits of approximately 5 mm diameter were sliced with sterile borer tube and picked from mother culture slants and transferred to fresh agar slants in such a way that the mycelium end touches the surface of agar. The PDA slants were then incubated at 30±2°C for 10 days to let the mycelium run on agar slants. The mycelial run slants were then stored in the refrigerator at 4°C for 6 weeks before next sub-culturing.

3.2 MYCELIAL GROWTH

3.2.1 Preparation of standard inoculum on agar plates.

About 20 ml of each media was poured in a sterile petri plates and allowed to solidify. At room temperature, these plates were incubated for two days. The plates free from contamination were used for inoculum preparation. With the help of inoculating needle, a mycelial bit was cut from the culture slant and was placed aseptically in the center of the agar plate. Parafilm was used to seal the inoculated plates and incubated at 30±2°C in an inverted position till the growth of the mycelium was complete.

3.2.2. Linear growth

The Wheat straw was wetted overnight, heated at 80°C for 30 minutes, allowed to cool and then filled in the test tubes to 2/3rd of their capacity. 7-10 spawn grains were inoculated into each test tube. Then incubate the test tubes at 30±2°C for 10 days to observed linear growth (mm/d)

3.2.3 Radial Growth

Mycelial bits for radial growth studies were cut from peripheral part of fully grown mycelial petriplates. 5mm mycelial bits were placed in the centre of petriplates containing PDA, CYM and MMM media. These inoculated petriplates were then incubated at 30±2°C for 10 days. After interval of every 3,7&10 days radial growth of strains were recorded as colony diameter (mm/d).

3.2.4 Biomass production

About 50ml of CYM was dispensed in each flask. The flasks were autoclaved at 15 psi for 20 mins. Each flask was inoculated with two bits of 5 mm dia of respective strains and for each strain, three replicate flasks were taken. After 10 days, content of each flask was filtered on pre-weighed Whatman filter paper No. 1. The filter paper along with mycelia biomass was then dried at 60°C for 8 hrs and weighed. The weight of biomass (gl^{-1}) was calculated as:

Biomass= weight of (filter paper+ biomass) – weight of filter paper

3.3 CULTIVATION OF *Calocybe indica* STRAINS

Cultivation trials were conducted using nine strains of *C. indica* and one *Macrocybe* strain during the summer season (May-September). The cultivation was carried out indoor under natural climatic conditions with temperature ranging from 25 to 40° C and relative humidity of 75-90 percent at Dr. HS Garcha Mushroom Laboratories, Department of Microbiology, Punjab Agricultural University, Ludhiana. Proper ventilation and diffused light in growing rooms was maintained.

3.3.1 Spawn preparation

Wheat grains were boiled in water for 40 mins (wheat grains: water, 1:2 v/v) so that they become soft enough to get pressed within the fingers. Extra water was sieved out and the grains were allowed to cool. The grains were then mixed with 2% $CaCO_3$ and 4% $CaSO_4$ and filled in glucose bottles @ 250 g per bottle, plugged with non-absorbent cotton, wrapped with paper and then autoclaved at 20 psi for 1.5h. The autoclaved bottles were cooled overnight and then inoculated aseptically with *C. indica* strains. The bits were placed in such a way that the mycelium touched the grains. These bottles were incubated at 30±2°C for 10-15 days and result in the production of 'Master cultures' bottles. After 10 days 'Master cultures' were shaken so that grains with mycelium were homogeneously mixed and then from it grains were transferred to 10-15 freshly prepared bottles. These bottles were the 'Generation I' spawn bottles ready for sowing into the substrate.

3.3.2 Cultivation

Cultivation trial of *Calocybe indica* was conducted during the month of April to October 2018. The cultivation was carried out under indoor natural conditions of about 25°C to 40°C and relative humidity of about 75-90%.

3.3.2.1 Substrate, pretreatment and spawning

Wheat straw was used as substrate for growing nine strains of *C. indica* and one *Macrocybe* strain. Wheat straw was spread on pucca floor and wetted with clean, fresh water for 14-20 hrs to attain 65-70 per cent moisture. Wetted substrate was boiled for 45 to 50 minutes and cooled. Pretreated substrate was thorough spawned @ 10 per cent on dry weight basis and was filled in polybags (12″ x 16″). The bags were kept in growing rooms for spawn run and subsequent casing. The cultivation process of milky mushroom has been outlined in Fig. 1.

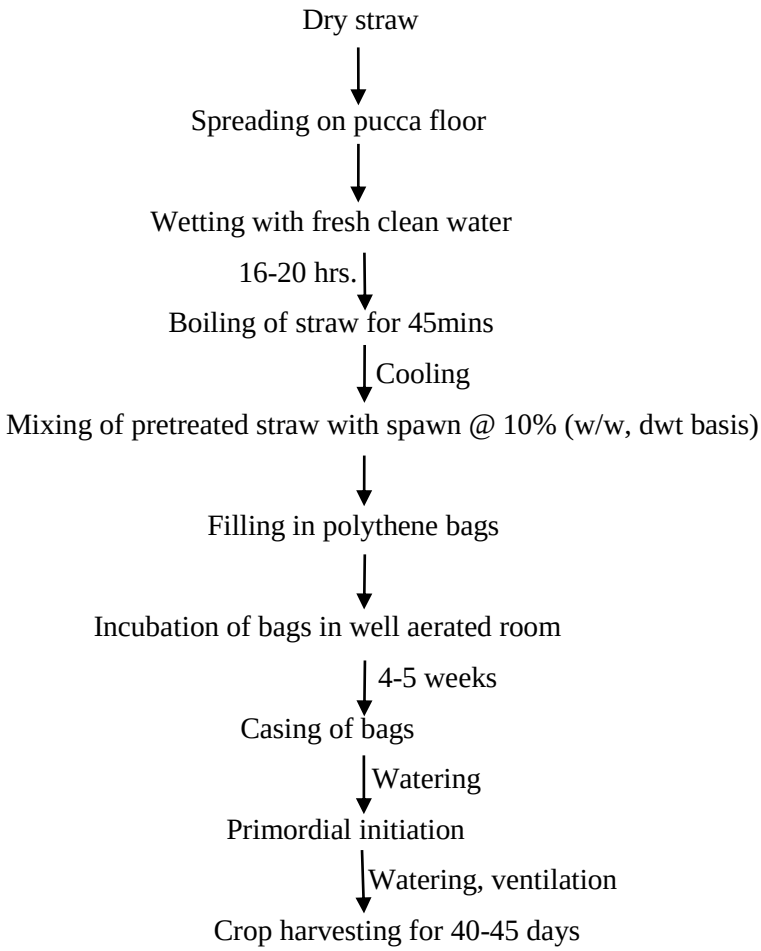


Fig. 1 Cultivation process of milky mushroom, *Calocybe indica*

3.3.2.2 Casing

After complete impregnation of bags (4-5 weeks), the bags were cased with steam sterilized casing mixture consisting of farm yard manure (FYM) and sandy soil in the ratio 4:1. About 2.5-3.0 cm thick uniform layer of casing mixture was used. After casing, the bags were watered twice daily using nap-sac pump fitted with nozzle to give misty spray.

3.3.2.3 Fruiting and harvesting

After 10-15 days of casing, mushrooms started appearing in flushes for about 40 days. The mushrooms were harvested by gentle twisting of the fruit bodies. Water was sprayed regularly over the surface of casing twice a day till the end of the crop. Time taken for spawn run, case run, days of first harvest, number and weight of fruit bodies from each bag was recorded. Percent biological efficiency (B. E.) was calculated by the formula:

$$\text{B. E. (\%)} = \frac{\text{Fresh wt. of fruit bodies}}{\text{Dry wt. of substrate}} \times 100$$

3.4 POST HARVEST OBSERVATION

3.4.1 Yield

Yield data was recorded as the number of fruiting bodies harvested and the fresh weight of mushrooms for a time period of about 30 days.

3.4.2 Quality characteristics

Quality characteristics like diameter of pileus, stipe length, stipe diameter, total length and colour were observed. Colour is one of the important quality parameters, white colour indicates its good commercial value. The main purpose of colour management to get idea of comparative change in colour due to temperature or some environmental condition. Miniscan XE plus Hunter Lab Calorimeter was used to measure the colour of freshly harvested mushroom. The L value (lightness) was recorded to represent the colour of fresh mushrooms.

3.5 ENZYME ASSAY

C. indica strains were tested for their ability to produce lignocellulolytic enzymes. Every week, the culture filtrate was collected for endoglucanase, endoxylanase and laccase estimation.

3.5.1 Endoglucanase (E.C.3.2.1.4)

Endoglucanase activity was measured by the method of Mandels *et al* (1976). The reducing sugars were estimated using DNS reagent (Miller 1959).

Reagents

1) 0.1M citrate buffer (pH 4.8)
   A) 0.1M citric acid
   B) 0.1M tri sodium citrate
   Mix 23 ml of A and 27 ml of B and volume was made to 100ml with the help of distilled water
2) Carboxymethyl cellulose (CMC)- 1g of CMC was dissolved in 90 ml of 0.1M citrate buffer of pH 4.8 and volume was made to 100 ml.
3) Dinitrosalicylic acid (DNS) solution- 10 gms of DNS and 0.5g sodium sulphite in 500 ml of 2% NaOH solution already prepared in distilled water were added. The solution was allowed to cool, 2g of phenol was dissolved in it and final volume was made to 1000ml. The solution was filtered and stored in dark bottles in refrigerator.
4) Sodium potassium tartrate solution (40%)- 40g of Na-K-tartrate was dissolved in 100ml distilled water. The solution was filtered and stored at room temperature.

Assay

Test tubes containing a mixture of 0.5 ml CMC solution and 0.5 ml of culture filtrate were incubated at 50° C for 30 mins in water bath. Controls devoid of enzyme extract were also run simultaneously.

Reducing sugar produced during this reaction was measured by using DNS method. 3 ml of DNS was added to each tube and kept in boiling water bath for 15 mins. While still hot 1 ml of Na-K-tartrate solution was added, the contents were cooled to room temperature followed by addition of 2 ml of distilled water in each test tube. Enzyme extract was added to control after adding DNS. The percent light absorbance by the resulting solution was recorded

at 575nm in spectronic 20. The corresponding enzyme activity was calculated from standard curve (Fig. 2).

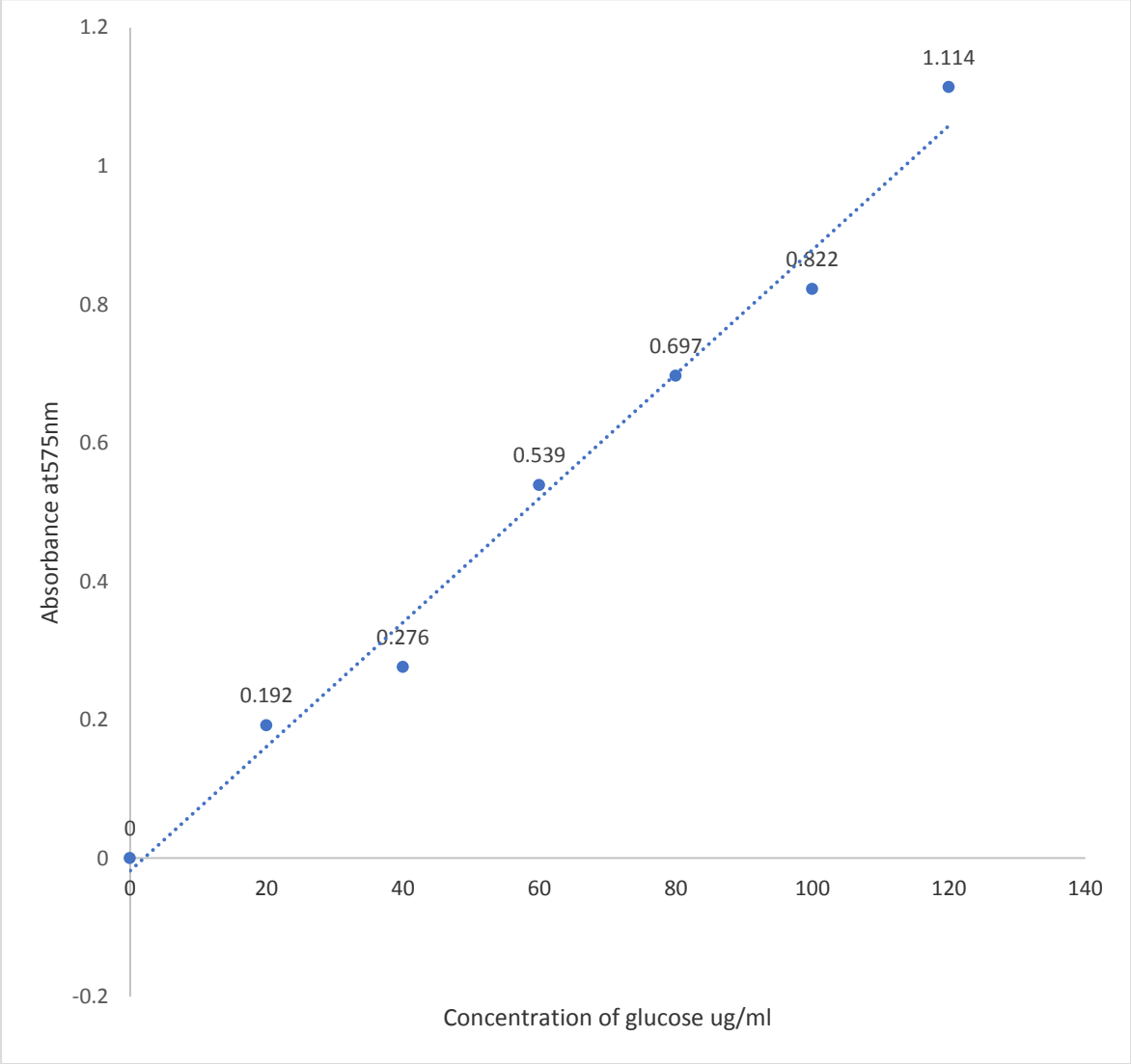


Fig. 2: Standard curve of Glucose

3.5.2 Endoxylanase (E.C.3.2.1.8)

Xylanase activity was determined by the method described by Bucht and Eriksson (1968).

Reagents:

1) Xylan solution- 1 gm of xylan was dissolved in 0.05M citrate buffer, pH 4.8 and volume made 100ml
2) Dinitrosalicylic acid reagent
3) Sodium potassium tartrate solution (40%)

Assay

One ml of sample of culture filtrate was mixed with 1ml of 1% xylan solution and incubated for 30 mins at 50° C. The reaction was stopped by adding 3ml of DNS reagent. Reducing sugar was measured as xylose equivalents by DNS method (Fig. 3).

On the basis of growth studies and enzyme activity *Calocybe indica* strains were selected for further analysis

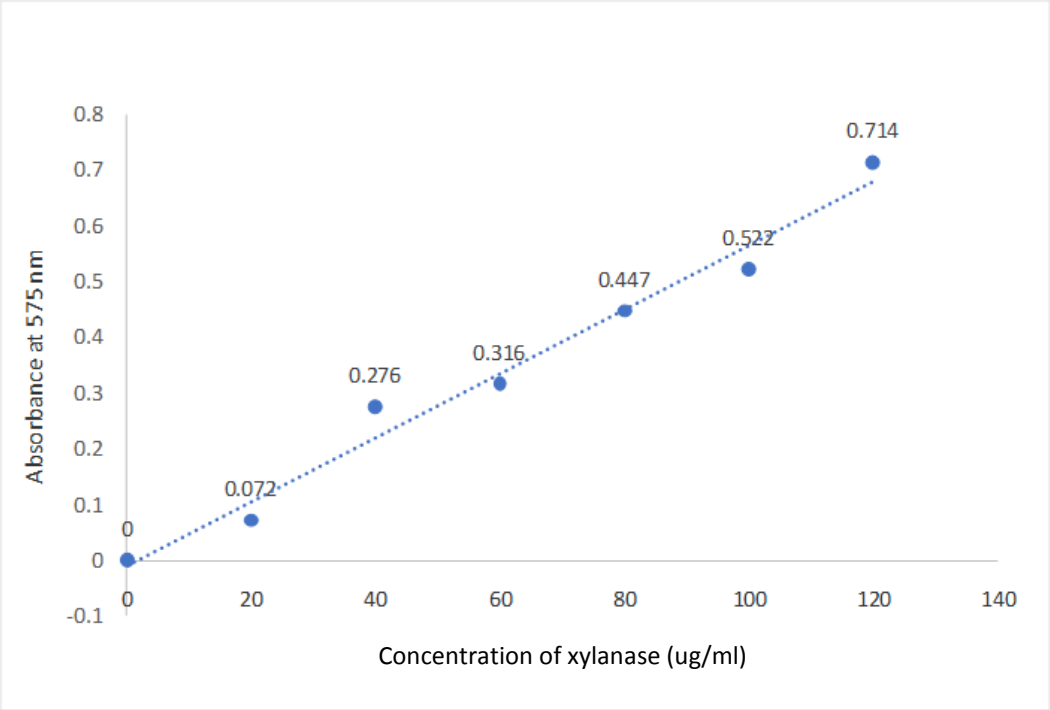


Fig. 3: Standard curve of Xylanase

3.5.2 Laccase (E.C.1.10.3.2)

Laccase estimate was carried out according to the method of Turner (1974) with some modification as described by Singh *et al* (1988).

Reagents

1) 0.1M phosphate buffer (pH 6.0)

   A) 0.2M KH_2PO_4

   B) 0.2M K_2HPO_4

   87.7ml of A and 12.3ml of B and diluted to 200ml.

2) 0.22M Guaiacol (pyrocatechol monoethyl ether $C_7H_8O_2$) solution- 0.22M solution of guaiacol was prepared in 0.1M phosphate buffer (pH 6.0) and stored in refrigerator in a dark colour bottle.

Assay

One ml of enzyme extract and 3 ml of buffered guaiacol were added to colorimeter cuvette, mixed and tube was placed in colorimeter immediately. The change in absorbance was recorded for every 15 sec upto 120 secs at 495 nm. The constant change in absorbance was plotted for laccase activity.

Enzyme units: an increase in OD by 0.01 in 60 sec was taken as one unit. Enzyme activity was expressed as units/ ml of cultural filtrate.

3.6 ESTIMATION OF TOTAL PROTEIN CONTENT

The total protein content of culture supernatant was estimated by the method given by Lowry *et al* (1951).

Reagents

Reagent A: 2% sodium carbonate in 0.1 N sodium hydroxide

Reagent B: 0.5% copper sulphate solution in 1% sodium citrate

Reagent C: Freshly prepared by mixing 50 ml of A and 1 ml of B

Reagent D: Folin Ciocalteau reagent diluted with distilled water (1:1, v/v)

***Assay*:** Culture filtrate (0.5ml) was diluted to 1 ml by adding 0.5 ml of distilled water. To each tube, 5 ml of reagent C was added and incubated for 10 minutes at room temperature and vortexed. Controls devoid of culture filtrate were also run simultaneously. Then, 0.5 ml of reagent D was added and tubes were incubated for 30 minutes at room temperature and vortexed. Absorbance was read at 520 nm. The corresponding enzyme activity was recorded from standard curve as U/mg.

***Preparation of standard curve*:** 0.2 ml, 0.4 ml, 0.6 ml, 0.8 ml and 1.0 ml of standard Bovine Serum Albumin (BSA) was taken in different tubes. The total volume was made to 1 ml by adding distilled water. To each tube, 5 ml of reagent C was added and incubated for 10 minutes at room temperature and vortexed. Then, 0.5 ml of reagent D was added and incubated for 30 minutes at room temperature and vortexed. Absorbance was read at 520 nm. The protein content calculated by above method was used to determine the specific activity of extracellular enzymes (Fig. 4).

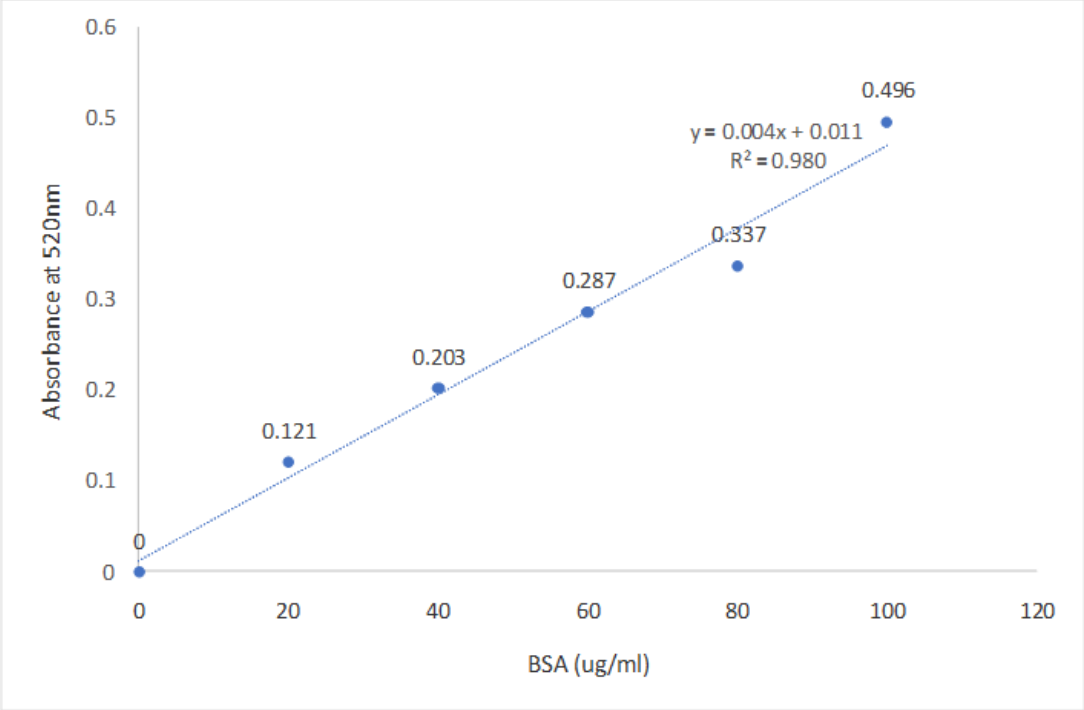


Fig. 4: Standard Curve for BSA

3.7 STATISTICAL ANALYSIS

Statistical analysis of the data was carried out using CPCS1 software developed by the Department of Mathematics, Statistics and Physics, Ludhiana, Punjab. The treatments were compared by using ANNOVA (CRD). Further the correlation of biomass and enzyme was carried out by using Microsoft Excel software.

CHAPTER IV

RESULTS AND DISCUSSION

The observations made out of experiments conducted during the study have been presented under the following headings and sub-headings:

4.1 Mycelial growth

4.1.1 Radial growth

4.1.2 Biomass production

4.1.3 Linear growth

4.2 Cultivation of *Calocybe indica* strains

4.2.1 Quality parameters

4.3 Enzyme Assay

4.3.1 Culture filtrate

4.3.2 Spawn run

4.3.3 Pinhead

4.3.4 Fruiting body

4.4 Correlation of biomass and enzyme with yield

4.5 Optimization of biomass and endoglucanase enzyme for the yield of different strains of *C. indica.*

4.1 Mycelial growth

In the mycelial growth study, radial growth, biomass and linear growth were observed. Colony diameter (mm) was observed for *C. indica* strains on Potato dextrose agar, Complete yeast extract agar media and Mushroom minimal media.

4.1.1 Radial growth

The colony diameter of the strains after 3 days on PDA ranged between 10-22mm, on CYM 8-18mm and on MMM was 5.6-11mm. The maximum colony diameter was observed with Ci-09 on PDA, CI-07 on CYM as well as in MMM. On 7^{th} day the colony diameter of *Calocybe indica* strains on PDA ranged between 42-63mm, on CYM 42-60mm and on MMM 38-44mm. The maximum colony diameter was that of Ci-09 on PDA & Ci-07 and *Macrocybe* on CYM and on MMM, the *Macrocybe*. On 10^{th} day the range of colony diameter was between 68-87.6mm on PDA, 65-85.3mm on CYM, 60-70.6mm on MMM. Maximum growth on PDA was observed in Ci-06 and Ci-09, on CYM it was on Ci-07 while on MMM, maximum growth was observed with Ci-09 (Table 1; Plate 1).

The characteristics of *C. indica* have been studied and reported for 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 (Kaur 2003). Growth of *C. indica* on wheat dextrose agar, rice straw extract agar, potato dextrose agar and wheat straw extract agar showed maximum linear growth on wheat dextrose agar medium (7.03 cm on 10^{th} day) followed by potato dextrose agar (5.10 cm) (Singh *et al* 2009).

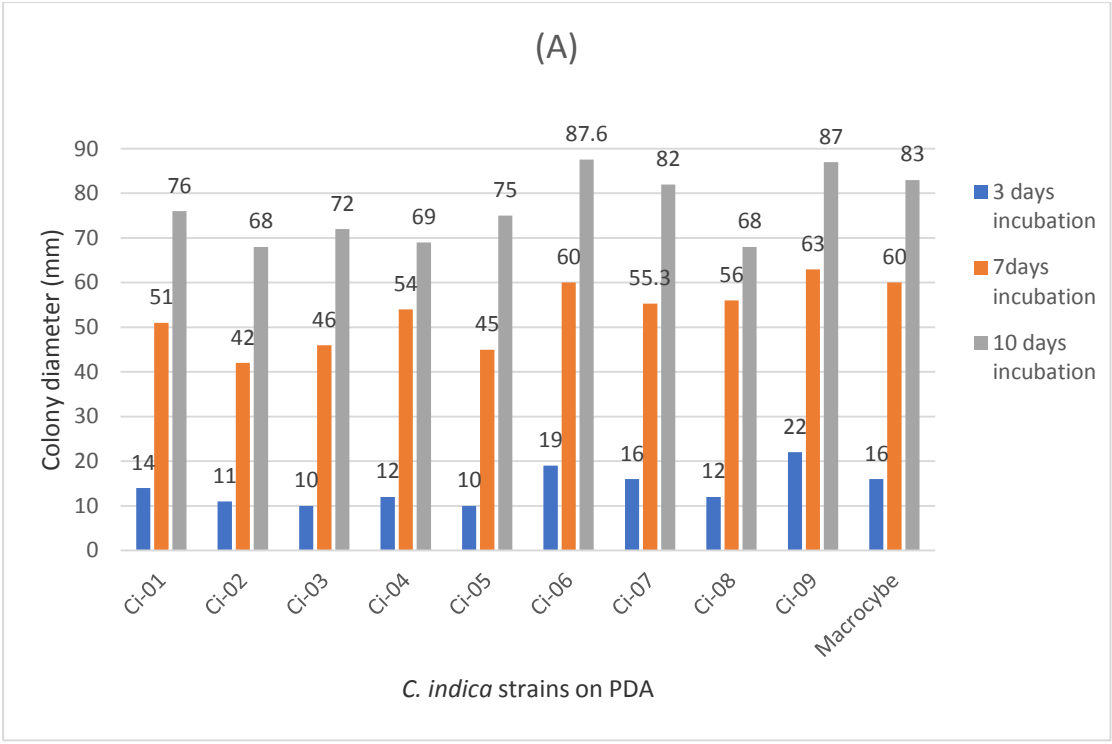
Table: 1 Radial growth of *Calocybe indica* strain on agar media

Strain	Colony diameter(mm)								
	Media								
	PDA			CYM			MMM		
	3d	7d	10d	3d	7d	10d	3d	7d	10d
Ci-01	14	51	76	11	49	72	8.0	42	67.3
Ci-02	11	42	68	9.0	44	67	5.6	38	60.0
Ci-03	10	46	72	8.0	42	66	9.0	46	61.6
Ci-04	12	54	69	10	48	67	8.6	43	63.6
Ci-05	10	45	75	8.0	44	65	8.3	38	62.0
Ci-06	19	60	87.6	16	53	82	9.6	42	68.6
Ci-07	16	55.3	82	18	59	85.3	11	44	61.3
Ci-08	12	56	68	11	42	74.6	8.6	42	58.6
Ci-09	22	63	87	15	53	78.3	10.3	43.3	70.6
Macrocybe	16	60	83	16	60	83.3	9.3	49	61.3
CD (p=0.05)		1.4			1.3			4.2	

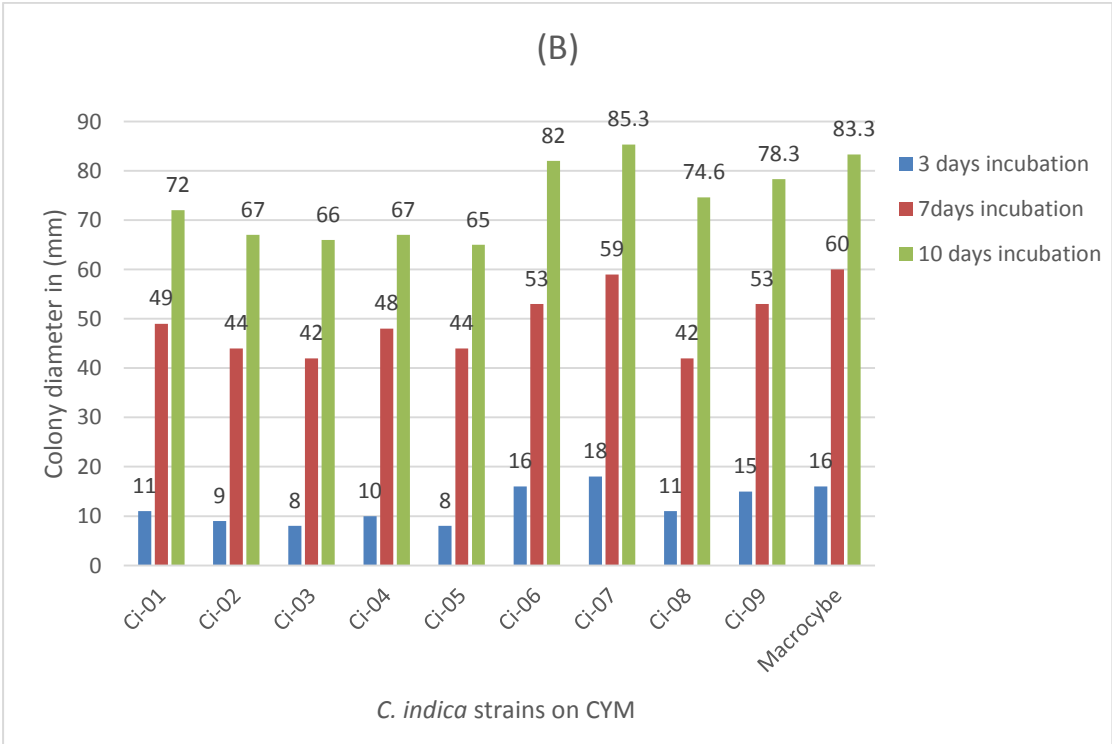
Media: Potato dextrose agar (PDA), Complete yeast extract (CYM), Mushroom minimal media (MMM)

Incubation temperature: 30°C

Inoculum size: 5mm agar bit per plate.



C. indica strains on PDA



C. indica strains on CYM

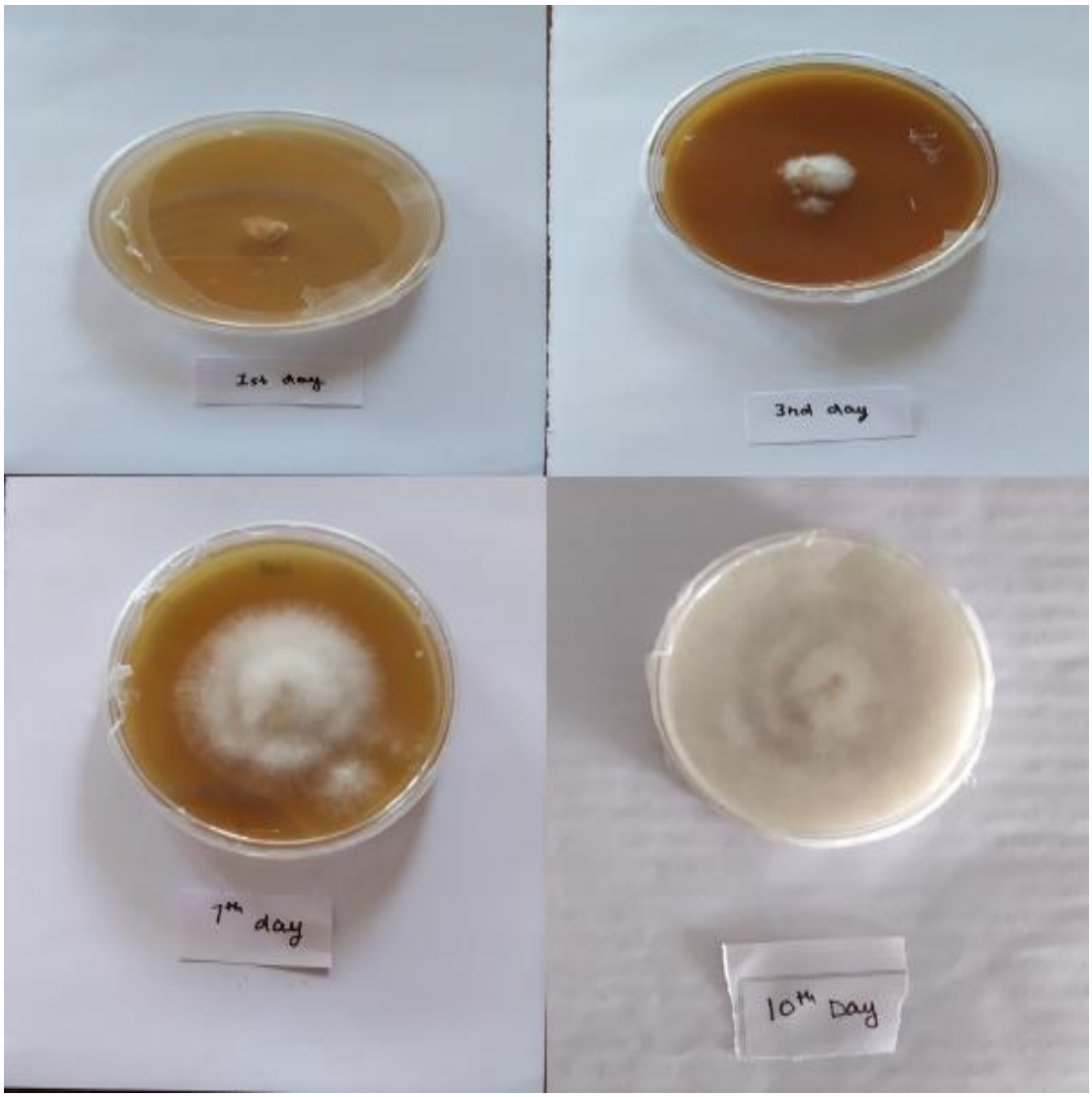


Plate 1. Radial growth of *C. indica* on PDA (1st, 3rd, 7th, and 10th day)

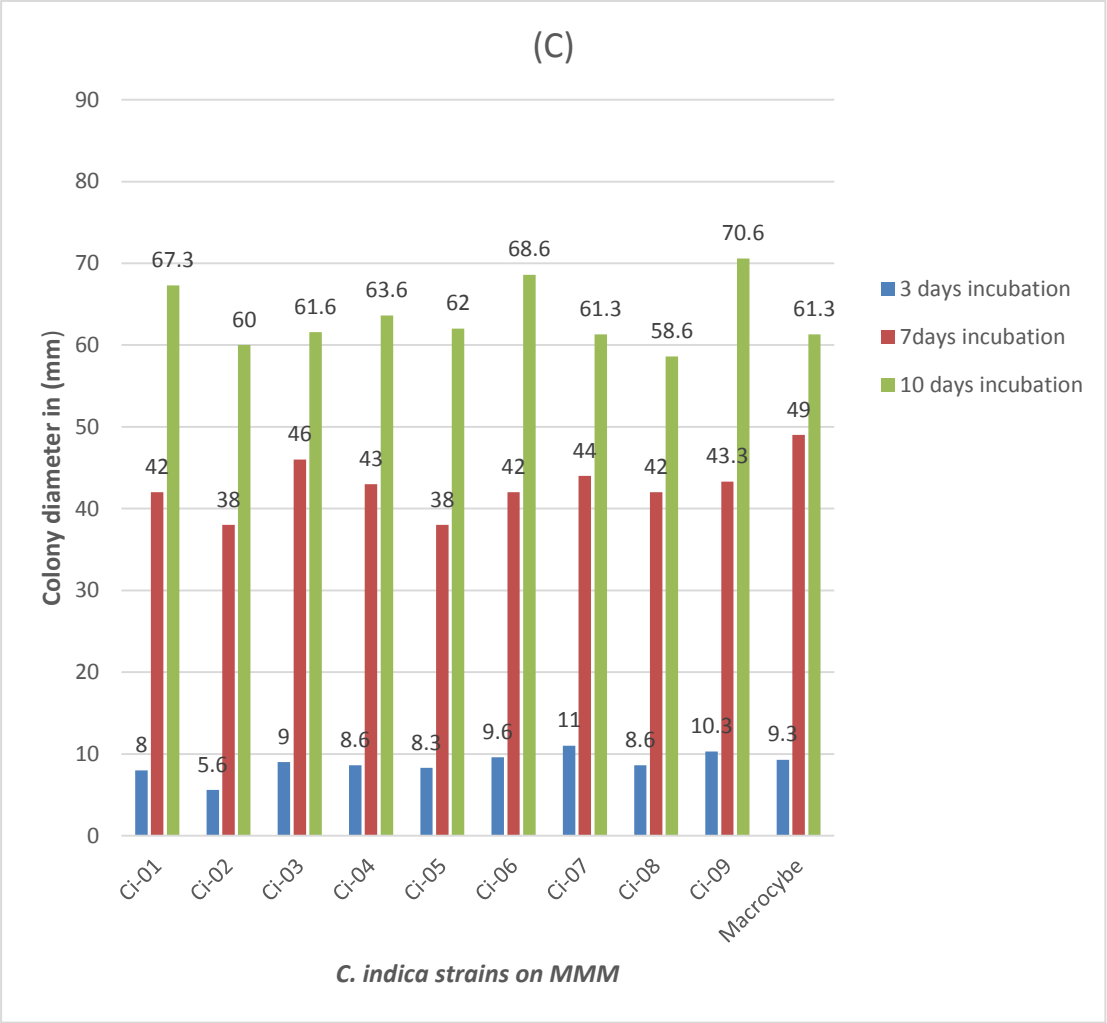


Fig. 5 Mycelial growth of *C. indica* strains

(A) Radial growth of *C. indica* on (PDA)

(B) Radial growth of *C. indica* on (CYM)

(C) Radial growth of *C. indica* on (MMM)

4.1.2 Biomass production

Calocybe indica strains were grown in Potato dextrose broth (PDB), Complete yeast extract (CYM), Mushroom minimal media (MMM) broth. The mycelial biomass (g/l) of *C. indica* strains were observed after 10 days of incubation. The mycelial biomass ranged between 9.6 to 16.1 g/l in PDB, 9.2 to 16.1 in CYM and 8.3 to 12 in MMM. The maximum biomass in PBD was that of Ci-06 and Ci-07, while on CYM, it was maximum in Ci-06 and Ci-09. The maximum biomass in MMM was observed in Ci-04, Ci-06, Ci-07 and Ci-09. (Table2; Fig. 6)

Growth of *Calocybe indica* in 11 different broth media had been studied with maximum mycelial biomass harvested from wheat seed extract medium followed by malt extract medium and potato dextrose medium (Pani 2010).

Table2: Growth study of Biomass in broth

Strains	**Mycelium biomass(g/l)**		
	MEDIA		
	PDB	**CYM**	**MMM**
Ci-01	11.1	10.3	8.3
Ci-02	11.8	12.1	8.6
Ci-03	9.6	10.2	9.3
Ci-04	10.4	9.2	11.2
Ci-05	12.3	12.4	9.5
Ci-06	14.7	16.1	10.4
Ci-07	16.1	13.7	12
Ci-08	12.3	13.8	9.3
Ci-09	13.9	15.5	11.7
Macrocybe	10.8	9.7	8.5
CD (p=0.05)	1.4	1.3	1.6

Medium: Complete yeast extract (CYM) broth, Potato dextrose broth (PDB), Mushroom minimal media (MMM) broth.

Incubation temperature: 30°C

Incubation period 10 days.

Inoculum: 5mm bits (two each per 50 ml broth flask)

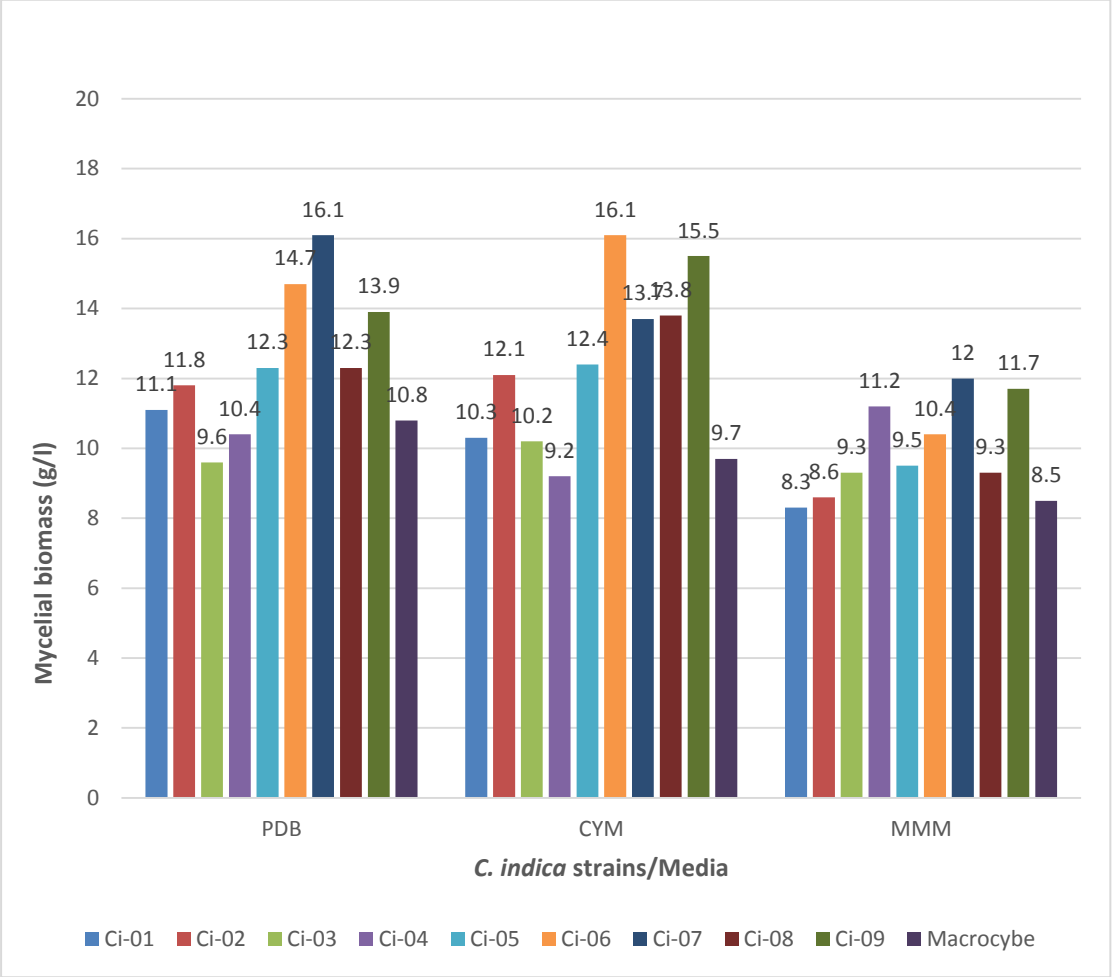


Fig. 6 Mycelial biomass of *C. indica* on Potato dextrose broth (PDB), Complete yeast extract broth (CYB), Mushroom minimal media broth (MMB).

4.1.3. Linear growth

The mycelial impregnation from grain spawn to the hot water treated wheat straw was observed for *C. indica* strains upto 10 days at 30±2°C. the linear growth after three days of incubation in *C. indica* strains range between 7.6 to 13mm, 24-36.3mm on 7th day and 51-66mm on 10th day of incubation. Maximum linear growth after 3 days was observed in Ci-06 and Ci-07. After 7 days maximum linear growth was that of Ci-03, Ci-07, Ci-09 and after 10 days maximum growth was found in Ci-07 and Ci-09 (Table 3; Fig. 7).

It was observed that the highest linear growth (87mm) was observed in response to the tissue culture obtained from the mushroom which consisted of stipe with well differentiate pileus (Pani 2016)

Table 3: Linear Growth on wheat straw

Strains	**Linear growth (mm)**		
	3d	**7d**	**10d**
Ci-01	8	24	53.6
Ci-02	7.6	28	51
Ci-03	10	36.3	59
Ci-04	9.3	31.3	55.3
Ci-05	8.3	26	58.3
Ci-06	10.6	31	58
Ci-07	13	35	63
Ci-08	8	30	60
Ci-09	10	33.6	66
Macrocybe	8	29.6	56
CD (p=0.05)	2.4	4.2	4.7

Tube size: 20×2 cm.

Substrate: Wheat straw wetted heated 80°C for 30 min.

Incubation time: 30±2°C

Inoculum: 7-10 spawn grains in each tube.

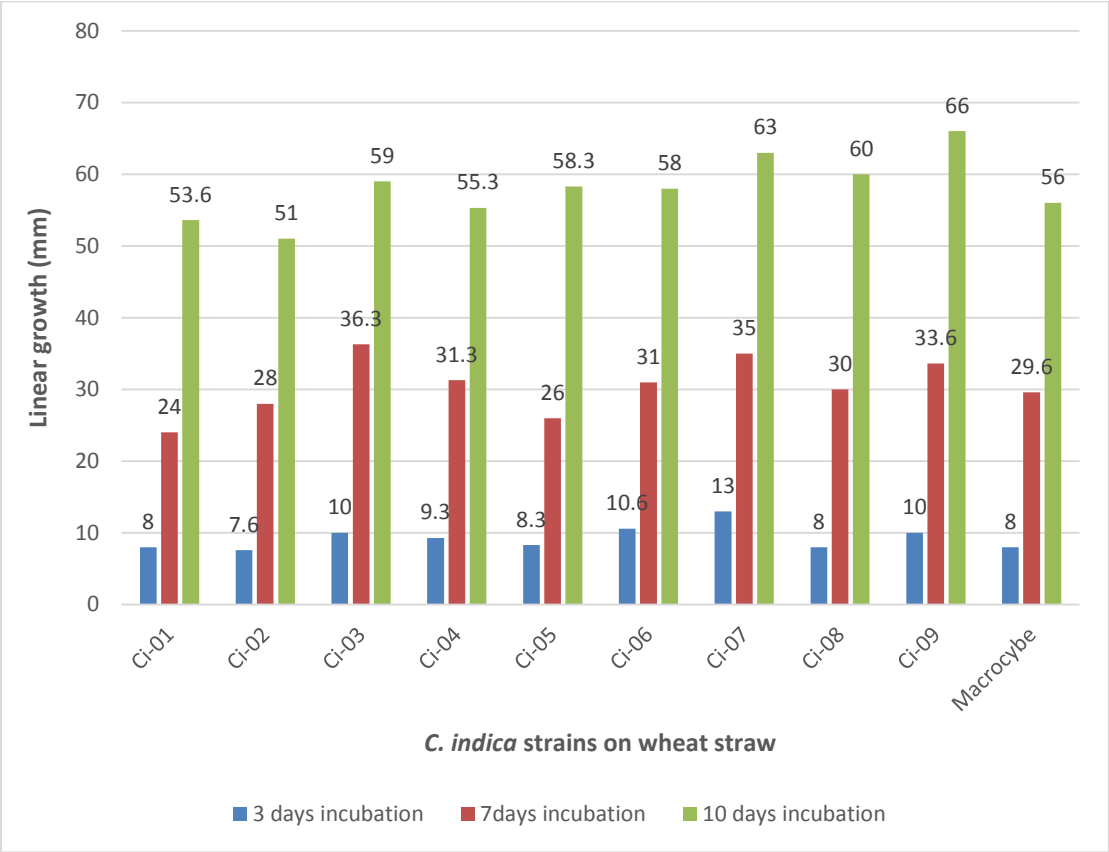


Fig. 7 Linear growth of *C. indica* strains on wheat straw

4.2 Cultivation of *Calocybe indica* strains

Nine strains of *Calocybe indica* Ci-01 to Ci-09 and one *Macrocybe* were grown on wheat straw following the standard adapted cultivation technology. Observations were recorded on days of spawn run, number of fruit bodies, yield (percent) and average weight of fruit body along with stipe length and pileus diameter of the different sample of each strain.

Spawn run period ranged between 15-21 days. The number of fruit bodies harvested were maximum for Ci-06 (1552) followed by Ci-07 and Ci-09. The biological efficiency estimated from the harvested yield (kg/q dry straw) was found to show maximum yield potential for the strain Ci-06 (57.3%). Two strains Ci-07 and Ci-09 were found to give yield at par with each other whereas Ci-03 showed low biological efficiency (20.2%), whereas two strains did not fruit (Ci-02 and Ci-05). Average weight of fruit body ranged between 35-53g with maximum for Ci-06, Ci-07 and Ci-09 strains and lowest for Ci-03 strain.

The pileus diameter of strains ranged between 6.9 to 8.8 cm and stipe length ranged between 10.8 and 14.3 cm. The maximum pileus diameter and stipe length was recorded in the strain Ci-09. The maximum total length of the fruiting body and stipe diameter was observed in Ci-06. During the crop no disease and pest infestation was recorded. Considering the yield potential of *C. indica* strains only one strain Ci-06 indicated maximum biological efficiency. (Table 4; Plate 2-4)

Maximum biological efficiency had been reported from the strain Ci-06 (62%) followed by Ci-04 (58.1%) (Kumar *et al* 2011). It has been observed that maximum biological efficiency ranged from 51.57% -146.3%. The highest biological efficiency of *C. indica* was observed with the treatment of wheat straw followed by the treatment of paddy straw which is 132.4% whereas soybean straw, coconut pith oil and cotton waste showed 126.1%, 108.7% and 92,.07% (Vijaykumar *et al* 2014). Mangat (2005) reported that the boiling of wheat straw for about 45-50 minutes was the best pretreatment for obtaining high yield with the biological efficiency (41.6-62.9%). It has been observed that on spraying 50ppm Gibberellic acid on the emerging primordial increased the number as well as the size of mushroom and produced higher yield (82.2% BE) compared to control (67.8% BE) (Pani 2011b). Kaur (2012) observed maximum biological efficiency for the strain Ci-06 (81.82%) and lowest biological efficiency was observed in the strains Ci-01, C-02 and Ci-09 (47.8-51.28%).

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

Strains	Spawn run (days)	NFB (no./q dry straw)	Yield (kg/q dry straw, % B. E.)	Av. Wt. of fruit body (g)
Ci-01	18	753	25.5	40.3
Ci-02	Did not fruit			
Ci-03	17	452	20.2	51.5
Ci-04	19	1015	30.4	35.6
Ci-05	Did not fruit			
Ci-06	15	1552	57.3	42.5
Ci-07	17	1515	48.5	38.5
Ci-08	15	890	32.3	43.5
Ci-09	18	1030	45.7	51.4
Macrocybe	Did not fruit			
CD (p=0.05)		151.1	5.67	

No. of replicates: 10 (5+5)

Substrate: Wheat straw boiled for 45mins

Substrate/bag: 2.5 kg wet straw/bag

Spawn rate: 10% dry wt. Basis

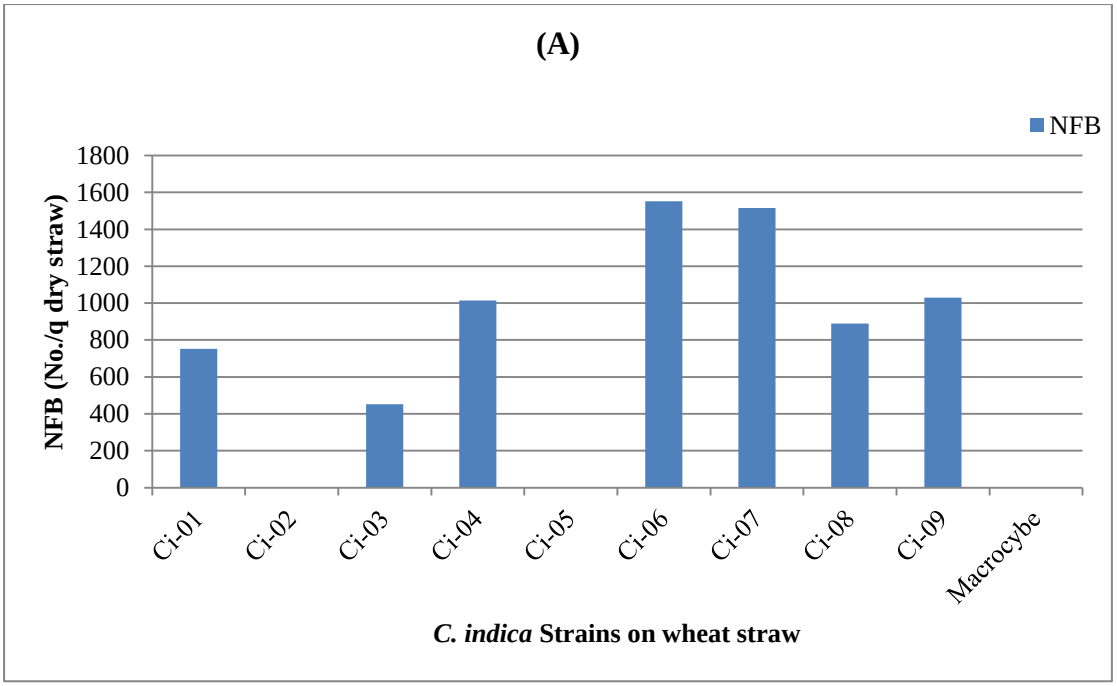
Casing: FYM + sandy loam soil (4:1)

NFB: Number of fruit bodies

Date of sowing: 5th June 2018, 12th July 2018

Date of casing: 8th July 2018, 17th Aug 2018

Date of termination: 23rd Aug 2018, 4th Oct 2018



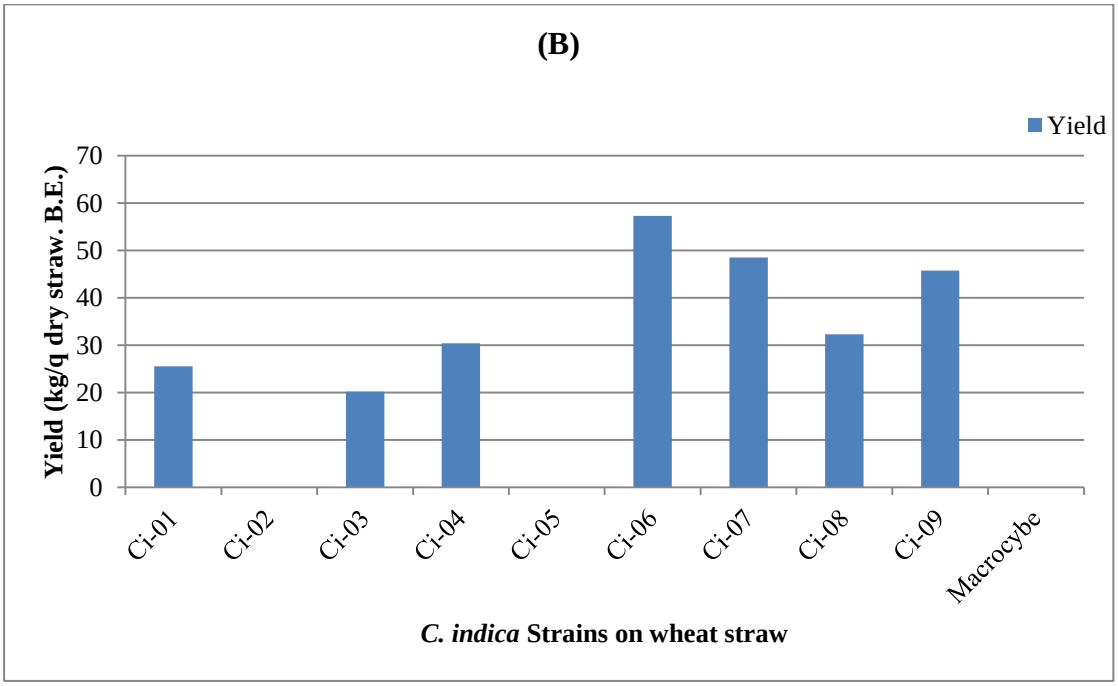


Fig. 8 Yield performance of *C. indica* strains on wheat strain

(A) Number of fruiting bodies.

(B) Yield

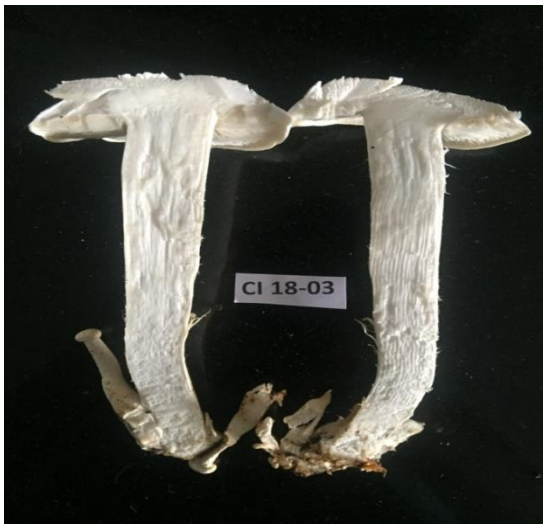


Plate 2: *Calocybe indica* strains, Ci-01 and Ci-03 (in bag and cross section)

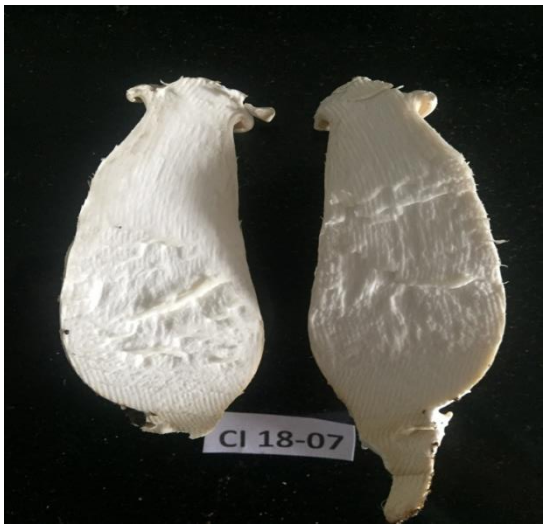


Plate 3: *Calocybe indica* strains, Ci-06, Ci-07 (in bag and cross section)

Plate 4: *Calocybe indica* strains, Ci-08, Ci-09 (in bag and cross section)

4.2.1 Quality parameters

In fruit body dimension, pileus diameter of strains ranged between 6.9 to 8.8 cm and stipe length ranged between 10.8 to 14.3 cm. The total length and stipe thickness ranged between 13.2-16.4 cm and 1.6-2.2 cm. The maximum pileus diameter was observed in the strain Ci-09 followed by Ci-03 and Ci-08. The maximum stipe length was observed in the strain Ci-09 followed by Ci-08 and Ci-06. Maximum total length was observed in the strain Ci-06 followed by Ci-09 and Ci-08. The maximum stipe thickness was that of strain Ci-06. The stipe thickness of the strain Ci-09 and Ci-04 was at par with each other. The L- value ranged between 58-78. The maximum L-value was observed in Ci-07 followed by Ci-06 and Ci-09(Table 5; Fig. 9)

The maximum pileus diameter and stipe length was observed in Ci-03(9.4cm) and APK-2 (14.2cm) strain of *Calocybe indica*. The maximum total length and stipe thickness was observed in same strain APK-2 16.8 and 3.1cm of *C. indica* (Kaur 2012). The L value was observed by (Kaur 2016). She observed that L-value of mushroom ranged between 68-82.

Table 5: Quality check of *Calocybe indica* strains

Strains	**Fruit body dimension(cm)**				**Colour**
	PD	**TL**	**SL**	**ST**	**L- Value**
Ci-01	7.5	13.6	11.2	1.8	58
Ci-02	Did not fruit				
Ci-03	8.6	15.2	10.8	1.6	65
Ci-04	6.9	13.2	11.6	2.1	63
Ci-05	Did not fruit				
Ci-06	8.2	16.4	13.6	2.2	75
Ci-07	7.4	14.4	12.4	1.6	78
Ci-08	8.5	15.8	14.0	1.8	62
Ci-09	8.8	16.0	14.3	2.1	72
Macrocybe	Did not fruit				
CD (p=0.05)	0.6	0.6	0.5	NS	6.1

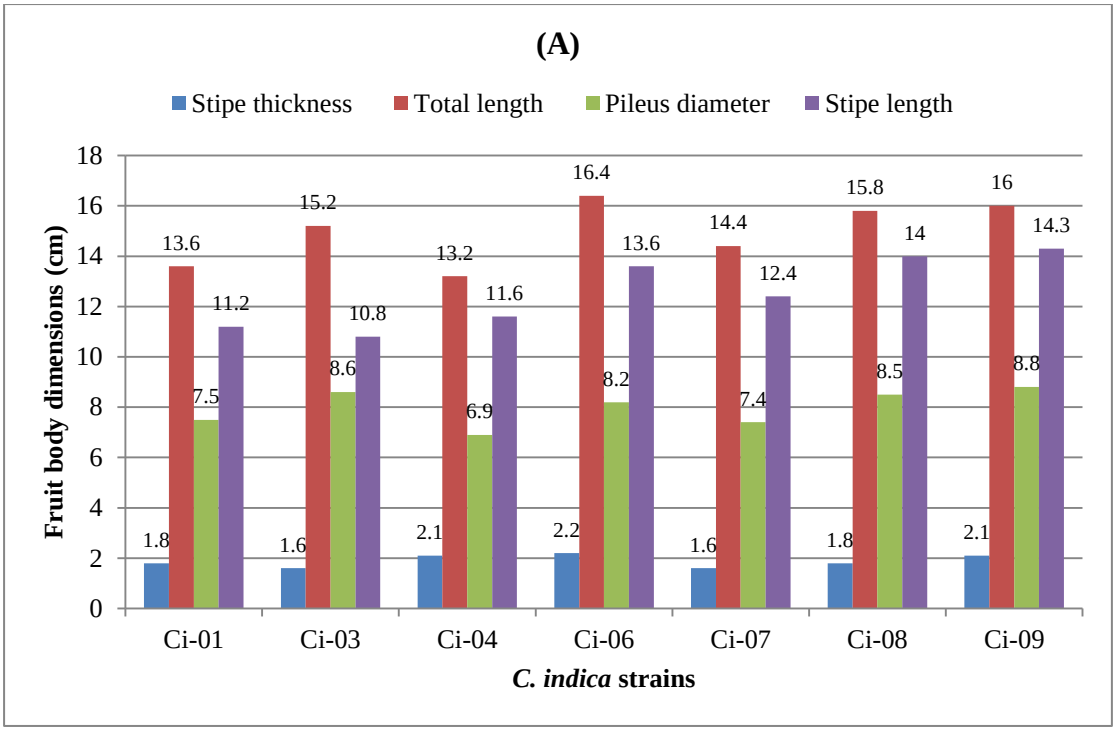
No. of replicate: 3

PD = Pileus Diameter

TL = Total Length of Fruit Body

SL = Stipe Length

ST = Stipe Thickness



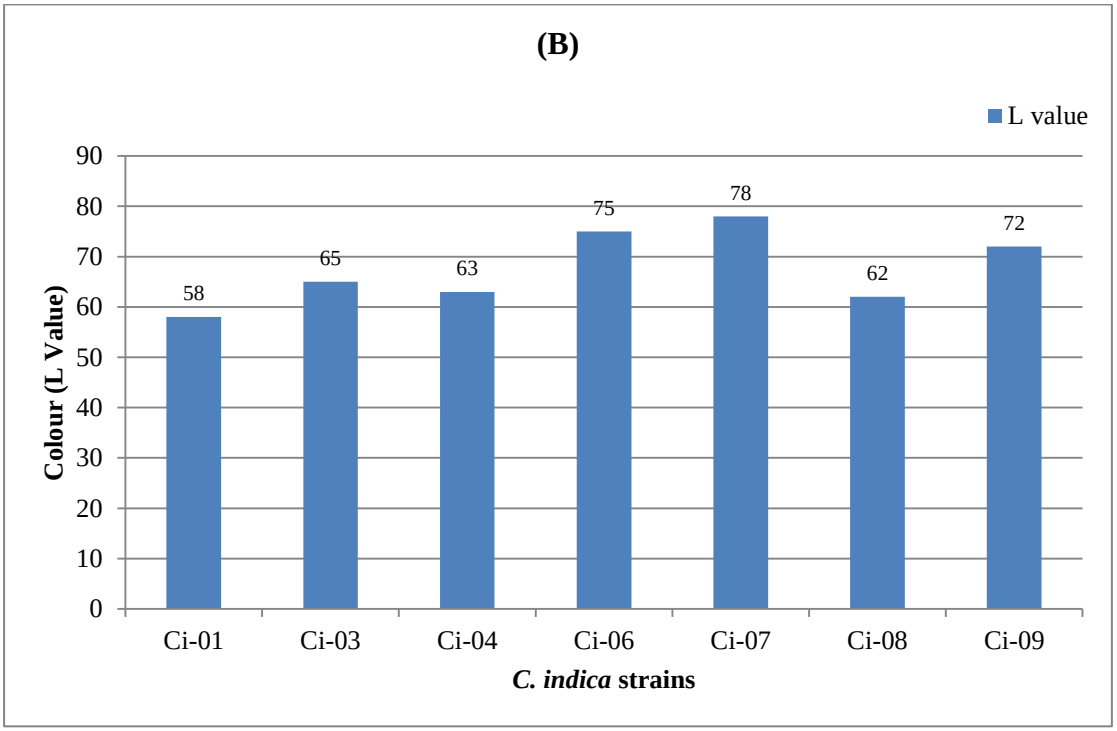


Fig. 9 Quality assessment of the *C. indica* strains

(A) Fruit body dimensions

(B) Colour (L value)

4.3 Enzyme assay

4.3.1 Enzyme assay of Culture filtrate

C. indica strains Ci-01 to Ci-09 and a *Macrocybe* were grown in CYM broth at 30±2°C for 10 days to harvest the culture filtrate for enzyme assay. Endoglucanase enzyme activity range between 1.91-4.42 U/mg with maximum enzyme activity in strain Ci-08 followed by that of Ci-09 (2.83 U/mg) while Ci-06 and Ci-01 were at par with each other.

Endoxylanase activity ranged between 2.73-4.76 U/mg and maximum endoxylanase activity in Ci-07 followed by Ci-04, Ci-06 & Ci-09. The lowest activity was observed in Ci-08. Laccase activity of the strains culture filtrate ranged between 6.94 to 10.2 U/mg with maximum activity in Ci-03 followed by Ci-04, Ci-07, Ci-06. (Table 6; Fig. 10)

Table 6: Specific enzyme activity of Culture filtrate

Strain	Endoglucanase (U/mg)	Endoxylanase (U/mg)	Laccases (U/mg)
Ci-01	2.23	3.16	7.23
Ci-02	1.72	2.84	7.38
Ci-03	1.91	2.73	10.2
Ci-04	1.96	3.59	8.22
Ci-05	1.44	2.96	7.19
Ci-06	2.77	3.74	7.97
Ci-07	1.93	4.76	8.61
Ci-08	4.42	2.81	7.13
Ci-09	2.83	3.97	6.94
Macrocybe	1.80	2.88	7.15
CD (p=0.05)	0.45	0.64	1.40

Replicates: 3

Media: Complete yeast extract media (CYM)

Incubation temperature: 30°C

Incubation period: 10 days.

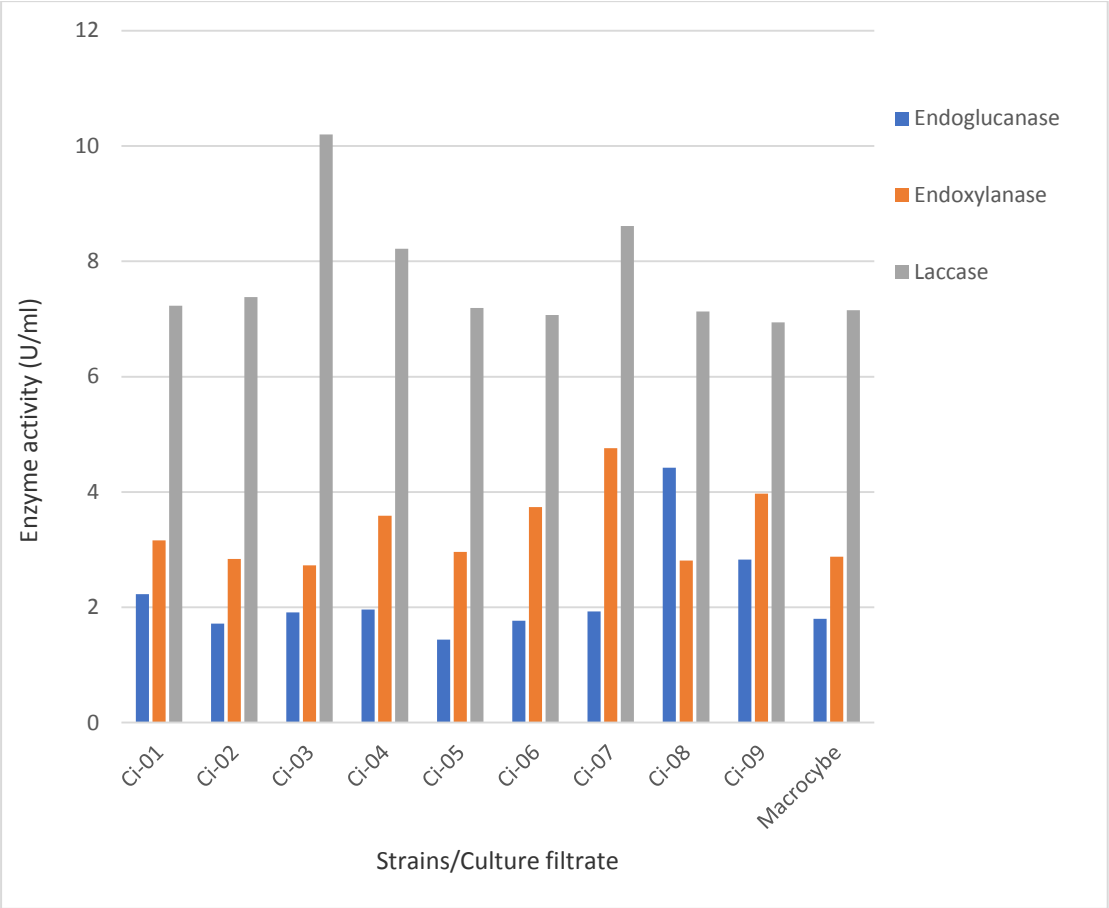


Fig. 10 Specific enzyme activity of *C. indica* strains during culture filtrate

4.3.2 Spawn run

The enzyme extracted from the mycelium impregnated straw of different strains was subjected to the estimation of enzyme activities. Endoglucanase enzyme ranged between 0.58-1.03 (U/mg) with maximum activity from the strain Ci-04, Ci-06 & Ci-08. The endoxylanase activity ranged between 0.74 to 2.11 (U/mg) with maximum activity again in the culture Ci-04, Ci-06 & Ci-08. The laccase activity was maximum in the culture Ci-03 & Ci-09 (Table 7; Fig. 11)

Table 7: Specific enzyme activity of Substrate collected at the time of spawn run

Strain	**Endoglucanase (U/mg)**	**Endoxylanase (U/mg)**	**Laccases (U/mg)**
Ci-01	0.81	0.74	1.51
Ci-02	0.66	1.08	2.13
Ci-03	0.78	1.13	4.54
Ci-04	1.03	1.81	2.96
Ci-05	0.87	1.41	2.44
Ci-06	0.95	1.70	3.77
Ci-07	0.58	1.13	3.24
Ci-08	0.91	2.11	2.03
Ci-09	0.65	1.42	4.11
Macrocybe	0.61	1.25	2.64
CD (p=0.05)	0.19	0.43	0.65

Mean of three replicates

Incubation temperature: 30°C

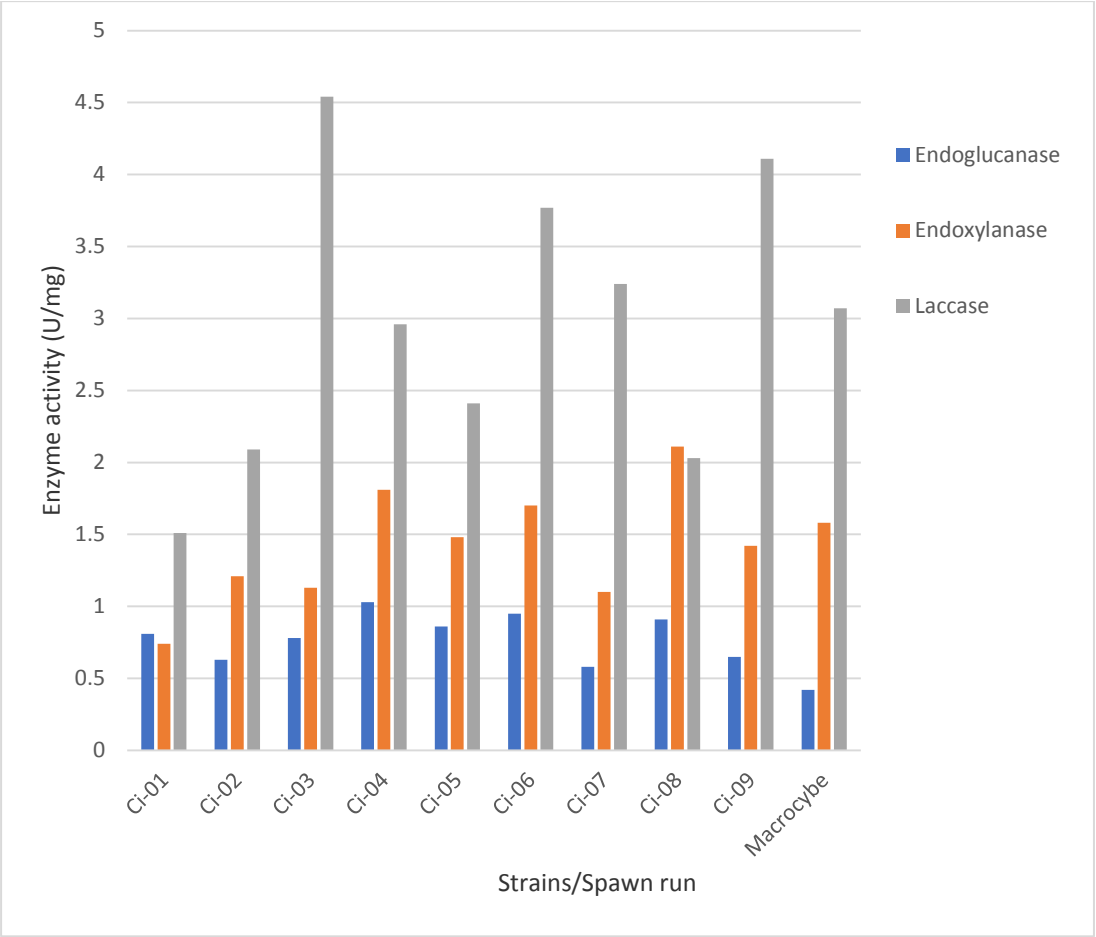


Fig. 11 Specific enzyme activity of *C. indica* strains during spawn run

4.3.3 Pinhead

The enzyme extracted from the substrate underneath the mushroom pinheads indicated endoglucanase activity 0.61-1.59 (U/mg) with maximum activity in Ci-06 & Ci-07 followed by Ci-04, Ci-01 & Ci-09. The endoxylanase activity ranged between 1.57-2.96 (U/mg) with maximum activity from Ci-01, Ci-06 Ci-08 followed by Ci-04 & Ci-09. The laccase activity range between 6.53-10.6 U/mg with maximum activity in Ci-04 & Ci-09 followed by Ci-03, Ci-06 & Ci-08 (Table 8; Fig. 12)

Table 8: Specific enzyme activity of substrate at the time of Pinhead formation

Strain	Endoglucanase (U/mg)	Endoxylanase (U/mg)	Laccases (U/mg)
Ci-01	1.25	2.96	6.96
Ci-03	1.29	1.81	8.82
Ci-04	1.12	2.88	10.6
Ci-06	1.59	2.29	7.75
Ci-07	1.43	1.57	6.53
Ci-08	0.61	2.93	8.32
Ci-09	1.09	1.86	10.4
CD (p=0.05)	0.40	0.47	1.18

Mean of three replicates

Incubation temperature: 30°C

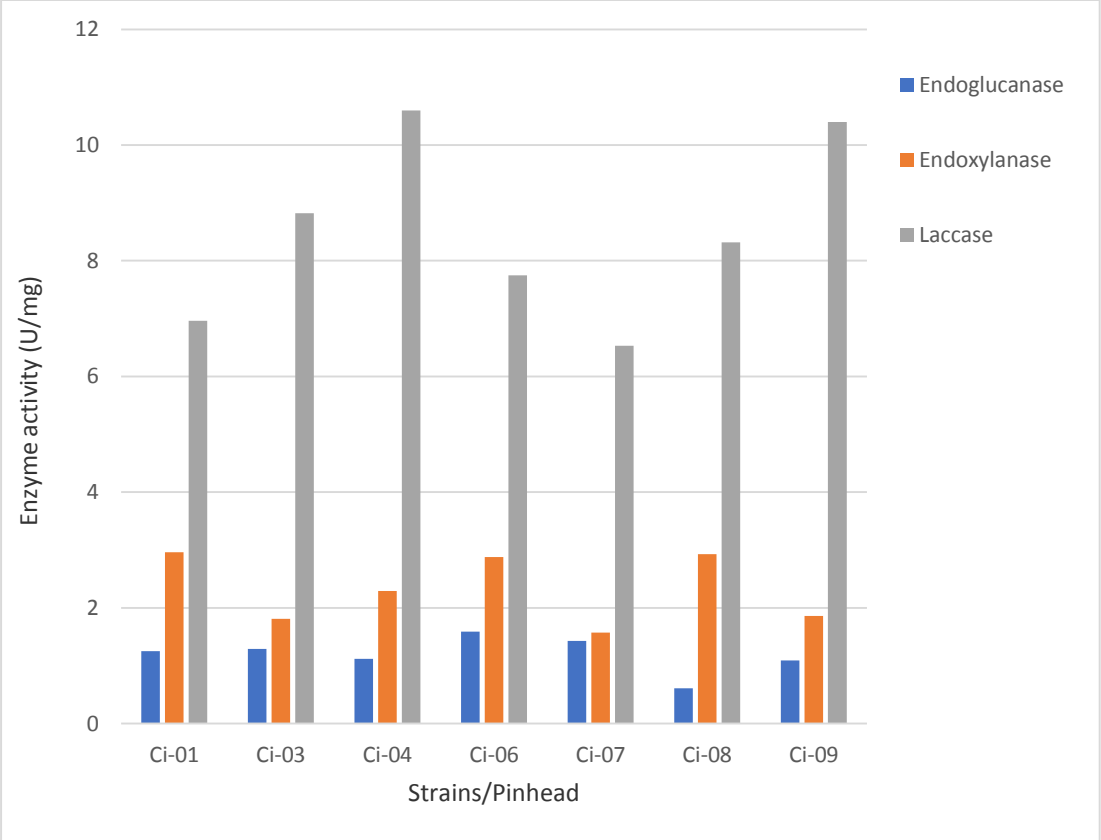


Fig. 12 Specific enzyme activity of *C. indica* strains during pinhead

4.3.4 Fruiting body

The endoglucanase and endoxylanase activity of the strain ranged between 0.94-1.88 U/mg and 0.93-2.36 U/mg, but statistically the enzyme activity of the harvested mushroom fruit body was at par with each other. The laccase activity ranged between 5.43-9.21 U/mg with maximum activity in the fruiting body of Ci-08 & Ci-09 followed by Ci-04, Ci-06 and Ci-07 (Table 9; Fig. 13)

Table 9: Specific enzyme activity of Fruiting body

Strain	Endoglucanase (U/mg)	Endoxylanase (U/mg)	Laccases (U/mg)
Ci-01	1.36	2.11	5.43
Ci-03	0.94	2.36	5.98
Ci-04	1.31	2.21	7.21
Ci-06	1.88	1.98	7.03
Ci-07	1.71	1.59	6.33
Ci-08	1.22	1.83	9.21
Ci-09	1.43	0.93	8.24
CD (p=0.05)	NS	NS	1.40

Mean of three replicates
Incubation temperature: 30°C

Cellulase activity in *Calocybe indica* (0.49 U/ml) and *Pleurotus ostreatus* (0.60U/ml) was observed maximum at 70°C (Karthikeyan 2015). It has been reported that wheat straw produced maximum laccase (187.38 U/ml) after 24 days at 30°C (Upadhyay and Tripathi 2014). The maximum Xylanase activity was observed in the strain VvS-4 (147 U/100ml) (Choudhary *et al* 2009). Maximum activity of Endoglucanase and Endoxylanase was recorded for the strain Ci-6 (2.72, 2.29 U/mg) and Ci-2 (1.70,1.88) (Redhu 2010).

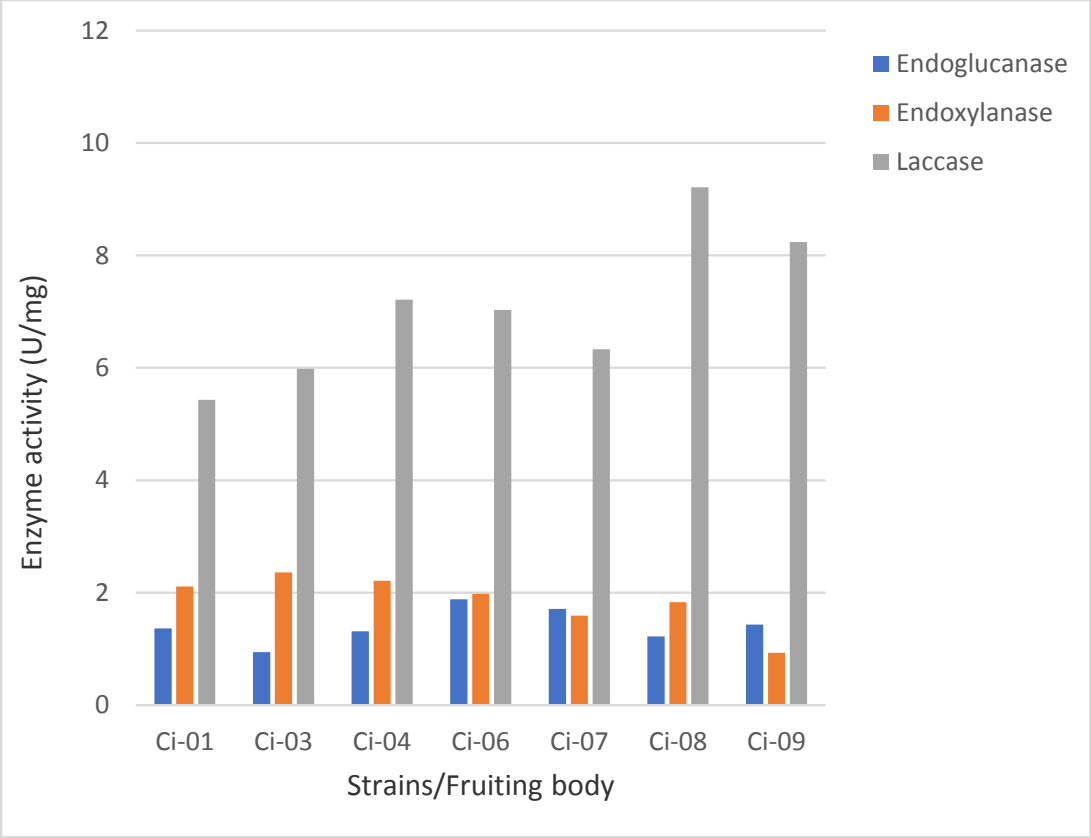


Fig. 13 Specific enzyme activity of *C. indica* strains during fruiting body

4.4 Correlation of biomass and enzyme with yield

Table 10: Correlation of Yield with Biomass and Enzymes

Strains	Correlation		
	Yield (% B.E.)	Biomass	Endoglucanase
Ci-01	25.5	-0.252	-0.625
Ci-03	20.2	0.366	0.896
Ci-04	30.4	-0.186	0.401
Ci-06	57.3	0.044	0.670
Ci-07	48.5	0.259	0.619
Ci-08	32.3	-0.869	-0.326
Ci-09	45.7	0.490	0.562

The correlation of yield with biomass and endoglucanase had indicated positive correlation in the strains Ci-06, Ci-07, Ci-09 and Ci-03. These strains with the maximum yield had a positive relation with biomass as well as endoglucanase enzyme production (Table 10; Fig 14)

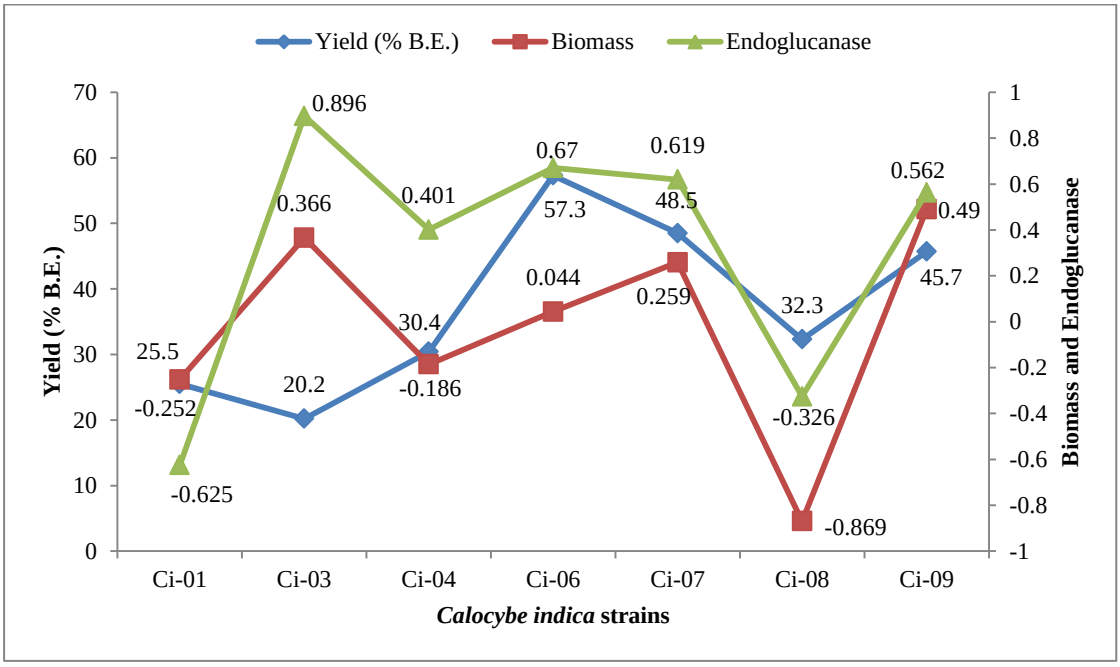
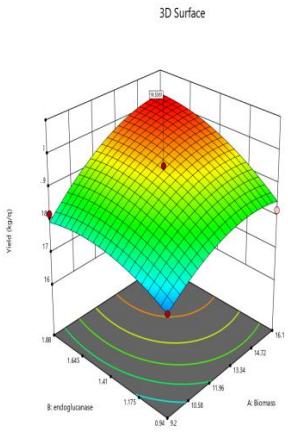


Fig. 14 Correlation of yield with biomass and enzyme

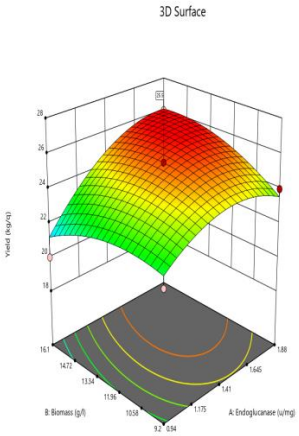
4.5 Optimization of biomass and endoglucanase enzyme for the yield of different strains of *C. indica.*

The three-dimensional (3D) response surface graph of yield based on RSM depicted model were generated in pairwise combination of two factors. Response at the central point corresponds to maximum achievable yield for that factor.

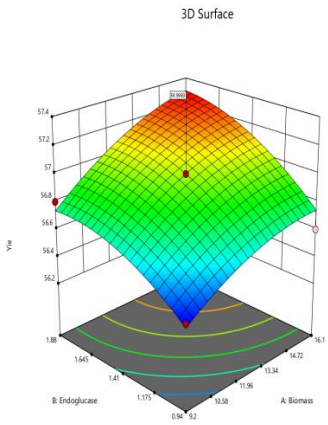
Ci-01



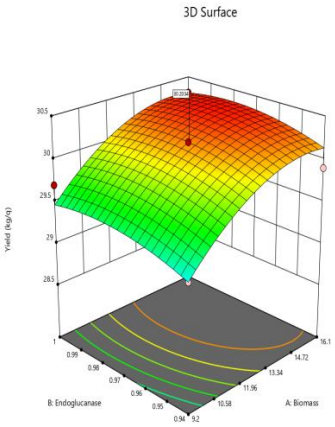
Ci-03



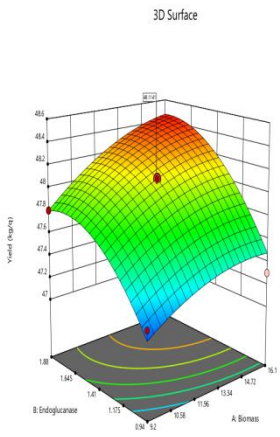
Ci-06



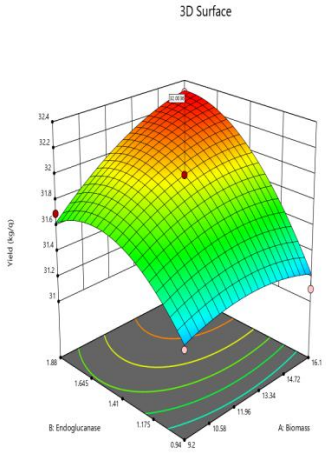
Ci-04



Ci-07



Ci-08



Ci-09

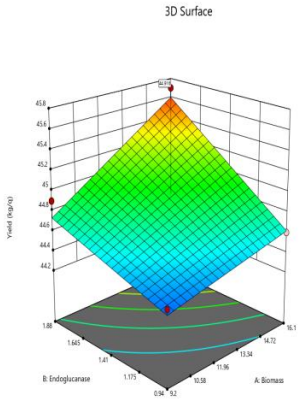


Fig 15: Response surface plot of biomass and endoglucanase with respect to the yield production of *Calocybe indica* strains

CHAPTER V

SUMMARY

Calocybe indica commonly known as milky mushroom, an edible fungal species originated from India (P&C 1974). *Calocybe* genus has about 40 species. This mushroom grows well in the months of May to September**.** It is primarily cultivated in India. It is a heat loving mushroom require a temperature of about 28-35°C and relative humidity of 80-90%. The objective of the study was to evaluate of *Calocybe indica* strains for cellulase, xylanase and laccase enzyme producing capability and to observe a possible correlation of the enzyme production from different strains with their yield potential. Nine strains of *Calocybe indica* Ci-01 to Ci-09 and one *Macrocybe* were used.

Calocybe indica was studied for mycelium growth on solid as well as on liquid media. In the mycelial growth study, radial growth and biomass growth were observed on Potato dextrose, Complete yeast extract and Mushroom minimal media. Linear growth was also studied on the wheat straw. Observations were recorded as colony diameter (mm), biomass (g/l) in broth and linear growth (mm/day). The maximum colony diameter was observed with Ci-09 on PDA, CI-07 on CYM as well as in MMM. On 7^{th} day the colony diameter of *Calocybe indica* strains on PDA ranged between 42-63 mm, on CYM 42-60 mm and on MMM 38-44 mm. The maximum colony diameter was that of Ci-09 on PDA & Ci-07 and *Macrocybe* on CYM and on MMM, the *Macrocybe*. On 10^{th} day the range of colony diameter was between 68-87.6 mm on PDA, 65-85.3 mm on CYM, 60-70.6 mm on MMM. Maximum growth on PDA was observed in Ci-06 and Ci-09, on CYM it was on Ci-07 while on MMM, maximum growth was observed with Ci-09. The mycelial biomass ranged between 9.6 to 16.1 g/l in PDB, 9.2 to 16.1 in CYM and 8.3 to 12 in MMM. The maximum biomass in PBD was that of Ci-06 and Ci-07, while on CYM, it was maximum in Ci-06 and Ci-09. The maximum biomass in MMM was observed in Ci-04, Ci-06, Ci-07 and Ci-09. Maximum linear growth after 3 days was observed in Ci-06 and Ci-07. After 7 days maximum linear growth was that of Ci-03, Ci-07, Ci-09 and after 10 days maximum growth was found in Ci-07 and Ci-09.

Nine strains of *Calocybe indica* Ci-01 to Ci-09 and one *Macrocybe* were grown on wheat straw. Observations were recorded on days of spawn run, number of fruit bodies, yield (percent) and average weight of fruit body along with stipe length and pileus diameter of the different sample of each strain. The number of fruit bodies harvested were maximum for Ci-06 (1552) followed by Ci-07 and Ci-09. The biological efficiency estimated from the harvested yield (kg/q dry straw) was found to show maximum yield potential for the strain Ci-06 (57.3%). Two strains Ci-07 and Ci-09 were found to give yield at par with each other whereas Ci-03 showed low biological efficiency (20.2%), whereas two strains did not fruit

(Ci-02 and Ci-05). Average weight of fruit body ranged between 35-53g with maximum for Ci-06, Ci-07 and Ci-09 strains and lowest for Ci-03 strain.

The pileus diameter of strains ranged between 6.9 to 8.8 cm and stipe length ranged between 10.8 and 14.3 cm. The maximum pileus diameter and stipe length was recorded in the strain Ci-09. The maximum total length of the fruiting body and stipe diameter was observed in Ci-06. During the crop no disease and pest infestation was recorded. Considering the yield potential of *C. indica* strains only one strain Ci-06 indicated maximum biological efficiency.

To study the enzyme activity of endoglucanase, endoxylanase and laccase, the enzymes were extracted from culture filtrate, at the time of spawn run, pinhead formation and fruiting bodies of *Calocybe indica.* In culture filtrate endoglucanase and endoxylanase activity ranged between 1.44-4.42 (U/mg) and 2.73-4.76 (U/mg) maximum endoxylanase activity was found in Ci-07 followed by Ci-04, Ci-06 & Ci-09. The lowest activity was observed in Ci-08. Laccase activity of the strains culture filtrate ranged between 6.94 to 10.2(U/mg) with maximum activity in Ci-03 followed by Ci-04, Ci-07, Ci-06. At the time of spawn run endoglucanase enzyme ranged between 0.58-1.03 (U/mg) with maximum activity from the strain Ci-04, Ci-06 & Ci-08. The endoxylanase activity ranged between 0.74 to 2.11 (U/mg) with maximum activity again in the culture Ci-04, Ci-06 & Ci-08. The laccase activity was maximum in the culture Ci-03 & Ci-09.

The enzyme extracted from the substrate underneath the mushroom pinheads indicated endoxylanase activity 0.61-1.59 (U/mg) with maximum activity in Ci-06 & Ci-07 followed by Ci-04, Ci-01 & Ci-09. The Endoxylanase activity ranged between 1.57-2.96 (U/mg) with maximum activity from Ci-01, Ci-06 Ci-08 followed by Ci-04 & Ci-09. The laccase activity range between 6.53-10.6 (U/mg) with maximum activity in Ci-04 & Ci-09 followed by Ci-03, Ci-06 & Ci-08. At the time of fruiting bodies, endoglucanase and endoxylanase activity of the strains ranged between 0.94-1.88 U/mg and 0.93-2.36 (U/mg), but statistically the enzyme activity of the harvested mushroom fruit body was at par with each other. The laccase activity ranged between 5.43-9.21 (U/mg) with maximum activity in the fruiting body of Ci-08 & Ci-09 followed by Ci-04, Ci-06 and Ci-07. The correlation of yield with biomass and endoglucanase had indicated positive correlation in the strains Ci-03 Ci-06, Ci-07, and Ci-09 of *Calocybe indica.*

REFERENCES

Ahlawat O P, Raj D, Indurani C, Sagar M P, Gupta P and Vijay B (2009) effect of spent mushroom substrate recomposed by different methods and on different age on vegetable growth, yield and quantity of tomato. *Indian J Horti* **66:** 208-14

Akhmedova Z R (1994) Cellulolytic, xylanolitic and lignolytic enzymes of the fungus *Pleurotus ostreatus. Prikladnaya Biokhimiya i Microbiologiy* **30:** 42-48.

Alam N, Amin R, Khan A, Ara I, Shim M J, Lee M W and Lee T S (2008) Nutritional analysis of cultivated mushrooms in Bangladesh: *Pleurotus ostreatus, Pleurotus sajor-caju, Pleurotus florida and Calocybe indica. Mycobiology* **36:** 228-32.

Alam N, Amin R, Khair Aand Lee T S (2010) Influence of different supplements on the commercial cultivation of milky white mushroom. *Myco biol* **38(3):** 184-88.

Amin R, Khair A, Alam N and Lee T S (2010) Effect of the substrate and casing material on the growth and yield of *Calocybe indica. Mycobiol* **38(2):** 97-101.

Anandh K and Prakasam V (2002) Studying the post-harvest biology of the mushrooms *Tricholoma lobayense* and *Calocybe indica. Souvenir and abstracts 3rd IndianMush Conf.* pp 132. TNAU, Coimbatore.

Beelman R B, Guthrie B D and Royse D J (1989) Shelf life extension of mushroom *Agaricus bisporus* by application of hydrogen peroxide and browning inhibitors. *Mush Sci* **12(2):** 655-65.

Beyer M, Koch H, Fischer K (1996) Photoyellowing of high yield pulps-looking for oxygen radicals.8th International Symposium, Finland. *Wood Pulp Chem* **3:** 5-8.

Bhavana A K and Thomas G V (2002) Yield pattern and nutrient composition of milky white mushroom *Calocybe indica*- Cultivation on fermented Coir pith. I: *Souvenir-cum-Abstracts*, 3rd*Indian Mushroom Conf*erence pp 87 TNAU, Coimbatore.

Bhupinder K and Ibitwar B B (2007) Mushroom cultivation and technology. *Sci Technol Enterpreneur* pp 1-12.

Bokaria k, Balsundram S K, Bhattarai I and Kaphle K (2014) Commercial production of milky mushroom (*Calocybe indica*). *Merit Res J Agric Sci Soil Sci* **2(2):** 032-037.

Breene W M (1990) Nutritional and medicinal value of specialty mushrooms. *J Food Protec* **53:** 883-94.

Bucht B and Eriksson K E (1968) Extracellular enzyme system utilized by the rot fungus *Stereum sanguinolentum* for the breakdown of cellulose. *Arch Biochem Biophys* **124**:134-41.

Burton K S and Noble R (1993) The influence of flush number, bruising and storage temperature on mushroom quality**.** *Postharv Biol Technol* **3:** 39-47.

Buswell J A, Cai Y J, Chang S T, Pederdy J F, Fu S Y and Yu H S (1996) Lignocellulytic enzyme profiles of edible Mushroom fungi. *World J Microb Biotechnol* **12(5):** 537-42.

Cavazzoni V and Manzoni M (1994) Extracellular cellulyticcomplex from *Morchella conica*: production and purification. *Lebensmttel-Wissenschaft Technologie* **27:**73-77.

Chakravarty D K, Sarkar B B and Kundu B M (1981) Cultivation of tropical edible mushroom *Calocybe indica. Indian Agri* **25**: 57-60.

Chang Ho Y (1980) Some factors affecting cellulose Utilization by Vorvariella volvacea Singer. Presented at Mycological Workshop at the New York University, Toronto, Ontario, Jan. 26, 1980.

Chang S T (2001) A 40-year journey through bioconversion of lignocellulosic waste to mushrooms and dietary supplements. *Int J Med Mush* **3:** 299-310.

Chaudhary M, Shashi D, Kapoor S and Giridhar S (2009) Lignocellulolytic enzyme activities and substrate degradation by Volvariella volvacea, the paddy straw mushroom/Chinese mushroom. *Indian J Agril Res* **43:** 466-70.

Chauhan S (2009) *RAPD marker assisted development of improved strains of Agaricus bisporus (Lange) Sing.* Ph. D Dissertation, Punjab Agricultural University, Ludhiana.

Coskuner Yand Ozdemir Y (2000) Acid and EDTA blanching effects on the essential element content of mushrooms *(Agaricus bisporus). J Sci Food Agri* **80:** 2074-76.

Crisan E V and Sands A (1987) Nutritive value. In: Chang S T and Hayes W A (eds) *The Biology and Cultivation of edible mushrooms*. Pp 137-68. Academic Press, New York, London.

Czapski J (1994) Dynamics of sorption of sodium metabisulfite solution in aspect of sulphur dioxide residue in mushrooms. *Biul Warz* **41:**169-76.

Czapski J (2002) Mushrooms quality as affected by washing and storage condition. *Veg Crops Res Bull* **56:** 121-28.

Czapski J and Bakowski J (1995) The influence of different concentrations of sodium metasulfite and time of storage on whiteness and sulphur dioxide residues in frozen mushrooms. *Biul Warz* **43:**103-08.

Czapski J and Szudyga K (2000) Frozen mushrooms quality as affected by strain, flush, treatment before freezing, and time of storage. *J Food Sci* **65:** 722-25.

Das M and Kalita M C (2006) *Value addition of mushroom.* Sci Tech Entrepreneur, Directorate of Extension Education, Assam Agriculture University, Jorhat, Assam, India.

Devece C, Rodriguez-Lopez J N, Fenoll L G, Tudela J, Catala J M, de los Reyes E and Garcia-Canovas F (1999) Enzyme inactivation analysis for industrial blanching applications: comparison of microwave, conventional, and combination heat treatments on mushroom polyphenoloxidase activity. *J Agril Food Chem* **47:** 4506-11.

Dickinson C and Lucas J (1979) The encyclopedia of mushrooms. Lucea Orbis Publishing, London.

Doshi A and Sharma S S (1995) Production technology of specialty mushrooms. In: Chada K L and Sharma S R (ed). *Advances in Horticulture*.**13**: 135-54. Mushrooms, MPH, New Delhi.

Doshi A, Munot J F and Chakarvarti B P (1987) Pectinolytic and Celluolytic enzyme production at various stages of spawn growth of *Calocybe indica. Mush J Tropics* **7:** 83-85.

Doshi A, Munot J F and Chakarvarti B P (1988) Nutritional status of an edible mushroom *Calocybe indica* P&C. *Indian J Mycol Pathol* **18:** 3301-02.

Doshi A, Sharma S S and Trivedi A (1993) A promising edible mushroom for the tropics: *Calocybe indica* (P&C). *Mush Info* **10**: 14-22.

Doshi A, Sidana N and Chakravarti B P (1989) Cultivation of summer mushroom, *Calocybe indica* Purkayastha and Chandra in Rajasthan. *Mush Sci* **12**:395-400.

Edelstein L and Segel L A (1999) Growth and metabolism in mycelia fungi. *J Theor Biol* **104:**187-90.

Eggert C, Temp U, Dean J F and Eriksson K E (1996) A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Letters* **391:**144-48.

Eichlerova I and Homolka L (1999) Preparation and crossing of basidiospores-derived monokaryons- a useful tool for obtaining laccase and other ligninolytic enzyme higher producing dikaryotic strains of *Pleurotus ostreatus. Antonie van Leeuwenhoek* **75:** 321-27.

Fox R and Chorover J (1999) Seeking a cash crop in spent substrate. Retrieved from http:/aginfo.psu.edu/psa/sgg/mushrooms.html cited on 11-7-19.

Fritsche G (1983) Breeding *Agaricus bisporus* at the Mushroom Experimental Station, Horst. *Mush J* **122:** 49-53.

Girdhari L, Siddappa G S and Tandon G L (1998) *Preservation of fruits and vegetables.* Indian Council of Agricultural Research, publication and information division, New Delhi 110012, pp 73-74.

jawoHofrichter M and Ullrich R (2010) New trend in fungal biooxidation. In: Hofrichter, M (Ed.). industrial applications. 2nd Edition.Spingerverlag, Berlin Heideberg pp.425-449.

Jalali S, Pandey M, Gowda I N D and Tewari R P (2003) Development of value-added products from milky mushroom of *Calocybe indica*, In: Upadhyay R C, Singh S K and Rai R D (ed.) *Current vistas in mushroom biology and production* pp 237-41, Mushroom Society of India, NRCM, India.

Jaworska G, Gebezynski P and Golyszny A (2003) The use of mushrooms (Agaricus bisporus) in the production of frozen and canned stuffings. *Zywnosc Supl* **36:** 63-71.

Joseph K, Kothandaraman R and Mathew J (1998) Rubber wood saw dust - an ideal substrate for summer mushroom cultivation. *Planter* **74:** 527-29.

Kalmis E, Yasa I, Kalyoncu F, Pazarbasi B and Kocyigit A (2008) Ligninolytic enzyme activities in mycelium of some wild and commercial mushrooms. *Afr J Biotechnol* **7:** 4314-20.

Kapoor M, Sodhi H S and Dhanda S (1996) Strategies for strain improvement in *Pleurotus* species. *Mush Res* **5:** 57-66.

Karthikeyan P (2015) Optimisation of cellulase enzyme production from *Pleurotus ostreatus* and *Calocybe indica. IJPBS* **5:**11-16.

Kaur J (2012) *Molecular characterisation of mutant strains of Calocybe indica using RAPD-PCR.* M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Kaur K (2005) *Studies on Calocybe indica strains for improving biological efficiency and shelf life.*M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Kaur N (2010) *Studies on genetic variability and biomolecules production in Lentinus edodes strains.* M.Sc. Thesis. Punjab Agricultural University, Ludhiana, India.

Kaur P (2003) *Nutritional and Cultivational AspectsCalocybe indica.* M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Kaur R (2016) *Effect of growth regulator on biomass production and yield potential of milky mushroom Calocybe indica* (P&C). M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Khurdiya D S (1995) Non-thermal methods for preservation of fruits and vegetables: A critical appraisal. *J Food Sci Technol* **32:** 441-52.

Kochthresiamma J (1998) Rubber wood saw dust- An ideal substrate for summer mushroom cultivation. *Planter* **74**: 527-29.

Krick P M, Cannon P F, David J C and Stalpers J A (2001) *Ainsworth and bisby's dictionary of fungi.* Biddles, London. 655pp.

Krishnamoorthy A S (2003) Commercial prospects of milky mushroom (*Calocybe indica*) in the tropical plains of India. In: Upadhyay R C, Singh S K and Rai R D (ed) *Current Vistas in Mushroom Biology and Production*. Pp 131-35. Mushroom Society of India, NRCM, Solan, India.

Krishnamoorthy A S and Muthuswamy M (1997) Yield performance of *Calocybe indica* (P&C) on different substrate. *Mush Res* **6:**29-32.

Krishnamoorthy A S, Marimuthu T and Muthusamy M (2002) Enzymes related biodegradative potential of *Calocybe indica. Souvenir and Abstracts 3rd Indian Mushroom Conference.*pp 36. TNAU, Coimbtore.

Krishnamoorthy A S, Muthuswamy M T and Nakkeeran S (2000) Technique for commercial production of milky mushroom *Calocybe indica* P&C. *Indian J Mush* **18:**19-23.

Kuhad R C, Singh A and Eriksson K E L (1997) Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In K.-E. L. Eriksson (ed.), *Advances in Biochemical Engineering Biotechnology*, vol. 57. Springer-Verlag, Germany. p. 46-125.

Kumar R, Singh G, Pandey P and Mishra P (2011) Cultural, physiological characteristics and yield attributes of strains of milky mushroom (*Calocybe indica*). *J Mycol Pl Pathol* **41(1):** 67-71.

Kumar R (2010) *Interspecific hybridization between Pleurotus florida and Pleurotus sajorcaju to develop hybrid dikaryon(s) and their molecular characterization.* Ph. D Dissertation, Punjab Agricultural University, Ludhiana, India.

Kumara K LW and Edirimann I C S (2009) Improvement of strains of two oyster mushroom cultivars using duel culture technique. *World Appl Sci J* **7:** 654-60.

Lewicki P P and Jakubczyk E (2004) Effect of hot air temperature on mechanical properties of dried apples. *J Food Eng* **59:** 191-98.

Lonergan G, Jones C and Mainwaring D (1993) The effect of pH and temperature on radial growth rate and biomass production for selected Australian white rot fungi in comparison with two strains of *Phanerochaete chrysosphorium. Material and Organism* **28:** 309-17.

Lopez M J, Nicholas N N, Dien B S, Moreno J and Bothast R J (2008) Isolation of microorganisms for biological detoxication of lignocellilosic hydrolysates. *Appl Microbial Biotechnol* **64**: 125-31

Lowry O H, Rosebrough N J, Farr A L and Randall R J (1951) Protein measurement with folin phenol reagent. *J Biol Chem* **193:** 265-75.

Madan M and Bisaria R (1983) Cellulolytic enzymes for an edible mushroom. *Pleurotus sajor- kaju. Biotechnol Lett* **5(9):** 601-04.

Mallavadhani U V, Sudhakar A V, Satyanarayana K V, Mahapatra A, Li W, Van-Breemen R B (2006) Chemical and analytical screening of some edible mushrooms. *Food Chem* **95:** 58-64.

Mandels M, Andreotti R and Roche C (1976) Measurement of saccharifying cellulase. *Biotech Bioeng Symp* **6:** 21-23.

Mangat M (2005) *Evaluation of Calocybe indica Strains for their Enzymes and Yield Potential.* M.Sc. thesis, Punjab Agricultural University, Ludhiana, India.

Mangat M, Khanna P K, Kapoor S and Sohal B S (2008) Biomass and Extracellular Lignocellulolytic Enzyme Production by *Calocybe indica* Strains. *Global J Biotechnol Biochem* **3**: 98-104.

Manning K and Wood D A (1983) Production and regulation of extracellular endo-cellulase by *Agaricus bisporus. J General Microbiol* **129**:1839-47.

Marimuthu T and Krishnamoorthy A S (1991) Annual report of All India Coordinated mushroom Improvement Project 1991-91, Dept. of Plant Pathol, TNAU, Coimbtore **3**: 23.

Mayer A M and Staples R C (2002) Laccase: new functions for an old enzyme. *Phytochemistry* **60:** 551-65.

Miller G L (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugars. *Anal Chem* **31**: 426-28.

Mtui G Y S (2012) Lignocellulolytic enzymes from tropical fungi: types, substrate and application. *Sci Res Appl* **7(15):** 1544-55.

Naraian R, Sahu R K, Kumar S, Garg S K, Singh C S and Kanaujia R S (2009) Influence of different nitrogen rich supplements during cultivation of *Pleurotus florida* on corn cob substrate. *Environmentalist* **29:** 1-7.

Ohga S, Smith M, Trurston C and Wood D A (1999) Transcriptional regulation of laccase and cellulase genes in the mycelium of *Agaricus bisporus* during fruit body development on a solid substrate. *Mycol Res* **103:** 1557-60.

Ohm R A, Jong J F, Lugones L G, Aerts A, Baker S E,Bartholomew S, Erdmann S, Fowler T J, Gathman A C, Lombard V,Henrissat B, Lilly W and Raudaskoski M (2010) Genome sequence of the model mushroom *Schizophyllum commune. Nature Biotechnol* **28:** 957–63.

Palmieri G, Bianco C, Giardiana P, Scaloni A, Capasso A and Sannia G (1997) A novel white laccase from *Pleurotus ostreatus. J Biol Chem* **272:** 301-07.

Pandey M (1998) *Cultivation Technology of Specialty Mushroom (Calocybe indica).* Ph.D. dissertation, Himachal Pradesh University, Shimla, India.

Pandey M and R P Tewari (1993) Milky mushroom grown on paddy straw. *The Hindu* **93:** 24.

Pandey M and Tewari R P (2003) *Tricholoma giganteum* – A new potential species for tropical regions. In: Upadhyay R C, Singh S K and Rai R D (ed) *Current Vistas in Mushroom Biology and Production*. Pp 115-20. Mushroom Society of India, NRCM, Solan, India.

Pani B K (2010) Growth of white summer mushroom (*Calocybe indica)* in submerged culture in Orissa. *J Environ Eco* **28:** 965-66.

Pani B K (2011) Effect of age and quantity of spawn on milky mushroom production. *Asian J Exp Biol Sci* **2:**769-71.

Pani B K (2011b) Influence of some growth regulator on biomass production and sporophore yield of milky mushroom (*Calocybe indica). Asian J Exp Biol Sci* **2(2):** 328-31.

Pani B K (2016) Studies on tissue culture as a biotechnological tool for improving production of milky mushroom *Calocybe indica. IJPAES* **6:** 11-17.

Pani B K and Das S R (1998) Seasonal productivity of summer white mushroom (P & C) in Orissa. *Sci Cult* **40(7&8):** 177-78.

Peng J T, Lee C M and Tsai Y F (2000) Effect of rice bran on the production of different king oyster mushroom strains during bottle cultivation. *J Agril Res China* **49:** 60-67.

Perez J, Martinez J and Rubia T D L (1996) Purification and partial characterization of a laccase from the white rot fungus *Phanerochaete flavido-alba*. *Appl Environ Microbiol* **62:** 4263–67.

Phutela U G and Phutela R P (2012) Effect of physical and chemical factors on growth of *Calocybe indica* (P & C). *Int J Adv Life Sci* **2:** 8-16.

Purkayastha R P (1984) cultivation of *Calocybe indica* (P & C). *Indian J Mush* **10-11**: 12-17.

Purkayastha R P and Chandra A (1974) New species of edible mushroom from India. *Trans Br Mycol Soc* **62:** 415-18.

Purkayastha R P and Chandra A (1976) A new technique for *in vitro* production of *Calocybe indica* – an edible white mushroom. *Mush J* **40**:112-13.

Purkayastha R P and Nayak D (1977) Effects of substrates and spawn density on the production and protein content of fruit body of *Calocybe indica*. *Mush J* **52**: 1-3.

Purkayastha R P and Roy S J (1982) Cultivation of *Calocybe indica*: a new source of vegetable protein. *Front Res Agri* pp. 580-86.

Rai R D and Saxena S (1990) Extracellular enzymes and non-structural components during growth of *Pleurotus* on rice straw. *Mush J Tropics* **10**: 69-72.

Redhu N (2010) *Studies of extracellular lignocellulolytic enzymes in relation to Calocybe indica (P&C) strains.* M.Sc. Thesis, Punjab Agricultural University, Ludhiana, India.

Rinker D L (2002) Handeling and using spent mushroom substrate around the world. In J E Sanchez, G Huerta and E Monitel (eds.), *Proc fourth International Conference on Mush Biol Mush Products* Pp:43-60.

Rodrigo M, Calvo C, Sanchez T, Rodrigo C and Martinez A (1990) Quality of canned mushrooms acidified with glucono-δ-lactone. *IntJ Food Sci Technol* **34:** 161-66.

Salam A S, Geetha D, Suharban M and Nair K H (2004) Coirpith- a non- conventional substrate for *Calocybe indica* (milky mushroom) production. *Mush Res* **13:** 60-64.

Sandhu K S and Aggarwal P (2001) Steeping preservation of mushrooms (*A. bisporus*). *J Res* **38:** 53-57.

Sapers G M, Miller R L, Pilizota V and Kamp F (2001) Shelf-life extension of fresh mushrooms (*Agaricus bisporus*) by application of hydrogen peroxide and browning inhibitors. *J Food Sci* **66:** 362-66.

Saxena S and Rai R D (1992) Effect of pretreatment of wheat straw on biodegradation by *P. Sajor-caju* (Fr). Sing. *Mush Res* **1(2):** 131-33

Schisler L C and Sinden J W (1962) Nutrient supplementation of mushroom compost spawning. *Mush Sci* **5**: 150-64.

Selman J D (1998) The blanching process. In: *Development in Food Preservation.* Thorne S (ed). Elsevier Applied Science Publisher, Barking, UK.

Selvi S , Devi P U, Suja S, Murugan S and Chinnaswamy P (2007) Comparison of non-enzymic antioxidant status of fresh and dried form of *Pleurotus florida* and *Calocybe indica. Pak J Nutr* **6:** 468-71.

Sharma J P and Kumar S (2007) Evaluation of strains of milky mushroom *Calocybe indica* for cultivation in Jharkhand (Abstract). *Int Conf Mush Bio Biotech* Pp.135-36.

Sharma S R and Upadhyay R C (1998) Scope and cultivation of specialty mushrooms in India. In: *Compendium of Lectures on Recent Advances in Mushroom Cultivation*. pp 177-202.HAU, Hisar.

Sharma S S, Doshi A, Trivedi A and Kothari K L (1994) Factors affecting sporophore production of *Calocybe indica*. *National Symp Mush* **3**: 57.

Sharma S S, Doshi A, Trivedi A and Kothari K L (1997) Studies on spawn research of *Calocybe indica* (P&C). *Indian Bot Reporter* **14**: 97-100.

Sharma V P, Annepu K, Gautam Y, Singh M and Kamal S (2017) Status of mushroom production in India. *Mush Res* **26(2):** 111-20

Sharma V P, Sharma S R and Kumar S (2006) Comparative studies on substrate treatment methods for cultivation of *Calocybe indica. J Mycol Plant Pathol* **36:** 145-48.

Shashirekha MN, Rajarathnam S and Bano Z (2002) Enhancement of bioconversion efficiency and chemistry of the mushroom, *Pleurotus sajor-caju* (Berk and Br.) Sacc. produced on spent rice straw substrate, supplemented with oil seed cakes. *Food Chem* **76:** 27-31.

Singh M (2011) Mushroom production: an agribusiness activity. In: M. Singh, B Vijay, S Kamal, and G C Wakchaure (Eds.), Mushroom cultivation, marketing and consumption. Solan, HP, India: Directorate of Mushroom Research (ICAR). pp: 1-10

Singh M, Singh A K and Gautam R K (2009) Screening of substrate for growth and yield of *Calocybe indica. J Indian Phytopath* **62(1):** 109-11.

Singh R P, Garcha H S and Khanna P K (1988) Laccase production by *Pleurotus* spp. *Indian J Microbiol* **28**: 38-41.

Sivaparkasam K, Balasubrimanian T, Sadasivam S and Shanmugam N (1986) Nutritive value of sporophores of *Calocybe indica. Mush Newslett Trop* **6:**15-25.

Sivaprakasam K and Shanmugam N (1984) *Calocybe indica* grows well at Coimbatore. *TNAU Newslett* **14**:3.

Tan Y H and Wahab M N (1997) Extracellular enzyme producing during anamorphic growth in the edible mushroom *Pleurotus sajor*-caju. *World J Microbiol Biotech* **13:** 613-17.

Tandon G and Sharma V P (2006) Yield performance of *Calocybe indica* on various substrates and supplements. *Mush Res* **15:** 37-39.

Tano K, Arul J, Doyon G and Castaigne F (1999) Atmospheric composition and quality of fresh mushrooms in modified atmospheric packages as affected by storage temperature abuse. *J Food Sci* **64:** 1073-77.

Teeri T (1997) Crystalline cellulose degradation: new insight into the function of cellobiohydrolases. *Trends in Biotechnology* **15:**160-67.

Tewari R P (2004) Ingredients, formulations and methods of substrate preparation and crop management of milky mushroom (*Calocybe indica*). *National Training Course on Mushroom Production Technology Entrepreneurs.* pp 77-79. NRCM, Solan.

Theradimani M, Meena B and Krishnamoorthy A S (2001) Innovative techniques for improvement of sporophore size and yield of Milky mushroom. *Mush Res* **10:**23-26.

Thurston C F (1994) The structure and function of fungal laccases. *Microbiology* **140:** 19-26.

Trivedi A, Sharma S S and Doshi A (1991) Cultivation of *C. indica* under semi-arid conditions. *Indian Mush* **1991**:166-69.

Turner E M (1974) Phenol oxidase activity in relation to substrate and development stage in

mushroom *Agaricus bisporus. Trans Br Mycol Soc* **63:** 541-47.

Uddin M J, Nasiruddin K M, Haque M E, Biswas A K and Islam M S (2012) Influence of different media variety and growth regulators on mycelial colony proliferation of mushroom. *J Environ Sci Nat Res* **5**(1): 223-27.

Upadhyay R C, Tripathi A (2014) Lignolytic enzymes of *Calocybe indica Int Conf Mush Bio and Mush Prod ICMBMP* **8:** 225-30.

Upamanya G K and Rathaiah Y (2000) Effect of different methods of pasteurization on yield of oyster mushroom. *Ind J Hill Farming* **13(1/2):** 108-09.

Usha S (2007) Energy value of different agro-residues used for cultivation of *Calocybe indica* (P and C). *Agril Sci Digest* **27(1):** 75-76.

Vijaykumar G, John P and Ganesh K (2014) Selection of different substrate for the cultivation of milky mushroom (*Calocybe indica*). *Ind J Traditional Knowledge* **13(2):** 434-36.

Wuest P J and Fahy H K (1991) Mushroom compost: traits and uses. *Mulch News* **39:** 9-15.

Yu M Y and Chang S T (1987) Effect of osmotic stabilizers on the activity of mycolytic enzymes used in fungal protoplasts liberation. *Mircen J* **3:** 161-67.

APPENDIX-I

Radial growth

PDA

NR = 3 NT = 10 NE = 1

Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	11.100000	11.800000	9.5999990	10.400000	12.300000
6	14.600000	16.100000	12.300000	13.900000	10.800000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	12.376300	17.29	1.44096	
Error	20	.71599120		12.290000	6.88

CYM

NR = 3 NT = 10 NE = 1

Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	10.300000	12.100000	10.200000	9.2000000	12.400000
6	16.100000	13.766670	13.833330	15.500000	9.7000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	18.003800	26.65	1.39982	
Error	20	.67568360		12.310000	6.68

MMM

NR = 3 NT = 10 NE = 1

Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	8.3000000	8.5999990	9.3000000	11.200000	9.5000000
6	10.400000	12.000000	9.3000000	11.733330	8.5000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	5.5994470	5.69	1.68869	
Error	20	.98333740		9.8833330	10.03

Biomass

PDB

NR = 3 NT = 10 NE = 1

Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	11.100000	11.800000	9.5999990	10.400000	12.300000
6	14.600000	16.100000	12.300000	13.900000	10.800000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	12.376300	17.29	1.44096	
Error	20	.71599120		12.290000	6.88

CYB

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	10.300000	12.100000	10.200000	9.2000000	12.400000
6	16.100000	13.766670	13.833330	15.500000	9.7000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	18.003800	26.65	1.39982	
Error	20	.67568360		12.310000	6.68

MMM

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	8.3000000	8.5999990	9.3000000	11.200000	9.5000000
6	10.400000	12.000000	9.3000000	11.733330	8.5000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	5.5994470	5.69	1.68869	
Error	20	.98333740		9.8833330	10.03

---

linear growth on wheat straw
3rd day

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	8.0000000	7.6666670	10.000000	9.3333330	8.3333330
6	10.666670	13.000000	8.0000000	10.000000	8.0000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	8.4037280	4.13	2.42830	
Error	20	2.0333250		9.3000000	15.33

7th day

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	24.000000	28.000000	30.333330	31.333330	26.000000
6	31.000000	35.000000	30.000000	33.666670	29.666670

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	32.448570	5.21	4.25162	
Error	20	6.2332030		29.900000	8.35

10th day

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	53.666670	51.000000	59.000000	55.333330	58.333330
6	58.000000	63.000000	60.000000	66.000000	56.000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	57.663190	7.39	4.75605	
Error	20	7.8000000		58.033330	4.81

Cultivation
NFB

NR = 5 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	633.00000	392.00000	854.00000	1348.0000	1266.0000
6	742.00000	889.00000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	572324.00	42.12	151.094	
Error	28	13588.860		874.85710	13.32

Yield

NR = 5 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	25.500000	20.200000	30.400000	57.300000	48.500000
6	32.300000	45.680000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	916.42650	47.85	5.67238	
Error	28	19.152340		37.125710	11.79

Quality parameter
PD

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	7.5000000	8.5999990	6.9000000	8.2000000	7.4000000
6	8.5000000	8.8000000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	1.5542810	13.11	.603015	
Error	14	.11857390		7.9857140	4.31

TL

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	13.600000	15.200000	13.200000	16.400000	14.400000
6	15.800000	16.000000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	4.5887050	31.20	.671593	
Error	14	.14707730		14.942860	2.57

SL

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	11.200000	10.800000	11.600000	13.600000	12.400000
6	14.000000	14.300000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	6.0385340	53.51	.588268	
Error	14	.11284530		12.557140	2.68

ST

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	1.8000000	1.6000000	2.1333330	2.2000000	1.6000000
6	1.8000000	2.1000000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	.19190850	1.57	NS	
Error	14	.12190520		1.8904760	18.47

L value

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	58.000000	65.000000	63.000000	75.000000	77.666660
6	62.000000	72.000000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	163.42840	13.41	6.11421	
Error	14	12.190290		67.523810	5.17

Culture filtrate
Endoglucanase

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	2.2133330	2.2066670	1.9400000	2.0733340	1.4466670
6	1.7400000	1.0433330	3.6066670	2.7666670	1.8000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	1.5084470	20.90	.457465	
Error	20	.72163390E-01		2.0836670	12.89

Endoxylanase

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	3.1366670	3.9633330	2.9600000	3.3500000	2.9600000
6	3.7233330	4.2333340	2.8333330	3.8700000	2.8833330

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	.79451160	5.56	.643833	
Error	20	.14293820		3.3913340	11.15

Laccase

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	7.2633340	7.7966670	10.276670	8.1366660	7.7766670
6	7.1466670	8.5733330	7.2533340	6.6033330	7.1266670

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	3.2482100	4.74	1.40905	
Error	20	.68463130		7.7953340	10.61

Spawn run
Endoglucanase

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	.69666670	.69333330	.63000000	.95333330	.87333330
6	.96333330	.67000000	.64333340	.72333340	.61000000

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	.53474000E-01	4.00	.197007	
Error	20	.13383390E-01		.74566670	15.51

Endoxylanase

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	.84666660	1.0866670	1.0733330	1.6233330	1.4066670
6	1.6466670	1.4466670	1.8500000	1.4400000	1.8166670

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	.33204140	5.01	.438506	
Error	20	.66306110E-01		1.4236670	18.09

Laccase

NR = 3 NT = 10 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	1.8466670	2.1333330	3.8100000	2.8700000	2.4433330
6	3.3900000	3.2766670	2.0566670	3.9200000	2.6466670

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	9	1.6319260	11.12	.652317	
Error	20	.14673000		2.8393340	13.49

Pinhead
Endoglucanase

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	1.5733330	.76333330	1.3366670	1.5266670	1.5400000
6	1.1700000	1.2400000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	.24659350	4.52	.408836	
Error	14	.54504390E-01		1.3071430	17.86

Endoxylanase

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	2.7666670	1.6933330	3.0466670	2.1533330	1.8700000
6	2.5733330	2.0000000			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	.75722500	10.16	.478195	
Error	14	.74566430E-01		2.3004760	11.87

Laccase

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	6.9066670	8.8366670	10.466670	7.6300000	7.2733330
6	8.2966660	9.4466670			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	4.8299160	10.46	1.18976	
Error	14	.46158270		8.4080950	8.08

---

fruiting body
Endoglucanase

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	1.4600000	1.2700000	1.3433330	1.7433330	1.7800000
6	1.7866670	1.4133330			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	.14688490	1.02	NS	
Error	14	.14437650		1.5423810	24.64

Endoxylanase

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	2.3933330	2.3866670	2.1333330	2.0133330	1.7400000
6	1.8466670	1.8433330			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	.20983510	2.72	NS	
Error	14	.77157700E-01		2.0509520	13.54

Laccase

NR = 3 NT = 7 NE = 1
Analysis of CRD with equal no. of replications

TREATMENT MEANS

1	5.5433340	6.0300000	7.2066670	7.0933340	6.4499990
6	9.0100000	8.3533330			

ANOVA TABLE

SOURCE	d.f.	M.S.	F-Ratio	CD(5%)	G.M.C.V.
Treatments	6	4.6104330	7.14	1.40701	
Error	14	.64555140		7.0980960	11.32

VITA

Name of the student	:	Karanpreet Singh
Father's name	:	S. Gurpreet Singh
Mother's name	:	Smt. Narinder Kaur
Nationality	:	Indian
Date of birth	:	10.12.1990
Permanent home address	:	B-25, 316/9, Netaji Nagar Salem Tabri, Ludhiana
E-mail ID	:	karanpreet1990@gmail.com

EDUCATIONAL QUALIFICATION

Bachelor degree	:	B.Sc. (Med.)
University and year of award	:	Panjab University, Chandigarh 2017
%age	:	64.15
Master's degree	:	M.Sc. (Microbiology)
University and year of award	:	Punjab Agricultural University, Ludhiana, 2019
OCPA	:	6.83/10.00
Title of Master's Thesis	:	*Calocybe indica* strain evaluation for lignocellulolytic enzymes in relation to mushroom yield