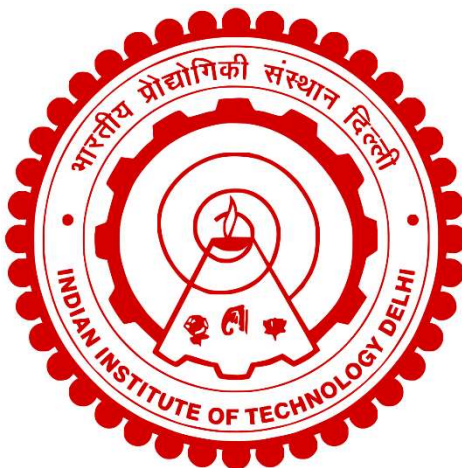


**BIOPROCESS DEVELOPMENT FOR THE LARGE-SCALE
PRODUCTION OF VITAMIN D ENRICHED MUSHROOM
MYCELIA**

UMESH SINGH



CENTRE FOR RURAL DEVELOPMENT AND TECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY DELHI

DECEMBER 2023

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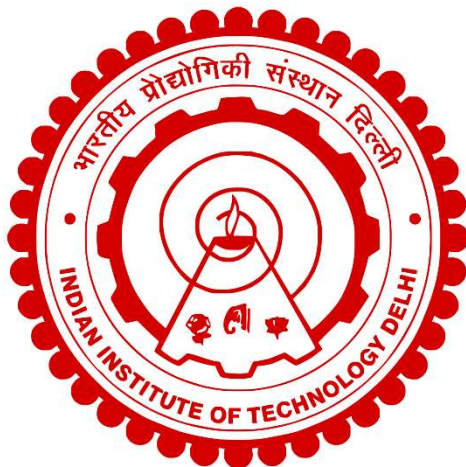
by

UMESH SINGH

Centre for Rural Development and Technology

Submitted

**in fulfillment of the requirements of the degree of philosophy
to the**



INDIAN INSTITUTE OF TECHNOLOGY DELHI

DECEMBER 2023

DEDICATED TO MY FAMILY

CERTIFICATE

This is to certify that the thesis entitled "**Bioprocess development for the large scale production of vitamin D enriched mushroom mycelia**" being submitted by **Mr. Umesh Singh** to the **Indian Institute of Technology Delhi** for the award of "**Doctor of Philosophy**" is a record of bonafide research carried by him. He has worked under my supervision and guidance and has fulfilled the requirements for the submission of the thesis. To the best of my knowledge, the results presented in this thesis have not been submitted in part or full to any other institute or university for the award of any degree or diploma.



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(Umesh Singh)

Abstract

Mushrooms are rich in various nutritious and biologically active compounds such as polysaccharides including β -glucans, peptides, proteins, terpenoids, fatty acid esters, organic acids, sterols, alkaloids, phenolic compounds and are responsible for antioxidative, anticancer, antiviral, anti-inflammatory, antihyperlipidemic, immunomodulating and hypocholesterolemic properties. These properties can modify the physiological and metabolic functions of the human body and also have pro-health effects. However, the cultivation of mushrooms is tedious, requires a specific substrate approach, labour-intensive and takes 2 to 3 months. Moreover, its growth is season dependent and if the temperature is not controlled in the interior of the mushroom unit, it could only be grown during specific months of the year and hence makes it unsuitable for the commercialization of mushroom fortified products. In this context, the production of mushroom mycelia through submerged culture technique can be used as an alternative solution to produce higher mycelial biomass of mushrooms at industrial scale in a shorter period (8-10 days) in a fermenter enriched with nutraceutical properties. Also, growing mushroom mycelium under submerged culture conditions ensures its availability throughout the year, which requires limited space and fewer chances of contamination. At the same time, there exist certain limitations in this process e.g. the need for rather sophisticated equipment, expensive infrastructure and highly skilled personnel. In this study, mass production of fungal mycelial biomass of *Pleurotus eryngii* and *Calocybe indica* with enhanced nutritional values through submerged cultivation technique was achieved using one factor at a time (OFAT) approach. Both the physical (Temperature, pH, and RPM) and chemical (carbon sources, nitrogen sources, and C/N ratio) parameters were studied in shake flasks levels and further the optimized parameters were used for mass production of mushroom mycelia in fermenters (15 L, 100 L, 350 L). The harvested mushroom mycelia were then exposed to UV-B irradiation for enhancement of vitamin D₂. The highest biomass yield of *P. eryngii* in 15 L fermenter in batch mode

under optimized conditions of agitation 200 rpm, aeration rate 0.1 vvm, temperature 28 °C, pH 6.5 and inoculum size 4% was 13.44 ± 0.82 g/l. The batch phase was extended to fed-batch phase to increase biomass yield and the final biomass was increased up to 22.68 ± 0.8 g/l. *P. eryngii* mycelial biomass was further produced in 100 L fermenter in which biomass yield obtained was 12.54 ± 0.81 g/l. The highest biomass yield of *C. indica* in 15 L fermenter in batch mode under optimized condition of agitation 150 rpm, aeration rate 0.1 vvm, temperature 28 °C, pH 6.5 and inoculum size 4% was 9.54 ± 0.35 g/l. Further, *C. indica* mycelia was also produced in large scale fermenter 350 L and the final biomass yield was 9.34 ± 0.81 g/l. The ideal UV-B irradiation conditions were determined for vitamin D2 synthesis in harvested mycelia using RSM. The effects of three independent variables namely, temperature (20-40 °C), ultraviolet-B intensity (0.6-1.2 W/m²), and time of exposure (10-30 min) on vitamin D2 were investigated. Under optimal conditions of ambient temperature, 24.29 °C, UV-B intensity, 1.099 W/m², and exposure time, 23.88 min, the vitamin D2 got increased to 329.43 µg/g dry wt. Similarly, for *C.indica* the vitamin D2 was improved to 247 ± 3.28 µg/g dry wt. The freeze-dried *P. eryngii* mycelia contained protein 24.52±0.53%, crude fat 6.78±0.81%, crude fiber 18.54±0.57%, total ash 7.82±0.61%, and carbohydrate 55.36±1.24% and rich in various amino acids like glutamic acid (3.05%), arginine (2.28%), threonine (2.03%), glycine (2.01%), and alanine (1.74%). While, freeze-dried *C.indica* mycelia was found to have protein 28.21±0.31%, crude fat 5.78±0.55%, crude fiber 14.24±0.37%, total ash 6.72±0.22%, and carbohydrate 57.06±1.13%. *C.indica* mycelia showed higher content of glutamic acid (4.01%), alanine (2.77%), leucine (1.89%), lysine (1.74%), isoleucine (1.68%), aspartic acid (1.68%), glycine (1.49%), Threonine (1.33%) and valine (1.33%). The effect UVB irradiation on nutritional quality of produced mycelia was also studied and compared with their non UV-treated (control) mycelia. Ergosterol content in both the mycelia (*P. eryngii* & *C.indica*) got decreased significantly from 2.48 to 1.63 mg/g (*P. eryngii*) and from 2.11 to 1.46 mg/g (*C. indica*).

UV irradiation caused a significant ($p < 0.05$) improvement in mycelial beta glucans (%), i.e., from 26.18 to 32.21% (w/w) in *P. eryngii* and from 24.11 to 31.46 % (w/w) in *C. indica*. The antioxidant activity including FRAP, DPPH scavenging ability, TPC and TFC in UVB treated samples was also improved significantly ($p < 0.05$) of both the mycelial extracts when compared with their control (non UVB treated) samples. Nutraceutically enriched mycelia were utilized for the preparation of two different types of food products namely instant noodles and meat analogues. The physico chemical properties, sensory evaluation and self-life studies were conducted for both the products. The noodle samples with 6% *P. eryngii* mycelia powder and with 15% *C. indica* mycelia powder were found acceptable. It was observed that mushroom mycelia fortified food products are well accepted by the rural consumers (both adults and children).

सार

मशरूम विभिन्न पौष्टिक और जैविक रूप से सक्रिय यौगिकों जैसे बीटा ग्लूकेन्स, पेप्टाइड्स, प्रोटीन, टेरपेनोइड्स, फैटी एसिड एस्टर, कार्बनिक अम्ल, स्टेरोल्स, अल्कलॉइड्स, फेनोलिक यौगिकों से भरपूर होते हैं और एंटीऑक्सीडेंट, एंटीकैंसर, एंटीवायरल, एंटी-इंफ्लेमेटरी, एंटीहाइपरलिपिडेमिक, इम्यूनोमॉड्यूलेटिंग और हाइपोकोलेस्ट्रॉलेमिक गुण के लिए जिम्मेदार होते हैं। ये गुण मानव शरीर के शारीरिक और चयापचय कार्यों को संशोधित कर सकते हैं और स्वास्थ्य-समर्थक प्रभाव भी डाल सकते हैं। हालाँकि, मशरूम की खेती कठिन है, इसके लिए एक विशिष्ट सब्सट्रेट दृष्टिकोण की आवश्यकता होती है, श्रम-गहन होता है और इसमें 2 से 3 महीने लगते हैं। इसके अलावा, इसकी वृद्धि मौसम पर निर्भर है और यदि तापमान को बाहरी रूप से नियंत्रित नहीं किया जाता है, तो इसे केवल वर्ष के विशिष्ट महीनों के दौरान ही उगाया जा सकता है और इसलिए यह मशरूम फोर्टिफाइड उत्पादों के व्यावसायीकरण के लिए अनुपयुक्त है। इस संदर्भ में, जलमग्न कल्चर तकनीक के माध्यम से मशरूम माइसेलिया का उत्पादन, मशरूम फलने के बराबर न्यूट्रास्युटिकल गुणों के साथ किण्वक में कम अवधि (8-10 दिन) में औद्योगिक पैमाने पर मशरूम के उच्च माइसेलियल बायोमास का उत्पादन करने के लिए एक वैकल्पिक समाधान के रूप में इस्तेमाल किया जा सकता है। इसके अलावा, जलमग्न संस्कृति परिस्थितियों में मशरूम माइसेलियम उगाने से पूरे वर्ष इसकी उपलब्धता सुनिश्चित होती है, जिसके लिए सीमित स्थान की आवश्यकता होती है और संदूषण की संभावना कम होती है। इस अध्ययन में, एक समय में एक कारक (ओएफएटी) दृष्टिकोण का उपयोग करके जलमग्न खेती तकनीक के माध्यम से बढ़े हुए पोषण मूल्यों के साथ प्लुरोटस एरिंजि और कैलोसाइब इंडिका के फंगल माइसेलियल बायोमास का बड़े पैमाने पर उत्पादन हासिल किया गया था। भौतिक (तापमान, पीएच, और आरपीएम) और रासायनिक (कार्बन स्रोत, नाइट्रोजन स्रोत, और सी/एन अनुपात) दोनों मापदंडों का अध्ययन शेक फ्लास्क

स्तरों में किया गया था और इसके अलावा किण्वकों में मशरूम मायसेलिया के बड़े पैमाने पर उत्पादन के लिए अनुकूलित मापदंडों का उपयोग किया गया था (15) एल, 100 एल, 350 एल)। विटामिन डी₂ की वृद्धि के लिए काटे गए मशरूम मायसेलिया को यूवी-बी विकिरण के संपर्क में लाया गया। दोलन (200 आरपीएम), वातन दर (0.1 वीवीएम), तापमान (28 डिग्री सेल्सियस), पीएच (6.5) और इनोकुलम आकार (4%) की अनुकूलित स्थितियों के तहत बैच मोड में 15 एल किण्वक में पी. इरिंजि की उच्चतम बायोमास उपज 13.44 ± 0.82 ग्राम/लीटर था। बायोमास उपज बढ़ाने के लिए बैच चरण को फेड-बैच चरण तक बढ़ाया गया था और अंतिम बायोमास को 22.68 ± 0.8 ग्राम/लीटर तक बढ़ाया गया था। पी. इरिंजि मायसेलियल बायोमास को 100 एल किण्वक में उत्पादित किया गया था जिसमें बायोमास उपज 12.54 ± 0.81 ग्राम/लीटर प्राप्त हुई थी। दोलन (150 आरपीएम), वातन दर (0.1 वीवीएम), तापमान (28 डिग्री सेल्सियस), पीएच (6.5) और इनोकुलम आकार (4%) की अनुकूलित स्थिति के तहत बैच मोड में 15 एल किण्वक में सी. इंडिका की उच्चतम बायोमास उपज 9.54 ± 0.35 ग्राम/लीटर था। इसके अलावा, सी. इंडिका मायसेलिया का उत्पादन भी बड़े पैमाने पर किण्वक (350 लीटर) में किया गया था और अंतिम बायोमास उपज 9.34 ± 0.81 ग्राम/लीटर थी। आरएसएम का उपयोग करके कटे हुए मायसेलिया में विटामिन डी₂ संश्लेषण के लिए आदर्श यूवी-बी विकिरण की स्थिति निर्धारित की गई थी। विटामिन डी₂ पर तीन स्वतंत्र चर, तापमान (20-40 डिग्री सेल्सियस), पराबैंगनी-बी तीव्रता (0.6-1.2 डब्ल्यू/एम²), और एक्सपोजर का समय (10-30 मिनट) के प्रभावों की जांच की गई। इष्टतम परिस्थितियों (परिवेश तापमान, 24.29 ओसी, यूवी-बी तीव्रता, 1.099 डब्ल्यू/एम², और एक्सपोजर समय, 23.88 मिनट) के तहत विटामिन डी₂ बढ़कर $329.43 \mu\text{g/g}$ शुष्क डब्लूटी हो गया। इसी प्रकार, सी.इंडिका के लिए विटामिन डी₂ को 247 ± 3.28 माइक्रोग्राम/ग्राम शुष्क वजन तक सुधारा गया था। फ्रीज़-सूखे पी. इरिंजि मायसेलिया में प्रोटीन $24.52 \pm 0.53\%$, अपरिष्कृत वसा $6.78 \pm 0.81\%$, अपरिष्कृत फ़ाइबर $18.54 \pm 0.57\%$, कुल राख $7.82 \pm 0.61\%$, और कार्बोहाइड्रेट 55.36 ± 1.24 शामिल थे। %) और ग्लूटामिक एसिड (3.05%), आर्जिनिन (2.28%), थ्रेओनीन (2.03%), ग्लाइसिन (2.01%), और एलानिन

(1.74%) जैसे विभिन्न अमीनो एसिड से भरपूर है। जबकि, फ्रीज-सूखे सी.इंडिका मायसेलिया में प्रोटीन $28.21 \pm 0.31\%$, अपरिष्कृत वसा $5.78 \pm 0.55\%$, अपरिष्कृत फाइबर $14.24 \pm 0.37\%$, कुल राख $6.72 \pm 0.22\%$, और कार्बोहाइड्रेट $57.06 \pm 1.13\%$ पाया गया। सी.इंडिका मायसेलिया में ग्लूटामिक एसिड (4.01%), ऐलेनिन (2.77%), ल्यूसीन (1.89%), लाइसिन (1.74%), आइसोल्यूसीन (1.68%), एसपार्टिक एसिड (1.68%), ग्लाइसिन (1.49%) थ्रेओनीन (1.33%) और वेलिन (1.33%) की उच्च मात्रा देखी गई। उत्पादित मायसेलिया की पोषण गुणवत्ता पर यूवीबी विकिरण के प्रभाव का भी अध्ययन किया गया और उनके गैर यूवी-उपचारित (नियंत्रण) मायसेलिया के साथ तुलना की गई। दोनों मायसेलिया (पी. एरिंगी और सी. इंडिका) में एर्गोस्टेरोल की मात्रा 2.48 से 1.63 मिलीग्राम/जी (पी. एरिंगी) और 2.11 से 1.46 मिलीग्राम/जी (सी. इंडिका) तक काफी कम हो गई।

यूवी विकिरण के कारण मायसेलियल बीटा ग्लूकेन्स (%) में महत्वपूर्ण (पी < 0.05) सुधार हुआ, यानी, पी. इरिंजि में 26.18 से 32.21% (डब्ल्यू/डब्ल्यू) और सी. इंडिका में 24.11 से 31.46% (डब्ल्यू/डब्ल्यू) तक सुधार हुआ। यूवीबी उपचारित नमूनों में एफआरएपी, डीपीपीएच सफाई क्षमता, टीपीसी और टीएफसी सहित एंटीऑक्सीडेंट गतिविधि में भी उनके नियंत्रण (गैर यूवीबी उपचारित) नमूनों की तुलना में दोनों मायसेलियल अर्क की काफी सुधार हुआ (पी < 0.05)। न्यूट्रास्यूटिक रूप से समृद्ध मायसेलिया का उपयोग दो अलग-अलग प्रकार के खाद्य उत्पादों अर्थात् इंस्टेंट नूडल्स और मीट एनालॉग्स की तैयारी के लिए किया गया था। भौतिक रासायनिक गुण, संवेदी मूल्यांकन और स्व-जीवन अध्ययन आयोजित किए गए। 6% पी. इरिंजि मायसेलिया पाउडर और 15% सी. इंडिका मायसेलिया पाउडर वाले नूडल्स के नमूने स्वीकार्य पाए गए। यह देखा गया कि मशरूम मायसेलिया फोर्टिफाइड खाद्य उत्पादों को ग्रामीण उपभोक्ताओं (वयस्कों और बच्चों दोनों) द्वारा अच्छी तरह से स्वीकार किया जाता है।

TABLE OF CONTENTS

Acknowledgments	ii
Abstract	iv
Contents	x
List of Figures	xiv
List of Tables	xvii
Abbreviations	xix
Chapter 1: Introduction	1-8
1.1. Background	1
1.2. Medicinal potential of mushrooms	1
1.3. Mushroom production scenario	2
1.4. Mushrooms: Sources of vitamin D	3
1.5. Mycelia production through submerged fermentation	5
1.6. Mushroom mycelia as meat alternatives	6
1.7. Cultivation of <i>Calocybe indica</i> and <i>Pleurotus eryngii</i> mycelia	7
Chapter 2: Review of Literature	9-58
2.1. Mushrooms	9
2.2. Life cycle of mushroom	9
2.3. Mushroom mycelia	12
2.4. Mushroom mycelia cultivation process	14
2.5. Factors affecting submerged cultivation of mushrooms in bioreactors	20
2.5.1. Temperature	20
2.5.2. Dissolved oxygen	21
2.5.3. pH	23
2.5.4. Agitation	24
2.5.5. Inoculum	26
2.6. Enhancement of nutraceutical attributes in mycelia	27
2.7. Nutraceutical properties of mushroom mycelia	29
2.7.1. Vitamin D	30
2.7.2. Proteins	36
2.7.3. Polysaccharides	38
2.7.4. Lipids	41
2.7.5. Terpenes	43
2.8. Functional food products developed from mushroom mycelia	43
2.9. Market availability of mushroom mycelia based functional food products	52
Chapter 3: Materials and methods	59-85
3.1. Production of <i>Pleurotus eryngii</i> mycelia	60
3.1.1. Procurement of micro-organism culture	60

3.1.2. Chemicals	61
3.1.3. Growth media	61
3.1.4 Culture cultivation	61
3.1.5. Growth profiling experiments of mycelia (solid media)	62
3.1.6. Inoculum preparation strategy for submerged cultivation	62
3.1.7. Growth profiling study of <i>P.eryngii</i> under submerged culture conditions	63
3.1.8. Screening and optimization of physical parameters for submerged cultivation of <i>P. eryngii</i>	65
3.1.9. Cultivation of <i>P. eryngii</i> mycelia in 1L shake flasks	65
3.1.10. Fermentation	65
3.2. Production of <i>C. indica</i> in liquid medium	65
3.2.1. Procurement of micro-organism culture	65
3.2.2. Growth media substrates	66
3.2.3. Inoculum preparation for <i>C.indica</i> mycelia production	66
3.2.4. Screening and optimization of physical parameters for submerged cultivation of <i>C.indica</i>	68
3.3. UV irradiation for vitamin D2 synthesis	68
3.3.1. UV Exposure Set-up	68
3.3.2. RSM experimental design	69
3.4. Characterization of harvested mushroom mycelia	70
3.4.1. Determination of mycelial dry biomass	70
3.4.2. Determination of residual sugar concentration	71
3.4.3. Assay of ergosterol and vitamin D2	71
3.4.4. Proximate analysis	72
3.4.5. Quantification of mineral elements in mycelia	73
3.4.6. Assay of ergothioneine	73
3.4.7. Estimation of amino acids	74
3.4.8. Quantification of beta glucans (β -glucans)	74
3.4.9. GC-MS analysis	75
3.4.10. Antioxidant activities of mycelia	75
3.4.10.1. Free radical scavenging activity	76
3.4.10.2. Ferric reducing antioxidant power (FRAP)	76
3.4.10.3. Total phenol content (TPC)	76
3.4.10.4. Determination of total flavonoid content (TFC)	77
3.5. FTIR Spectroscopy	77
3.6. SEM Analysis	77
3.7. Development of functional food products from mushroom mycelia	77
3.7.1. Instant noodles preparation fortified with dry mycelium powder	77
3.7.2. Physical and textural characteristics of noodles	78
3.7.2.1. Process recovery	78

3.7.2.2. Cooking time	78
3.7.2.3. Water absorption	79
3.7.2.4. Solid loss	79
3.7.2.5. Noodles extensibility	79
3.7.3. Nutraceutical analysis of instant noodles	79
3.7.4. Storage studies and shelf-life evaluation of instant noodles	80
3.7.4.1. Determination of free fatty acid	80
3.7.4.2. Determination of peroxide value	80
3.7.4.3. Sensory evaluation	81
3.7.5. Meat-analogue preparation using fresh mycelia	81
3.7.6. Nutraceutical analysis of meat analogues	83
3.7.7. Storage studies and shelf-life (micro-biological) evaluation of meat analogues	83
3.7.7.1. Sample preparation	83
3.7.7.2. Standard/ total plate count	83
3.7.7.3. Yeast and molds count	84
3.7.8. Texture profile analysis (TPA)	84
3.8. Field studies	84
3.9. Statistical analysis	85
Chapter 4: Result and discussion	86-168
4.1. Production of <i>Pleurotus eryngii</i> mycelia	87
4.1.1. Screening and identification of favorable media for cultivation (petri-plate studies)	87
4.1.2. Screening of media under submerged culture conditions (shake flask studies)	89
4.1.3. Selection of carbon, nitrogen sources and CN ratios	91
4.1.4. Optimization of growth conditions in liquid media (pH, Temperature and RPM)	94
4.1.5. Mycelia production under optimum growth conditions in shake flask (up to 1l)	96
4.1.6. Biomass yield of <i>P. eryngii</i> in 15 L fermenter	97
4.1.7. Biomass yield of <i>P. eryngii</i> in 100 L fermenter	100
4.2. Production of <i>Calocybe indica</i> mycelia	105
4.2.1. Growth profiling of <i>C. indica</i>	105
4.2.2. Growth of <i>C. indica</i> in liquid medium	105
4.2.3. Development of production media for <i>C. indica</i> mycelia	106
4.2.4. Modifications in production media for increased biomass yield of <i>C. indica</i>	108
4.2.5. Development of food grade media for the production of mycelial biomass of <i>C. indica</i>	111
4.2.6. Mycelial cultivation under optimum growth conditions in shake flask (up to 1l)	114
4.2.7. Biomass growth of <i>C.indica</i> in 15 L fermenter	115
4.2.8. Biomass yield of <i>C. indica</i> in 350 L fermenter	116
4.3. Vitamin D2 enhancement in mushroom mycelia	122
4.3.1. Parameter selection for enhancing vitamin D2 in mycelia	122

4.3.2. Design of Experiments and Statistical Optimization using RSM	124
4.3.3. Validation of the Model	129
4.4. Nutraceutical characterization of harvested mushroom mycelia	129
4.4.1. Proximate composition	129
4.4.2. Minerals content	130
4.4.3. Amino acid content	131
4.4.4. Beta glucans, ergosterol and ergothioneine quantification	132
4.4.5. Antioxidant properties	133
4.4.5.1. Total phenolic content (TPC)	134
4.4.5.2. Total flavonoid content (TFC)	135
4.4.5.3. DPPH (2, 2-Diphenyl-1-picrylhydrazyl) radical scavenging activity	135
4.4.5.4. FRAP (Ferric reducing antioxidant power) activity	136
4.5. Selenium bioaccumulation in <i>P. eryngii</i> mycelia	136
4.5.1. FTIR spectroscopy of Se treated mycelia	137
4.5.2. SEM analysis	139
4.5.3. Effect of Se supplementation on nutraceutical properties of mycelia	140
4.5.4. GC-MS analysis of Se treated mycelia	143
4.5.5. Effect of Se enrichment on antioxidant properties	145
4.6. Effect of UVB irradiation on nutritional quality of mycelia	146
4.6.1. Ergosterol	146
4.6.2. Beta glucans	147
4.6.3. Antioxidant properties	148
4.6.4. Bioactive components	148
4.7. Value added products from harvested mycelia	152
4.7.1. Instant noodles	152
4.7.1.1. Physical and textural characteristics of noodles	152
4.7.1.1.1. Process recovery	152
4.7.1.1.2. Cooking time	154
4.7.1.1.3. Water absorption	154
4.7.1.1.4. Solid loss	155
4.7.1.2. Selection of instant noodles composition (wheat flour- mycelia ratio) based on sensory characteristics	155
4.7.1.3. Nutritional analysis of noodles	157
4.7.1.4. Shelf-life evaluation of instant noodles	159
4.7.2. Meat analogues	160
4.7.2.1. Characteristics of fresh mycelia based meat-analogues	161
4.7.2.1.1. Texture profile (TP)	161
4.7.2.1.2. Nutritional and nutraceutical properties of meat analogues	162
4.7.2.2. Storage studies and shelf-life evaluation of developed meat analogues	164
4.8. Field studies among the rural population	166

Chapter 5: Summary, conclusions and future prospective	169-180
References	181-182
Annexure	183-201

LIST OF FIGURES

Fig.No	Title	Page no.
1.1.	Mushroom species cultivated majorly worldwide	2
1.2.	Major mushroom-producing states in India	3
1.3.	Nutraceutical qualities provided by mushrooms to meat analogues	7
2.1.	Different stages of the mushroom life cycle	11
2.2.	Schematic representation of mushroom mycelium physiology. (A) Branched micro-filaments (hyphae) (B) hypha with visible septum (C) Components of cell wall of hypha (proteins, chitins and glucose)	13
2.3.	Mushroom mycelia production approach under liquid culture conditions	16
2.4.	Fermented mycelial pellets of <i>L. edodes</i> (A) and <i>P. ostreatus</i> (B), dispersed inside the culture vessel	17
2.5.	The scheme of ergosterol to vitamin D ₂ conversion along with its photo-isomers	32
2.6.	A possible mechanism for ergosterol-induced antitumor activity against cancer cells	35
2.7.	Health impacts of dietary intake of meat and mushroom based food products	44
3.1.	Schematic diagram of the work plan	60
3.2.	Culture plate of <i>P.eryngii</i>	61
3.3.	Growth profiling of <i>P. eryngii</i> in PDA medium at 28°C.	62
3.4.	Inoculum development strategy for submerged cultivation of mushroom cultures	63
3.5.	Growth profiling experiment of <i>P.eryngii</i>	64
3.6.	Incubator shaker (Scigenic biotech stackable shaker, LE4676-H)	64
3.7.	Culture plate of <i>C.indica</i>	66
3.8.	Schematic representation of inoculum development process for <i>C. indica</i>	67
3.9.	In-house designed and developed UV exposure Set-up.	69
3.10.	Instant noodles fortified with <i>C. indica</i> mycelia powder. (a) Control, without mycelia (b) with 5% (c) 10% (d) 15% (e) 20% dried <i>C. indica</i> mycelia.	78
3.11.	Instant noodles fortified with <i>P. eryngii</i> mycelia powder. (a) Control, without mycelia (b) with 2% (c) 4% (d) 6% (e) 8% (f) 10% dried <i>P.eryngii</i> mycelia.	78
3.12.	Mycelia produced through fermentation process before heat treatment (A) and after heat treatment (B).	82
3.13.	Patties fortified with mushroom mycelia (A) <i>P.eryngii</i> (B) <i>C.indica</i>	82
4.1.	Radial Growth of <i>P. eryngii</i> on different agar medium at pH 6.0 at different time point.	88
4.2.	Growth profile of <i>P. eryngii</i> on different agar medium at pH 6.0 at different time	88
4.3.	Growth profile of <i>P. eryngii</i> on SFx medium at 28°C at 120 rpm. The graph represents dry biomass (g/l), pH, residual glucose concentration (g/l) during the process.	89

4.4.	Growth profile of <i>P. eryngii</i> on VSPE medium at 28°C at 120 rpm. The graph represents dry biomass (g/l), pH, residual glucose concentration (g/l) during the process	90
4.5.	Box plot of dry mycelial biomass (g/l) of <i>P. eryngii</i> under submerged growth condition on SFx and VSPE media after harvest on day 12. Lowercase letters indicate significant/ non-significant differences with $p < 0.05$	90
4.6.	Comparative growth profile of <i>P.eryngii</i> mycelia in VSPE and SFx media	91
4.7.	Effect of carbon sources on <i>P.eryngii</i> mycelial biomass under submerged condition in shake flask. Lower case letters indicate differences with $p < 0.05$ (n=3). (12 days study)	92
4.8.	Effect of nitrogen sources on <i>P.eryngii</i> mycelial biomass yield under submerged condition in shake flask. Lowercase letters indicate significant/ non-significant differences with $p < 0.05$ (n=3)	93
4.9.	Effect of carbon to nitrogen (C/N) ratios on <i>P.eryngii</i> mycelial biomass under submerged condition in shake flask. Lowercase letters indicate significant/ non-significant differences with $p < 0.05$ (n=3)	94
4.10.	Effect of (A) initial pH (B) temperature and (C) agitation (rpm) on mycelial growth of <i>P.eryngii</i> under the submerged condition in shake flask studies. Lowercase letters indicate significant/ non-significant differences with $p < 0.05$ (n=3)	95
4.11.	In-situ 15 l lab scale fermenter installed in our lab at IIT Delhi	98
4.12.	Growth profile of fed-batch production of <i>P. eryngii</i> mycelia in 15 L fermenter. Various process parameters are shown in different colors and symbols: pH (red), temperature (blue), DO (pink), agitation (black), dry mycelial biomass (-□-), and glucose (-○-)	100
4.13.	Batch production of <i>P. eryngii</i> in 100 L fermenter: Growth of <i>P. eryngii</i> in VSPE medium at 28°C at 120 rpm. The graph representing dry biomass (g/l), pH, residual sucrose concentration (g/l) and temperature (°C) during the process	101
4.14.	Production of mycelial biomass of <i>Pleurotus eryngii</i> in 100 L fermenter at industrial (CPB) fermenter site. (A & B) Inoculum preparation in flask, (C) Inoculum in transfer bottle, (D) Transferring of inoculum to 100 L Fermenter, (E) Mycelia harvesting and filtration through muslin cloth, (F) Production team with harvested mycelia, (G) Fresh harvested mycelia and, (H) Packaged fresh mycelia	102
4.15.	Overall scheme for large scale production of <i>P.eryngii</i> mycelia under submerged culture condition	104
4.16.	Growth profiling of <i>C.indica</i> in PDB media at pH 6.0 and 28 0C	105
4.17.	Growth of <i>Calocybe indica</i> in liquid media (A) In absence of peptone & elicitor, (B) In presence of peptone only, (C) In presence of Peptone & elicitor, (D) Fresh pellets and (E) Dried mycelial pellets	106
4.18.	Batch production of <i>C. indica</i> mycelia in broth (1L flask). The graph representing dry mycelial biomass (g/l), residual glucose concentrations (g/l), and pH during the production process (Process parameters)	115
4.19.	(A) Growth profile of batch production of <i>C. indica</i> mycelia in 15 L fermenter. Various process parameters are shown in different colors and	116

	symbols: pH (red), temperature (blue), DO (pink), agitation (black), dry mycelial biomass (-□-), and glucose (-○-) (B) Fresh harvested mycelial biomass and, (C) Dried mycelial biomass harvested from fermenter	
4.20.	Growth profile of batch production of <i>C. indica</i> mycelia in 350 L fermenter. Various process parameters are shown in different colors and symbols: pH (green), temperature (blue), dry mycelial biomass (-□-), and glucose (-○-)	117
4.21.	R & D and production teams harvesting the mycelial biomass of <i>Calocybe indica</i> from 350 L fermenter at TERI Gram site	119
4.22.	Response surface plots for conversion to vitamin D as a function of temperature (°C) and exposure time (min) is shown in A & B; temperature and UV-B light intensity is shown in C & D; and exposure time (min) and UV-B light intensity is shown in E & F.	126
4.23.	FTIR spectra of untreated Se0 (A), and Se treated samples Se2.5 (B), Se5 (C) Se10 (D) Se20 (E). Y-axis represents transmission percentage and X-axis represents units in centimeters	138
4.24.	SEM micrographs (at magnification 2500X and EHT20 kV) of <i>P. eryngii</i> mycelia: (a) Se0 (b) Se2.5 (c) Se5 (d) Se10 (e) Se20	139
4.25.	Contents of (a) β-glucan (b) ergothioneine (c) ergosterol (d) protein, in different Se supplemented media (Se0, Se2.5, Se5, Se10, Se20)	141
4.26.	Content of ergosterol in UV irradiated and non irradiated <i>P. eryngii</i> (Pe) and <i>C.indica</i> (Ci) samples	146
4.27.	Content of beta glucans in UV irradiated and non irradiated <i>P. eryngii</i> (Pe) and <i>C.indica</i> (Ci) samples	147
4.28.	Comparison of un-cooked (R1) and cooked (R2) meat analogues patties formed; A & D= meat-analogues fortified with <i>C. indica</i> mycelia; B & E= meat-analogues fortified with <i>P. eryngii</i> mycelia; and C & F= goat meat patties	162
4.29.	Various activities during the rural interaction and awareness programme	167

LIST OF TABLES

Table No.	Title	Page no.
2.1.	Various studies reported for mushroom mycelia production	18
2.2.	Nutraceutical enhancement during submerged cultivation of mushroom mycelia	27
2.3.	Enhancement of therapeutic properties during submerged cultivation of mushroom mycelia	28
2.4.	Therapeutic attributes of vitamin D	31
2.5.	Vitamin D2 content in irradiated mushrooms from different studies	33
2.6.	Mushroom based commercialized functional foods	45
2.7.	Mushroom based meat analogues using non-meat ingredients	49
2.8.	Companies involved in producing mycelia based food products	53
4.1.	Lab formulated production media for <i>P. eryngii</i>	96
4.2.	Influence of shear force on mycelial growth of <i>P. eryngii</i>	99
4.3.	An overview of mycelial biomass yield of <i>P.eryngii</i> in shake flasks and fermenters	101
4.4.	Effect of different carbon sources on mycelial biomass yield	107
4.5.	Effect of different nitrogen sources on mycelial biomass yield	107
4.6.	Effect of buffering capacity on yield of the mycelial biomass of <i>C. indica</i>	109
4.7.	Effect of split N-source (peptone and urea) on yield of the mycelial biomass of <i>Calocybe indica</i> (on day-9 harvest)	109
4.8.	Combined effect of buffering capacity and split N-source on pH of media and mycelial yield	110
4.9.	Cost implications on increasing buffer strength.	111
4.10.	Effect of plant-based N-source on yield of the mycelial biomass of <i>C. indica</i>	112
4.11.	Effect of variable concentration of C-substrate (glucose) on yield of the mycelial biomass of <i>C. indica</i> with CSL and soya flour as N-source in the media (day-9)	112
4.12.	Designed food grade production media for the production of food grade mycelial biomass of <i>C. indica</i>	113
4.13.	An overview of mycelial biomass yield of <i>C. indica</i> in shake flasks and fermenters	118
4.14.	Effect of different parameters on conversion of ergosterol to vitamin D2 under experimental conditions	124
4.15.	Center composite design (CCD) matrix of experimental runs with three levels (−1, low; 0, middle; +1, high) of each variable	125
4.16.	Analysis of variance (ANOVA) for conversion of Vitamin D2 model	126

4.17.	Proximate composition of the harvested mushroom mycelia of <i>P.eryngii</i> and <i>C.indica</i>	130
4.18.	Mineral composition of the harvested mushroom mycelia of <i>P.eryngii</i> and <i>C.indica</i>	131
4.19.	Amino acid content (g/100 g dry biomass) of the harvested mushroom mycelia of <i>P.eryngii</i> and <i>C.indica</i>	132
4.20.	Antioxidant properties of harvested mycelia	134
4.21.	Content of Se (µg/g) in mycelia and its bioaccumulation in mycelial fractions (proteins, polysaccharides and nucleic acids) and yield of mycelial biomass	136
4.22.	Amino acids profile of different Se supplemented PE mycelia.	141
4.23.	Metabolite profiling in the control and selenium enriched lyophilised PE Mycelia	143
4.24.	Antioxidant properties of Se supplemented and control PE mycelia	145
4.25.	Effect of UV-irradiation on antioxidant properties of the harvested mushroom mycelia of <i>P.eryngii</i> and <i>C.indica</i>	148
4.26.	Bioactive molecules in UV irradiated and non-irradiated <i>P. eryngii</i> mycelia	149
4.27.	Bioactive molecules in UV irradiated and non irradiated <i>C.indica</i> mycelia	151
4.28.	Physical and texture characteristics of <i>P. eryngii</i> mushroom mycelia supplemented instant noodles	153
4.29.	Physical and texture characteristics of <i>C. indica</i> mushroom mycelia supplemented instant noodles	153
4.30.	Sensory characteristics of cooked instant noodles fortified with mycelium powder	156
4.31.	Nutritional characteristics of noodles supplemented with mycelia powder	158
4.32.	Effect of storage at 30 °C on physicochemical properties of developed instant noodles	159
4.33.	Comparison of texture profiling of mycelia-based patties (meat analogues) with control (goat meat) patty	161
4.34.	Nutraceutical characteristics of developed meat analogues	163
4.35.	Changes in microbial count (log10 cfu/g) in developed meat analogue at different storage temperatures	165
4.36.	Shelf life (Days) of developed meat analogues	167
4.37.	Summary of rural interactions and awareness programme	168

ABBREVIATIONS

FAO: Food and Agricultural Organization

PE: *Pleurotus eryngii*

CI: *Calocybe Indica*

SSF: Solid state fermentation

SmF: Submerged fermentation

EPS: Exopolysaccharide

vvm: Volume of air per unit volume of media

SFx: Soya flour extract

CSL: Corn steep liquor

UV: Ultraviolet

C/N: Carbon to nitrogen ratio

VDR: Vitamin D receptor

LLC: Lewis Lung Carcinoma cells

MDA: Malondialdehyde

ROS: Reactive oxygen species

HYP: Hydroxyproline

TLR: Tolllike receptors

KCR: Kupffer cell receptor

GLUT4: Glucose transporter 4

RIPs: Ribosomal inactivating proteins

GABA: gamma-aminobutyric acid

GI: Glycaemic index

%: percent

°C: degree celcius

h: hour

min: minutes

s: seconds

PDA: Potato Dextrose Agar

g: gram

mg: milligram

µg: microgram

L: Litre
mL: milliliter
ppm: Parts per millions
rpm: Revolutions per minute
cm: centimeter
m: meter
nm: nanometer mm: millimeter
dw: Dry weight
N: Nitrogen
C: Carbon
K: Potassium
P: Phosphorus
Fe: Iron
Zn: Zinc
Mn: Manganese
FTIR: Fourier Transform Infrared Spectroscopy
HPLC: High Performance Liquid Chromatography
GC-MS: Gas Chromatography-Mass Spectrometry
ANOVA: analysis of variance
DMRT: Duncan's Multiple