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Preface

Madan Bhandari University of Science and Technology Development Board (MBUSTDB) is undertaking preparations for the establishment of a research-oriented world-class university. In this context, MBUSTB is engaging experts for identification of potential areas for research and teaching, which has the potential of directly contributing to economic development of the country.

This publication presents the outcome of a study related to the identification of research areas in the field of Yarsagumba and other cordyceps mushrooms, the outcomes of which have the potential of directly contributing to country's economic development. This study is a part of wider studies aimed at exploring the potential of natural products for biomedical, technological and agricultural applications.

MBUSTDB highly appreciates the remarkable hard work and dedication of the author – Dr. Bhushan Shrestha – for preparing this publication. MBUSTB appreciates and thanks all other individuals and institutions who contributed to bringing out this publication.

Prof. Rajendra Dhoj Joshi
Chairperson
Madan Bhandari University of Science and Technology Development Board

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Executive summary

This report is prepared with the objective of incorporating mycological study of Yarsagumba (*Ophiocordyceps sinensis*) for teaching and research in the proposed Madan Bhandari University of Science and Technology (MBUST) for biomedical, technological and agricultural applications. The report is divided into ten chapters dealing with different topics and sub-topics of Yarsagumba and other commercial mushrooms.. Chapter 1 deals with the occurrence, distribution and diversity of Yarsagumba and conservation policies in naturally Yarsagumba-growing countries including Nepal. Chapter 2 deals with the traditional use of Yarsagumba in local communities of Nepal and the surrounding countries. Chapter 3 deals with Yarsagumba-based products and its value chain in local and international trade channels. Chapter 4 deals with the bio-activity of wild and cultivated Yarsagumba and its potential products. Chapter 5 deals with finished Yarsagumba products and actions needed to utilize the potential of product development. Chapter 6 deals with the recent developments in cultivation of Yarsagumba and other *Cordyceps* fungi. Chapter 7 deals with the formulation of research projects to develop Yarsagumba products and their patenting, production and marketing. Chapter 8 deals with the development of national and international networking for research on Yarsagumba as well as promotion of its products. Chapter 9 deals with the topics for teaching and research of Yarsagumba in Madan Bhandari University of Science and Technology at the graduate level. Chapter 10 deals with the estimation of cost to establish a lab for research on Yarsagumba. This report is largely based on literature survey and personal experience of nearly 25 years in research on Yarsagumba and other *Cordyceps* mushrooms of Nepal. It is sincerely believed that the report will be valuable for developing teaching and research plans on Yarsagumba and other important *Cordyceps* mushrooms of Nepal in Madan Bhandari University of Science and Technology. Any comments on the report are highly welcome and will be sincerely taken into consideration for improvement in the research plans and models to develop technologically feasible and commercially viable bio-medical products from Yarsagumba. Finally, the author takes all the responsibility for any shortcoming in the report.

Bhushan Shrestha, PhD

Table of Contents

List of Tables and Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 6

Chapter 7

Chapter 9

Chapter 10

Chapter 1. Study of global and national occurrence and distribution, diversities and conservation of Yarsagumba

1) Distribution of Yarsagumba

Yarsagumba (*Cordyceps sinensis*, currently renamed as *Ophiocordyceps sinensis*) is a highly prized medicinal herb that is endemic to the alpine regions of the east and central Hindu Kush-Himalayas and Tibetan Plateau (Figs 1, 2). It is found in the cold, grassy, alpine pasturelands/grasslands of high Himalayan region between 3,000 and 5000 m asl of Nepal, Bhutan, the Indian Himalayas, and Tibetan Plateau and mountain ranges in interior China (DMP 1970, Kobayasi 1980, 1981, Chen *et al*. 1999, Chen *et al*. 2004, Kinjo and Zang 2001, Winkler 2005, Shrestha and Sung 2005, Amatya 2008, Chhetri and Lodhiyal 2008, Li *et al*. 2010, Singh *et al*. 2010, Weckerle *et al*. 2010, Pradhan 2016).

Yarsagumba infects larvae of *Thitarodes* moth and produces fruiting bodies after the death of its host (Kobayasi 1941) (Figs. 1, 3). The upper part of Yarsagumba is the fungal part and the lower part is the larval host (Fig. 1). Spores are released from apical part of the fungal body in summer and infect living insects by penetrating through the host's cuticle layer and then germinate inside the host body, ultimately killing the larva. The fungal body sprouts from the host cadaver next spring and develops into full fruiting body in summer again.

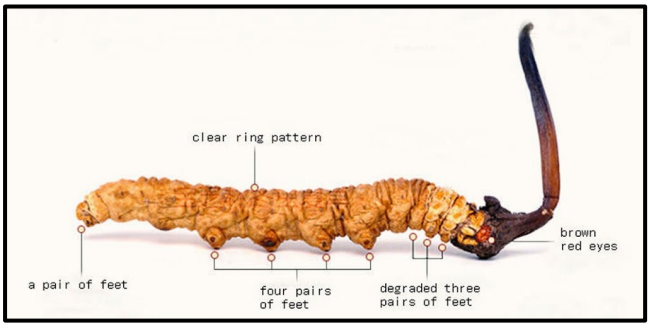

Fig. 1. Morphological features of Yarsagumba (https://yarsagumba-selling-center-nepal.business.site/).

Historically, this medicine was mentioned as Bhu-Sanjivani in old Ayurvedic literature (Shrestha *et al*. 2010). It is well known as a tonic and aphrodisiac herb in folk herbal medicine in the alpine region of Nepal (Sacherer 1979, Bhattarai 1993). This herb has been recognized as a medicinal product in China for the last 2000 yrs (Jones 1997, Zhu *et al*. 1998, Halpern 1999, Holliday *et al*. 2005, Winkler 2008). In China, it is regarded as a tonic and was introduced to Japan through Nagasaki. Yarsagumba is traditionally called dongchong xiacao in Chinese, Totsu Kasoo in Japanese and dhongchunghacho in Korean, its equivalent

English term being summer-plant winter-worm (Shrestha *et al*. 2010). It is also known as Chinese caterpillar fungus or Cordyceps mushroom in English.

Fig. 2. Distribution of Yarsagumba in Himalaya region and Tibetan Plateau (Winkler 2008).

Fig. 3. *Thitarodes* moth as host of Yarsagumba. A, Male adult moth; B, female adult moth; C, lateinstar larva; D, pupa and E, light-trapping for adult moths in Bhutan (Cannon *et al*. 2009).

Yarsagumba is also locally known as 'JibanButi' (life tonic) or 'Chyau' (mushroom). Other popular names for Yarsagumba are Saram Buti, Saram Buti Jadi, Jivan Buti, Kira Jhar, Kira Chhyau, Jingani etc (Shrestha *et al*. 2010). It is used by Amchis and other local healers as a tonic and sexual stimulant as well as to cure

several diseases including diarrhea, headache, cough, rheumatism and liver disease (Devkota 2010). Chinese government has officially included Yarsagumba in Chinese pharmacopeia (Jones 1997, Zhu *et al*. 1998, Halpern 1999, Mizuno 1999, Holliday *et al*. 2005, Winkler 2008).

i) Distribution in Nepal

Yarsagumba is distributed in northern region of Nepal (Balfour-Browne 1955, Devkota 2006, Adhikari 2008) and has been reported from high mountain ranges of 29 districts (Fig 4). They are, from east to west, Taplejung, Sankhuwasabha, Solukhumbu, Dolakha, Ramechhap, Sindhupalchok, Rasuwa, Dhading, Gorkha, Lamjung, Kaski, Myagdi, Baglung, Manang, Mustang, Dolpa, Rolpa, Eastern Rukum, Western Rukum, Jajarkot, Jumla, Mugu, Kalikot, Humla, Bajura, Bajhang, Darchula, Doti, Dailekh (थापा तथा क्षेत्री २०६८, DFO Dolpa 2067, Amatya 2008, Chhetri and Lodhiyal 2008, Devkota 2006, 2010, Sherchan *et al*. 2005, Sigdel 2009, Thapa *et al*. 2014) (Table 1). Along the northern region of Nepal, the greatest concentration of Yarsagumba is found in the mid-western districts (Shrestha and Bawa 2014).

Fig. 4. Distribution of Yarsagumba in Nepal (थापा तथा क्षेत्री, २०६८).

ii) Distribution in China

This mushroom herb is mainly found in alpine grasslands and shrublands of vast areas of Tibetan Plateau, northern Yunnan, eastern Qinghai, western Sichuan, and southwestern Gansu provinces of China (Pegler *et al*. 1994, Boesi 2003, Winkler 2005, 2008) (Table 1). Li *et al*. (2011) and Dai *et al*. (2020) have provided detailed information on distribution of Yarsagumba in China (Fig. 5).

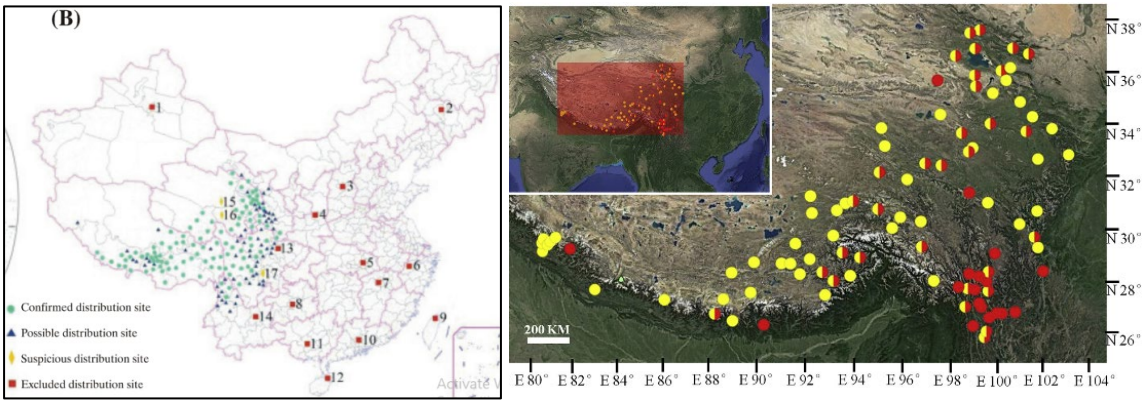

Fig. 5. Distribution of Yarsagumba in China (Li *et al*. 2011, Dai *et al*. 2020).

iii) Distribution in India

Yarsagumba is found in higher altitudes of northern states of India such as Sikkim, Arunachal Pradesh, Himachal Pradesh and alpine meadows of Kumaun Himalaya and Garhwal Himalaya of Uttarakhand (Rana 2004, Sharma 2004, Negi *et al*. 2006, Garbyal *et al*. 2004, 2007, Negi 2009, Panda 2010, Singh *et al*. 2010, Panda and Swain 2011, Pradhan 2016, Yadav *et al*. 2016) (Table 1) (Fig. 6). For example, Yarsagumba is reported from Johaar, Darma, Vyas, Gori Paar, Choudas, Kanar, Metali, Ranthi, and Jumma villages in Kumaun Himalaya of Uttarakhand (Negi *et al*. 2015). Similarly, it is reported from Lachen, Lachung, and Gnathang villages of Sikkim (Pradhan *et al*. 2020). Among northern states of India, Uttarakhand has the highest volume of collection and trade of Yarsagumba (Negi *et al*. 2020).

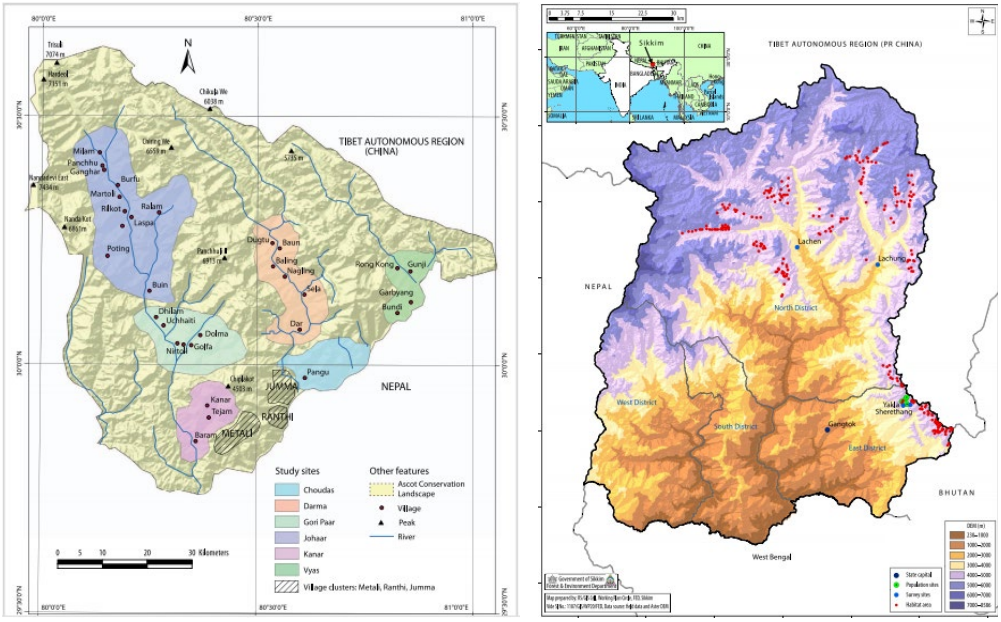

Fig. 6. Distribution of Yarsagumba in India; right, Uttarakhand (Negi *et al*. 2015) and left, Sikkim (Pradhan *et al*. 2020).

iv) Distribution in Bhutan

Yarsagumba is found in northern Bhutan between 4,070m and 4,800 m in the high alpine mountain valleys (Balfour-Browne 1955, Namgyel 2008, Cannon *et al*. 2009, Wangchuk and Wangdi 2015) (Table 1) (Fig. 7). It is found in the frozen high alpine meadows/pristine alpine Himalayan landscapes or yak pastures in isolated and unpopulated valleys such as Lunana, Bumthang Wangduephodrang etc near the border of Tibetan Autonomous Region of China (Gould 2007, Wangchuk *et al*. 2012). It is traditionally collected by local yak herders in grazing pastures.

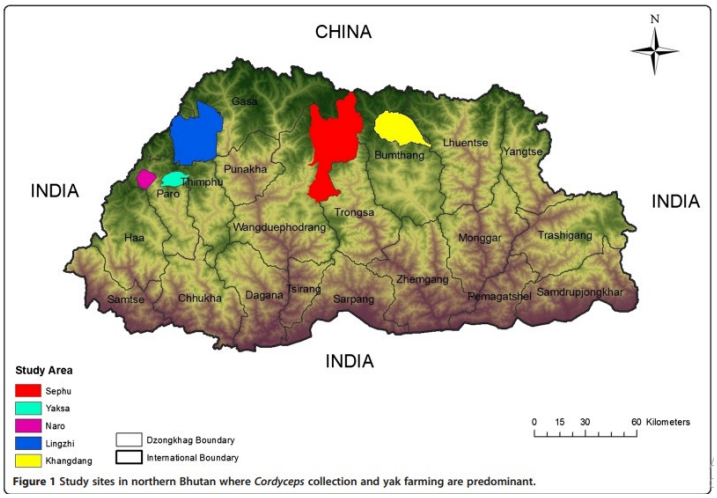

Fig. 7. Yarsagumba growing areas of Bhutan (Wangchuk and Wangdi 2015).

Table 1. Reported distribution of Yarsagumba in Bhutan, China, India and Nepal (Negi *et al*. 2020)

Country	Reported regions of occurrence	Altitudinal range (m asl)	References
Bhutan	Namna (North WesternBhutan), Bumthang Valley (North Central Bhutan), and Bumdeling Wildlife Sanctuary	4200-5200	Balfour-Browne (1955), Kobayasi (1980), Cannon et al. (2009)
China	Xinjiang, Yunan, Jilin, Shanxi, Shaanxi, Hubei, Zhejiang, Jiangxi, Guizhou, Taiwan, Guangdong, Guangxi, Sichuan and Hainan Province, and Lhasa and Shannan in Tibet	2260-5000	Winkler (2009), Li et al. (2011)
India	Uttarakhand (Darma valley, Choudans valley, Ralamdhura, Panchachuli base, Moist alpine areas of Dharchula and Munsyari Blocks especially, Pindari catchment in Bageshwar district, Niti valley, Nanda Devi Biosphere Reserve, Sutol, Kanol in chamoli district, Sikkim (North aand East Sikkim i.e. Luchung, Khangchendzanga national Park and Wildlife Sanctury, etc. and Arunanchal Pradesh	3200-4800	Negi (2003), Negi et al. (2006, 2009, 2014), Kuniyal and Sundriyal (2013), Sharma (2004), Pradhan (2016)
Nepal	Dolpa, Darchula, Jumla, Bajura, Kalikot, Mugu, Humla, Rukum, Bajhang, Manang, Mustang, Gorkha, Lamjung, Dhading, Rasuwa, Dolakha, Sindhupalchowk, Solukhumbu, Sankhuwasabha, and Taplejung districts	3540-5050	Shrestha and Sung (2005), Adhikari (2008), Devkota (2008, 2010)

2) Diversity of Yarsagumba in Nepal

Though Yarsagumba is known from the alpine regions of the Himalaya range, similar *Cordyceps* species have been reported from alpine regions of China and Nepal such as *C. nepalensis*, *C. kangdingensis*, *C. multiaxialis* and *C. gansuensis* (Zang and Kinjo 1998). They all grow on lepidopteran larvae. Since these species are very similar to Yarsagumba and grow on high altitudes, they are mixed up during collection and supply. There are many scientific studies on biological description of Yarsagumba and is reviewed by Shrestha *et al*. (2010). Besides Yarsagumba and Yarsagumba-like herbs, many other *Cordyceps* mushrooms have been collected from low and mid-hills of Nepal such as *C. liangshanensis*, *C. coccinea*, *C. ishikariensis*, *C. militaris*, *C. martialis*, *C. pruinosa*, *C. formicarum*, *C. gracilis*, *C. nutans*, *C. sphecocephala* and *C. tricentri* (Otani 1982, Tanda and Nagase 1994, Shrestha and Sung 2005, Adhikari 2008, Shrestha 2011) (Fig. 8). Morphologically, they are distinct from Yarsagumba and grow on different insects. For example, *C. liangshanensis* grows on lepidopteran larva, *C. coccinea* on coleopteran and lepidopteran larvae, *C. ishikariensis* on cicada nymph, *C. militaris* on lepidopteran larva and pupa, *C. martialis* on coleopteran larva, *C. pruinosa* on lepidopteran pupa, *C. formicarum* on adult ant, *C. gracilis* on lepidopteran larva, *C. nutans* on adult bug, *C. sphecocephala* on adult wasp and *C. tricentri* on adult spittlebug (Shrestha and Sung 2005, Shrestha 2011). Actually, Yarsagumba and *Cordyceps* mushrooms are among the first mushrooms studied scientifically since last 300 yrs (Shrestha *et al*. 2014). They grow on various species of insects such as coleopterans, lepidopterans, hymenopterans, hemipterans, spiders etc (Shrestha *et al*. 2016, 2017, 2019).

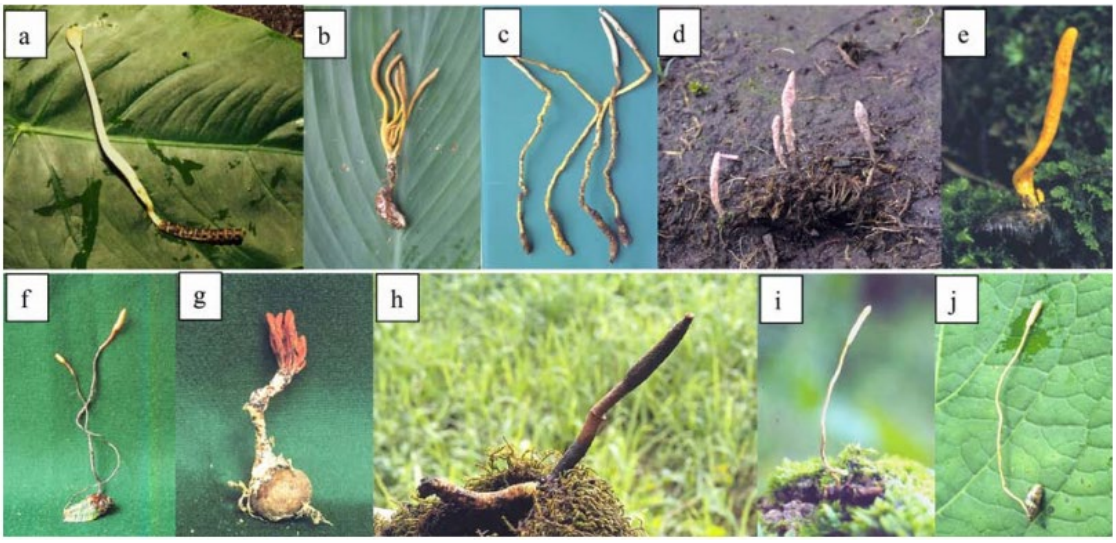

Fig. 8. Different *Cordyceps* mushrooms collected from Nepal. a, *C. gracilis*; b, *C. ishikariensis*; c, *C. liangshanensis*; d, *C. martialis*; e, *C. militaris*; f, *C. nutans*; g, *C. pruinosa*; h, *C. sinensis*; i, *C. sphecocephala* and j, *C. tricentri* (Shrestha and Sung, 2005).

3) Conservation of Yarsagumba

All of the Yarsagumba producing countries have implemented some measures to regulate harvest such as limiting collection period, number of collectors, areas of harvest etc. The common strategies implemented by the governments to conserve valuable, rare natural resources such as Yarsagumba, Panchaule, Kutki etc are banning their collection, transportation and use.

i) Nepal

In Nepal, the collection of panchaunle and yarsagumba was banned until February 2001. The government removed the ban on the collection and trade of Yarsagumba in the fiscal year 2001/2002 (Devkota 2010). Yarsagumba is now collected in areas where it was previously not known to occur, and it seems that lifting the ban has drawn collectors' attention to this product.

In order to sustainably harvest Yarsagumba, Nepal Government has recently formulated Yarsagumba Management Guideline (2073). According to the guideline, the collection period has been fixed from 2nd Week of Jestha to Ashar for harvesting. The Yarsagumba collection permit will be valid for 30 days and a person can have single permit at a time. The guideline (2073) has guided instructions for camping site management, drinking water facility management, waste management, temporary health facility at camping site and security at camping site. Camping site management should be done in coordination with local institutions like local administration office, Nepal Army, Armed police force, Nepal police, District Health Office, User Committees etc. The Guideline has forbidden certain activities in Yarsagumba collection and camping sites such as camping outside allocated area, carrying and use of plastic materials, doing activities that are detrimental to forest and wildlife, littering, firing and digging pits unsystematically, playing loud music and movies, using trails other than prescribed by the conservation area, activities that disturbs social peace and any other activities that are defined illegal by rule and regulations.

ii) Bhutan

Bhutan’s approach to managing Yarsagumba harvest includes (1) relaxing the laws on gathering of Yarsagumba in order to help local people with incentive to protect their natural resources, (2) delegating the power to restrict the number of harvesters and (3) establishing a law that Yarsagumba can only be sold at authorized auctions by authorized collectors and that buyers must be Bhutanese nationals. The government imposes a 4.9% levy on sales to cover the expenses of auctions and to support environmental protection programs (Cannon *et al.* 2009).

iii) India

In Sikkim of northeast India, the commercial collection and trade of Yarsagumba was legalized in 2009. Acoordingly, in Lachen and Lachung areas of Sikkim, it is mandatory for the collectors to get permission from Dzumsa and non-local residents are strictly prohibited from collecting Yarsagumba (Pradhan *et al*. 2020). Activities such as cutting trees and shrubs, harvesting of other medicinal herbs and wildlife poaching are restricted by Dzumsa. Collection of Yarsagumba is also restricted in the national park.

iv) China

In China, no activity of Yarsagumba collection is allowed in protected areas. In other areas, authorities regulate adaptive measures to harvest Yarsagumba in a sustainable way. This includes allocation of collection areas to local communities and banning non-locals from collecting Yarsagumba (Weckerle *et al*. 2010).

Chapter 2. Literature survey on traditional use of Yarsagumba in local communities of Nepal and other naturally Yarsagumba occurring countries

1) Introduction

The use of plants and plant products as medicine can be traced back to the beginning of human civilization. The earliest written record of medicinal plant use in the Himalayas is found in the Rigveda. This work, written between 4500 BC and 1600 BC, is supposed to be the oldest repository of human knowledge (MPN 1970, Singh et al. 1979, Malla and Shakya 1984). Charak Samhita (900 BC) and Susruta Samhita (500 BC) are the oldest literature that describe the art of surgery, therapeutics and medicine on the basis of Atharvaveda written about 4000 yrs ago (Nambiar 2002) and Ayurveda written about 2,500 yrs ago (Kunwar *et al*. 2006). Ayurvedic physicians incorporated medicinal plants in traditional Ayurvedic formulations that made Ayurvedic system reputed all over the Indian subcontinent (Olsen and Helles 1997, IUCN Nepal 2004). In addition to the Ayurvedic system, plants are also used in Homeopathic, Amchi (Tibetan traditional medicine), Chinese (Gurung *et al*. 1996, Thomas *et al*. 2002), and folklore (Kunwar 2006) systems. Himalaya is regarded traditionally as a secret heaven of potent medicinal herbs in Vedas, Samhitas and various folklores (Singh *et al.* 1979). Nepal Himalaya, occupying the central third of the entire length of the Himalayan ranges, has the most distinctive wild flora of the world.

2) Rasaayana in Ayurveda

There are eight branches or specialties in Ayurveda such as Kayachikitsa (कायचिकित्सा, general medicine or internal medicine), Shalyatantra or Shalyachikitsa (शल्यतन्त्र, शल्यचिकित्सा, major surgery), Shalakya tantra (शालाक्यतन्त्र, Ophthalmology and ENT), Bhuta Vidya (भूत विध्या, psychiatry or spiritual healing), Kaumarya Bhritya (काौमार्य भृत्य, gynaecology, obstetrics and paediatrics), Agada tantra or Visha Vidya (अगदतन्त्र, विषविध्या, toxicology), Rasaayana tantra (रसायनतन्त्र, nutrition, rejuvenation, immunology or tonics therapy or drugs, Geriatrics), and Vajikaran tantra (वाजीकरणतन्त्र, science of aphrodisiacs or aphrodisiac therapy). The objective of Rasaayana tantra is to prolong human life, and invigorate memory and vital organs of man. It deals with recipes which enable a man to retain his manhood or youthful vigour till old age, and which generally serves to make the human system invulnerable to disease and decay. Rasaayana drugs have the property to clear the channels by which they act as immunomodulators, adaptogenic etc. and delay the aging process (Panda 2007).

Charak Samhita, the first and most important of Ayurvedic text, has eight sections (sthana). Chikitsa sthana (चिकित्सा स्थान) is one of them. It deals with therapeutics such as Rasaayana Chikitsa (Rasaayana therapy or

Rejuvenation therapy) and Vajikarana Chikitsa (वाजीकरण चिकित्सा, Aphrodisiac therapy). Other sections of Charak Samhita are Sutra sthana (सुत्र स्थान), Nidana sthana (निदान स्थान), Vimana sthana (विमान स्थान), Sharira sthana (शारीर स्थान), Indriya sthana (इन्द्रिय स्थान), Kalpa sthana (कल्प स्थान) and Siddhi sthana (सिध्दि स्थान). In 6th and 7th verse (श्लोक ६, ७) of the first Chapter of Chikitsa sthana, Rasaayana is mentioned as

दीर्घमायु: स्मृतिं मेधामारोग्यं तरुणं वय: । प्रभावर्णस्वरौदार्यं देहेन्द्रियबलं परम् ॥६॥

वाक्सिद्धिं प्रणतिं कान्तिं लभते ना रसायनात् ।

लाभोपायो हि शस्तानां रसादीनां रसायनम् ॥७॥

Dirghamaayu smritimedhamaarogyam tarunam vaya. Prabha warna swaraudaaryam dehendriyam balam param //6//

Waaksiddhimpranatimkaantilavate na rasayaanat.

Labho paayo hi sastaanaam rasaadinaam rasayanam //7//

It means Rasaayana therapy (rejuvenation therapy) helps one attain longevity, memory, intelligence, good health, youthfulness, excellence of luster, complexion and modulated voice, optimum development of the body and sense organs, mastery over phonetics, respectability and brilliance. Gangadhar's commentary on "Labho paayo hi shastaanaam rasaadinaam rasaayanam" reveals that the word "Shastaanaam" is interpreted as persistent youthfulness and the word "Rasaadi" is taken as those originated from Rasa (Deshpande and Deshpande 2018). Based on Charak Samhita, Gangadhar comments that Rasaayana therapy is that treatment which produces excellent Dhatus and thereby promotes longevity, memory, etc.

Acharya Dalhana in 12th century compiled the views of a large number of authors and commentators in Nibandha Samgraha.

Bhesajashritaanam rasaviryavipaakaprabhavaparamaayurbalaviryaanaam vaya sthyairyakaranaamayanam laabhaopaayo rasaayanam.

Su. Su. 1/15

भेषजश्रीतानां रसवीर्यवीपाकप्रभावपरमायुर्वलवीर्यानां भाया

स्थ्याइर्याकरणामयानां लाभोपायो रसायानां

It means those dravyas which are capable of utilizing all the rasa (taste), guna (properties), veerya (potency), vipaak (post-digestion metabolic effect) and prabhava (specific property) to strengthen the body and develop immune mechanism are known as Rasaayana. Rasaayana increases a healthy person's mental and physical capabilities. Rasaayana or vitalizers replenish the vital fluids of our body, thus keeping us away from diseases. The word Rasaayana (Rasa +Ayana) refers to nutrition and its transportation in the body.

"Rasa" means nutrition and "Ayana" means microcellular channels and hence, Rasaayana may be taken as the way or the measure to attain replenished and excellent dhatus.

According to Mishra and Upadhyay (2011), the Rasaayana branch of Ayurveda deals specifically with Rasaayana herbs and formulations that bestows upon the user, the longevity with age stabilization and retaining youth for longer. From the Rasaayana treatment, one attains longevity, memory, intelligence, freedom from disorders, youthful age, excellence of luster, complexion and voice, oratory, optimum strength of physique and sense organs, respectability and brilliance.

3) Yarsagumba as a Rasaayana

Most of the medicinal plants (herbal drugs) in Sikkim Himalaya have Rasaayana and tridoshagna properties and are used for common health problems (Panda 2007). The Himalaya has a great wealth of medicinal plants and traditional knowledge. According to Panda (2010), more than 50% of the ethno-medicinally important species of India are from the Himalayan region, which number nearly 1748 plant species (Samant *et al*. 1998). Most of the rare and endogenous Himalayan medicinal plants used in Ayurveda are Rasaayana (rejuvenation drug) (Panda 2007). Yarsagumba is a true Rasaayana (rejuvenator/ antiaging) herb that functions as an immunomodulator, adaptogen, antioxidant, anti-cancer, neuroprotective, nootropic, aphrodisiac and hepatoprotective (Mishra and Upadhyay 2011). It is a well-known high-altitude herb (mushroom growing on a moth larva), and traditionally used as an aphrodisiac and tonic in North Sikkim and other Himalayan regions. The traditional healers of Sikkim have been using this herb for eighteen diseases, maximum use in form of self-medication and folk healer's recommendation is for aphrodisiac use (Table 1, Panda 2010).

Yarsagumba is categorized as Syabar Jangam Buti (स्यावर जङ्गम वुटी) (शर्मा, २०५३), 'Syabar'' meaning tree or plant and 'Jangam' meaning insect or animal. It is also known as Sanjeevani buti (संजीवनी बूटी) (शर्मा २०५३, Panda 2010) or BhuSanjeevani (भूसंजिवनी) (Shrestha *et al*. 2010), Sanjeevani (संजिवनी) (Gyawali and Gyawali 2006, Das *et al*. 2010, Kunwar et al. 2010, Mishra and Upadhyay 2011), Jeebanbuti (जीवनबूटी) (Bhattarai 1992, Gyawali and Gyawali 2006, Amatya 2008, Ghimire et al. 2008, DFO 2067). In Aatraya Samhita, the use of Yarsagumba is defined as सान्निपातिक बृक्क महफिरंग. It means it can cure complicated diseases related to kidney and sexual organs (Shrestha et al. 2010). In Sanskrit, सान्निपातिक means a complicated disease caused by त्रिदोष and बृक्क means kidney.

Table 1. Traditional uses of Yarsagumba in North Sikkim (Panda 2010)

S.N.	Illness	Strength of the claims
1	Erectile Dysfunction	++++
2	Female aphrodisiac	+++
3	Infertility	++
4	General weakness	+++
5	Tuberculosis	++
6	Bronchitis	++
7	Malignant Tumor	+++
8	Rhinitis	++
9	Rheumatism	+
10	Arthritis	++
11	Jaundice	++
12	Prostate enlargement	++
13	Liver diseases	+++
14	Kidney diseases	+++
15	Coronary Heart Disease	+
16	Chronic pain	++
17	Sciatica & backache	++
18	Low BP & dizziness	+++

There are three likely contributing factors of Yarsagumba to have an effect on sexual dysfunction. Firstly, it has been shown to improve physical vitality and stamina in general. In 1993, a group of nine Chinese women set world records at the Chinese National Games. When asked about the secret to their success, they attributed it to their use of Yarsagumba (Steinkraus and Whitfield 1994). Secondly, it helps dilate blood vessels and increase blood flow, which is certainly an important factor of erectile function (Panda 2010). Thirdly and perhaps most important of all is that two studies have shown Yarsagumba to "significantly increase" the production of testosterone in males (Panda 2010). Main ingredients of Yarsagumba are β-glucans, polysaccharides, sterols, cordycepin, cordycepic acid, vitamins etc with different bioactivities (Miller 2009, Shrestha *et al*. 2012, 2013).

4) Traditional uses of Yarsagumba

Yarsagumba is popularly used as a tonic for elderly people as well as for maintaining youth and restoring health after deep fatigue or illness in alpine and hilly regions of Nepal and surrounding countries. Various uses of Yarsagumba are briefly given below in different countries.

i) Traditional uses in Nepal

Yarsagumba is a high-valued highland herb of Nepal, which is a source of income for local communities in the northern parts of Nepal (Shrestha and Bawa 2014). Potential areas for Yarsagumba collection in Nepal are alpine grasslands or meadows of northern Nepal, ranging from 3000 m asl up to 5000m where the area is covered by snow for at least six months a year (Shrestha *et al*. 2010). Yarsagumba is found to be effective for asthma and other bronchial conditions (Miller 2009). It is also found effective against tuberculosis, leprosy and human leukaemia. It improves heart, liver and kidney functions as well as

enhances immunity, and hence considered a general health tonic (Miller 2009). There is a belief in Manang that if one takes Yarsagumba with 13 other medicinal herbs for 3 years or so, he will be as thick as elephant, as fast as a horse and as pretty as a peacock (Pohle 1990).

In Upper Langtang of Rasuwa district, Yarsagumba is taken as a tonic in combination with Panch Aule (*Dactylorhiza hatagirea*, Orchidaceae), honey and cow's milk (MPN 1970). It is also believed to be tonic to yaks and sheep in Langtang (MPN 1970). In Rolwaling valley of Dolakha district, Yarsagumba is regarded as a very popular medicine, vitamin or aphrodisiac (Sacherer 1979). According to Bhattarai (1989), the whole fruiting body is considered a good aphrodisiac and a tonic in Helambu. It is mostly taken with milk or honey, 1 - 2 fruiting bodies per day. According to a study in Karnali region of Nepal, two to three fruiting bodies of Yarsagumba are eaten as tonic with honey or milk for their alleged aphrodisiac property (Bhattarai 1992).

ii) Traditional uses in India

It was found that this fungus is used for male and female sexual dysfunction, to restore the general health and appetite and to promote longevity in Lachung & Lachen area of North Sikkim traditionally (Panda 2010). Both male and female usually take one piece of Yarsagumba with one cup of milk for enhancing their sexual potency and desire. The Bhutia communities put one piece of Yarsagumba in one cup of locally made alcohol (*chang*) and leave for one hour and drink in the morning and evening as an aphrodisiac. Some use hot water instead of alcohol. They say it is more potent than Ginseng and also use in the treatment of cancer, fatigue, to relieve chronic pain, tuberculosis and to treat liver and kidney ailments. The people of North Sikkim believe that this medicine has been used by their ancestors before Chogel period in between 1200-1600 BC. But the awareness about its uses has doubled after 1995 only. From 1997 to 2009 prices have increased by 500%, on average of over 20% per year in Tibet.

iii) Traditional uses in China

Yarsagumba was first mentioned in China in 620 AD during the Tang dynasty (618-907 AD), which described it as a magical creature that transformed from animal to plant in summer and then again from plant to animal in winter. From the middle of the Qing dynasty (1644-1912 AD), Yarsagumba had been in use as a tonic and supplement for court cuisine, and was also introduced to Japan through Nagasaki (Ono Ranzan, the Edo era (1603-1868)). Yarsagumba was also mentioned in "The Compendium of Materia Medica" in 1596 during Ming dynasty (1368-1644 AD) and later in 1694 during Qing dynasty (1644-1912 AD). The medical text "Ben Cao Bei Yao" (Synopsis of Materia Medica) listed the traditional usage of Yarsagumba as entering the lung and kidney meridian and being useful as a "lung protectorate" for "kidney improvement" and as a "Yin/Yang double invigorant".

The earliest objective and scientifically reliable description of Yarsagumba was written in New Compilation of Materia Medica. In Chinese medicine, Yarsagumba is considered sweet and warm that enters the lung and kidney channels; the typical dosage being 3-9 grams. Nyamnyi Dorje, a Tibetan physician and Lama, mentioned Yarsagumba in "An Ocean of Aphrodisiacal Qualities" in 15th century. At present, it is officially classified as a drug in the Chinese Pharmacopoeia by the Committee of Pharmacopoeia, Chinese Ministry of Health (1964, 2005). In Bhutan, the traditional use of Yarsagumba is influenced by Tibetan and Chinese systems.

Chapter 3. Literature survey on commercial products of Yarsagumba and its use, value chain in local and international trade channels and marketing of Yarsagumba

1) Value chain in local and international trade channels and marketing of Yarsagumba

In 2014, one piece of Yarsagumba was sold for an average price of NRs. 332 in Darchula (Karki *et al*. 2020). It generated the highest amount of revenue (i.e. 41% of the total revenue) out of 62 species traded in Nepal as non-timber forest products (NTFPs) (Karki *et al*. 2020). Because of the high market price, the income from Yarsagumba harvesting has improved local economy and provided employment opportunity for the people in this region. In Nepal, the market of wild Yarsagumba is attractive and the economic return is very high due to its price and demand.

The market of Yarsagumba is described as oligopsony, with several thousand collectors and few traders in higher level of the supply chain (Pant *et al*. 2020). The reasons for oligopsony are i) inability of local collectors and traders to afford investment, ii) exclusively international market of Yarsagumba, and iii) high risk of security, among others (THT 2013). In addition, there is a dramatic reduction in Yarsagumba collection and recently it is enlisted as vulnerable species in the IUCN's Red List of Threatened species (Yang 2020).

Two types of supply chains are in practice: i) open supply chain and ii) close supply chain (Pant *et al*. 2020) (Fig 1). The open supply chain is linked with national Yarsagumba trade and Kathmandu is the final destination for export to international market. There are at least four layers in its trade system such as collectors, local contractors, district-level traders and national traders (Fig. 1). The close supply chain is defined as a customary trade system with the involvement of migratory communities who collect and trade with the traders in Tibetan Autonomous Region (Pant *et al*. 2020). The collection sites are usually different for migratory and local collectors. The open supply chain is usually complex and often nontransparent. The marketing channel of Yarsagumba originating from Dolpa is documented by Thapa and Chhetri (2068 B.S.) (Fig. 2).

Few national traders ranging from 15 to 20 in number are involved in international trade of Yarsagumba from Kathmandu (Pant *et al*. 2020). They have good linkages with the international buyers, particularly Chinese (Pradhan *et al*. 2020) (Fig. 3). These traders also receive advance payment from international buyers which they circulate to the collectors via district traders. The traders follow informal system since

the formal system increases transaction costs. The policy and regulations of collection and trade of Yarsagumba differ from country to country.

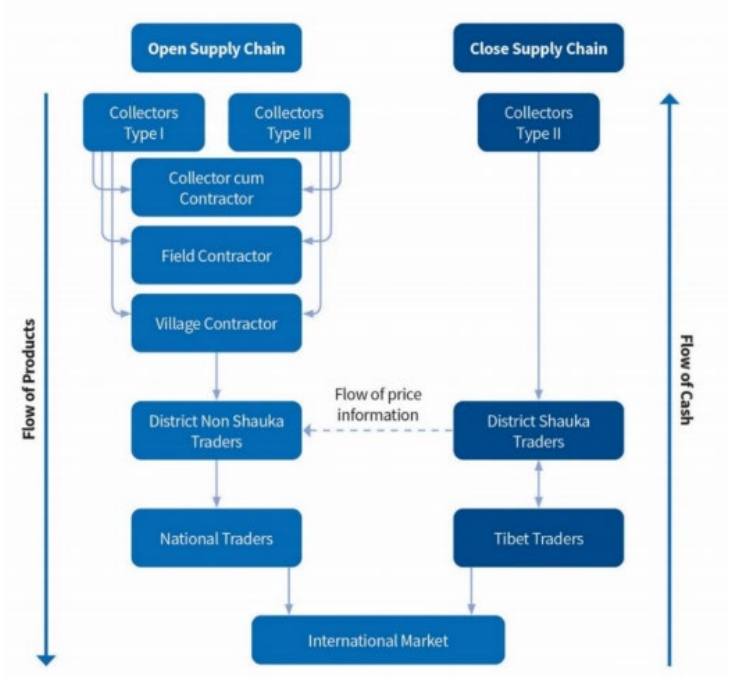

Fig. 1. Trade and money flow of Yarsagumba market chain related to Darchula district, Nepal (Pant *et al*. 2020).

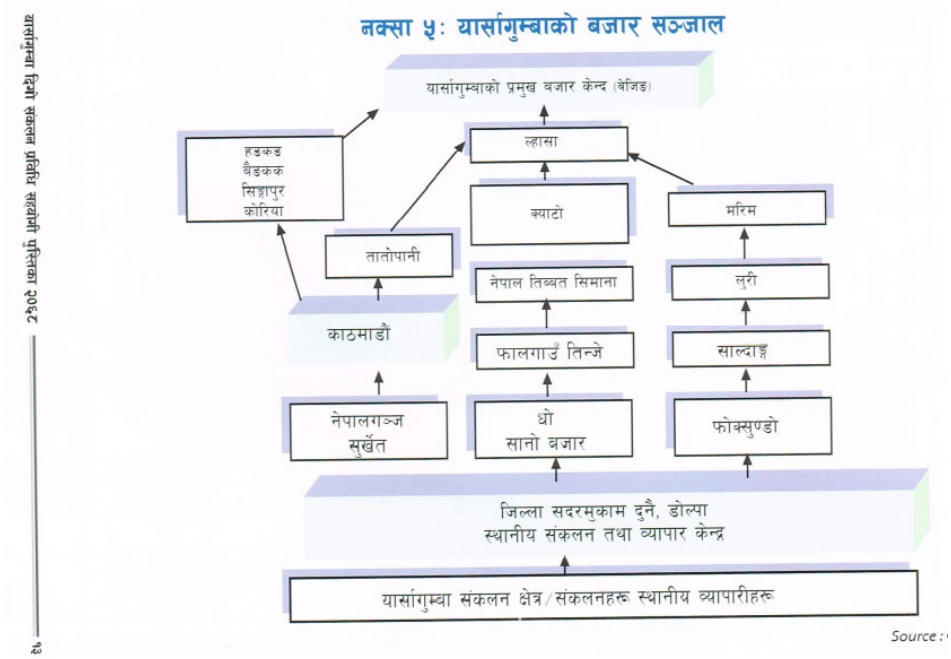

Fig. 2. Marketing network of Yarsagumba, starting from collection site to international market (थापा तथा क्षेत्री २०६८).

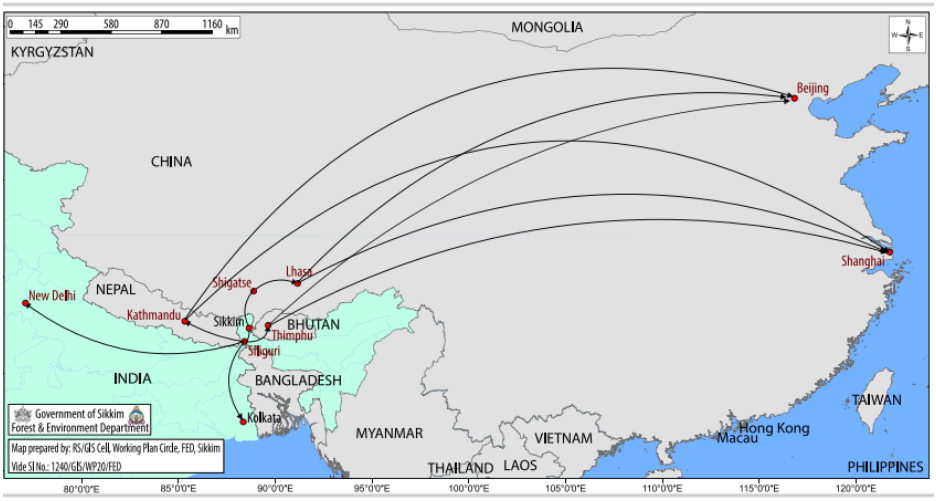

Fig. 3. National and international trade routes of Yarsagumba (Pradhan *et al*. 2020).

i) Bhutan

The Government of Bhutan had prohibited collection and sale of caterpillar fungus till 2004. In 2004, the Bhutanese government created a new policy to regulate collection and sale of caterpillar fungus (Wangchuk *et al*. 2012). The policy allowed local people to obtain harvesting permits against a nominal fee. In order to prevent over-exploitation of the species, a limited harvest regime was deployed, which included prohibition on collection except during the month of June and only one member from each of the permitted households was allowed for collection (Cannon *et al*. 2009). In 2009, a new rule was enforced that allows only three persons from one household to collect Yarsagumba. The herb is sold by the collectors at legally administered auction centers to Bhutanese buyers (Cannon *et al*. 2009).

ii) China

China is the main market for Yarsagumba. Also, it is the largest collector of the herb in the region. Due to increasing harvest, Yarsagumba is listed as an endangered species under the second class of state protection in China (Li *et al*. 2011). The harvesting of the species is forbidden from protected areas. However, authorities regulate adaptive management strategies for harvesting of Yarsagumba. This includes allocation of collection areas to communities and controlling of harvesters from outside (Weckerle *et al*. 2010). The trade flow of Yarsagumba in China is shown by Winkler (2008) (Fig. 4).

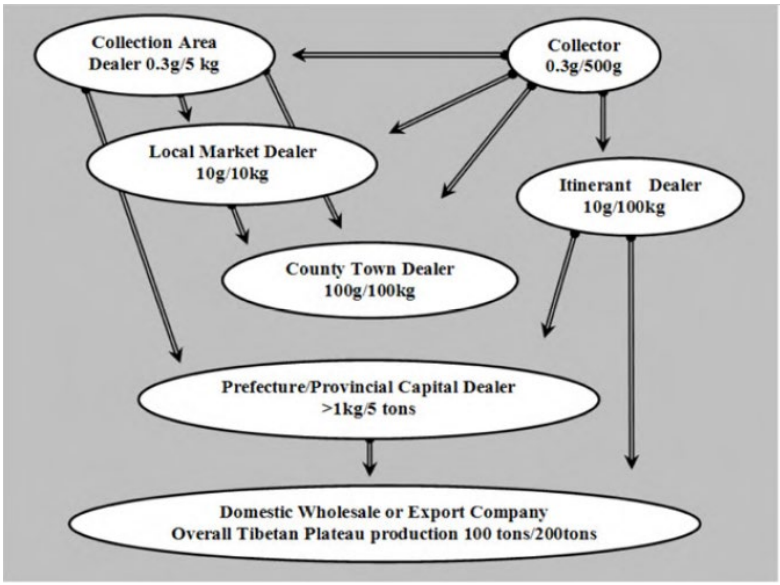

Fig. 4. Yarsagumba trade flow chart in China (Winkler 2008).

iii) Nepal

Nepal's Forest Act 1993 and Forest Regulations 1995 are the main legislations to protect medicinal plants including caterpillar fungus. It was legalized in 2001, with the provision of revenue of 20,000 Nepalese rupees per kg to the government (Devkota 2010). In 2006, the revenue amount was reduced to 10,000 Nepalese rupees per kg (Shrestha and Bawa 2013). After the ban was lifted, the caterpillar fungus became one of the most sought-after commodities in the natural medicine sector of Nepal. Under the current management regime, the caterpillar fungus is an open access common pool natural resource. The government is generating substantial revenue from the trade, which occurs mostly in unprocessed form. Thus, the collection and trade is economically beneficial for both the local people and the Government of Nepal. As such, there is no control over number of harvesters resulting in increasing pressure on this resource. In recent time the collection of caterpillar fungus per unit per person has declined due to a large number of people getting involved in this process (ICIMOD 2014).

iv) India

In India, the sale of Yarsagumba was not regulated by law, which led to its smuggling/illegal trade to Nepal and China (Yadav *et al*. 2016) (Fig. 5). In order to address the problems associated with unregulated trade, the Uttarakhand Government issued certain guidelines on the collection and trade of Yarsagumba from reserve forests through village level forest councils (Van Panchayat), while commercial exploitation from wildlife sanctuaries and national parks is completely prohibited. Protected areas in reality remain open to all for harvesting of caterpillar fungus because access is not strictly enforced (Negi *et al*. 2015). On top of

all this, ambiguities and complexities remain as there is no single policy of the Government pertaining to the trade and harvest of caterpillar fungus in India. The trade chain starts from the harvesters and is destined toward international markets, primarily China, where the demand is fuelled by the utilization of caterpillar fungus as an ingredient in traditional medicines.

The impact of trade on natural populations needs to be scientifically assessed for Yarsagumba (Cannon *et al*. 2009, Shrivastava *et al*. 2010, Shrestha and Bawa 2013, Wangchuk and Wangdi 2015). Policy to promote sustainable harvesting and conservation of the species require sufficient knowledge on ecological status, harvesting pattern and legal status. Therefore, intensive surveys, involving a longer research period and human resource, are also needed to assess species status in natural habitat, price scale escalation at various levels and the socio-economic linkages of stakeholders at each level.

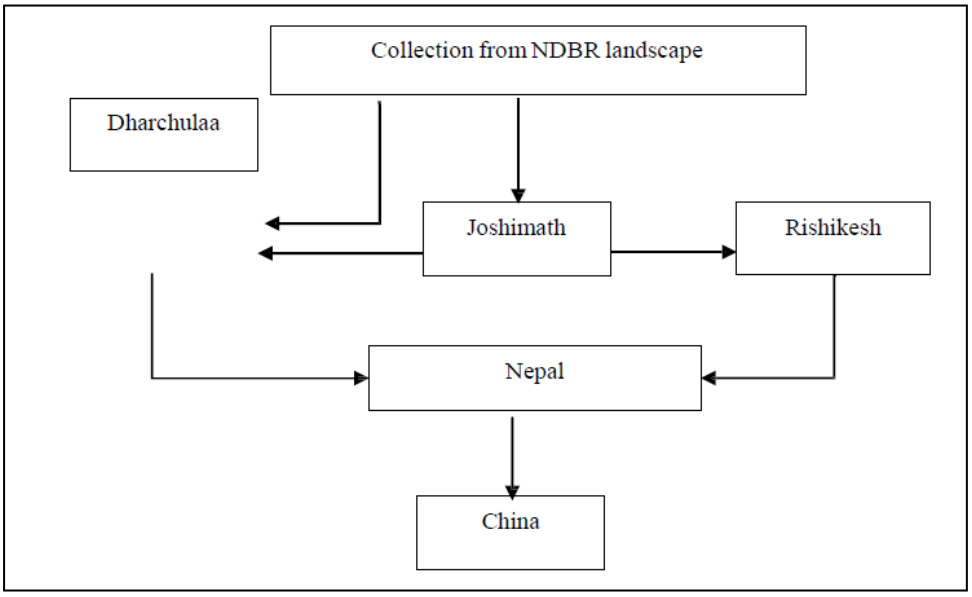

Fig. 5. Indicative trade route of Yarsagumba in India (Yadav *et al*. 2016).

2) Commercial products of Yarsagumba

Functional foods such as nutraceuticals, medical foods or nutritional foods are expected to play a leading role in the food industry. Among natural resources, Yarsagumba and other *Cordyceps* mushrooms are important ones. Due to high price of Yarsagumba, many people are collecting them. Hence, researchers have been trying to develop methods to grow mycelium from wild Yarsagumba to meet the growing demand. Other *Cordyceps* mushrooms, including *C. militaris*, are currently cultivated and introduced to market as substitutes of Yarsagumba.

At present, development of Yarsagumba and other *Cordyceps* mushrooms is mainly focused on the compounds originating from the *Cordyceps* mushrooms. Some major companies have started producing cosmetics containing *C. sinensis* and *C. militaris* extracts (Elkhateeb *et al*. 2019). Global annual production of Yarsagumba is estimated to be in the range of 85–185 tons (Winkler 2009) with further tonnage provided by other *Cordyceps* species. The harvesting and sale of wild *Cordyceps* can have a significant impact on household incomes in the regions in which it is collected (Winkler 2008). The intense global interest and value assigned to *Cordyceps* has led to a large range of commercial products (Elkhateeb *et al*. 2019) (Fig. 6).

Fig. 6. Commercial products of Yarsagumba and *Cordyceps* mushrooms (Elkhateeb *et al*. 2019).

Being functional foods, mushrooms represent a prolific source of bioactive compounds with countless therapeutic capabilities working toward preventing and controlling many diseases. A large number of biologically active compounds originating from mushrooms have been isolated and reported previously. Further researches are required in order to isolate and identify bioactive compounds responsible for such biological activities.

i) Health food

Health food, also called 'functional food', is widely known as food or food component that helps to modulate the human body and prevent diseases. There are different Yarsagumba and *Cordyceps* health foods on the market as shown in Fig. 6. These have been used by different groups such as young people, the elderly, pregnant women, people with nutritional imbalance etc (Zhou *et al*. 2009). It is worth noting that, in recent years, increasing attention is given to the function of *Cordyceps militaris*. Presently, Japan, Korea, Malaysia and other countries are increasing research and development of *Cordyceps* functional foods.

ii) Drug development

The most bioactive components of *Cordyceps* such as cordycepin, cordycepic acid and polysaccharide have been authorized as new medicinal resources by the government of China. Therefore, many products can be developed using Yarsagumba in combination with other traditional herbs or nutrient resources. These products have many functions such as enhancing physique, anti-aging, protecting the heart, improving sleep, increasing appetite, increasing immunity etc. For example, *Cordyceps militaris* mycelia powder and capsule are authorized as Chinese national drugs. So far, there are approximately twenty types of TCM preparation related to Yarsagumba and *Cordyceps* mushrooms, many of which are polysaccharide products (Zhou *et al*. 2009). The development of the curative and health-care products from *Cordyceps* mushrooms is more and more favourable among people in China (Zhou *et al*. 2009).

iii) *Cordyceps militaris* industry

Of all *Cordyceps* species, *C. militaris* is the only species that has been successfully cultivated and most intensively studied. It has been increasingly viewed as a substitute for Yarsagumba because of their similar chemical profiles and medicinal properties (Yue *et al*. 2008; Dong *et al*. 2012). Unlike Yarsagumba, *C. militaris* has a wider distribution from sea level to up to 2000 m or above (Shrestha *et al*. 2012). However, its occurrence is very low in nature. Currently, the fruit bodies of this fungus have been successfully cultivated and commercialized for medicinal and health products, and even for direct consumption as an edible mushroom. In addition to the medicinal and health products from the *C. militaris* mycelia, the

bioactive compounds from the fermented mycelia such as cordycepin, cordycepic acids, polysaccharide and carotenoids, have become sources for major industrial uses (Holliday and Cleaver 2008).

According to Dong *et al*. (2015), there are at least 36 approved health foods made from *C. militaris* in China. In addition, it can also be used as general foodstuff since it was listed as Novel Foods by the Chinese government (http:// www.moh.gov.cn/publicfiles/business/htmlfiles/zwgkzt/ pgg/200903/39591.htm) in 2009. Now, a huge industry based on *C. militaris* has been developed and it was estimated that the annual sale was about 3 billion in China (Dong *et al*. 2015).

Chapter 4. Literature survey on bio-activity of wild and cultivated Yarsagumba and identification and listing of potential Yarsagumba products of scientific and economic significance

1) Literature survey on bio-activity of wild and cultivated Yarsagumba

In the early Chinese medical books, Yarsagumba is shown to function as a lung protectorate and as a Yin/Yang double invigorant or Yin-nourishing and Yang-invigorating (Tsim and Li 2005, Song *et al*. 2010). Song *et al*. (2010) have also demonstrated that Yarsagumba extract can ameliorate renal function injury in glomerular sclerosis and reverse extracellular matrix accumulation, which show that the extract possibly could be one of the therapeutic drugs for chronic kidney diseases. Yarsagumba has also been shown to have a variety of pharmacological effects such as anti-inflammatory actions, anti-apoptotic effects, stimulating natural killer cells, anti-tumor activities etc (Shahed *et al*., 2001, Song *et al*. 2010). Other therapeutic effects of Yarsagumba include stimulating immune response, inhibiting cancer growth, protecting the kidney and liver, stimulating cardiovascular circulation, lowering blood glucose and acting against free-radical formation (Tsim and Li 2005) (Fig. 1).

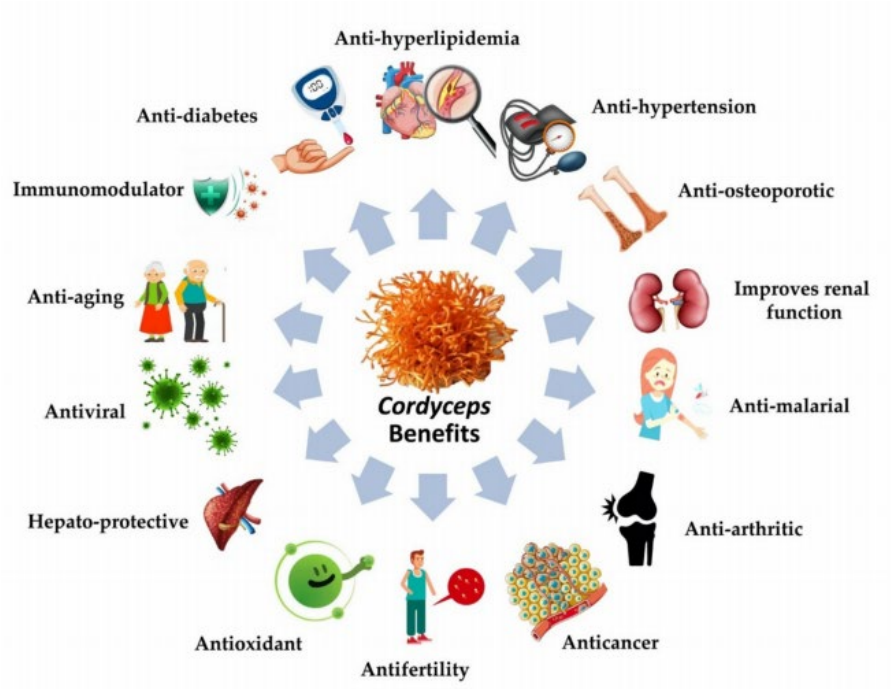

Fig. 1. Therapeutical potentials of *Cordyceps* (Ashraf *et al*. 2020).

Yarsagumba is commonly used in China to replenish kidney and soothe the lung for the treatment of fatigue, night sweating, hyposexualities, hyperglycemia, hyperlipidemia, asthemia, respiratory disease, renal

dysfunction and renal failure, arrhythmias, heart disease, liver disease etc (Zhu *et al*. 1998). Recent studies have demonstrated its multiple pharmacological actions in potentiating the immune system (Xu *et al*. 1992) and the antitumor activity (Chen *et al*. 1997). The anti-oxidation activity was reported in Yarsagumba, which has been used in China for preventing aging and improving physical performance (Zhu *et al*. 1998).

2) Major components of Yarsagumba

Adenosine, cordycepin, cordycepic acid (D-mannitol) and polysaccharides are four major and effective components of Yarsagumba. Among these components, adenosine is used as a marker for quality control of Yarsagumba (Fig. 2) (Zhou *et al*. 2019). It is known to depress the excitability of CNS neurons, to inhibit the release of various neurotransmitters and to possess anticonvulsant activity (Li *et al*. 2006). Cordycepin content in wild and cultivated Yarsagumba is confirmed in various studies (Lei *et al*. 2006, Yu *et al*. 2006, Zhou *et al*. 2019). Adenosine and its derivatives, including cordycepin, possess powerful bactericidal, antiviral, fungicidal, and anticancer properties, presenting strong pharmacological and therapeutic potential to cure many dreadful diseases (Fig. 2) (Tuli *et al*. 2013). Zhou *et al*. (2019) have shown that the contents of nucleosides (cordycepin, adenosine, etc) in cultivated Yarsagumba are significantly higher than in the natural type, as shown previously by Lei *et al*. (2006), Yang *et al*. (2009) and Wang *et al*. (2015). Nucleosides are believed to be one of the key active components of Yarsagumba (Li *et al*. 2001b). Cordycepin contributes to the anti-tumor, insecticidal, and antibacterial activity of Yarsagumba and is used as a marker for quality control (Li *et al*. 2001b, Ng and Wang 2005).

Cordycepic acid, also known as D-mannitol, is mainly used as an anhydride and diuretic in medical treatment (Fig. 2). It has pharmacological effects such as increasing plasma osmotic pressure, antitussive and anti-free radical activities (Li *et al*. 2006), and cerebrovascular dilation (Andrews 1993). It can be employed in the treatment of meningioma as a liquid chemoembolization agent (Feng *et al*. 2005), in the treatment of intracerebral hemorrhage (Seyfried *et al*. 2008), and for downregulating intracranial pressure (Lorenzl *et al*. 1996). Wild Yarsagumba contains higher amount of free mannitol and a small amount of glucose, while mannitol in cultured Yarsagumba is much less and free glucose is only detected in a few samples (Guan *et al*. 2010). Cordycepic acid was isolated from Yarsagumba in 1957, and for this reason, it had been used as a quality control marker for Yarsagumba for a number of years. Mannitol is a major component of carbohydrate in natural Yarsagumba and contributes to over 3.4% of the total dry weight. It is used to treat many diseases. The content of mannitol in natural Yarsagumba was higher than that in the cultured one (Tsim and Li 2005).

Polysaccharides are one of the most abundant components of Yarsagumba (Li and Li 2013). They have been extracted and isolated from the fruiting bodies, cultured mycelium, and fermentation broth, and are

structurally diverse biomacromolecules with varying physiochemical properties (Wu *et al*. 2015). They contribute to the anti-inflammatory, antioxidant, antitumor, anti-metastatic, immunomodulatory, antidiabetic, steroidogenic and hypolipidemic effects of Yarsagumba (Nie *et al*. 2013), and they have been the target of the development and quality control of natural Yarsagumba health products. It is significantly higher in cultured than those of natural samples (Wang *et al*. 2015).

Cordyceps fungi contain a large amount of polysaccharides, which could range from 3 to 8% of the total dry weight (Tsim and Li 2005). In cultured *Cordyceps*, functional polysaccharides are known to be secreted by the mycelium. *Cordyceps* polysaccharide was found to have different biological activities, which include anti-oxidation, immuno-modulation and tumor-inhibition (Tsim and Li 2005). Until now, the pharmacological profile of *Cordyceps* correlates very well with the amount of polysaccharide in the herb. Based on the activity-guided fractionation, several polysaccharides have been isolated from cultured *Cordyceps*; these *Cordyceps* polysaccharides could stimulate our immune system and prevent free radical formation (Tsim and Li 2005).

Proteins in Yarsagumba play role in biological processes such as ribosome formation, stress adaptation for temperature reduction and cell cycle control (Rajput *et al*. 2016). However, they are not the main effective components.

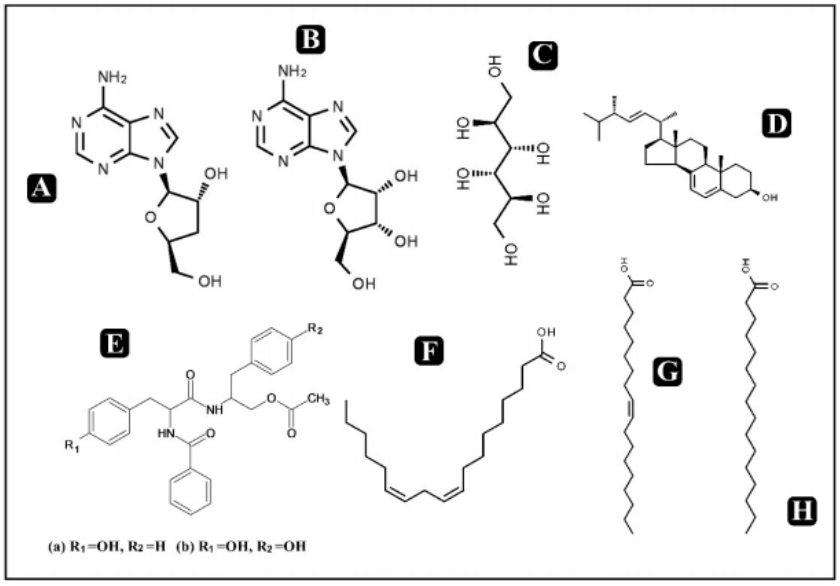

Fig. 2. Chemical structures of some known and potent bioactive compounds in Yarsagumba. A, cordycepin; B, adenosine; C, cordycepic acid; D, ergosterol; E, structure of cordyceamides (a) and Cordyceamides B; F, Linoleic acid; G, Oleic acid and H, Palmitic acid (Ashraf *et al*. 2020).

Ergosterol is a unique component in fungi, and it is required for vitamin D2 synthesis (Fig. 2). Sterols and their derivatives have been isolated from natural and cultured Yarsagumba. Ergosterol exists as free and or

in combined forms in Yarsagumba. The content of ergosterol is higher in cultured mycelia than in natural collection, and indeed the level of ergosterol reflects the amount of cultured mycelia (Tsim and Li 2005). Pharmacological study showed that ergosterol possesses anti-arrhythmia effect. In addition, the ergosterol derivatives also have multiple pharmacological activities, such as cytotoxic activity and anti-viral activity; these activities vary in accordance with the quality of Yarsagumba for both natural and cultured products (Tsim and Li 2005).

3) Bioactivities of Yarsagumba

There are different bioactivities of Yarsagumba (Table 1). Among them, few are discussed in brief below.

i) Anti-inflammatory activity

Treatment with water extract of Yarsagumba (0.1 or 0.2 g) brings about a down-regulation of inflammation-related genes in the rat kidney following ischaemia/reperfusion (Shahed *et al*. 2001). It improves lung function in sensitized guinea pigs and airway inflammation in sensitized rats, suggesting that it could be used in the prevention and treatment of asthma (Lin *et al* 2001).

ii) Antioxidant and neuroprotective activity

Aqueous and ethanolic extracts of Yarsagumba inhibit MDA generation via hydroxyl radicals induced by the peroxynitrite generator SIN-1, and suppress the oxidation of low-density lipoprotein (LDL) and macrophage accumulation of esterified cholesterol (Yamaguchi *et al*. 2000a, b). Tsai *et al* (2001) demonstrated the hydroxyl radical scavenging activity of an aqueous extract of Yarsagumba. Li *et al* (2001a) noted that water extracts as well as a partially purified polysaccharide fraction of cultured Yarsagumba mycelia manifest antioxidant activity in the xanthine oxidase, haemolysis and lipid peroxidation assay systems.

Subsequently, Li *et al* (2002) showed that the fruiting body part and the caterpillar part of Yarsagumba are similar in chemical composition and antioxidant activity because the caterpillar is fully invaded by the mycelia of Yarsagumba. Wang *et al* (2005) also noted that the ethanol extract of Yarsagumba exhibits free radical scavenging activity.

iii) Anti-tumour, anti-proliferative and anti-metastatic activity

A water extract of Yarsagumba increases the median survival time of ICR mice inoculated with allogeneic Ehrlichascites carcinoma cells and BALB/c mice inoculated with syngeneic Meth A fibrosarcoma cells (Yoshida *et al.* 1989). The water extract of Yarsagumba inhibits spontaneous liver

metastasis of Lewis lung carcinoma cells and B16 melanoma cells in syngeneic mice. It demonstrates potent cytotoxicity against these tumour cells, while cordycepin is devoid of cytotoxicity, suggesting that the anti-metastatic activity is not attributed to cordycepin (Nakamura *et al*. 1999). Co-administration of the water extract and methotrexate prolongs the survival period of mice inoculated with B16 melanoma cells compared with administration of the water extract alone and administration of methotrexate alone (Nakamura *et al.* 2003). Chen *et al* (1997) observed that the conditioned medium from blood mononuclear cells stimulated with the polysaccharide fraction from Yarsagumba inhibits the proliferation of human leukaemic U937 cells, and induces about half of the cells to differentiate into mature monocytes/macrophages expressing nonspecific esterase activity and CD116, CD14 and CD68 surface antigens.

Table 1. Biological activities of Yarsagumba (Ng and Wang 2005)

Biological activity	Chemical constituent/fraction	Representative reference(s)
Anti-inflammatory	Polysaccharide	Yu et al 2004a
	Methanolic extract	Kim et al 2003a
	Ethanolic extract	Woo & Park 2005
Antioxidant	Aqueous and ethanolic extracts	Yamaguchi et al 200oa, b
	Polysaccharide fraction	Li et al 2001a
Anti-tumour, anti-proliferative and anti-metastatic	Glucan	Yamada et al 1984
	Protein-bound-polysaccharide	Ohmori et al 1986
	Ergosterol peroxide	Bok et al 1999
	Galactosaminoglycan	Ohmori et al 1989a, b
	Exopolysaccharide	Zhang et al 2004b
	Cordycepin	Yoshikawa et al 2004
	Aqueous extract	Yoshida et al 1989
	Ethanolic extract	Xu et al 1992
Immunomodulatory	Aqueous methanol and methanol extracts	Went et al 2002
	Hot-water extract	Yu et al 2003
	Ergosterol peroxide	Kuo et al 2003
	Exopolysaccharide	Zhang et al 2004b
Steroidogenic	Polysaccharide	Huang et al 2001b
Hypoglycaemic	Polysaccharide	Kiho et al 1990
Hypolipidaemic	Polysaccharide	Kiho et al 1996
Anti-thrombotic	Water extract	Yamaguchi et al 2001a
Hepatic effects	Alcoholic extract	Zhao et al 1991
Renal effects	Hot-water extract	Nan et al 2001
Anti-lupus	HI-A	Lin et al 1999
		Yang et al 1990
Insecticidal	Cordycepin	Kim et al 2002
Antimalarial	Cordypyridones A and B	Isaka et al 2001b
Antibacterial	Cordycepin	Ahn et al 2000
Antifungal	Ophiocordin	Kneifel et al 1977
DNAse	DNAse	Ye et al 2004

iv) Immunomodulatory activity

Yarsagumba extract enhances the antibody response as judged by plaque-forming cells against T-dependent and T-independent antigens, like ovine red blood cells and bacterial lipopolysaccharide, respectively. The extract also restored the phagocytic activity of macrophages in tumour-bearing mice that received cyclophosphamide several days after tumour transplantation, and lengthened the survival period (Yamaguchi *et al.* 1990).

4) Potential Yarsagumba products of scientific and economic significance

Wild Yarsagumba is rare and expensive in the local market. Many researchers have extensively examined its life cycle with the aim of developing techniques to grow Yarsagumba in culture and manufacture large quality by fermentation technology (Yin and Tang 1995). The mycelial fermentation products have been demonstrated to have a stronger pharmacological efficacy than the natural Yarsagumba. At present, both natural and cultured Yarsagumba products are commonly sold as health food products in South East Asia. By using different assay methods, the water extracts of natural Yarsagumba and cultured mycelia were tested for their antioxidative activities.

On the average, Yarsagumba contains 25% protein, 8.4% fat, 18.5% fiber, 29% carbohydrate and 4.1% ash (Tsim and Li 2005). In addition, it contains uracil, adenine, adenosine, trehalose, mannitol, ergosterol and stearic acid. Indeed, Yarsagumba contains a high concentration of adenosine, guanosine and uridine. Amongst the nucleosides, adenosine is deemed to play a key role in many pharmacological effects, which include the widespread effects on coronary and cerebral circulation, prevention of cardiac arrhythmias, and effects on functions in nervous system e.g. the inhibition of neurotransmitter release and the modulation of adenylate cyclase activity (Tsim and Li 2005). As mentioned earlier, fresh natural Yarsagumba contains very little nucleoside, as compared with dry and processed ones, and more interestingly cultured mycelium contains high levels of nucleosides (Tsim and Li 2005).

Wild Yarsagumba has been used in traditional Chinese medicine for centuries, mainly as a tonic to invigorate the lungs and nourish the kidneys (Wu *et al*. 2015). Several components of wild Yarsagumba have been found to be related to its various pharmacological activities; of these, cordycepin (3′-deoxyadenosine) and polysaccharides are specific components (Shashidhar *et al*. 2013). Nucleosides are believed to be one of the key active components of wild Yarsagumba (Li *et al*. 2001b). Cordycepin contributes to the anti-tumor, insecticidal, and antibacterial activity of Yarsagumba and is used as a marker for quality control (Li *et al*. 2001b, Ng and Wang 2005). Nepal is projected to be one of the global leaders in cordycepin production due to availability of natural resources (Fig. 3). Polysaccharides are one of the most abundant components of the fungus and a major group of bioactive constituents; they have been extracted and isolated from the fruiting bodies, cultured mycelium, and fermentation broth, and are structurally diverse biomacromolecules with varying physiochemical properties. They contribute to the anti-inflammatory, antioxidant, antitumor, anti-metastatic, immunomodulatory, antidiabetic, steroidogenic and hypolipidemic effects of Yarsagumba (Nie *et al*. 2013), and they have been the target of the development and quality control of Yarsagumba-based health products (Nie *et al*. 2013). SOD is an

important active protein that contributes to the antioxidant activity of Yarsagumba. DNase is another enzyme that has been identified in Yarsagumba.

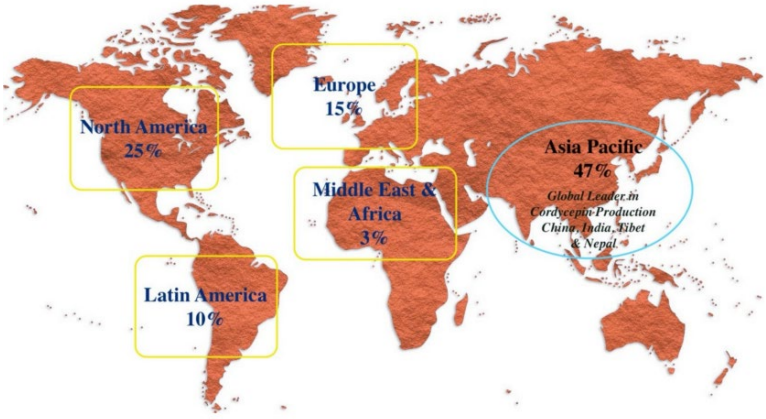

Fig. 3. Nepal is shown to be one of the global leaders for production and extraction of cordycepin (Ashraf *et al*. 2020).

Chapter 5. Evaluation of potential for production of finished Yarsagumba products in Nepal, and actions needed for utilization of this potential

1) Collection of wild Yarsagumba

Yarsagumba is distributed in the alpine grasslands of the Himalayan range throughout the northern region of Nepal (थापा तथा क्षेत्री, २०६८) (Fig. 1). However, it is mostly collected in western districts of Nepal such as Dolpa, Jumla, Humla, Mugu, Manang, Darchula, Bajhang etc. (Uprety *et al*. 2016). There is also a perception that Yarsagumba of east Nepal is not as good quality as that of west Nepal, causing no conflict among the collectors in east Nepal (Byers *et al*. 2020). Yarsagumba is also an important herb in Kailash Sacred Landscape, the largest portion of which is covered by four districts of west Nepal (Humla, Bajhang, Baitadi and Darchula), and the rest by the adjoining regions of India and China (Pandey and Pokhrel 2020). The trade of Yarsagumba is trans-boundary among the adjoining countries, the major market being China (Wallrapp *et al*. 2019, Pandey and Pokhrel 2020). Most of the larger, more expensive specimens are sold in eastern China and abroad, where discretionary income is greater (Winkler 2008).

Fig. 1. Distribution of Yarsagumba in Nepal (थापा तथा क्षेत्री, २०६८).

Due to heavy collection, there are reports of scarcity of Yarsagumba in Manang district (Koirala *et al*. 2017). Also, there are limited health care resources for the collectors who face poor health causing the risk of illness, injury and death (Koirala *et al*. 2017). Local actions for sustainable harvest of Yarsagumba have not been much success in Nepal whereas locally focused resource management has played a key role for the sustainability of Yarsagumba collection in Bhutan (Adkhikari 2017, Pouliot *et al*. 2018). Childs and Choedup (2014) have, however, shown some sustainable utilization of communal resources in Nepal

including Yarsagumba in northern Gorkha. Lack of transportation facilities, health facilities, fuel wood and not getting appropriate price of Yarsagumba etc. are some of the major problems faced by collectors in west Nepal (Budha 2017). Government interventions, improper implementation of rules and regulation, huge royalties, unfavorable weather and climate, conflicts, improper pricing system, inadequate awareness about market and prices along with price variation are major problems of Yarsagumba collection in Nepal (Karki *et al*. 2020). The incidence of conflicts among different communities has also recently increased (Pant *et al*. 2017, Pouliot *et al*. 2018). Hence, more research is needed on resource management of Yarsagumba such as access and management rules, ownership of Yarsagumba habitat, enforcement of rules, conflict resolution mechanisms etc. (Shrestha *et al*. 2019). It is important that the natural vegetation in the Yarsagumba habitat also be protected in order to conserve Yarsagumba (Sigdel *et al*. 2017).

2) Cleaning and drying of Yarsagumba

After Yarsagumba is dug and uprooted, it is gently cleaned to remove soil particles adhering to larvae as well as fruiting body with a toothbrush and later with water (थापा तथा क्षेत्री २०६८, Childs and Choedup 2014, Negi *et al*. 2020) (Figs. 2-4). Great care is taken during cleaning so that each piece of Yarsagumba is not damaged or broken and remains intact (Negi *et al*. 2020) (Fig. 4). The broken samples of Yarsagumba cannot fetch their price as they lose their morphological identity. The cleaned samples of Yarsagumba are then air-dried in shade. Incompletely dried Yarsagumba gets rotten or damaged by other molds.

Fig. 2. Picking up Yarsagumba (top left), cleaning Yarsagumba (top right), fresh Yarsagumba samples picked up from soil (bottom left) and cleaned Yarsagumba samples (bottom right) (थापा तथा क्षेत्री २०६८).

Fig. 3. Cleaning Yarsagumba in Tsum, northern Gorkha (Childs and Choedup 2014).

Fig. 4. Samples of Yarsagumba before cleaning (left) and after cleaning (right) (Negi *et al*. 2020).

3) Product and sale of wild Yarsagumba

It is a common practice to sell wild Yarsagumba in a mass in Nepal (Furger 2008) (Fig. 5), Bhutan (Cannon *et al*. 2009) (Fig. 6), India (Negi *et al*. 2020) (Fig. 7) as well as China (Boesi and Cardi 2009, Yeh and Lama 2013) (Figs. 8, 9). Sorting of Yarsagumba pieces based on color of their larvae or size of the piece is also done and graded as high, moderate or low quality (Negi *et al*. 2020) (Fig. 7). However, Yarsagumba stores are well established in China for all year round sale (थापा तथा क्षेत्री, २०६८) (Fig. 10) whereas such stores are still not easily visible in Nepal as well as in India and Bhutan. Pieces of Yarsagumba are packed in a bundle or are arranged in a circle as a product for customers in China (Holliday and Cleaver 2004, Winkler 2008) (Figs. 5, 11).

Fig. 5. Yarsagumba for trade in Dolpa (left) and China (right) (Furger 2008, Holliday and Cleaver 2004).

Fig. 6. Yarsagumba auction in Bhutan. Left, Yak herders waiting for their lot to be auctioned and right, collection of good-quality Yarsagumba ready for sale (Cannon *et al*. 2009).

Fig. 7. Collection of Yarsagumba in Uttarakhand Himalaya of India and sorting into different categories (Negi *et al*. 2020).


Fig. 8. Yarsagumba on sale in Chengdu market (left), a Tibetan lady weighing some Yarsagumba specimens (middle left), Yarsagumba traders waiting for customers in Lithang (middle right) and Kandging (right) of Tibet (Boesi and Cardi 2009).

Fig. 9. A traditional Chinese medicine store of Yarsagumba in Zhongdian (left) (Stewart 2009) and Yarsagumba advertisement in Xining (right) in China (Yeh and Lama 2013).

Fig. 10. Yarsagumba sale in a high-end Oriental medicine shop in Xining, China (थापा तथा क्षेत्री, २०६८).

Fig. 11. Yarsagumba arranged in a circle in a Tibetan market (left) and bundles in Kunming (right) (Winkler 2008).

4) Consumption of wild Yarsagumba

The main consumers of Yarsagumba are Chinese. Traditional system of consuming Yarsagumba is well described in western literature (Shrestha 2010, 2011). Accordingly, Yarsagumba is cooked with duck meat and eaten, as illustrated by Jensen (1982) and थापा तथा क्षेत्री (२०६८) (Figs. 12-14). Specially, Chinese runners have attracted attention of Yarsagumba after they were found taking Yarsagumba regularly as a part of diet during their training (Martel *et al*. 2017) (Fig. 14).

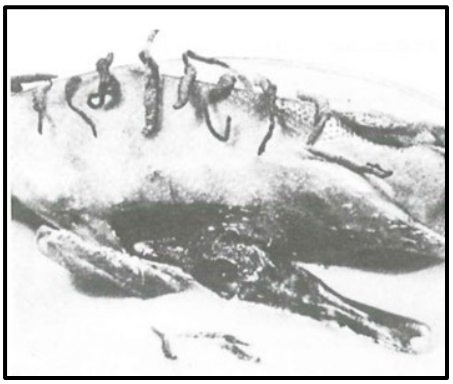

Fig. 12. Cooking Yarsagumba with duck meat in China (Jensen 1982).

Fig. 13. Cooking duck meat (left) and other items (middle) with Yarsagumba and manufacturing of capsules and tablets from Yarsagumba (right) (थापा तथा क्षेत्री, २०६८).

Fig. 14. Tibetan villagers harvesting Yarsagumba (top left), merchants inspecting Yarsagumba at a local market (top right), a soup containing Yarsagumba in a Chinese restaurant (bottom left) and a Chinese runner celebrating gold at the 1993 World Championships in Germany (bottom right) (Martel *et al*. 2017).

5) Adulteration in Yarsagumba

Yarsagumba is not free from adulteration. Due to its high price, different malpractices are conducted to cheat customers by mixing small plant materials with Yarsagumba such as roots, stems etc. As mentioned before, the broken samples of Yarsagumba fetch no value as they lose their morphological identity. Hence, it is also a practice of merchants to join the broken parts with a pin (Holliday and Cleaver 2004) (Fig. 15). Such practice should be discouraged and punished as they can result in fatal consequences.

Fig. 15. Pins inserted inside larvae of Yarsagumba (Holliday and Cleaver 2004).

6) Mycelium cultivation of Yarsagumba

Due to scarcity of wild Yarsagumba in natural habitat and its high price, a lot of research has been carried out to grow mycelium of Yarsagumba in artificially prepared media under appropriate environmental conditions (Dong and Yao 2011, Cao *et al*. 2015) (Figs. 16, 17). It has been found that the mycelium growth of Yarsagumba is too slow, just growing few cm in a period of 100 or more days (Dong and Yao 2011, Cao *et al*. 2015). Hence, great care should be taken while growing mycelium of Yarsagumba on artificial media in order to protect it from contaminating molds or other microorganisms. *Cordyceps militaris*, another insect fungus growing on lepidopteran larvae and pupae in sub-alpine or temperate region, grows much faster covering 9 cm plate within 3-4 weeks (Shrestha *et al*. 2016). For more rapid growth of Yarsagumba mycelium, liquid culture is a viable option. For this, fermentation technology using liquid medium has been developed for large scale mycelium cultivation (Martel *et al*. 2017) (Fig. 18). Continuous aeration and optimum nutritional requirements are supplied to fermentation tanks so that mycelia can continuously grow.

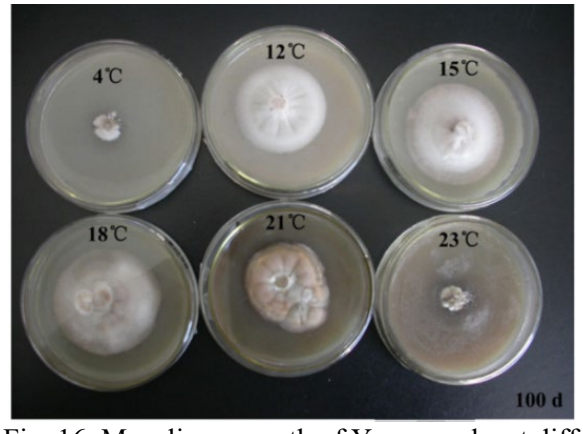

Fig. 16. Mycelium growth of Yarsagumba at different temperatures after 100 days (Dong and Yao 2011).

Fig. 17. Mycelium growth of Yarsagumba on Potato dextrose agar plate supplemented with 10% peptone for 60 days at 9-13°C and later for 6 months at 4°C (Cao *et al*. 2015).

Fig. 18. Large-scale mycelium culture of Yarsagumba (Martel *et al*. 2017).

7) Fruiting body cultivation of Yarsagumba

Though mycelium culture technique was developed long before, fruiting body cultivation of Yarsagumba is relatively a recent achievement in biological science. The first report of fruiting body development was reported in rice medium by Cao *et al*. (2015) (Fig. 19). But, more recent studies have successfully reported fruiting body development of Yarsagumba on larvae (Wei *et al*. 2016, Qin *et al*. 2018, Li *et al*. 2019, Liu *et al*. 2019) (Figs. 20-23). It is still early to commercialize fruiting body production of Yarsagumba as many studies have been conducted at experimental level. Nevertheless, it has opened door for large-scale cultivation of Yarsagumba on larvae or alternative substrates as substitute for wild Yarsagumba.

Successful development of fruiting body of Yarsagumba encompasses different steps. First of all, successful isolation of pure mycelium of Yarsagumba is a prerequisite. As shown by Dong and Yao (2011) and Cao *et al*. (2015), extremely slow growth of Yarsagumba mycelium is a big hindrance. There is a high chance that other contaminating fungi grow faster and outpace the growth of Yarsagumba mycelium. Hence, the

environment of the working place should be very sterile and no other activities should be carried out nearby the place.

Growth of Yarsagumba mycelium inside the larvae is the next step. During this stage, larvae can decay easily due to the presence of other microorganisms in the growing area. Hence, the substrate should be completely sterilized. Besides this, environmental conditions such as temperature, light, humidity etc should be optimized in order to induce fruiting body from larvae.

Fig. 19. Fruiting body development of Yarsagumba on rice medium (Cao *et al*. 2015).

Fig. 20. Industrial cultivation of Yarsagumba and its product. Young stage of Yarsagumba (left), fruiting bodies of cultivated Yarsagumba (middle left), larva filled with compact, hard mycelia of Yarsagumba (middle right) and capsules made from cultivated mycelia of Yarsagumba (right) (Wei *et al*. 2016).

Fig 21. Fruiting bodies of Yarsagumba growing out from base material (left) and samples of uprooted Yarsagumba (right) (Qin *et al*. 2018).

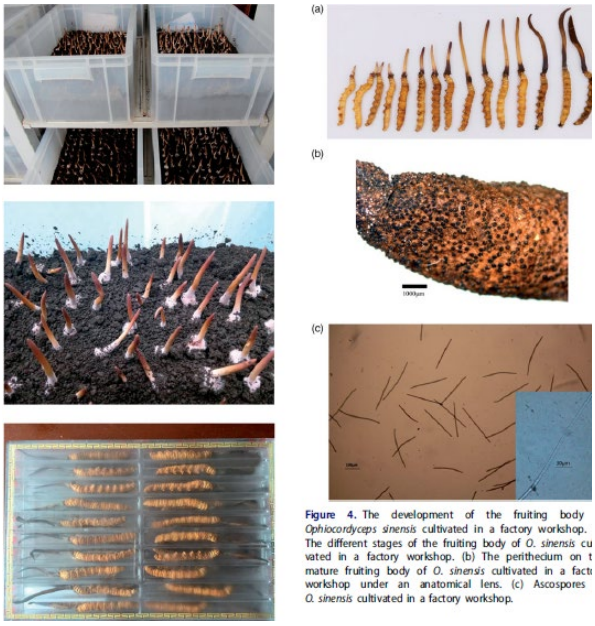

Figure 4. The development of the fruiting body *Ophiocordyceps sinensis* cultivated in a factory workshop. The different stages of the fruiting body of *O. sinensis* cu vated in a factory workshop. (b) The perithecium on t mature fruiting body of *O. sinensis* cultivated in a fact workshop under an anatomical lens. (c) Ascospores *O. sinensis* cultivated in a factory workshop.

Fig. 22. Development of the fruiting body of Yarsagumba on larvae (Li *et al*. 2019).

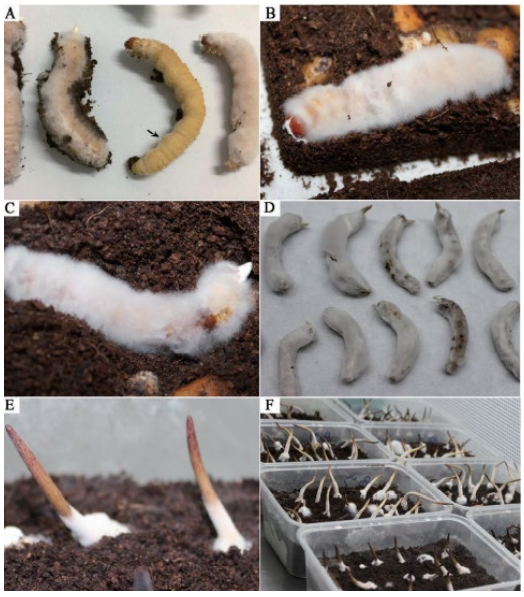

Fig. 23. Stromatal induction from the mummified larvae after Yarsagumba infection (Liu *et al*. 2019).

8) Actions for industrial production of Yarsagumba and product development in Nepal

Shrestha (2013, 2015) has urged for timely action by Nepal to gain benefit from sustainable collection of Yarsagumba as well as its cultivated products utilizing recent technologies. Similarly, श्रेष्ठ (२०६७ क,ख, २०६९ क,ख,ग,घ, २०७० क,ख, २०७१) has also pointed out to formulate necessary policy for development of appropriate technologies to cultivate Yarsagumba and other *Cordyceps* mushrooms.

As Nepal has been exporting wild Yarsagumba, it can formulate policies to improve marketing product of wild Yarsagumba in a bundle or in a packet with a particular trade name. This can be done through Govt-registered small scale industries, based on rural products. The government should facilitate and promote such industries so that locally harvested products can be conserved at community level.

China has developed cultivation technologies of Yarsagumba. Other neighboring countries India and Bhutan have also initiated research on technological innovation for cultivation of Yarsagumba. Nepal has not yet initiated research on cultivation of Yarsagumba. University or research institutes in Nepal should lead in this field. Cordyceps militaris is another commercially cultivated insect mushroom (Fig. 24).

Chapter 6. Literature survey on current status of cultivation techniques of Yarsagumba and *Cordyceps* fungi

1) Introduction

Among *Cordyceps* species, Yarsagumba is the most popular (Adhikari 2008, Shrestha et al. 2010). It is collected in large quantities from alpine regions of Nepal due to its high price. Another species that is popularly used as a medicinal mushroom in East Asia is *Cordyceps militaris* (Shrestha et al. 2012, Cui 2015). Techniques of spore isolation, mycelium growth and mating system have been developed (Shrestha et al. 2004, 2006). Short description of *Cordyceps* is given below, followed by current mycological techniques to grow in culture.

2) Morphological features of *Cordyceps*

In nature, *Cordyceps* infects insect and produces fruiting bodies after the death of its host (Kobayasi 1941). The upper part of *Cordyceps* is the fungal part called stroma and the lower part is the insect (Figs. 1, 2). Spores are released from apical part of stroma during summer and infect insects by penetrating into the host's cuticle, germinating inside the host body and ultimately killing the host. The fungus sprouts from the host body the next spring and develops its full fruiting bodies in summer (Figs. 1, 2).

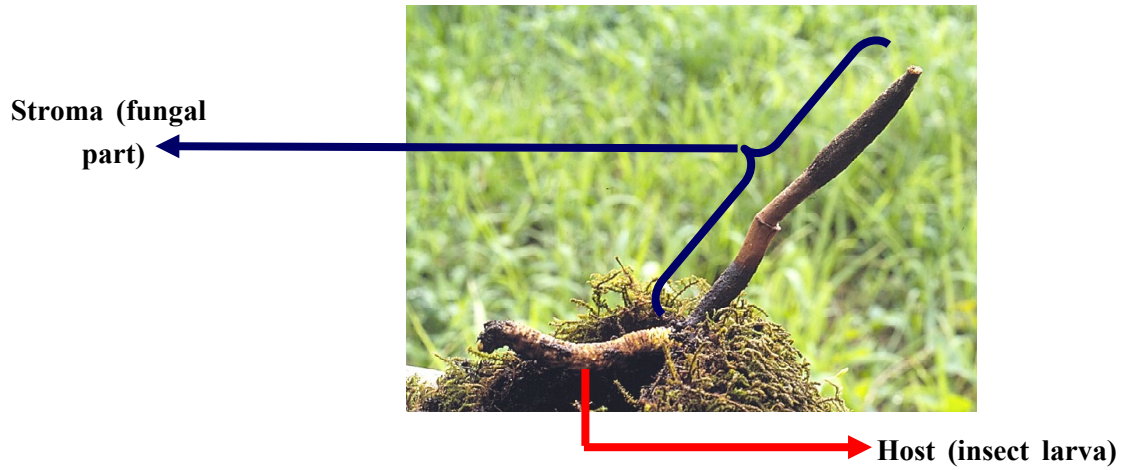

Fig. 1. Morphological features of Yarsagumba (Shrestha and Sung 2005).

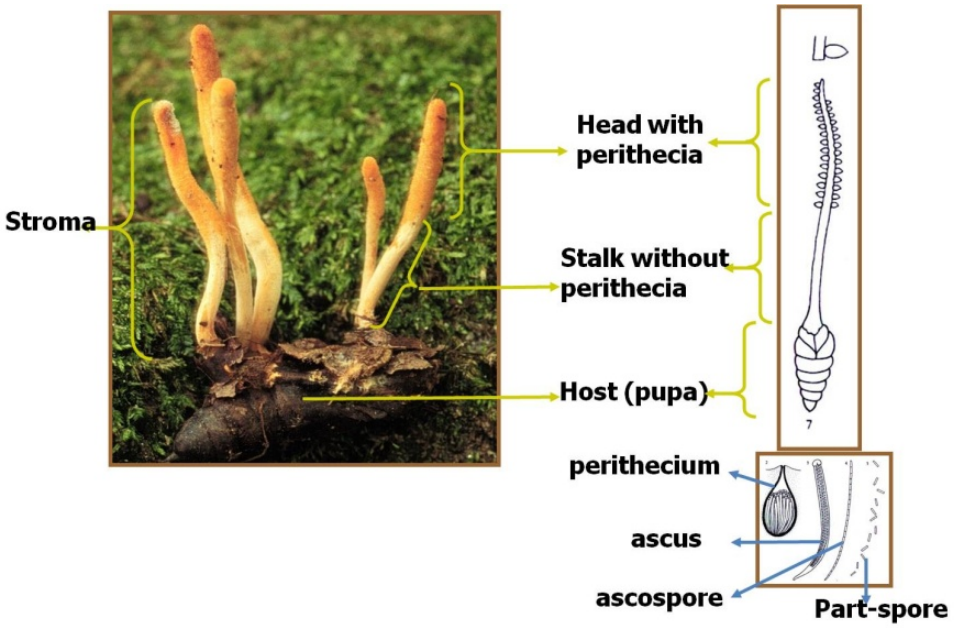

Fig. 2. Morphological features of *Cordyceps militaris* (Kobayasi 1941, Sung 1996).

3) Isolation of spores from mature fruiting bodies of *Cordyceps*

After collection of mature specimens of *Cordyceps*, stromata are cleaned and fixed on the inside of the lid of Petri dish. It is then placed over water agar (WA) medium and left overnight for discharge of spores (Fig. 3). The discharged spores are picked up with care using dissecting microscope and transferred to nutrient medium. Various types of mycological media are used to grow *Cordyceps* mycelia under *in vitro* condition (Shrestha et al. 2006).

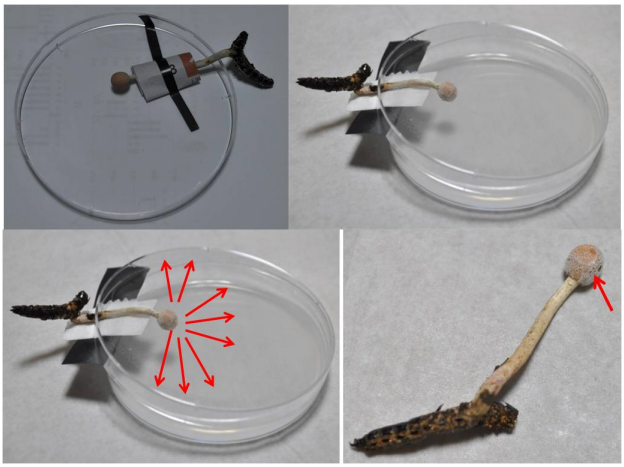

Fig. 3. Stroma of *Cordyceps* fixed on the inside of Petri dish lid (upper left), placed over WA (upper right), left for spore discharge (bottom left) and discharged cotton-like spores on head of stroma (bottom right) (Shrestha 2019).

4) *In vitro* fruiting body production of *Cordyceps*

Small mycelial discs are punched and inoculated in liquid medium and incubated at 25°C (Fig. 4). During early phase, small mycelial pellets are formed which later develop into thick pellets and cover the liquid surface (Fig. 4). Brown rice medium is prepared in plastic bottle by mixing brown rice with water and is inoculated with liquid culture of *Cordyceps* (Fig. 4). It is then incubated at 25°C for 10 to 14 days and then at 20±1°C till harvest, which lasts further about 30~40 days (Fig. 5).

Fig. 4. Agar culture (upper left), early liquid culture (bottom-left) and late liquid culture (bottom-middle) of *Cordyceps militaris*. Brown rice medium in plastic bottle (right) (Shrestha 2019).

Fig. 5. Large scale cultivation of *Cordyceps* (left) and harvest of fruiting bodies (right) (Shrestha 2019).

5) Recent development in Yarsagumba culture and cultivation

After the development of *in vitro* growth techniques of Cordyceps, many studies have been carried out especially in China to grow Yarsagumba mycelia in lab in small and then in large fermenters. The techniques for *in vitro* cultivation of Yarsagumba are recently described (Li et al. 2019, Liu et al. 2019; Fig. 6). Researchers and entrepreneurs are taking great interest in commercial cultivation of Yarsagumba and

C. militaris due to their beneficial bioactivities. Since Nepal is rich in *Cordyceps* diversity (Adhikari 2008, Shrestha 2011, 2013, Shrestha and Sung 2005), joint academic and entrepreneurship efforts can make their best utilization for the benefit of human health and economic uplift of the country.

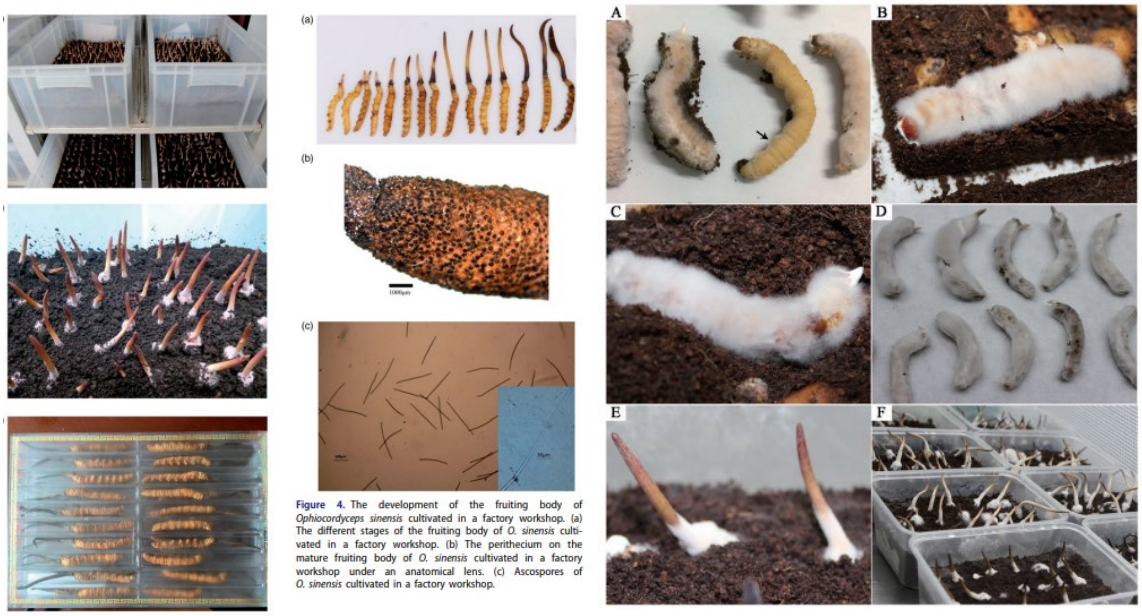

Figure 4. The development of the fruiting body of *Ophiocordyceps sinensis* cultivated in a factory workshop. (a) The different stages of the fruiting body of *O. sinensis* cultivated in a factory workshop. (b) The perithecium on the mature fruiting body of *O. sinensis* cultivated in a factory workshop under an anatomical lens. (c) Ascospores of *O. sinensis* cultivated in a factory workshop.

Fig. 6. Cultivation of Yarsagumba on moth larvae under optimized indoor environment (left, Li *et al*. 2019 and right Liu *et al*. 2019).

Chapter 7. Formulation of research projects for development of identified Yarsagumba products, including technology development, proto-type development, patenting, production and marketing

1) Introduction

Around 2,467 fungal species have been listed in Nepalese fungal flora (Adhikari 2018, Khadka and Aryal 2020). Among them, 1,271 species are described as macrofungi, that means, some of them overgrow in the form of mushrooms on soil or trees (Adhikari 2014). Among mushroom species found in Nepal, Christensen et al. (2008) have recorded 228 species as edible and Adhikari (2014) has recorded 100 species as poisonous and 89 as medicinal. Yarsagumba is one of the most valued medicinal mushrooms of Nepal (Adhikari 2014, Shrestha and Sung 2005, Shrestha et al. 2010), naturally distributed in alpine regions of northern Nepal.

2) Research components and products based on Yarsagumba

Following are some of the research components and products based on Yarsagumba.

i) Collection of fresh samples of Yarsagumba in alpine grasslands of west Nepal

West Nepal (Dolpa, Jumla, Humla, Mugu, Manang, Darchula, Bajhang etc) is a potential area for Yarsagumba (Uprety *et al*. 2016) and most of the collections and exports are done from this part of the country. Fresh samples of Yarsagumba can be collected in high altitudes of west Nepal to initiate *in vitro* culture and cultivation of Yaragumba in controlled-lab condition.

ii) Isolation of spores from Yarsagumba in nutrient medium in field and lab

Before collection trips are made, agar plates are prepared and taken to the field. Fungal isolates should be prepared in the field or nearby area soon after collection using spore discharge method. During spore discharge and further growth in culture, there is a high chance that other surrounding microorganisms grow and overcome the growth of Yarsagumba. Thus, pure cultures of Yarsagumba should be developed without interference of other microorganisms.

iii) Development of pure culture of Yarsagumba in controlled temperature and light conditions

Development of pure, uncontaminated culture of Yarsagumba is the prime step for further work. After the discharged ascospores are collected in an agar plate, they are transferred to fresh, nutrient media and incubated at optimum temperature for further growth. This is also termed as establishment of culture of Yarsagumba.

iv) Multiplication of culture of Yarsagumba in lab-scale fermenter

Solid state (agar) culture is done at first to initiate and develop cultures of Yarsagumba. After cultures are prepared and confirmed through observations of primary growth characteristics and microscopic structures, they are transferred to liquid state culture to enhance growth rapidly. For lab-scale cultures, small conical flasks up to 2000 ml volume can be used. For bigger volume, fermenters with continuous air supply can be used.

v) Production of dry Yarsagumba powder from lab-scale fermenter

Mycelium can be filtered from liquid culture and dried. This is the safest method of mycelium production. However, great care should be taken that the powders are pure and not contaminated by other microorganisms and molds.

vi) Study on secondary metabolites and bioactive compounds from Yarsagumba mycelium and culture filtrate

Mycelium as well as culture filtrates are sources of many bioactive compounds and secondary metabolites. The nutritional sources and culture environments can be tested in order to study their effects on bioactive compounds. Carbon and nitrogen sources are the main components of nutrition and their ratio play an important role in growth and bioactivity of Yarsagumba mycelium. Similarly, high and low temperature and light and dark conditions are prime environmental factors that affect mycelium growth and bioactivity of Yarsagumba.

vii) Mycelium culture of Yarsagumba in solid-state medium such as rice, wheat, barley etc. and production of health foods

Cereal grains are rich sources of nutrition for mushroom growth. The grains can be inoculated with liquid inoculum of Yarsagumba and incubated at optimum temperature. This will induce mycelium growth of Yarsagumba on the surface of grains. The grains can be dried and consumed in various ways. Such types of products are commonly known as Yarsagumba rice, Yarsagumba wheat etc, which are categorized as health foods. The dried products can be stored for a longer period and transported easily. श्रेष्ठ (२०७०ख) has shed some light on development of Yarsagumba rice as an exportable product from Nepal to rich, rice-consuming countries.

viii) Survey of other Yarsagumba-like *Cordyceps* mushrooms in Nepal and testing their lab-based growth for alternative sources of bio-active compounds

Besides Yarsagumba, many *Cordyceps* mushrooms are found all over the world including Nepal. Specially, east and southeast Asian countries have already done a lot of research on *Cordyceps* mushrooms and their

cultivation practices. *Cordyceps militaris* is one of such potential mushrooms (Shrestha et al. 2012). Major bioactive compounds such as adenosine and adenosine-derivatives, polysaccharides, cordycepic acid, ergosterols etc are commonly found in Yarsagumba and other *Cordyceps* mushrooms.

ix) Patenting of cultivation techniques of Yarsagumba and *Cordyceps* mushrooms.

Successful cultivation techniques of Yarsagumba and other *Cordyceps* mushrooms can be developed and patented. Because Yarsagumba cultures are extremely slow in growth in culture, there are prospects of doing research on how to achieve rapid growth for its successful cultivation.

x) Training to the local communities of highlands of Nepal to promote Yarsagumba trade in domestic and international markets as well as to conserve Yarsagumba

Collection and trade of wild Yarsagumba is a great late spring and early summer activity in northern regions of Nepal. Thousands of people are involved in collection of Yarsagumba as their seasonal high-earning occupation. However, continuous collection without preserving the natural habitat of Yarsagumba will diminish the amount that can be collected every year. Hence, local people and local authorities should be made aware of the impact of excessive collection on survival of Yarsagumba. Trainings and group discussions should be conducted in coordination with local bodies and communities.

3) Research components for improvement of mushroom cultivation in Nepal

Besides Yarsagumba and *Cordyceps* mushrooms, research projects on following areas related to commercial mushroom cultivation in Nepal such as oyster mushroom, white button mushroom, shitake mushroom and others can be developed

- Spawn improvement using tissue isolation and spore isolation techniques
- Increasing mushroom productivity and bioconversion rate using different types of substrates
- Strain preservation of commercial varieties of mushrooms
- Identification of pests and diseases in mushroom cultivation and their control
- Introduction and popularization of other new mushroom varieties such as King oyster or trumpet mushroom, morel etc.
- Development of cold storage, drying, powdering and canning technology for mushroom
- Product development from mushroom such as soup, achar, nutraceuticals etc.
- Substrate improvement using straw, sawdust, agro-wastes etc.
- Mechanical improvement for substrate pasteurization (efficient boiler) and mushroom balling etc.

- Re-use of spent substrates with fresh raw material
- Development of biofertilizer or compost for crops using spent substrates
- Use of AI for early detection of contamination, optimum harvesting timing etc.
- Database of commercial mushroom strains cultivated in Nepal
- To strengthen microbial culture collection of Nepal

4) Requirements for implementation of research projects

A mushroom research facility with several compartments is a prerequisite. Several facilities for medium preparation and washing, sterilization of medium and instruments, inoculation in solid or liquid substrate, incubation at high and low temperatures and humidity levels, harvesting, cold storage, drying etc should be constructed. A list of equipment, glassware and chemicals necessary for mushroom research facility are shown in Table 1, which can be modified according to the needs of individual lab or production facility.

Table 1. List of equipment, glass wares and chemicals for mushroom research facility

Equipments/Instruments

1. Biosafety cabinet/Laminar air flow cabinet
2. Stereo/dissection microscope + illuminator with imaging system
3. Compound microscope w/wo imaging system
4. Autoclaves of different sizes such as portable small cooker-type, automatic vertical type (100-150 l) or fixed vertical or horizontal type
5. Lab refrigerator
6. BOD incubator with illumination (LED light) (150 l - 250 l) and removable trays
7. Microbiological incubator (with trays)
8. Deep freeze (-20C/-80C)
9. Hot air oven
10. Water distillation double unit/water filter system
11. Shaking incubator
12. Precision balance (100 g/0.001 g)
13. Trolley (multiple-storey)
14. pH meter
15. Digital balance or digital weighing scale: 30 kg/1 gm
16. Magnetic stirrer with hot plates
17. Hot water bath
18. Vortex
19. Humidifier

Glasswares/plasticwares

20. Conical flasks

21. Beakers
22. Funnel
23. Measuring cylinders
24. Polypropylene (PP) bottle, glass bottles and PP bags
25. Test tubes
26. Test tube racks
27. Petri dish
28. Disposable Petri dish
29. Falcon tubes

Other lab items

30. Camera SLR
31. Parafilm tape/plastic wrap
32. Digital thermo/hygrometer with probe
33. Stainless steel forceps with pointed tips/tweezers
34. Syringe with needle
35. Nichrome loops holder/inoculation needle
36. Micropipette (100-1000 µl)
37. Micropipette tips (100-1000µl)
38. Pipettes with rubber bulb
39. Cryotubes (1 ml)
40. Scalpel
41. Lab spatula
42. Aluminium foil
43. Rubber hand gloves
44. Water spray bottles
45. Plastic sqeeze bottles, wash bottles
46. LPG gas cylinder and Bunsen burner
47. Spirit lamp
48. Bottle brush
49. Cotton (absorbent and non-absorbent)
50. Tissue paper
51. Face mask and laboratory safety cap/disposable head covers/bouffant head cap
52. Lab coat
53. Knives and scissors
54. Chopping board
55. Laboratory glassware drying rack

Chemicals

56. Ethanol
57. Glycerol
58. Formalin
59. Sterile Mineral oil (100 ml)
60. Medium components such as PD, PDA, yeast extract, peptone, dextrose, agar, agarose

61. Mineral salts

5) Proto-type development of Yarsagumba products

i) Yarsagumba powder

Liquid fermentation culture supports growth of mycelium, which, afterwards, can be filtered and dried to powder form. The pure, powdered form of Yarsagumba functions as the first raw product from which value-added products can be prepared. The powders can also be added to herbal tea to take it as a Yarsagumba tea or a herbal drink.

ii) Yarsagumba rice

Brown rice covered by growing mycelium of Yarsagumba can be dried and labeled as Yarsagumba rice. It can be added in little amount to normal rice during cooking that can give taste and flavor of Yarsagumba. Rice-consuming societies like ours can benefit from this product when produced in large quantity.

iii) Yarsagumba cultivation

Yarsagumba cultivation is an area of intensive, time-consuming research. Though Chinese scientists have developed Yarsagumba fruiting bodies in controlled, indoor environment, it has not yet been cultivated in large scale. Once Yarsagumba cultivation is achieved in MBUST lab facility, it can be multiplied to a greater scale by maintaining optimum conditions in sterile environment.

Chapter 8: Development of national and international networking and communication for Yarsagumba research and development as well as promotion of Yarsagumba technology and products

1) Introduction

For research and development in any field, many institutions at national and international levels are directly or indirectly involved. In similar way, for research and development on Yarsagumba and other commercially cultivated mushrooms, many government and private institutions at national and international levels are involved and networking with them will be very important and fruitful. Here, different institutions are listed below with their brief introduction and role that are related with research and development of Yarsagumba and other mushrooms as well as for promotion of Yarsagumba technology and products.

2) National network

Many government institutions are involved for conservation and sustainable harvesting of Yarsagumba in alpine grasslands of northern Nepal. No research is continuously going on at present in Nepal for Yarsagumba or *Cordyceps* mushrooms although some occasional researches were carried out in the past. Some governmental institutions are listed below that are associated with conservation as well as research and development of Yarsagumba.

i) Department of National Parks and Wildlife Conservation (DNPWC), Babar Mahal, Kathmandu

http://www.dnpwc.gov.np/en/

Department of National Parks and Wildlife Conservation (DNPWC) was established in 1980 to conserve and manage wildlife and biodiversity of the country. It manages a network of Protected Areas under 12 National Parks, 1 Wildlife Reserve, 1 Hunting Reserve, 6 Conservation Areas and 13 Buffer Zones extending from lowland Terai to high mountains, covering 23.39 % of the total country's land, which contribute to *in-situ* conservation of ecosystems and biodiversity across the country. Conservation efforts made by the government of Nepal is worldwide popular and highly recognized by the international community.

The primary objective of the Department is to conserve the country's major representative ecosystems, unique natural and cultural heritage and give protection to the valuable and endangered wildlife species. It encourages scientific research for the conservation of genetic diversity. In achieving its objectives, the

Department implements regular programs, National Parks Management, Wildlife Reserve Management, Conservation Area Management, Hunting Reserve Management, Buffer Zone Management, Hattisar Management and Non Timber Forest Products Management that includes key activities such as habitat management, species conservation, anti-poaching operations, conservation education, ecotourism promotion in and around the Protected Areas.

Since Yarsagumba naturally grows in alpine grasslands and pastures, most of the lands lie within the boundary of protected areas managed by National Parks or wildlife reserves under DNPWC. Hence, a network with DNPWC will be helpful for research on conservation and sustainable harvest of Yarsagumba in high mountains and alpine regions of the country.

ii) Department of Forests and Soil Conservation, Babar Mahal, Kathmandu
http://www.dofsc.gov.np/

Yarsagumba also grows naturally in alpine grasslands that are near to or outside of the protected areas. Those areas are protected either under the supervision of district offices of Department of Forests or by community forests registered at district forest offices of the Department for preservation, utilization and management of forest resources of the country. Nepal is worldwide known as an example for successfully managing community forests. The Department has a network of forest offices spreading all over seventy seven districts of the country. Hence, any work for sustainable utilization of forest resources including Yarsagumba and Cordyceps mushrooms involves collaboration and coordination with Department of Forests.

iii) National Trust for Nature Conservation (NTNC), Khumaltar, Lalitpur https://ntnc.org.np/

The National Trust for Nature Conservation (NTNC), established in 1982, is an organization working in the field of nature conservation. NTNC's mission is to conserve nature and natural resources in Nepal while meeting the needs of the people in sustainable way. The Trust activities have spread from the sub-tropical plains of Chitwan, Bardia and Kanchanpur to the Annapurna and Manaslu region of the high Himalayas, including Upper Mustang and Manang. The Annapurna Conservation Area Project (ACAP), the Manaslu Conservation Area Project (MCAP) and Gaurishankar Conservation Area Project (GCAP) are some of the protected areas that harbor Yarsagumba and other medicinal mushrooms. Any research work on Yarsagumba can be carried out in collaboration with NTNC, especially in high altitude areas managed by it.

iv) Nepal Agricultural Research Council (NARC), Singh Durbar Plaza, Kathmandu
https://narc.gov.np/

Nepal Agricultural Research Council (NARC) was established in 1991 as an autonomous organization to conduct agricultural research in the country to uplift the economic level of the people. NARC is the central institute of Nepal Government for research on agriculture and livestock in Nepal ranging from lowland to the high mountain regions. Its central research centers are located in Khumaltar, Lalitpur that are directly or indirectly involved with conservation and utilization of Yarsagumba.

a) National Plant Pathology Research Center https://narc.gov.np/plant-pathology/

This research center is the main Government agency to carry out research on cultivation and production of edible and medicinal mushrooms in Nepal. Historically, it was the institute that developed suitable protocols for household mushroom cultivation. It also introduced new varieties of mushrooms to Nepal from abroad, mostly from Japan. Mushroom research facilities developed at this center and the current on-going researches there in the center will be helpful to formulate plan for research and development on Yarsagumba and other commercial mushroom varieties in Nepal.

Problems of pests and diseases are also emerging in mushroom farms in Nepal as the growers are increasing. Hence, timely identification of pests and diseases and their control is very important before the huge losses are incurred. This center has played a vital role in control of diseases of crop plants as well as mushrooms and this is the area where collaborative research work is highly solicited.

b) National Entomology Research Center https://narc.gov.np/entomology/

Since Yarsagumba and other *Cordyceps* mushrooms grow on insect larvae and pupae as well as adult insects, entomological knowledge of this research center will be very helpful not only for identification of insects but also for rearing of insects for commercial purpose. Some insect larvae and pupae can be infected with Yarsagumba or *Cordyceps* cultures and incubated for production of mushrooms. Hence, rearing of insects should be carried out in indoor environment and this can be done based on technical knowledge and experience of this center.

c) National Soil Science Research Center https://narc.gov.np/soil-science/

For any research on organic agriculture or natural habitat of Yarsagumba and *Cordyceps* mushrooms, essential soil testing is a routing work. Improvement of soil characteristics without use of chemical fertilizers and pesticides is a must for organic agriculture. Similarly, improvement of forest soil with no or limited interference of human activities should be given priority for natural growth of Yarsagumba and *Cordyceps* mushrooms. Soil testing protocols developed by this center will be practically very important to test soil health of Yarsagumba habitat as well as maintaining soil health for organic agriculture.

d) National Agricultural Engineering Research Center https://narc.gov.np/agri-engineering/

For Yarsagumba and especially commercially cultivated mushrooms, many mechanical modifications of the equipment are deemed necessary. For instance, the steamer used to pasteurize mushroom substrate uses firewood individually as a source of fuel. Recently, bigger size steamers such as 1000 or 2000 liters are used instead of small ones in order to efficiently use firewood. More recently, steamers are connected to a common boiler that supplies steam to all the steamers connected to it, using the firewood more efficiently. However, no electricity is used as source of energy for large scale steamers and instead firewood or gas is the main source of energy for the steamers in Nepal. Hence, electrified system of boiler or steamer needs to be developed.

For mushroom cultivation, substrates are packed in plastic bags manually. That costs lot of time and human efforts. Baller machines are available that can make plastic bags with substrates and spawn uniformly and those machines are imported from either India or China. Nepali engineers and technicians are also engaged in developing prototypes of baller machines and should be encouraged.

As the mushroom production is increasing in Nepal, there is a dire need of cold storage as well as dryer to store and dry surplus production of mushrooms. Vehicles with cold storage facility are also essential to transport fresh mushrooms from farms to the markets or customers' areas. Besides, the fresh mushroom should be stored at low temperature to increase its shelf life. Similarly, dryers for agro-products should be a part of production for big producers. In Nepal, solar dryers are commonly used in many sunny areas of rural parts. Hence, this center is very important for development and innovation of agricultural machineries and equipment that are directly related to Yarsagumba and mushroom production and processing.

e) National Food Research Center https://narc.gov.np/food-research-division/

This center is involved in development of post-harvest technology and value-added processing of agriculture produces to uplift the socio-economic condition of the country. There are problems of post-harvest losses during storage, transportation, and packaging of highly perishable items such as mushrooms etc. The center is responsible for formulating the strategies to minimize post-harvest losses by modifying or manipulating existing handling processes and innovating or introducing new technologies. Future collaborative works can be done with this center to develop appropriate technologies to reduce post-harvest loss of mushrooms and to process and produce value-added products from mushrooms.

v) Nepal Academy of Science and Technology (NAST), Khumaltar, Lalitpur
https://nast.gov.np/biological-resource

Nepal Academy of Science and Technology (NAST) is an autonomous apex body established in 1982 to promote science and technology in the country for the upliftment of socio-economic condition of the country. The Academy is entrusted with four major objectives: advancement of science and technology for all-round development of the nation, preservation and further modernization of indigenous technologies, promotion of research in science and technology and identification and facilitation of appropriate technology transfer.

Biological Resource Unit under the Faculty of Science of NAST is actively engaged in mushroom research and development. It has also made available its facilities and space to research students, mainly Master's degree students of related subjects such as microbiology, biotechnology etc for research on mushrooms. Its experience on science and technology promotion in Nepal is vital for all the sectors of the country.

vi) Department of Food Technology and Quality Control (DFTQC), Babar Mahal, Kathmandu
http://www.dftqc.gov.np/

Department of Food Technology and Quality Control (DFTQC) is one of the three departments under the Ministry of Agriculture and Livestoks Development of Government of Nepal. It was established in 1961 as Department of Food in Singha Durbar. The Department was later renamed as Food Research Laboratory which was again converted as Central Food Research Laboratory (CFRL) and was known by this name until it became DFTQC in 2000.

From the very beginning of its establishment, DFTQC has played a pioneer role to lay down foundation stone for food quality control system, research and development in the field of food science and technology and nutrition support program. It is the apex organization responsible for the enforcement of Food Act and Regulations. It has been working for the implementation of Feed Act and Regulations as well. The main aim is to ensure and enhance the quality and safety of food and feed products in the country. Further, the department has a paramount role in augmenting appropriate food processing and post-harvest techniques to promote agribusinesses. Similarly, the department has been implementing various food and nutrition activities for the reduction of various forms and types of malnutrition in the country.

Any agro or forest-based edible products should be tested and verified by DFTQC for commercial production that does not pose harm to human body. When Yarsagumba or *Cordyceps* mushrooms are cultivated in controlled environment using appropriate substrates, its edibility should be tested and proven by DFTQC. For this, all the steps necessary for cultivation of Yarsagumba should be clearly submitted for verification by DFTQC.

vii) Department of Cottage and Small Industries, Tripureshor, Kathmandu http://dcsi.gov.np/en

Department of Cottage and Small Industries (DCSI) was constituted under Ministry of Industry, Commerce and Supplies (MOICS) in 1974 to promote and foster various kinds of cottage and small industries enhancing their industrial productivity increment along with making congenial environment for industrial investment in accordance with policy execution for contributing national economy.

viii) Department of Industry, Tripureshor, Kathmandu http://www.doind.gov.np/

Department of Industry is one of the major agencies at the Ministry of Industry which is responsible for implementation of policy, act, rules and regulations related to the industrial development, through which Government of Nepal plans to strengthened Nepalese economy. The department administers and facilitates Middle and Large scale industries which have fixed assets of more than 100 million Nepalese rupees. Furthermore, its scope of function also includes foreign investment and technology transfer in the industry as well as protection of industrial property.

Other functions of the Department include promotion of local and foreign investment for industrial development, support for formulation of acts and laws related to industrial as well as foreign investment, approval of industry registration and providing a license to the industry which requires permission, granting permission for foreign investment, technological transfer and foreign loan, registration of foreign investment based industries, recommendation of facilities and concessions accorded to industries, approval of Initial Environment Examination (IEE) report of the industry, preparation of raw material consumption norms for the industry, monitoring environmental compliance of the industry, recommending Certificate of Origin for the products for preferential entry into India as per Nepal-India Trade Treaty etc. Patenting of new technologies is also processed and registered at the Department.

3) International network

i) National Center for Genetic Engineering and Biotechnology (BIOTEC), Bangkok, Thailand
https://www.biotec.or.th/en/index.php

It is an institute under National Science and Technology Development Agency (NSTDA) of Thai Government. It is the largest collection of fungal resources of Thailand, including *Cordyceps* mushrooms. This institute is also a leading institute in the field of utilization of fungal resources in the world. This institute successfully organized 10th International Mycological Congress in 2014, the biggest gathering of mycologists from all over the world that is organized every four year. Biotech Culture Collection (BCC) under BIOTEC is one of the largest culture collections in the world that preserves fungal isolates for industrial production of novel compounds produced by fungi as well as utilizing fungi for other purposes such as biological control of harmful insects and pests in organic agriculture.

ii) Institute of Microbiology, Chinese Academy of Sciences, Beijing, China http://english.im.cas.cn/

The Institute of Microbiology of the Chinese Academy of Sciences (IMCAS) is the largest microbiological research institution in China, founded in 1958. In the past, there were eight divisions in IMCAS that included fungal classification, bacterial classification, virology, microbial ecology, microbial metabolism, microbial enzymology, microbial genetics and microbial strain preservation. In last two decades, IMCAS has made great strides in both basic and applied research, which are represented by a large number of scientific achievements of great significance to economic and social development.

At present, the scientific research system of IMCAS comprises of five laboratories such as State Key Laboratory of Microbial Resources, State Key Laboratory of Plant Genomics, State Key Laboratory of Mycology, CAS Key Laboratory of Pathogenic Microbiology and Immunology, and CAS Key Laboratory of Microbial Physiological and Metabolic Engineering. State Key Laboratory of Mycology has carried out extensive research on Yarsagumba, its cultivation and genomics. IMCAS is also the largest fungal herbarium in Asia with nearly 500,000 specimens and the largest microbiological culture collection in China with more than 41,000 strains. Three national academic societies, i.e., Chinese Society of Microbiology (CSM), Chinese Mycology Society and Chinese Society of Biotechnology (CSBT), are currently affiliated to IMCAS. The Institute also attaches great importance to cooperation with international scientific community and industries.

iii) Japan Collection of Microorganisms, Japan https://jcm.brc.riken.jp/en/

The Microbe Division in RIKEN-Bioresource Research Center (BRC) has been collecting, preserving, and distributing cultured microbial strains as one of the leading culture collections in the world since established as Japan Collection of Microorganisms (JCM) in 1981. JCM aims to contribute to scientific communities by maintaining and serving high-quality microbial resources useful for general microbial studies and various research fields particularly in health and environmental science. JCM has participated in the National BioResource Project supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan as the core facility for “General Microbes“. JCM maintains approximately 29,000 strains, and the approximate numbers of strains available at JCM are 12,600 strains of aerobic and anaerobic bacteria including actinomycetes, 560 strains of archaea, and 6,200 strains of fungi including yeasts. Information of the available strains is opened to the public through the JCM On-line Catalogue Database. Genomic DNA samples of some strains are also distributed in cooperation with RIKEN BRC-DNA Bank. JCM is working to continuously improve the functions as a microbial resource center to exploit new microbial resources, to describe novel microbial taxa, and to develop the methods for investigating and handling extremophiles, uncultured microorganisms, and microbial communities. In order to promote the

use of the microbial strains, JCM regularly issues newsletter and gives training courses for basic and advanced microbial techniques. JCM welcomes a deposit of microbial strains published or designed to be published in scientific papers as well as an order for microbial cultures.

iv) CBS Fungal Biodiversity Center, the Netherlands

https://www.gbif.org/publisher/6a430540-7f09-11d8-a1a0-b8a03c50a862

The Dutch Centraalbureau voor Schimmelcultures (CBS) is a research facility of the Royal Netherlands Academy of Arts and Sciences, accommodating a world-renowned collection of living filamentous fungi, yeasts and bacteria. It is the oldest microbial culture collection in the world established in 1907. The CBS-KNAW culture collections of micro-organisms are carried out in a scientific environment, which guarantees state of the art quality checks and allows the development of scientific programs to improve the quality of the material entrusted to them. CBS was actively involved in setting the standards for modern long-term preservation as well as for data storage, recognizing the necessity of data exchangeability. It was also actively involved in setting up criteria and minimal demands for culture collections within the CABRI framework. In short, CBS developed from a culture collection into a Biological Resource Centre (BRC). BRCs are an essential part of the infrastructure underpinning life sciences and biotechnology. They contain collections of culturable organisms (e.g. micro-organisms, plant, animal and human cells), replicable parts of these organisms, cells and tissues, as well as databases containing molecular, physiological and structural information relevant to these collections and related bioinformatics. BRCs must meet the high standards of quality and expertise demanded by the international community of scientists and industry for the delivery of biological resources on which R&D in the life sciences and the advancement of biotechnology depends.

The collections of CBS-KNAW offer a comprehensive coverage of the culturable biodiversity of the fungal Kingdom (over 100,000 strains), while the prokaryotes are represented by unique collections of bacterial mutants, hosts suitable for DNA research, genetically engineered plasmids, broad-host-range plasmids and phages. A large staff of scientists with expertise on every systematic group represented in the collections is involved as well as a dedicated staff of technicians for maintenance and distribution of the strains, guaranteeing their quality and the maintenance of all regulations concerning biosafety.

v) India

India has recently developed infrastructures for research on Yarsagumba and other *Cordyceps* mushrooms. According to Rathi et al. (2021 https://www.researchgate.net/profile/Neelam-Chaudhary-7/publication/350193906_A_THEMATIC_APPROACH_ON_CORDYCEPS/links/6055fb89458515e834595479/A-THEMATIC-APPROACH-ON-CORDYCEPS.pdf), following institutes are currently involved in Yarsagumba research in India.

a. Omcar India training center, Gwalior, M.P.

b. ICAR-Directorate of Mushroom Research, Solan, Himachal Pradesh

c. Khan Mushroom Farm & Training Centre, Una, Himachal Pradesh

d. Best *Cordyceps militaris* culture & Training in India, Anupshahar, Uttar Pradesh

e. Dr. Harisingh Gour Vishwavidhayalaya, Sagar, M.P.

Many mushroom growers of Nepal get training on mushroom cultivation techniques in India and also rely for mother spawns.

4) Private sector

Recently, private sectors have emerged as large scale commercial producers of mushroom in Nepal. They have installed modern facilities for culture development of mushrooms, their spawn production and cultivation in controlled environment. Followings are short descriptions for private mushroom producers of Nepal

i) Mush Nepal Pvt. Ltd., Balambu, Kathmandu http://mushnepal.com/

It is one of the oldest mushroom growers in Nepal. Also, it started modern mushroom growing facilities in the country. The spawn growing lab facilities are super. It is one of few mushroom compost producers of Nepal with high-tech facilities. It is mainly concentrated in oyster and white button mushroom production ans is also involved in mushroom training.

ii) Ares Mushroom Pvt. Ltd., Chandragiri, Kathmandu https://www.facebook.com/aresmushrooms/

It is also one of the oldest mushroom producers of Nepal. It is the only commercial producer of king oyster mushroom in Nepal. Also, it has the largest sterilizing facility in Nepal that can sterilize around 4500 mushroom bags at a time. It hs good spawn production facility.

iii) Agro Business Center for Research and Development, Rabi Bhavan, Kathmandu
https://www.facebook.com/AgroBusinessCentreForResearchDevelopment/

It has an experience of more than 2 decades of mushroom cultivation. It also has rich mushroom collection experience in Nepal. It has demonstrated cultivation protocol of different varieties of mushroom and is involved in mushroom training also. It has good spawn production facility.

iv) Bansun Agro Organics Pvt. Ltd., Chitwan https://organicnepalcoop.com/bansun-agro/

It has installed one of the largest modern mushroom production facilities in Chitwan for white button mushroom production. It has also production facility for oyster mushroom and has established its own spawn production facility.

v) Nepal Plant Disease Associates Pvt. Ltd., Balaju, Kathmandu

https://www.facebook.com/Nepal-Plant-Disease-Associates-NPDA-1880236945388785/

It is one of excellent private centers that work on identification and control of fungal and bacterial diseases in agriculture including mushrooms. It is involved in formulation of biopesticides to control plant diseases and also conducts regular trainings.

vi) Mushroom Seed Nepal and Research Center, Naya Thimi, Bhaktapur

https://www.facebook.com/mushroomresearchcenter/

This research center has both research facilities and commercial mushroom farm. The center produces different mushrooms such as oyster mushroom, shiitake mushroom, Ganoderma mushroom etc. It has its onw spawn production facility and also conducts trainings regularly.

vii) Maa Durga Mushroom Industry Ptc. Ltd., Godawari, Lalitpur

https://www.facebook.com/Maa-Durga-mushroom-industry-346173589421126/

It has a big modern hi-tech mushroom production facility for white button mushroom. It also has a compost production facility.

viii) Seedland Nepal Pvt. Ltd, Sipadol, Bhaktapur

https://www.facebook.com/Seedland-Nepal-Pvt-Ltd-683009388459467/

It has a big modern hi-tech mushroom production facility for white button mushroom and traditional mushroom tunnels for oyster mushroom production. It has also established a greenhouse for organic agriculture near the mushroom production facility.

ix) Indreni Mushroom Farm Public Ltd, Chitlang, Makawanpur

https://www.facebook.com/Indreni-Mushroom-Farm-Public-Limited-1442777675745768/

It has hi-tech facilities for production of white button mushroom and oyster mushroom. It has also a compost production facility of 20 ton and supplies compost to mushroom farms that grow white button mushroom. There is Indreni Mushroom Resort Pvt. Ltd. Near the mushroom farm that serve delicious mushroom items.

x) Sonitpur Mushroom and Spawn House Pvt. Ltd., Nagdhunga, Thankot, Kathmandu

https://www.facebook.com/sonitmushroom/

It has a modern mushroom production facility for oyster mushroom and white button mushroom production as well as a spawn production facility.

xi) Ultimate Mushroom Pvt. Ltd., Dhumbarahi, Kathmandu

https://www.facebook.com/TheKingOfMushrooms/

It has mushroom production facility for oyster mushroom and shiitake mushroom. It also produces commercial mushroom spawn.

xii) Everest Kinoko Pvt. Ltd., Chhauni, Kathmandu https://www.facebook.com/everestkinoko/

It has a good production and demonstration facility for high-yielding, multiple-year, log cultivation of shiitake mushroom. It also produces commercial mushroom spawn of shiitake and also conducts regular mushroom training.

5) Development of national and international network

MBUSTDB has organized several meetings with national stakeholders, both governmental bodies and private organizations, related to mushroom industry and product development in Nepal. MBUSTDB has also increased its international networking by appointing internationally renowned intellectuals such as Prof. Seeram Ramakrishna (https://www.eng.nus.edu.sg/me/staff/ramakrishna-seeram/) from National University of Singapore as Honorary Everest Chair, Prof. Ning Yan (https://academic.daniels.utoronto.ca/forestry/yan-n/) of University of Toronto, Canada as Forest Bio-Materials consultant and Prof. Andre van Amstel (https://www.researchgate.net/profile/Andre-Van-Amstel) of University of Wageningen, the Netherlands as Organic Agriculture consultant and Prof. Suresh Manandhar (https://www.naamii.com.np/teams/suresh-manandhar/) as IT consultant. Several rounds of meetings have been organized with the Honorary Chair and international consultants to develop academic programs of the MBUST. To deepen relationship between academia and industry, MBUSTDB has also signed a MOU with National Business Initiative (https://nbinepal.org.np/), a private enterprise, to do research on organic agriculture and mushroom industry. MBUSTDB will have to organize more meetings and discussions with other institutes to develop research plan for research and development on Yarsagumba and its products.

Chapter 9. Support Design of Master's and PhD level course in Madan Bhandari University of Science and Technology (MBUST) incorporating Yarsagumba studies. Formulation of topics for PhD research and Master's degree research

1) Design of Master's and PhD level courses in MBUST incorporating Yarsagumba studies

i) Design of Master's course

Master's course is designed to expose students to basic fungal culture techniques, also known as mycological techniques, which are different from *in vitro* techniques used for plant culture and other microorganisms such as bacteria. During Master's course, students will be able to apply the basic techniques to conduct their research. Following basic topics will be incorporated in the Master's course.

Fungal diversity in the world and Nepal. Major taxonomic groups of fungi are oomycetes, zygomycetes, ascomycetes and basidiomycetes etc. There are estimated 2.2 to 3.8 M fungal species existing in the world (Hawksworth and Lücking 2017), second to insects. Out of total fungal species, 10000 to 14000 species grow as mushrooms on soil or trees. In Nepal, around 2,467 fungal species have been listed in the local fungal flora (Adhikari 2018, Khadka and Aryal 2020), of which 1,271 species are identified as mushrooms (Adhikari 2014). Ascomycetes and Basiodiomycetes are two dominant groups within fungi to which mushrooms belong.

Habitats and modes of nutrition of fungi. Fungi grow on all the terrestrial habitats of the world (Yakop *et al*. 2019). The mode of nutrition varies from saprophytism to parasitism, pathogenic or symbiosis.

Human, plant and animal pathogenic fungi. Fungi are also notoriously known to cause diseases in humans, plants and animals. Among animals, insects are one of the groups that are prone to fungal infection, causing mortality of the host species. Yarsagumba is such an example (Shrestha *et al*. 2010, Shrestha 2011).

Types of mushrooms such as edible, medicinal, toxic, poisonous. Mushrooms are broadly characterized as edible, medicinal or poisonous from human health perspectives. Edible mushrooms are exclusively cultivated whereas medicinal mushrooms are mostly wild collected and also cultivated where cultivation techniques are available. Public awareness is highly important to avoid poisonous mushrooms from consumption and sale.

Saprophytic, pathogenic, symbiotic, endophytic, mycorhizal fungi. Fungi, including mushrooms, are connected to their habitat in different modes. Usually, edible mushrooms either grow on soil or in living or dead trees. Those growing on soil may have mycorhizal connection with plants/trees such as matsutake mushroom (*Tricholoma matsutake*), morel (*Morchella* spp.) etc which are sold in high price.

Common edible, medicinal and poisonous mushrooms of Nepal. Nepal is rich in mushroom diversity. Among 1,271 mushroom species (Adhikari 2014). Christensen *et al*. (2008) have recorded 228 edible species and Adhikari (2014) has recorded 100 poisonous species and 89 medicinal species. Among medicinal mushrooms, Yarsagumba is regarded as the most valued medicinal mushroom of Nepal (Adhikari 2014, Shrestha and Sung 2005, Shrestha *et al*. 2010), naturally distributed in alpine regions of northern Nepal.

Commercially cultivated mushrooms of Nepal. Mushroom cultivation has been practiced in Nepal since last 50 yrs (Raut 2019). Among cultivated mushrooms, oyster, white button and shiitake are the three major mushrooms. King oyster, straw mushroom and milky mushrooms are other mushrooms cultivated in limited areas in Nepal. Red (Reishi) mushroom is a medicinal mushroom cultivated in few farms in Nepal. Besides them, maitake mushroom, enokitake mushroom, lion's mane mushroom are also experimentally tried.

***In vitro* culture techniques of mushrooms and fungi.** Fresh mushrooms are either perishable or long lasting. In either case, in order to propagate mushrooms in natural habitat or in controlled environment, we need to isolate spores or small portion of the tissue part and grow *in vitro*. Different techniques are used to isolate and grow in large quantity.

Collection of fresh specimens of mushroom. Collection of fresh mushroom or fungal samples is a prerequisite for establishment of *in vitro* culture of mushrooms. Fresh samples can be collected in natural habitats such as forest, agriculture land or cultivation farms.

Sources of isolates. Freshly prepared isolates and isolates obtained from culture banks or microbial culture collections are the two main sources of isolates. Availability of trained human resources and lab facilities and equipments can lead to self-preparation of isolates. In case of failure or non-availability of fresh samples, purchasing of cultures from culture collections or culture banks can be the best option.

Tissue isolation and spore isolation. Fungal tissue and spores are the two sources for preparing isolates of mushrooms. Fungal tissue inside the mushroom cap or stem is a good source of isolation. This method is relatively simple and can be done as long as the tissue is healthy and not rotten. For spore isolation, mushroom caps are placed over a clean surface or directly over the medium for spore release.

Spore discharge technique: Spores are discharged by living, fresh mushroom specimens, either by active process or by passive process. Under the active discharge, spores are released forcefully by mushrooms when they are mature without external forces such as wind, rain etc. But, under passive discharge, external forces such as wind or rain are necessary for spore release from mature specimens. Hence, depending upon the nature of samples, mature specimens of mushrooms are either directly placed over medium for active discharge of spores or streaking method is applied using a sterile pin.

Single spore isolation and multiple spore isolation. Spores can be transferred to fresh media, either in single or in mass (also known as multispore). Single spores are genetically homogenous and carry monokaryotic characters such as single mating type (in case of heterothallism), pigmentation etc. Multispore isolates produce gross morphological characters, representative of the species but may not be suitable for mating type determination.

Dilution method for single spore isolation. For establishment of pure cultures, single spores should be isolated and inoculated. For single spore isolation, serial dilution method can be applied.

Major nutritional sources for growth of fungi. Carbon and nitrogen sources are two main nutritional components for fungal culture medium. Different carbon sources such as dextrose, maltose, sucrose or complex carbohydrates such as starch can be used although dextrose is the most preferred. Similarly, organic nitrogen sources such as yeast extract, peptone or inorganic sources such as $NaNO_3$, NH_4NO_3 etc are used. In addition, mineral salts in small amounts are also used. Complex organic substances such as oat, potato, carrot, vegetables and fruit juices etc are also used.

Concept of fungal culture medium. Concept of medium is very important for fungal culture. The fungal culture media are variously categorized according to the purpose such as complete and incomplete media, poor, moderate and rich media, synthetic medium or chemically defined medium etc. A complete medium is the one that consists of all the nutritional requirements for a given fungal species whereas incomplete medium lacks at least one or more nutritional requirements. Similarly, media can be poor, moderate or rich, depending upon the concentrations of nutritional sources. Generally, poor media produces low mycelium density but higher conidiation whereas rich media produce high mycelium density but less conidiation. Fungal culture media are less chemically defined than plant culture media. Usually one or more components of the culture media are derived from complex organic materials such as potato, oat, malt extract etc.

Effects of light and temperature on fungal growth. Besides quality and quantity of nutritional sources, other environmental factors such as light and temperature play vital roles for *in vitro* fungal growth. Light plays deciding roles in vegetative or reproductive phases of fungi. Temperature affects growth rate of fungi. A moderate range of temperature of 20-25C favor optimum growth, lower or higher temperatures slow down growth rate or can be even detrimental.

Microscopic techniques for observation of spore germination, conidiation and mycelium development. Mycelium growth resemble to each other among different groups of fungi. However, they contrast from each other in conidiation that help us to differentiate among them. Two methodologies are routinely used to induce conidiation. They are i) growing cultures in poor media such as WA and ii) slide

culture method. Conidiation takes place in nutritionally rich media also but due to thick mycelium growth, observation of conidiation becomes difficult.

Preservation of fungal cultures. Different types of preservation methods of fungal cultures are practiced such as room temperature, distilled water (DW) method, low temperature, freezing temperature (-20C, -80C), lyophilization, liquid nitrogen etc. Ultra-low temperature such as -80C or liquid nitrogen are considered ideal for indefinite period of preservation. However, such facilities are not always available and are difficult to ensure. Low temperature condition is easy to ensure and regular subcultures can be done for preservation of longer periods.

Factors that stimulate vegetative stage to reproductive stage. Mushrooms grow as mycelium mat in vegetative state over the substrate surface in a wide range of temperature under both light and dark conditions. But, reproductive stage develops under a narrow range of temperature, usually at low temperatures. Light may be a deciding factor for inducing reproductive stage from vegetative stage. High humidity is usually favorable for reproductive stage.

Yarsagumba and *Cordyceps* mushrooms as examples of medicinal mushrooms. Yarsagumba is regarded as a highly valued medicinal herb in Nepal, especially in high-altitude areas. Besides Yarsagumba, there are other *Cordyceps* mushrooms considered as medicinal mushrooms. Different fungal culture techniques are applied in order to cultivate Yarsagumba and other *Cordyceps* mushrooms.

Role of insect-pathogenic fungi as biocontrol agents. Besides medicinal values of insect-growing mushrooms, they are also equally valuable as potential sources of biological control agents. The insect mortality property of these fungi can be used to control undesired, harmful insects in agriculture lands or forests.

Production of biocontrol agents using insect-pathogenic fungi. Among insect-pathogenic fungi, few have wide host range such as *Beauveria* and *Metarhizium* species. They can infect and cause mortality in hundreds of different species of insects. Hence, such fungal resources can be multiplied in culture facilities and applied in the field that can control harmful insects.

Bio-assay of insect pathogenesis using fungi. Before application of fungal resources in the field, the bio-assay test can be performed in the lab to determine the dose of fungal spores, the stage of insect life cycle that is prone to mortality due to fungal infection, duration that is necessary to cause insect mortality and impact of such fungal resources on human health etc.

Establishment of microbial culture collection. Each and every biological resources are valuable either for economic or environment-safety purposes. Such resources should be preserved in living condition so that they can be propagated at time of need. Culture collections of microorganisms function as seed banks

of plants and trees. Preservative techniques such as low and ultra-low temperatures supplemented by regular subcultures as well as regular collection from nature are necessary to establish and run microbial culture collections.

ii) Design of PhD course

PhD students are trained to do research independently using advanced techniques to improve mushroom cultivation techniques and productivity. Following advanced topics are included in PhD course.

Types of mating system in fungi: Homothallic, secondary homothallic and heterothallic mating systems are three major mating systems in fungi. Fungi, including mushrooms, complete their life cycle with or without mating between individuals. Mating between two individuals, also known as crossing, is necessary for recombination of nuclear materials that can lead to production of sexual spores. There are other fungi which produce sexual spores without mating. There is a third type of fungi which externally do not mate with its counterpart but which always possess both types of mating genes within a single spore. Depending upon their mating system, crossing is either necessary or not for fruiting of fungi.

Sister crossing and out-crossing. When crossing is done between two individuals produced by the same mushroom sample, it is called sister-crossing whereas when two individuals from different samples are used, it is called out-crossing. Sister-crossing is usually highly successful; however, success of out-crossing depends on the genetic range of the participating samples.

Vegetative compatibility. Without vegetative compatibility, no mating can take place for heterothallic fungi. It can be demonstrated in an agar plate culture by inoculating individual strains on opposite sides and observing their behavior at the meeting line.

Detection of mating system in culture and in substrate. It is difficult to detect mating types in natural habitat. However, in culture, individual strains show their characters, including mating behavior. A mixture of individual strains can be inoculated in medium or substrate to observe its effect on fructification. Or, two individuals can be inoculated on opposite sides of the substrate to observe nature of fructification at the meeting line.

Mating system in Yarsagumba and other *Cordyceps* mushrooms. A lot of curiosity is generated in mating system of Yarsagumba and *Cordyceps* mushrooms. Research has shown homothallism in Yarsagumba, but heterothallism in other *Cordyceps* mushrooms such as *C. militaris* (Shrestha *et al*. 2004).

Nucleus and gene-based mating system. Some fungi show secondary homothallism or pseudo-homothallism. In this case, an individual carries both mating types, ie, produces binucleate spores, thereby

causing no necessity of crossing. In yet another rare case, a mononucleate individual carries genes of both mating types in haploid set of chromosomes.

Insect-fungus interaction. Fungi and insects are associated with each other in different models such as mutualism, commensualism, parasitism or saprophytism. Among them, Yarsagumba and *Cordyceps* mushrooms show parasitic life cycle on insects.

Host range of insect-pathogenic fungi. Two types of insect-pathogenic fungi are found in nature, one with wide host range and the other with narrow host range. The former is also known as generalist and the latter as specialist. It is directly related to host specificity. Yarsagumba is an example of specialist whereas *Beauveria* and *Metarhizium* fungi are examples of generalists.

Ecological and physiological host range of insect-pathogenic fungi. In nature, insect-pathogenic fungi usually grow on forest-dwelling insects. But, in controlled environment, they can grow on other insects too. It shows that physiological hosts are different from ecological hosts. This study will be helpful to cultivate insect fungi on insects that are different from natural hosts.

Mode of infection of insects by insect-pathogenic fungi. Insect-pathogenic fungi can infect insects by two modes. The common mode is penetration through the exoskeleton layer of insects, mainly cuticle. The fungal spores break down cuticle layer by enzymatic action and penetrate into the insect body. The other mode is through the elementary canal with food particles.

Mode of nutrition of insect-pathogenic fungi. Insect-pathogenic fungi show hemi-biotrophic mode of nutrition. The fungi show parasitism by devouring the living insects till their death. After the death of the host insect, the fungi continue growing but change their mode of nutrition from parasitism to saprophytism.

Antimicrobial property of insect-pathogenic fungi. When the insect hosts are dead after the growth of Yarsagumba, the larva change to a cadaver but do not decompose even after a long time. What makes the insect body intact and decomposition-proof? The causal organism is Yarsagumba fungus. The fungus produces different kinds of antimicrobial compounds that inhibit the surrounding microorganisms from decomposing the host larva. Such antimicrobial properties should be tested further that can be beneficial to human health and environment.

Growth rate of Yarsagumba mycelium at high and low temperatures. Yarsagumba is widely known as an extremely slow growing fungus (Dong and Yao 2011). It is a very peculiar fungus that grows on extreme climatic condition. Low temperature might be more favorable for Yarsagumba compared to other temperate and tropical mushrooms. Shrestha *et al*. (2006) have shown effect of light and nutrition on growth characteristics of *Cordyceps miliaris*.

2) Formulation of topics for Master's and PhD degree research

Following are some of the research topics for Master's and PhD courses on Yarsagumba.

i) Collection of Yarsagumba and other *Cordyceps* mushrooms in different types of forests from alpine to lowland areas of Nepal

West Nepal (Dolpa, Jumla, Humla, Mugu, Manang, Darchula, Bajhang etc) is a potential area for Yarsagumba (Uprety *et al*. 2016) and most of the collections and exports are done from this part of the country. Fresh samples of Yarsagumba can be collected in high altitudes of west Nepal to initiate *in vitro* culture and cultivation of Yaragumba in controlled-lab condition. Collaborative researches will be planned with related research centers of NARC such as National Plant Pathology Research Center and National Entomology Research Center for identification of fungi and insects associated with Yarsagumba.

ii) Tissue and spore isolation from fresh specimens

Before collection trips are made, agar plates are prepared and taken to the field. Fungal isolates should be prepared in the field or nearby area soon after collection using spore discharge method. During spore discharge and further growth in culture, there is a high chance that other surrounding microorganisms grow and overcome the growth of Yarsagumba. Thus, pure cultures of Yarsagumba should be developed without interference of other microorganisms.

iii) Growth of pure culture of Yarsagumba and other *Cordyceps* mushrooms in controlled temperature and light conditions

Development of pure, uncontaminated culture of Yarsagumba is the prime step for further work. After the discharged ascospores are collected in an agar plate, they are transferred to fresh, nutrient media and incubated at optimum temperature for further growth. This is also termed as establishment of culture of Yarsagumba. Growth of Yarsagumba mycelium is very slow in artificially prepared media under lab condition, just growing few centimeters in a period of 100 days or more (Dong and Yao 2011, Cao *et al*. 2015). Hence, research will be focused to achieve faster growth of mycelium using intrinsic components in culture medium.

iv) Multiplication of culture of Yarsagumba in lab-scale fermenter

Solid state (agar) culture is done at first to initiate and develop cultures of Yarsagumba. After cultures are prepared and confirmed through observations of primary growth characteristics and microscopic structures, they are transferred to liquid state culture to enhance growth rapidly. For lab-scale cultures, small conical flasks up to 2000 ml volume can be used. For bigger volume, fermenters with continuous air supply can be

used.

v) Production of dry Yarsagumba powder from lab-scale fermenter

Mycelium can be filtered from liquid culture and dried. This is the safest method of mycelium production. However, great care should be taken that the powders are pure and not contaminated by other microorganisms and molds. Collaboration with institute such as National Agriculture Engineering Research Center of NARC can be made to develop drying equipment for Yarsagumba powder.

vi) Study on secondary metabolites and bioactive compounds from Yarsagumba mycelium and culture filtrate

Mycelium as well as culture filtrates are sources of many bioactive compounds and secondary metabolites. The nutritional sources and culture environments can be tested in order to study their effects on bioactive compounds. Carbon and nitrogen sources are the main components of nutrition and their ratios play an important role in growth and bioactivity of Yarsagumba mycelium. Similarly, high and low temperature and light and dark conditions are prime environmental factors that affect mycelium growth and bioactivity of Yarsagumba.

vii) Mycelium culture of Yarsagumba in solid-state medium such as rice, wheat, barley etc. and production of health foods

Cereal grains are rich sources of nutrition for mushroom growth. The grains can be inoculated with liquid inoculum of Yarsagumba and incubated at optimum temperature. This will induce mycelium growth of Yarsagumba on the surface of grains. The grains can be dried and consumed in various ways. Such types of products are commonly known as Yarsagumba rice, Yarsagumba wheat etc, which are categorized as health foods. The dried products can be stored for a longer period and transported easily. श्रेष्ठ (२०७०) has shed some light on development of Yarsagumba rice as an exportable product from Nepal to rich, rice-consuming countries.

viii) Survey of other Yarsagumba-like *Cordyceps* mushrooms in Nepal and testing their lab-based growth for alternative sources of bio-active compounds

Besides Yarsagumba, many *Cordyceps* mushrooms are found all over the world including different parts of Nepal. Specially, east and southeast Asian countries have already done a lot of research on *Cordyceps* and other insect mushrooms and their cultivation techniques. *Cordyceps militaris* is one of such potential mushrooms (Shrestha *et al*. 2012). Major bioactive compounds such as adenosine and adenosine-derivatives, polysaccharides, cordycepic acid, ergosterols etc are commonly found in Yarsagumba and other

Cordyceps mushrooms. All year-round survey of *Cordyceps* mushrooms should be carried out in different climatic regions of Nepal although rainy season is the most favorable for growth of such mushrooms. The hosts of such mushrooms are mostly hypogeal, that means, buried under the soil surface. Besides soil, the hosts are also buried inside the tree trunks or branches, with fruiting body of the mushroom protruding outside through a hole.

ix) Patenting of cultivation techniques of Yarsagumba and *Cordyceps* mushrooms.

Qin *et al*. (2018), Li *et al*. (2019) and Liu *et al*. (2019) have recently demonstrated fruiting body production of Yarsagumba on larvae, which is a valuable achievement in modern biological science of fungi. However, modified cultivation techniques of Yarsagumba and other *Cordyceps* mushrooms can be tested. Because Yarsagumba cultures are extremely slow in growth in culture, there are prospects of doing research on how to achieve rapid growth for its successful cultivation. Academic institutions such as universities and research centers are appropriate places to initiate research in this area. Besides Yarsagumba, other medicinal *Cordyceps* mushrooms will also be experimented for their cultivation.

x) Training to the local communities of highlands of Nepal to promote Yarsagumba trade in domestic and international markets as well as to conserve Yarsagumba

Collection and trade of wild Yarsagumba is a great late spring and early summer activity in northern regions of Nepal. Thousands of people are involved in collection of Yarsagumba as their seasonal high-earning occupation. However, continuous collection without preserving the natural habitat of Yarsagumba will diminish the amount that can be collected every year. Hence, local people and local authorities should be made aware of the impact of excessive collection on survival of Yarsagumba. Trainings and group discussions should be conducted in coordination with local bodies and communities. Hence, study will also be focused on resource management such as access and management rules and conservation of natural vegetation of Yarsagumba growing areas. As Nepal has been exporting wild Yarsagumba, interaction will be made with local and central Govt. bodies to formulate policies to improve marketing product of wild Yarsagumba in a bundle or in a packet with a trade name through Govt-registered small scale industries. Collaborative approaches will be made to promote domestic market of Yarsagumba in Nepal by opening stores, especially in touristic area and souvenir shops of high-cost hotels.

xi) Techniques of spawn improvement of mushrooms using tissue isolation and spore isolation methods

Spawn production is a regular work for a large scale of mushroom cultivation and a daily routine for spawn suppliers. Methods of tissue isolation and spore isolation are described below.

a) Tissue isolation: It is recommended for mother culture production because genetic characteristics of the mushroom are preserved in the isolated mycelia. Tissue isolation is relatively a simple process, but needs a highly sanitized and sterile environment (Fig. 1).

b) Spore isolation: Spore culture easily brings about variation of character manifestation due to recombination of genes. It is a multi-step process and is briefly described. Spores are released by mature fruiting bodies of mushroom (Fig. 2) and are collected on agar plates by placing the cap above the agar plate. The spores are suspended in sterile water and diluted further after which the spores are again plated on an agar plate (Fig. 3). The plated agar plates are incubated at 24C under dark after which single colonies form (Fig. 3). Single colonies are picked up from the agar plate and transferred to fresh agar plates. The plates are incubated at 24C in dark and observed under microscope at regular intervals to make sure that no clamp connection has developed on them (Figs. 4 and 5). These isolates are called single spore isolates or monosporous isolates.

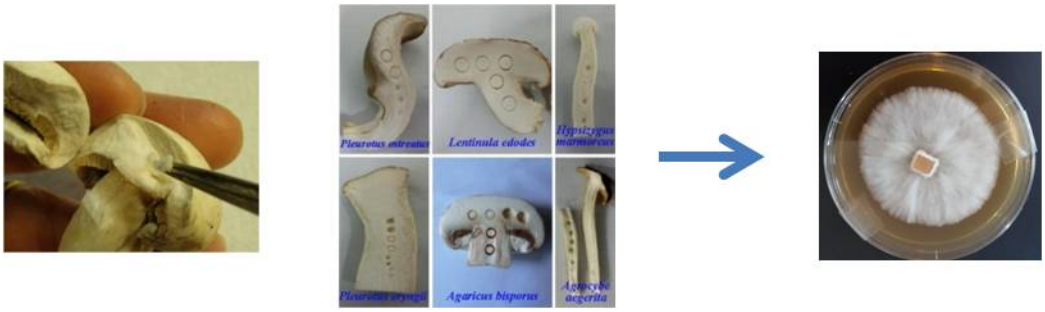

Fig. 1. Fresh fruiting bodies are cut into half and inner tissue is cut (https://plantpath.psu.edu/about/facilities/mushroom/cultures-spawn/how-to-start-a-culture) or punched (Liu *et al*. 2016) and transferred to agar plates.

Fig. 2. Life cycle of a mushroom (left, https://forestorigins.com/blogs/mushroom-blog-posts/the-mushroom-life-cycle) and spore discharge from cap of mature fruiting body (right, http://sietalab.com/mushroom-culture-from-spores/).

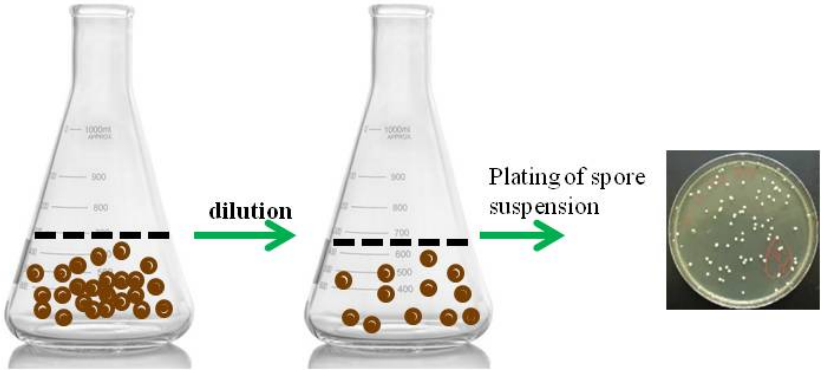

Fig. 3. Spores are suspended in sterile water (left), then diluted further (middle) and plated on a agar plate (right).

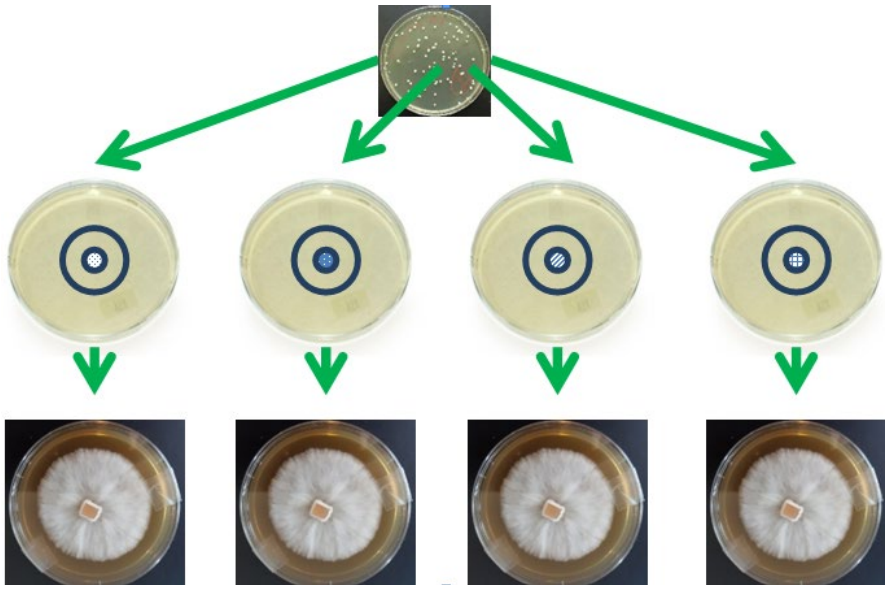

Fig. 4. Monocolony isolates incubated for 3 wks at 24C in dark.

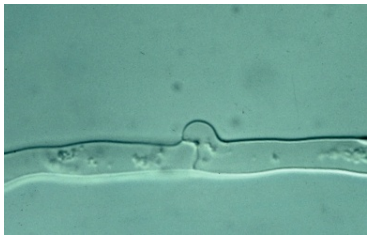

Fig. 5. Clamp connection on mushroom mycelium (https://www.quora.com/What-is-a-clamp-connection-in-botany).

The monosporous isolates are then inoculated on opposite sides or on four quarters of an agar plate and incubated at 24C in dark until the mycelia come in contact with each other (Fig. 6). Mycelia at the meeting point between isolates are again cut and transferred to fresh agar media (Fig. 7). The isolates are also observed for clamp connection (Fig. 5). The dikaryon isolates grow faster than their mother monokaryons (Fig. 8).

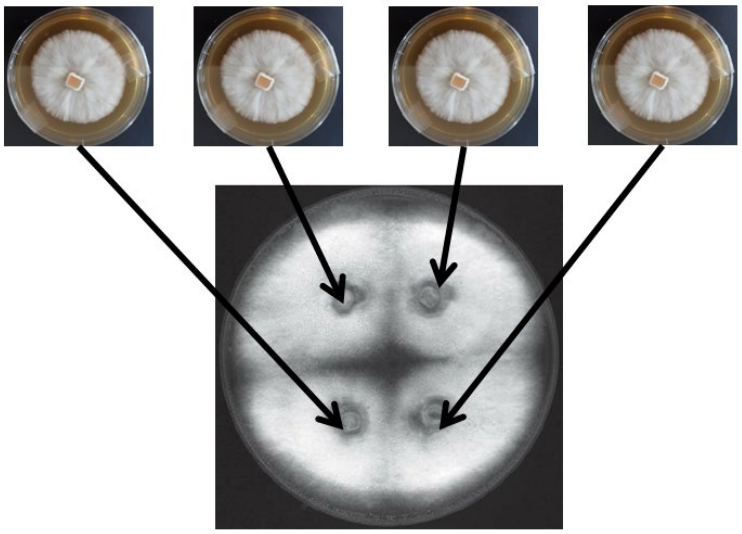

Fig. 6. Monokaryon isolates incubated on a single agar plate.

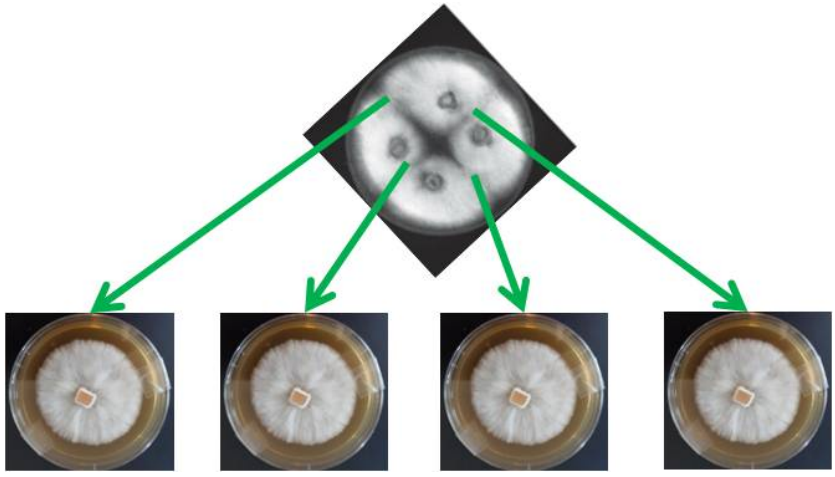

Fig. 7. Mycelia are cut from the meeting line between the isolates and transferred to fresh agar media.

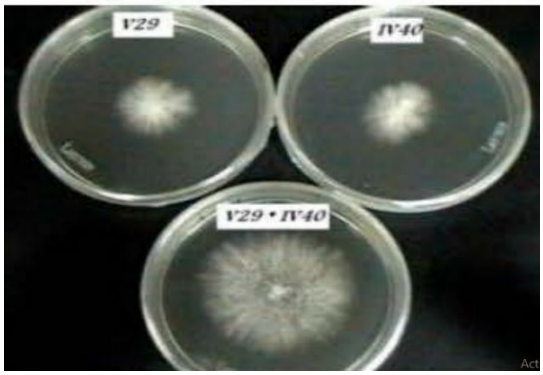

Fig. 8. Comparative growth rate of monokaryon isolate (upper) and a dikaryon isolate (lower) (Goltapeh *et al*. 2007).

3) Other topics for Master's and PhD degree research

- Observation on growth characteristics of temperate and tropical mushrooms at low and high temperatures
- Increasing mushroom productivity and bioconversion rate using different types of substrates
- Strain preservation of commercial varieties of mushrooms
- Identification of pests and diseases in mushroom cultivation and their control
- Introduction and popularization of new mushroom varieties such as king oyster mushroom, morel etc.
- Development of cold storage, drying, powdering and canning technology for mushroom
- Value-added product development from mushroom such as soup, achar, nutraceuticals etc.
- Substrate improvement using straw, sawdust, agro-wastes etc.
- Mechanical improvement for substrate pasteurization (efficient boiler) and mushroom balling etc.
- Improvement for industrial production of mushrooms such environment control, disease control etc.
- Alternative uses of mushroom substrates such as re-use for other mushrooms or as source for biofertilizer etc.
- Use of AI for early detection of contamination, optimum harvesting timing etc.
- Database of commercial mushroom strains cultivated in Nepal
- Mushroom-based entrepreneurship development
- To strengthen microbial culture collection of Nepal

4) Regular Graduate seminar

All Master's and PhD students will give presentations on topic of their choice or recommended by the supervisor every semester. The presentation skill, organization of materials, fluency in speaking, inclusion of correct references etc will be evaluated.

Chapter 10. Estimation of cost for lab establishment in MBUST for Yarsagumba research and development of Yarsagumba products

1) Mushroom facility for research on Yarsagumba

A mushroom facility is necessary for research on Yarsagumba. A design for mushroom facility is drawn as shown in Fig. 1. The purpose of the facility is to do basic research for strain improvement of commercial mushrooms such as oyster mushroom, white button mushroom and shiitake mushroom as well as for new mushroom varieties such as Yarsagumba, Cordyceps mushrooms, king oyster mushroom, milky mushroom, straw mushroom, Ganoderma mushroom, enokitake mushroom, maitake mushroom, lion's mane mushroom etc as well as to develop a pilot scale production unit as a center of demonstration and training for small and large scale mushroom growers. Other research areas include selection of optimum substrates for higher mushroom productivity and quality improvement, identification of diseases and insects and their control, optimization of cold storage techniques of fresh mushroom, reuse of spent substrates as alternative source of biofertilizer for organic farming etc. The facility consists of several rooms. These include a medium preparation room with working tables and washing space, sterilizing or autoclaving room, inoculation room, incubation rooms, cool chamber, harvest room, cold storage, storage room, drying room etc. Each room is briefly discussed below.

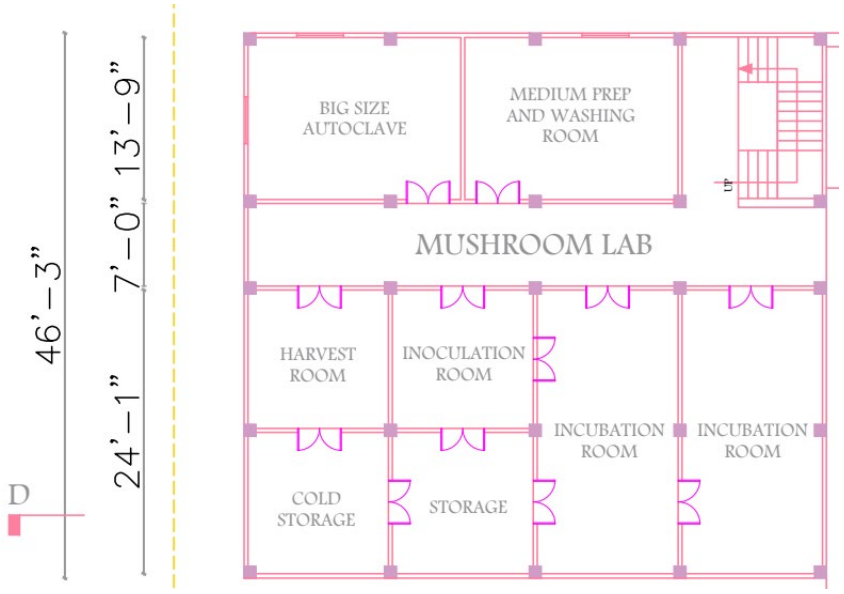

Fig. 1. A design of mushroom facility for research on Yarsagumba and mushrooms in MBUST, Chitlang.

i) Medium Preparation room with working tables and washing space (18 × 12 sq. ft)

The medium preparation room consists of storage space for chemicals, culture vessels and closures, and glasswares required for medium preparation and dispensing (Fig. 2). This room also consists of bench space for microscopes, hot plates/magnetic stirrers, pH meters, vortex, weighing balances, water baths, incubator, refrigerator, hot air oven and media dispensing equipments etc. Similarly, the working tables should consist of racks, drawers and cabinets (for personal use of researchers), cloth hanger etc. The space of working tables is also allocated for individual researchers with sitting chairs. The washing area should contain large wash basin sinks and draining boards. Hot air ovens and storage cabinets are placed near the washing area. The room also should have water sources, electric points and exhaust fans for air flow. Other necessary equipments include A/C, heater, water demineralization unit and water distillation unit, refrigerators for storing solutions and chemicals. The size of working tables and slabs are given in Fig. 3, 4 and 5.

ii) Sterilizing room (18 × 12 sq. ft)

It is a room for sterilizing/autoclaving all the medium components that are ready to use for mushroom culture. To autoclave small quantities, small portable vertical autoclaves ranging from 20 l to 150 l can be used using electricity (Fig. 6). However, for large quantities, bigger autoclave machines ranging from 200 l to 2000 l or even bigger are used. Usually, firewood is used as source of fuel for bigger autoclaves due to its cheap price and easy availability (Fig. 7). Gas is another source of fuel for small portable autoclaves (Fig. 6). Electricity is practically not used for bigger autoclaves in commercial mushroom farms due to its high cost as well as frequent interruption. A boiler is connected to big autoclave machines for supply of steam (Fig. 7). Big autoclave machines are usually vertical, but horizontal autoclaves are also used (Fig. 7). This room is built near the entrance gate and medium preparation room, but away from other rooms, and should be well-ventilated so that the heat released during sterilizing process can be diverted away from the building. Autoclaves are also used to sterilize contaminated materials before discarding them.

Fig. 2. A medium preparation room with working tables and washing space.

Fig. 3. Size of one-sided working table. Left, front view and right, side view.

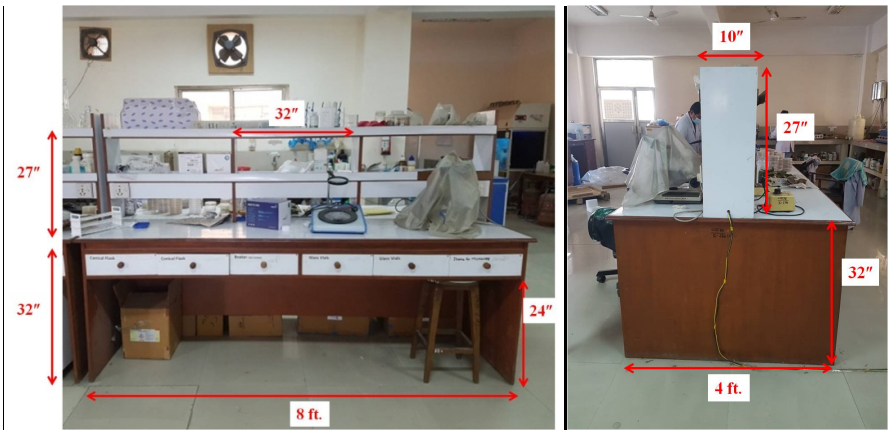

Fig. 4. Size of both-sided working table. Left, front view and right, side view.

Fig. 5. Size of table with drawers and cabinets (left) and slab with wash basin sink and storing space.

Fig. 6. Portable autoclave machines.


Fig. 7. Big-sized autoclaves with capacity ranging from 200 l to several thousand liters, using firewood as fuel source.

iii) Inoculation room (12 × 12 sq. ft)

After media and substrates are sterilized, they are left overnight for cooling on one side of the autoclaving room. The cooled substrates will be transferred to inoculation room. A laminar air flow cabinet or a biosafety cabinet with Hepa filter as well as germicidal tube (UV light) and fluorescent light will be fitted in the inoculation room (Fig. 8). Long sized cabinets (70-80 inch long) can be used by two persons at a time, whereas smaller cabinet is suitable for single person use. A LPG cylinder is connected to a Bunsen burner inside the cabinet for flame sterilization of small equipments such as inoculation loop, inoculation pin, scalpels, syringe, spatula, forceps, tweezers etc.

Fig. 8. Biosafety cabinet and laminar air flow cabinet for inoculation purposes.

iv) Incubation Room (24 × 12 sq. ft)

All types of cultures are incubated under controlled conditions of temperature, light, humidity, air circulation, CO_2 concentration etc after inoculation (Fig. 9). These environmental factors influence the growth and differentiation process during culture period.

Fig. 9. Incubation rooms for spawn production as well as mushroom cultivation.

Generally, two incubation rooms are used, one for high temp (25-30°C) and the other for low temp (18-20°C). Typically, the high temp incubation room is required for mycelium or vegetative growth of mushrooms and the low temp incubation room for fruiting body production. Steel racks with multiple shelves, each connected with LED tubes are fitted in the incubation rooms (Fig. 9). A humidifier or sprinkler or moisturizer is connected to maintain high humidity. A/C and CO_2 supplier is also connected to maintain optimum levels of temperature and CO_2. The temperature is adjusted according to the need of mushroom growing stage.

v) Cool chamber (12 × 12 sq. ft)

Cool chambers (10-15° C) are necessary to store mushroom spawns (seed cultures) for longer period of time. Besides, cool chamber is also used to observe effect of low temperature on growth of temperate or cold-resistant varieties of mushrooms. An A/C connected with CoolBot and run by wifi is used to lower temperature as low as 10C (Fig. 10).

Fig. 10. Application of CoolBot technology for lowing temperature of cold storage up to 4C or lower.

vi) Harvest room (12 × 12 ft)

Harvest rooms are used to harvest mushrooms. Generally, several flushes of mushrooms are harvested periodically for most of the mushrooms. However, Cordyceps mushrooms are harvested only one time. Hence, fully grown Cordyceps mushrooms are transferred from incubation room to harvest room for cutting fruiting bodies from the substrates.

vii) Cold storage and post-harvest technology (12 × 12 ft)

Cold storages are used to study impact of low temperature on preservation of mushroom fruiting bodies. An A/C connected with CoolBot and run by wifi can be used to lower temperature as low as 4C or lower (Fig. 10). Research on post-harvest technology of mushrooms is very important, given that more and more farmers are involved in mushroom cultivation and the necessity to increase the shelf-life of fresh mushrooms during over-production. Color changes (browning) is the most common post-harvest deterioration observed in mushrooms, especially oyster mushroom and white button mushroom. This is due to enzymatic reaction of mushrooms with oxygen which form brown pigments on mushroom surface, hence known as enzymatic browning. This parameter most affects consumers' acceptance. Other change is the loss of turgor. Mushrooms have no cuticle to act as a physical barrier against mechanical damage, water loss, or microbial attack. Mushroom texture is one of the most important attributes contributing to consumer satisfaction. Off-flavor is another deteriorating character. Mushroom fruiting bodies slowly lose its odor.

Another deteriorating character is microbial spoilage. Microorganisms in harvested mushroom become active and cause tissue rotting.

Fresh mushrooms have a short shelf life. After harvesting, the metabolic activities of mushrooms continue at 15-18C. Hence, storage in refrigerated conditions at 1-4C is recommended for extending shelf-life of mushrooms. Following post-harvest techniques are recommended for cold storage of fresh mushrooms.

- Storage of mushroom at 0C and 95% RH has been reported to be the optimum condition to extend marketable life of mushroom (Jayathunge and Illeperuma 2005).
- Mushrooms should be cooled to 0-2C within five hours of picking (Kim 2004).
- The best method for fresh storage of oyster mushroom is to keep them at 8-10C in packed container wrapped in plastic film (Kim 2004). It's called 'PVC film storage' (Figs. 11 and 12).
- Wrapping mushrooms with such microporous or perforated plastic film can improve their storage life, as this reduces the moisture loss and preserves the quality of mushrooms (Kim 2004).
- The choice of the cooling system depends upon the quantity to be handled; it may be a refrigerator for a small grower or consumer a cold room with all the facilities for a commercial grower (https://www.kisansuvidha.com/fresh-mushrooms-storage/?v=ad4f1670f142).
- The mushrooms should not be stored in the same cooler along with fruits as the gases produced by fruits cause discolouration of mushrooms (https://www.kisansuvidha.com/fresh-mushrooms-storage/?v=ad4f1670f142).
- The maturation and textural changes in button mushrooms are slowed down at 0C ensuring the maintenance of excellent quality. The shelf-life of the button mushroom is about 14-20 days at 1C, about 10 days at 6C and 2 to 3 days at 20C (https://www.kisansuvidha.com/fresh-mushrooms-storage/?v=ad4f1670f142).
- Optimum temperature and relative humidity for storage of button mushrooms as 0-2C and 85-90% respectively https://www.kisansuvidha.com/fresh-mushrooms-storage/?v=ad4f1670f142

Fig. 11. Button mushrooms packaged in the bionanocomposite-based laminate/EPS tray (a) and in the conventional PVC stretchable/EPS tray configuration (b) (Gholami *et. al*. 2017).

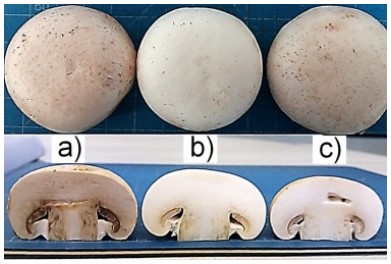

Fig. 12. Surface (up) and cross-section (down) images of uncoated mushrooms packaged using the control film (PVC stretch film) (a), the test film (PET/coating/ LLDPE film – EPS tray) with (b) and without (c) MAP after 22 days of storage at 4°C (Gholami *et al*. 2017).

viii) Drying room (12 × 12 ft)

Shelf-life of fresh mushrooms, especially oyster mushroom, does not last longer than 24 hrs. Mushroom is such a crop that probably has the shortest shelf-life of all the agricultural crops. White button mushroom can remain fresh longer than oyster mushroom at room temperature but soon deteriorates. Shiitake mushroom remains fresh for a longer period than oyster mushroom and white button mushroom. Due to fleshy nature, oyster and white button mushroom are not suitable for drying whereas shiitake mushroom is highly suitable for drying. An electric dryer with multi-racks can be used for drying mushroom fruiting bodies (Fig. 13). Electric dryer should be a food dryer or agro-product dryer. The moisture content is adjusted at 0% or 2% with a capacity to dry 5-10 kg of fresh mushroom. Some big dryers can dry as much as 100 kg of fresh mushroom at a time. Different methods of drying are practiced for mushrooms as shown below. Besides drying, canning and pickling are other methods for long-term preservation of fresh mushroom. Drying is a method of preserving edible mushrooms such as shiitake and wood ear mushrooms. It is not often used for button mushrooms or oyster mushrooms. Moisture of dried mushrooms is adjusted near to 10%.

- Solar drying. For solar drying, plastic tunnel and cabinet dryers can be used (Fig. 13).
- Thermal power drying. Mushrooms should be dried during sunny days at an initial temperature of 35C while mushrooms should be dried during damp days at an initial temperature of 30C. After five hours of heat for mushrooms under sunny conditions and seven hours of heat for those during the rainy season, the temperature can be raised gradually and then kept at 40-60°C for 12-18 hours.
- Hot air drying
- Canning and bottling
- Pickling

Fig. 13. Solar dryers (plastic house and cabinet model) and electric dryer.

ix) Storage room (12 × 12 ft)

Dried mushrooms will be stored in this room at room temp of 20-25C. A/C will be connected to maintain room temperature.

x) Store (12 × 12 ft)

A store room will be set up near the entrance gate for storing materials, equipments etc.

xi) Control panel

A control panel will be set up near the entrance gate to display temp., humidity etc (Fig. 14).

Fig. 14. Control panels showing temperature, humidity, CO_2 concentration etc outside of the incubation rooms.

xii) Disposal area

Organic wastes are produced after harvest of mushrooms and should be disposed in a distant place from the mushroom facility. Along with organic wastes, used plastic bags are also generated and should be disposed carefully. The organic wastes can be utilized as source of biofertilizer.

Fig. 15. Organic wastes disposed in mushroom farms.

Before harvesting, some of the organic wastes are generated due to disease and insect problems. White mycelium grows in uncontaminated poly-bags, but contaminated bags produce green, black, blue or other colors and should be immediately disposed safely so that the molds do not spread. Similarly, insects are another problem and can be controlled in limited amount by using stickers (Fig. 17).

Fig. 16. Uncontaminated and contaminated poly-bags used for mushroom cultivation.

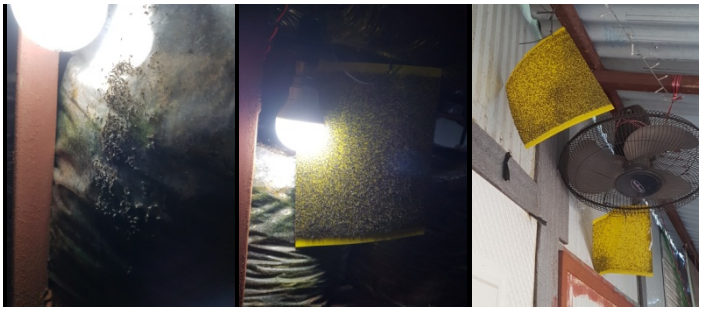

Fig. 17. Poly-bag covered by insects and use of trap for control of insects.

2) Estimation of cost for lab establishment for Yarsagumba research

A mushroom lab is necessary for research on Yarsagumba for making isolation and growing in cultures. A list of basic equipment, glass wares and chemicals are given along with their costs in Table 1, with total estimated fixed cost in the range of Rs. 48~50 lakh (price of small equipments, tools not shown). The estimated cost shown in Table 1 may vary extensively depening upon the quality and quantity. Pictures of some of the equipments are shown in Figs. 18 and 19. Further, electrification, water supply with tank, air conditioners etc also add additional costs.

Table 1. A list of equipment, glass wares and chemicals with estimated cost (Rs.)

1.	Biosafety cabinet: 2 pcs	8,00,000
2.	Autoclave (100-150 l): small cooker type, automatic medium and big size	7,00,000
3.	Lab refrigerator: 2 pcs	5,00,000
4.	Stereo/dissection microscope + illuminator with imaging system: 1 pc	4,00,000
5.	BOD Microbiological incubator (with 2 removable trays): 2 pcs	4,00,000
6.	Compound microscope (phase contrast or DIC) with imaging system: 1 pc	3,00,000
7.	BOD incubator with illumination (fluorescent or LED light) (150 l - 250 l), with 3 removable trays: 1pc	2,50,000
8.	Steel rack 4-storey with LED light in each storey: 10 pcs.	2.50.000
9.	Distillation double unit 5 LT/Hr all stainless steel 10 KW 440V 3 PH Biobase	2,20,000
10.	Deep freeze (-20C and -80C): 2 pcs	2,00,000

11.	Camera SLR: 1 pc	1,50,000
12.	Shaking incubator: 1 pc	1,10,000
13.	Hot air oven: 1 pc	1,00,000
14.	Polypropylene (PP) bottle: 1000 ml (1000 pcs)	50,000
15.	Precision balance (100 g/0.001 g): 1 pc	30,000
16.	Hot water bath	30,000
17.	Micropipette (100-1000 µl): (2 pcs)	30,000
18.	Glass test tubes: 18 × 150 mm (500 pcs), 25 × 150 mm (500 pcs)	20,000
19.	Trolley (2-storey): 2 pcs	20,000
20.	Glass Petri dish: 100 pcs	16,000
21.	Conical flasks: 3000 ml (2 pcs), 2000 ml (4 pcs), 1000 ml (8 pcs), 500 ml (20 pcs), 250 ml (100 pcs), 100 ml (5 pcs)	10,000
22.	Beakers: 3000 ml (1 pc), 2000 ml (2 pcs), 1000 ml (4 pcs), 500 ml (2 pcs), 200 ml (2 pcs), 100 ml (2 pcs)	10,000
23.	Measuring cylinders: 2000 ml (1 pc), 1000 ml (2 pcs), 500 ml (2 pcs), 250 ml (2 pcs), 100 ml (4 pcs), 50 ml (4 pcs)	10,000
24.	Disposable plastic 1000 pcs	10,000
25.	pH meter: 1 pc	10,000
26.	Magnetic stirrer with hot (heating) plates	10,000
27.	Stainless steel forceps with pointed tips: 2 pcs	6,000
28.	Digital balance or digital weighing scale: 30 kg/1 gm: 1 pc	5,000
29.	Parafilm (sealing tape): 1 pc	5,000
30.	Digital thermo hygrometer with probe: 2 pcs	3,000
31.	Bunsen burner: 2 pcs	2,000
32.	Pipettes: 10 ml, 50 ml, 100 ml	1,500
33.	Funnel: 2 pcs	1,000
34.	Micropipette tips (100-1000µl): 100 pcs	
35.	Micropipette stand	
36.	Cryotubes 1 ml size	
37.	Sterile Mineral oil (100 ml):	
38.	Syringe with needle: 1 ml, 2 ml, 5 ml, 10 ml, 20 ml	
39.	Electric extension line: 1 pc	
40.	Scissors: 1 pc	
41.	Scalpel: 2 pcs	
42.	Lab spatula: 2 pcs	
43.	Inoculation needle: 3 pcs	
44.	Test tube rack: 20 pcs, each with 50 capacities	
45.	Rubber gloves: 1 packet	
46.	Plastic spray: 750 ml (2 pcs), 2 liter (2 pcs)	
47.	Plastic baskets: 2 pcs	
48.	Knife, secateurs: 2 pcs	
49.	Tissue rolls	
50.	LPG gas cylinder: 2 pcs	
51.	Lab coat:	
52.	Chemicals such as PDA, yeast extract, peptone, dextrose	
53.	mineral salts, agar, ethanol and others	
Total estimated cost		48,00,000~50,00,000

Fig. 18. Microscopes, electric balance, magnetic stirrer with hot plates, vortex, water bath, water distillation unit etc.

Fig. 19. Hot air oven, orbital shaker, incubators, growth chambers, dehumidifier, deep freezes, BOD incubators etc.

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