

Mycelial Growth of *Termitomyces albuminosus* (Berk.) R. Heim in vitro Culture

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Abstract

Termitomyces albuminosus (Berk.) R. Heim, commonly known as termite's mushroom, is a fungus belonging to Basidiomycetes. It grows on termite mound and has food and medicinal values. This research was carried out to determine the effect of various carbon sources, nitrogen sources, amino acids, vitamins and carbon-to-nitrogen ratios on the *in vitro* mycelial growth of this fungus. The specimens of this species were collected from Chitwan National Park, Central Nepal and inoculated into culture plates. The mycelia so obtained were subjected to culture process using semi solid and liquid media. One-way ANOVA, followed by Tukey HSD test was performed to compare the results of different treatments. Among the six carbon sources used, the best growth was observed in maltose with somewhat compact mycelial density while the least growth was observed in lactose ($p < 0.05$). Among the nitrogen sources, the best growth was seen in yeast extract with compact mycelial density while the least growth was observed in urea. Glutamic acid was found to be the best amino acid for the mycelial growth with compact mycelial density while serine showed least growth with thin mycelial density. Out of 5 vitamins tested, thiamine showed the best growth stimulatory effect with compact mycelial density while folic acid showed the least growth effect with somewhat thin mycelial density. The carbon-to-nitrogen ratio of 5:1 favored the best growth with somewhat compact mycelial density while the least growth was observed in 1:1 ratio. Based on their popularity and capability to utilize lignocellulosic substrates, there is a need to pursue further work for cultivation of this mushroom.

Keywords: Artificial cultivation, Mycelial texture, Nutrients, Termitophilous fungi

Introduction

Termitophilous fungi comprise a monophyletic group of tropical gilled mushrooms with a single genus *Termitomyces*. They are unique, obligatory symbionts growing in close and intimate association with termites. They contain higher dry matter, protein and fiber, but contain lower amount of fat and carbohydrates. They are rich sources of important minerals, like phosphorus, potassium, calcium, copper, manganese, zinc, magnesium, sodium and iron. Besides nutrition, the species possess high medicinal value (Aryal, 2015). They are good sources of bioactive compounds (Aryal & Budhathoki, 2013). Due to high concentration of diverse phytochemicals, they are used in drug development (Aryal & Budhathoki, 2014).

Growth of fungi is measured in terms of changes in number of cells, in linear dimensions, in cell mass, in cell volume or in amount of some cellular components (Bilgrami & Verma, 1981). Various

environmental and biochemical factors affect growth; among them nutrients have significant influence on mycelial growth (Hawker, 1957; Bilgrami & Verma, 1981; Kaul, 1999). Fungi receive their food either parasitically or saprotrophically. Nutritional requirements for growth and reproduction are carbon, nitrogen, sulphur, phosphorus, vitamins and certain metallic or trace elements sources (Kaul, 1999).

The mycelium accumulates food materials and synthesizes complex substances such as proteins, polysaccharides, fats and enzymes (Hawker, 1957). Thus, to achieve maximum yield, an optimum culture medium containing the nutrients in suitable amounts and combination should be formulated. Cultural characteristics optimization procedure aims at developing such a medium.

Nutritional components of a medium can be varied and the impact of these changes assessed in terms of fungal growth. All of these factors must be

considered during optimization. The defined medium developed by such optimization process serves as a nutritional framework from which a production medium can be formulated. In a production medium, the nutritional components of the defined medium are replaced with low-cost, complex substrates. Use of this directed optimization method will be useful in developing production media for commercial mycelial production (Jackson, 1997). Thus, this study was conducted for the optimization of culture medium for mycelial growth of *Termitomyces albuminosus* (Berk.) R. Heim.

Materials and Methods

Study area

Exhaustive survey of various localities of Chitwan National Park, within the coordinate range of 27°35'08"N to 27°73'32"N latitude and 84°30'02"E to 84°59'27"E longitude, and altitudinal range of 220-330 m asl (Figure 1), was conducted for the collection of specimens of *Termitomyces albuminosus* (Berk.) R. Heim. The collected specimens were inoculated on culture plates and brought to the laboratory at Central Department of Botany (CDB), Tribhuvan University (TU) for further study.

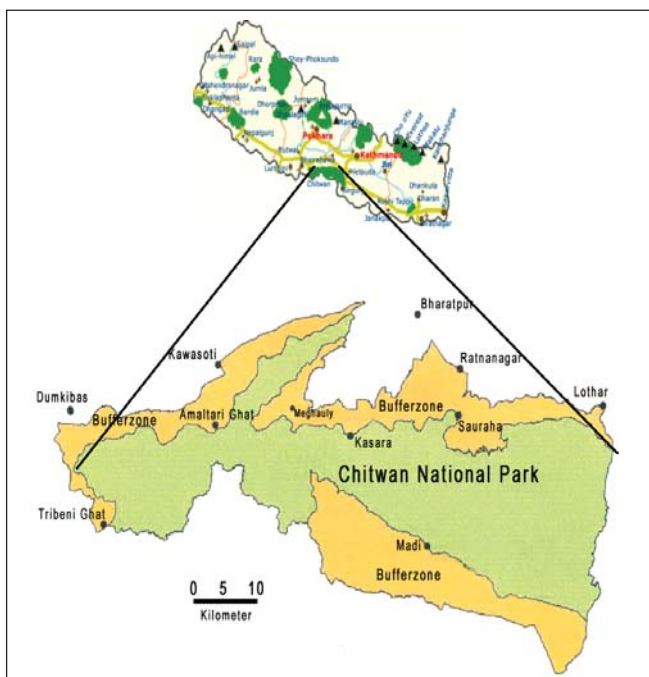


Figure 1: Chitwan National Park, Central Nepal.

Laboratory studies

The specimens were subjected to culture process using semi-solid and liquid media (Shim et al., 2005a) to observe the effects of various carbon sources, nitrogen sources, vitamins, amino acids and carbon-to-nitrogen ratios on mycelial growth at pH 6 and temperature $25\pm 2^\circ\text{C}$. Measurements of linear growths were done on semi-solid media by means of a standard scale. Weighing of mycelial mass growth in liquid medium was done using an analytical balance.

Sterilization of instruments: Glasswares were wrapped in aluminium foil after cleaning with water and sterilized in a hot air oven at $160\text{-}170^\circ\text{C}$ for 2 hrs. The culture media, cotton etc. were sterilized in an autoclave, at 121°C and 15 psi pressure for 30 min.

Isolation on potato dextrose agar (PDA) medium: Fresh and healthy fruiting body sample was surface sterilized by submerging in 0.4% sodium hypochlorite (NaOCl) for 1 min and was washed with sterilized water to remove residual NaOCl. Using a sterilized blade, its pileus and stipe were separated. The stipe was split longitudinally into two equal halves and approximately 3×6 mm pieces of tissue were taken from depth of $1/4^{\text{th}}$ thickness of the upper end of the stipe so that they contained neither outermost portion nor the central tissues of the stipe. The tissue pieces were inoculated on PDA plates. The plates were then sealed with parafilm tape and were covered with the help of aluminum foil.

The inoculated petriplates were incubated at $25\pm 2^\circ\text{C}$ in inverted position for seven days (Shim et al., 2005a; Shim et al., 2005b). At the end of the incubation period, mycelium was observed growing out of the inoculated tissue. Mycelial growth, colony diameter and colony texture were noted. Sub-cultures of the mycelium were done to obtain pure cultures. The pure cultures were transferred to PDA slants for further process.

Media preparation: Nutritional requirements for vegetative growth of mycelium of the species were studied by adopting the standard procedures

(Chandra & Purkayastha, 1977; Fasidi & Olorunmaiye, 1994; Shim et al., 2005a). The effects of variations in important components of culture media viz. carbon sources (dextrose, lactose, sucrose, fructose, mannitol, maltose and control), nitrogen sources (ammonium nitrate, peptone, urea, sodium nitrate, calcium nitrate, yeast extract and control), vitamins (ascorbic acid, nicotinic acid, folic acid, thiamine, D-riboflavin and control), amino acids (serine, leucine, valine, glutamic acid, arginine, aspartic acid and control) and C:N ratios were evaluated by maintaining five replicates for each treatment.

Statistical analysis

SPSS version 20.0 was used to analyze data. One-way analysis of variance (ANOVA) followed by Tukey's HSD test was performed to compare the results of different treatments.

Results and Discussion

Termitophilous mushrooms possess capability to produce lignocellulolytic enzymes, hence, has a potential to be efficient degrader of agro-wastes. Taprab et al. (2005) postulated that symbiotic fungi, viz. *Termitomyces* spp., produce laccases which are potentially involved in fungus combs and facilitate mushroom growth. Hence, for proper utilization of agro-wastes-biodegradation potential of *Termitomyces albuminosus* optimization of culture media for in vitro culture of this species would be beneficial. For the growth and reproduction of most fungi, the culture media must contain sources for carbon, nitrogen, sulphur, phosphorus, vitamins and certain trace or metallic elements (Kaul, 1999). Thus, various nutrients were compared to identify their efficiencies as the sources of these elements for the mycelial growth of *Termitomyces albuminosus*.

Effects of carbon sources

Linear mycelial growth varied significantly with change in carbon sources at 5% level of significance; *Termitomyces albuminosus* showed differential preferences for carbon sources for its metabolism (df = 6, 28; F = 25.88; P < .001). Utilization varied

from one carbon source to another. The best linear growth was observed in maltose with somewhat compact mycelial density while the least growth was observed on lactose with compact mycelial density. However, in control medium, mycelial growth was better than in lactose. Dextrose, fructose and sucrose also stimulated good growth with somewhat compact mycelial density and better growth than control (Figure 2). However, both fresh weights (df = 6, 28; F = 1.28; P = .298), and dry weights (df = 6, 28; F = 0.914; P = .499) of mycelium in submerged culture did not vary significantly with change in carbon sources at 5% level of significance (Figure 3). It might be due to the lack of agitation, orbital incubation and continuous back up.

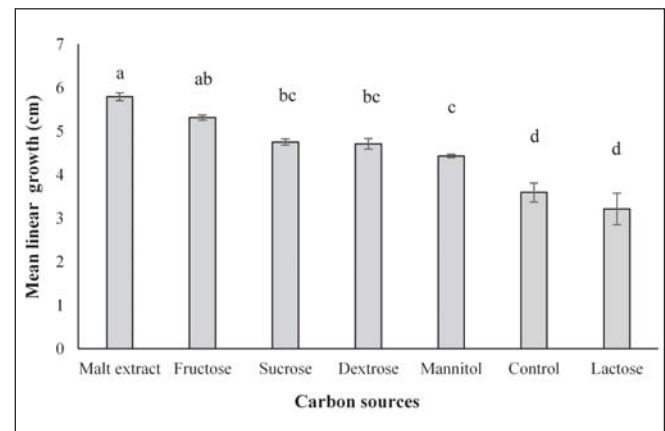


Figure 2: Linear mycelial growth on different carbon sources. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD test [$p < 0.05$].

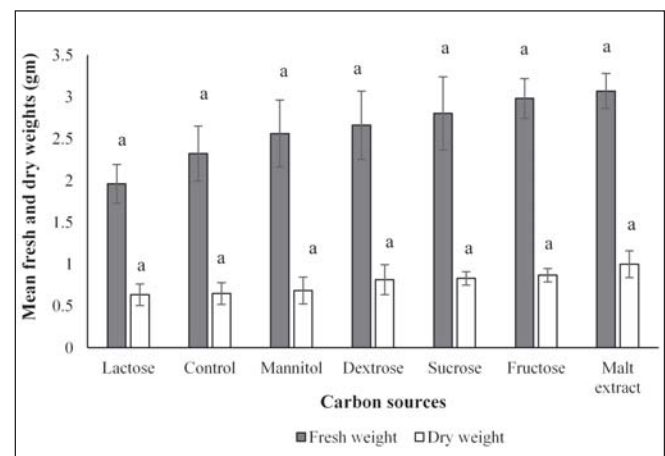


Figure 3: Mycelial growth on different carbon sources in submerged culture. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD test [$p < 0.05$].

According to Cochrane (1958), the ability of an organism to utilize carbohydrate depends on the types of enzymes produced by the organism. The results of the study showed that *Termitomyces albuminosus* produces enzymes which utilize maltose and fructose better than any other carbon sources. Shim et al. (2005b) reported that maltose was most suitable for mycelial growth in *Cystoderma amianthinum* var. *ruigosoreticulatum* while lactose was least suitable. According to Manjunathan and Kaviyaran (2011), for *Lentinus tuber-regium*, dextrose was most effective while lactose was least effective. Ayodele (2008) found that, in *Psathyrella atroumbonata*, glucose was the best carbon sources followed by maltose, starch and mannitol in decreasing order. The least growth was observed in lactose and control. Subba (1975) reported that, among the carbon sources, sucrose caused the best growth while lactose supported poor growth in *Choanephora infundibulifera* Chandra and Purkayastha (1977) reported that most of the tropical edible macro-fungi were in favor of utilizing glucose than other carbon sources. Jayasinghe et al. (2008) found that dextrose was the best carbon source on *Ganoderma lucidum*. This was closely followed by galactose and fructose which were not considerably different from each other. Jonathan et al. (2006) found that in *Pleurotus florida*, the most supportive sugars were among the monosaccharides. Aldohexose (glucose) stimulated greater biomass yield than ketohexose (fructose) under the same conditions. Generally, complex sugar and sugar alcohols produced little biomass with the exception of dextrin and mannitol. He attributed the lower mycelial production with polysaccharides and sugar alcohols to their complex nature since hydrolytic enzymes would be required to convert polysaccharides and sugar alcohols to simple sugar before they will enter respiratory pathways. Mannitol (a sugar alcohol) also supported good biomass yield of *P. florida*. The best carbon source suitable for promoting mycelial growth of *Lignosus rhinoceros* was glucose but with somewhat compact mycelial density according to Lai et al. (2011). Additionally, fructose and mannose also recorded a high radial mycelial growth rate. The combination of these three

carbon sources indicated that *L. rhinoceros* preferred monosaccharides. Imtiaj et al. (2008) found that, in *Schizophyllum commune*, the suitable mycelial growth was found in dextrin and fructose. However, the lowest growth of mycelium was obtained in lactose, mannose and sorbitol. Poudel (2012) reported that maltose and sucrose, as sources of carbon, were most suitable for in vitro mycelial growth in *Volvariella taylorii*. Acharya (2012) reported malt extract as the best carbon source for the optimum growth of *Amanita chepangiana*.

Effects of nitrogen sources

Linear growth varied significantly with change in nitrogen source at 5% level of significance ($df = 6, 28; F = 147.39; P < .001$). The best linear growth was seen with compact mycelium density in yeast extract while the least growth with compact mycelial density was observed in urea (Figure 4). Similarly, both fresh weights ($df = 6, 28; F = 29.87; P < .001$), and dry weights ($df = 6, 28; F = 2.53; P = .043$) of mycelium in submerged culture also varied significantly with change in nitrogen source at 5% level of significance. Maximum fresh weight was observed in yeast extract while minimum was observed in urea. However, in control treatment, mycelia had better growth than in urea with somewhat compact mycelial density (Figure 5).

Among the organic and inorganic nitrogen sources, *Termitomyces albuminosus* utilized organic nitrogen

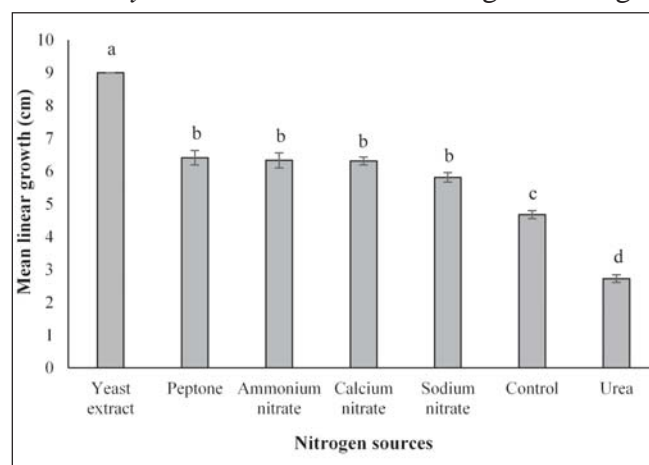


Figure 4: Linear mycelial growths on different nitrogen sources. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

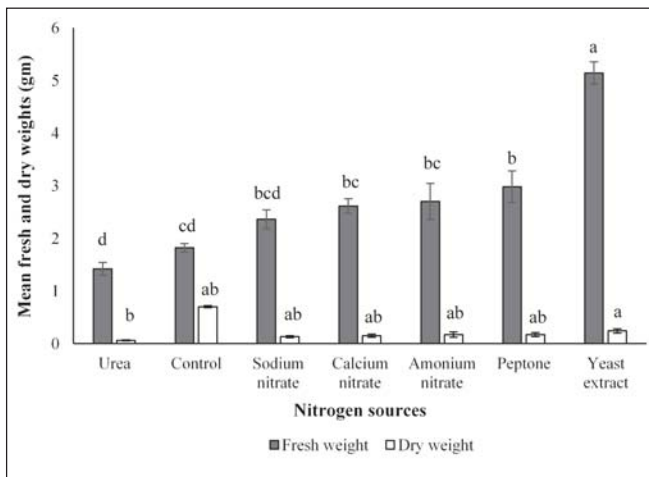


Figure 5: Mycelial growth on different nitrogen sources in submerged culture. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

better than inorganic nitrogen. This finding was similar to that of Ayodele (2008), who reported that *Psathyrella atroumbonata* showed preference for organic nitrogen than inorganic nitrogen with the best yield in yeast extract and the least yields in sodium nitrate and ammonium nitrate. Manjunathan & Kaviyarasan (2011) also found that *Lentinus tuber-regium* utilized organic nitrogen better than inorganic nitrogen, yeast extract being the best for its mycelial growth in the same line. They suggested that the stimulatory effect of yeast extract in their study may have been due to its amino acids, protein and vitamins. According to Adebayo et al. (2011), comparatively, organic nitrogen supported optimum production of mycelium by *Pleurotus ostreatus* but when inorganic nitrogen was used, poor biomass growth was yielded. They reported urea as the best nitrogen source for biomass production by *P. ostreatus*. Jayasinghe et al. (2008) reported that *Ganoderma lucidum* showed optimum mycelial growth on ammonium acetate, glycine, arginine and calcium nitrate. They observed that inorganic nitrogen sources also enhanced the mycelial growth of *G. lucidum*. Subba (1975) found that, among the nitrogen sources, ammonium nitrogen and aspartic acid caused the best growth while nitrate nitrogen was found to stimulate moderate growth in *Choanephora infundibulifera* Jonathan et al. (2006) found that, in *Pleurotus florida*, inorganic compounds supported moderate biomass production.

The best biomass yield was found with ammonium nitrate closely followed by potassium nitrate. Among the complex nitrogen compounds, yeast extract and casein hydrolysate supported significant biomass. Lai et al. (2011) observed that the best nitrogen source was potassium nitrate for promoting mycelial growth of *Lignosus rhinoceros*. Intiaj et al. (2008) found that the most suitable nitrogen sources were calcium nitrate, potassium nitrate, and alanine, and the most unsuitable were ammonium phosphate, histidine, urea and arginine for growth of *Schizophyllum commune* Shim et al. (2005a) found that the best nitrogen source was glycine for *Macrolepiota procera*. However, in this study, urea, despite being an organic nitrogen source, showed the least support for the mycelial growth of the mushroom species under study. Poudel (2012) reported yeast extract and peptone as the most suitable sources of nitrogen for *in vitro* mycelial growth in *Volvariella taylorii*. Acharya (2012) observed sodium nitrate as optimum nitrogen source for the mycelial growth of *Amanita chepangiana*.

Effects of vitamins

Linear growth varied significantly with change in vitamin in the culture medium at 5% level of significance ($df = 5, 24; F = 6.37; p < .001$). Out of five vitamins tested, the highest linear growth was found in thiamine and the lowest in folic acid ($p < .05$). Ascorbic acid and D-priotine also stimulated good growth with somewhat thin mycelia density and better growth than control (Figure 6).

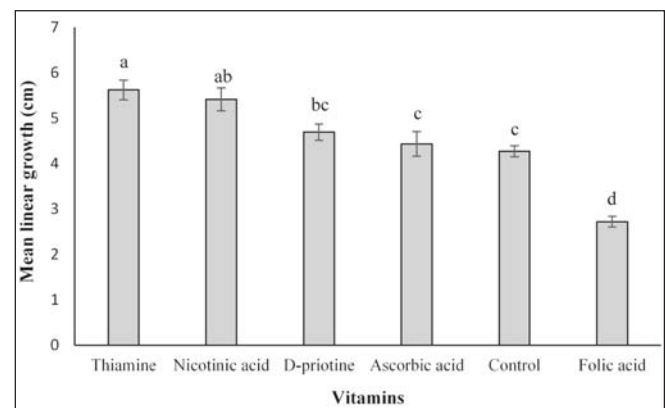


Figure 6: Linear mycelial growths on different vitamins. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

Likewise, fresh weight of mycelium in liquid medium also varied significantly with change in vitamin sources at 5% level of significance ($df = 5, 24$; $F = 8.87$; $p < .001$). Maximum weight was observed in D-priotine while minimum weight was observed in ascorbic acid. However, dry weight of mycelium in submerged culture did not vary significantly with change in vitamins source at 5% level of significance ($df = 5, 24$; $F = 2.28$; $p = 0.078$; Figure 7).

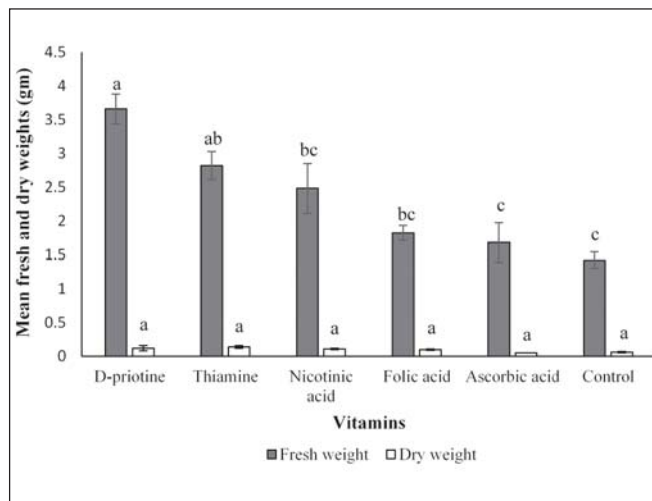


Figure 7: Mycelial growth on different vitamins in submerged cultures. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

Among different vitamins in this study, thiamine was observed to produce the best, linear growth in *Termitomyces albuminosus*. Control treatment with somewhat compact mycelia density, ascorbic acid with somewhat thin mycelial density and folic acid with somewhat thin mycelial density showed the least growth (Figure 4). This finding was in line with the report of Ayodele (2008), who proved that thiamine was the best vitamin, followed by nicotinic acid and riboflavin for *Psathyrella atroumbonata* while the least growth was observed in folic acid. This observation was also in line with the report of Landers (1964) who found that thiamine stimulated mycelial growth of *Cercospora arachidicola* in liquid culture. Manjunathan and Kaviyarasan (2011) also proved that thiamine was the best among the vitamins followed by biotin and tocopherol for *Lentinus tuber-regium* Adebayo et al. (2007) found

that riboflavin and pyridoxine promoted the mycelial growth for *Schizophyllum commune*. Adebayo et al. (2011) observed that among the vitamins tested, ascorbic acid and folic acid had the highest stimulatory effect on mycelia of *Pleurotus ostreatus*. Pokhrel et al. (2009a) reported that riboflavin and thiamine were the most stimulatory vitamins for mycelial growth of *Lyophyllum decastes*. Poudel (2012) observed that serine, as vitamin, caused the best in vitro mycelial growth in *Volvariella taylorii*. Acharya (2012) reported that, in *Amanita chepangiana*, ascorbic acid caused optimum mycelial growth.

Effects of amino acids

Linear growth varied significantly with change in amino acid in the culture medium at 5% level of significance ($df = 6, 28$; $F = 11.56$; $p < .001$). Among them, glutamic acid proved to stimulate the maximum linear growth with somewhat thin mycelial density while folic acid caused the least growth with thin mycelial density. The control treatment showed intermediate growth with somewhat thick mycelial density. Leucine, valine, aspartic acid and serine showed medium linear growths with somewhat thin mycelial densities (Figure 8). However, fresh weights ($df = 6, 28$; $F = 0.958$; $p = .471$) and dry weights ($df = 6, 28$; $F = 0.409$; $p = .866$) of mycelia in submerged cultures did not vary significantly with change in amino acid source at 5% level of significance (Figure 9).

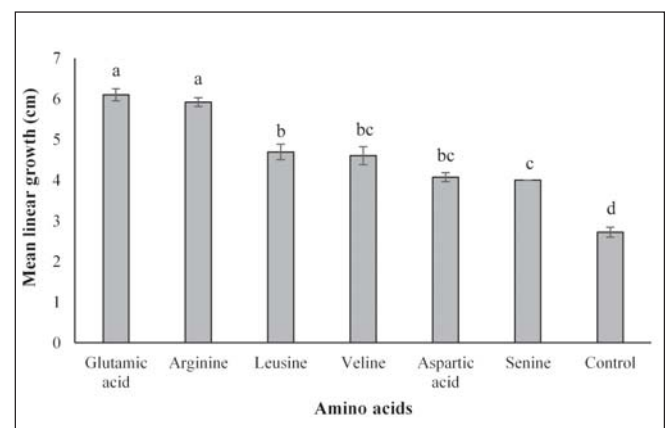


Figure 8: Linear mycelial growths on different amino acids. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

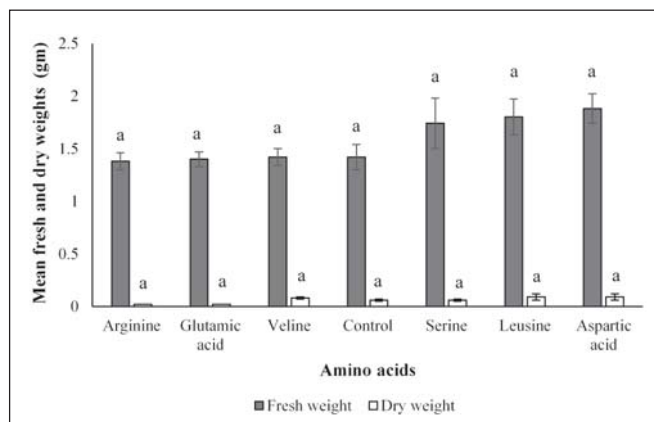


Figure 9: Mycelial growth on different amino acids in submerged culture. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

According to Ayodele (2008), asparagine proved to be the best amino acid followed by aspartic acid for *Psathyrella atroumbonata*. According to Chandra & Purkayastha (1977), amides such as asparagine and aspartic acid have been employed in increasing mycelial growth and fruiting body production in *Agaricus bisporus*. Hayes (1981) observed that both higher and lower concentrations of amino acids were found to be either ineffective or inhibitory for the growth of mycelia. According to Manjunathan & Kaviyaran (2011) glycine proved to be the best amino acid followed by L-ornithine monohydrochloride for the mycelial growth of *Lentinus tuber-regium* Hayes (1981) reported that higher and lower concentrations of these amino acids were found to be either ineffective or inhibitory for growth. Pokhrel et al. (2009b) investigated the effects of light, moisture, amino acids, vitamins and mineral nutrients on mycelial growth of *Lyophyllum decastes* on solid media. Glutamic acid was the best amino acid among the eight amino acids tested. Adebayo et al. (2011) observed that some of the amino acids had a stimulatory effect on mycelial yield of *Pleurotus ostreatus*. Optimum mycelial growth was recorded when glycine was used. Jonathan et al. (2006) found that in *Pleurotus florida*, although all the twelve amino acids used in his study enhanced biomass production, the most stimulatory amino acid was tryptophan. Pokhrel et al. (2009b) found that proline and glutamic acid showed significant growth than other amino acids tested in

Lyophyllum decastes. Poudel (2012) concluded that serine was most suitable amino acid for mycelial growth of *Volvariella taylorii*. According to Acharya (2012), arginine was found to cause maximum in vitro mycelia growth in *Amanita chepangiana*.

Effects of carbon-to-nitrogen (C:N) ratios

The effects of different carbon-to-nitrogen ratios on linear growths varied significantly with the change in carbon-to-nitrogen ratio in the culture medium at 5% level of significance ($df = 9,40$; $F = 8.656$; $p < .001$). The linear growth rate increased with the proportional increase in carbon up to C:N ratio of 5:1. C:N ratio of 1:1 showed the least linear growth with somewhat compact and thin mycelial densities respectively. In the same manner, when the ratio of nitrogen was increased, linear growth rate also increased gradually up to the C:N ratio of 1:5. Hence, the best linear growths were observed when the C:N was 5:1 and 1:5. However, better growth was observed in control than in 1:1 ratio (Figure 10). Similarly, both fresh weights ($df = 9,40$; $F = 7.44$; $p < .001$) and dry weights ($df = 9,40$; $F = 2.876$; $p = .010$) of mycelium in submerged culture also varied significantly with change in carbon-to-nitrogen ratio at 5% level of significance (Figure 11). Maximum fresh weight was observed in C:N ratio of 5:1 while minimum was observed in 1:1 ratio. However, mycelial fresh weight in control was observed to be better than in C:N ratios of 1:1, 1:2 and 1:5 while it was equal with 2:1 and 3:1 ratio. Similarly, maximum dry weight was observed in C:N ratio of 1:2 and minimum in 1:1 ratio.

It was observed that the mycelial growth of the fungi under study increased with the proportional increase of carbon in comparison to nitrogen up to C:N ratio of 5:1. Control treatment and C:N ratio of 1:1 showed the least growth with somewhat compact and thin mycelial density respectively. When the ratio of nitrogen was increased, mycelial growth was also found to increase gradually in the same manner, up to C:N ratio 1:4. However, the best growth observed was when the C:N ratio was 5:1 (Figure 10). Lai et al. (2011) reported that C:N ratio of 10:1 was favorable for mycelial growth of *Lignosus*

rhinoceros. Notably, no growth occurred at 40:1 C:N ratio. Shim et al. (2003) observed that the optimum C:N ratio suitable for favorable growth of *Paecilomyces fumosoroseus* were on culture media which were adjusted to C:N ratio of 40:1. Shim et al. (2005b) found the C:N ratio of 30:1 was the best for the mycelial growth of *Cystoderma amianthinum* var. *ruigosoreticulatum*. Chandra & Purkayastha (1977) observed C:N ratio of 3:1, 5:1 and 1:3 were favorable for the growth of *Lentinus subnudus*, *Volvariella volvacea* and *Termitomyces eurhizus* respectively. According to Manjunathan & Kaviyaran (2011), carbon-to-nitrogen ratios of 1:3 and 1:5 were the best for the growth of *Lentinus tuber-regium* (Fr.) Fr. Ayodele (2008) found that the C:N ratios of 4:1 and 1:4 supported the best growth

of *Psathyrella atroumbonata*. According to Poudel (2012), C:N ratios of 5:1, 1:4, and 1:5 induced optimum mycelial growths in *Volvariella taylorii* (Berk. & Broome) Singer. Acharya (2012) has concluded 4:1 as the optimum carbon-to-nitrogen ratio for the growth of *Amanita chepangiana* Tulloss & Bhandary.

Conclusion

In view of the popularity of *Termitomyces albuminosus* with rural masses and its capability to utilize lignocellulosic substrates, there is need to pursue further work for the cultivation of this mushroom species, so as to utilize its biological efficiency in converting agro-forestry by-products and other such substrates available in plenty in different parts of the country. Through domestication and bulk production, it can be made easily available to the consumers. This can generate revenue to the country and, at the same time, will reduce collection pressure on natural habitat. This is how the dual purpose of meeting the human demand and conservation aspects can be targeted simultaneously which is so vital for conservation of the natural ecosystem and sustainability. Therefore, there is need to domesticate this mushroom species. In this regard, the findings of this research indicate that the optimal medium, comprising of maltose as carbon source, yeast extract as nitrogen source, thiamine as vitamin, serine as amino acid and C:N ratio of 5:1, may be suitable for in vitro mycelial growth of this mushroom species.

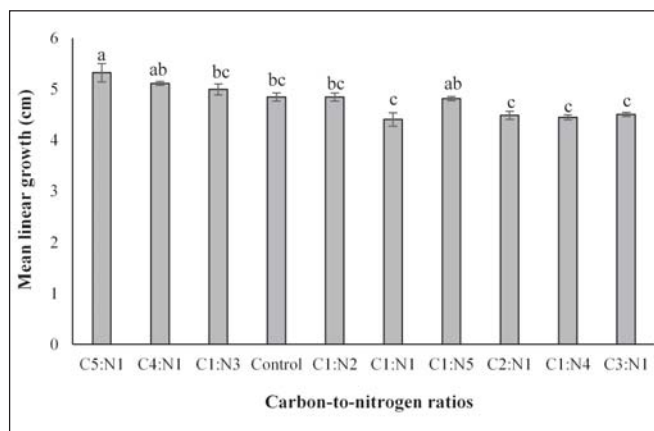


Figure 10. Linear mycelial growths on different carbon-to-nitrogen ratios. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

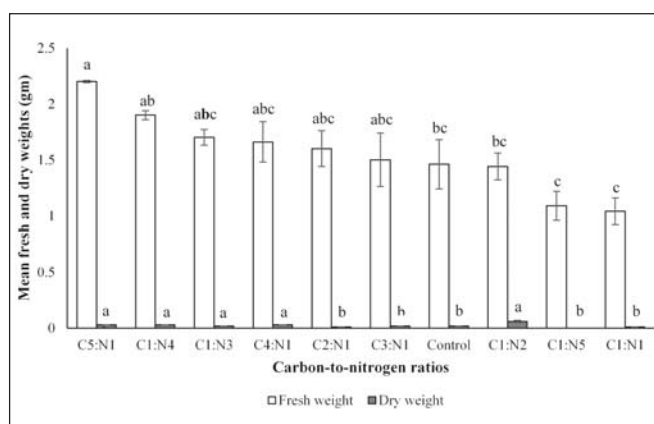


Figure 11. Mycelial growth on different amino acids in submerged culture. Different letters above the error bars indicate significant differences in mycelial growths among the treatments as determined by Tukey's HSD Test [$p < 0.05$].

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