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Investigation on mycelial growth requirements of *Cantharellus cibarius* **under laboratory conditions**

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Abstract

The golden chanterelle represents one of the commonly found, edible mushrooms that is highly valued in various cuisines. The present study focused on assessing the requirements of *Cantharellus cibarius* such as pH, temperature, as well as the carbon and nitrogen sources for mycelial growth. Optimization of the growth parameters was carried out by one-factor-ata-time method. The optimal pH and temperature were determined to be 6.0 and 22.5 °C, respectively. Among the various carbon sources studied, sucrose at a concentration of 2% gave maximum mycelial growth and proved to be the most suitable one. Amongst the nitrogen sources studied, peptone, ammonium sulphate, and sodium nitrate, gave the maximum mycelial growth at an optimized concentration of 0.5%. In the presence of beef extract and yeast extract, a change in colony pigmentation from yellow to dark grey was observed. Finally, the carbon to nitrogen ratio of 2:0.5 proved to be optimal for mycelial growth. This study is the frst report on the optimisation of in vitro growth requirements of *C. cibarius*.

Keywords *Cantharellus cibarius* · Carbon source · Nitrogen source · Pigmentation · Edible fungi · C:N ratio

Introduction

Wild mushrooms have been valued for their nutritious and appetising qualities for several decades in various regions of the world (Kozarski et al. [2015](#page-6-0)). *Cantharellus cibarius,* also known as the golden chanterelle is a commonly found, wild, edible, ectomycorrhizal mushroom (Pilz et al. [2003](#page-6-1)). It belongs to the phylum *Basidiomycota* and the family *Cantharellacea*e (Nyman et al. [2016\)](#page-6-2). It is well-recognised as a delicious food from Scandinavia to the Mediterranean in Europe (Kozarski et al. [2015](#page-6-0)). It ranks among the top fve selling mushrooms in Europe that is harvested from the wild

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for commercial purpose (Nowacka-Jechalke et al. [2018](#page-6-3)). The production of Chanterelle is estimated to be 1,50,000 to 2,00,000 tons per year; the marketplace value of which is around \$1.7 billion (Nowacka-Jechalke et al. [2018\)](#page-6-3). *C. cibarius* is considered as the most desirable amongst the edible wild mushrooms and is recognized as a delicious food. The color of *C. cibarius* ranges from orange to yellow and it gives a fruity, apricot-like aroma which is highly appreciated in cooking throughout Europe (Kozarski et al. [2015](#page-6-0)).

Owing to the signifcance of the fruiting bodies of mushroom in agriculture, ecology, and medicine, mushroombased functional foods have started gaining the attention of researchers (Wang et al. [2020\)](#page-6-4). For over 2000 years, several species of mushrooms have been used for medicinal purposes in the Asian countries (Nyman et al. [2016\)](#page-6-2). Today, several of them are used to treat diseases associated with the immune system, and also as supplementary medicines in the conventional treatment of cancer. These activities have been attributed to the presence of bioactives such as polysaccharides (Nyman et al. [2016\)](#page-6-2). Similar to these medicinal mushrooms, *C. cibarius* displays various promising biological activities such as antioxidant, neuroprotective (Lemieszek et al. [2018\)](#page-6-5), and antimicrobial (Kolundžić et al. [2017\)](#page-6-6) activities. Furthermore, *C. cibarius* has also been reported for its anticancer, immunomodulatory, anti-infammatory,

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and antiaging activities (Lemieszek et al. [2018](#page-6-5)). Chanterelle species have a rich macronutrient profle with high amounts of carbohydrates and proteins, while featuring only low amounts of fats (Kumari et al. [2011](#page-6-7); Kozarski et al. [2015](#page-6-0)). It contains 53.7% crude protein, 31.9% carbohydrates and 2.9% lipids of dry matter (Barros et al. [2008](#page-5-0)). With its high content of protein, carbohydrates, and dietary fbre, *C. cibarius* shows a substantial nutritive value. Furthermore, *C. cibarius* is also rich in minerals such as calcium, phosphorous, and potassium as well as vitamin D (Nowacka-Jechalke et al. [2018](#page-6-3)). Chanterelles exhibit good antioxidant and free radical scavenging activity (Ebrahimzadeh et al. [2010\)](#page-6-8) owing to the presence of phenolic acids, specifcally cafeic acid and catechins (Palacios et al. [2011](#page-6-9)). Owing to all the benefts that Chanterelles offer, studying their growth in controlled conditions to obtain maximum production becomes crucial. There has been a considerable interest in pigment production by edible mushrooms. In case of *C. cibarius*, the characteristic color is attributed mainly to the presence of carotenoid pigments. The pigment mixture consists of *β*-carotene as a major constituent along with lycopene, *α*-carotene, and the xanthophyll canthaxanthin (Haxo [1950;](#page-6-10) Sandmann and Misawa [2002\)](#page-6-11).

Although *C. cibarius* species grow in a variety of soils, the best growth is observed in well-drained forest soils (pH 4–5.5) with low nitrogen content. Despite the wide-spread occurrence of *C. cibarius*, cultivating it has been a difficult task, the reason behind which has been speculated to be the presence of bacteria and other foreign microbes within the sporocarp tissues (Kozarski et al. [2015\)](#page-6-0). The complex symbiotic association between chanterelle mushrooms and its host trees makes its natural cultivation nearly impossible (Kozarski et al. [2015](#page-6-0)).

Presently, the information on the utilization of various carbon and nitrogen sources by *C. cibarius* mycelium is scarce. Furthermore, the effect of nutritional components on pigment production by the mycelium has so far caught little attention and needs to be investigated.

The present investigation aims to determine the optimal mycelial growth conditions (pH, temperature, carbon and nitrogen source) of *C. cibarius* under laboratory conditions. To the best of our knowledge, this is the frst report on investigation of nutritional requirements of *C. cibarius* for its growth as well as its efect on the pigmentation.

Materials and methods

Materials

Murashige and Skoog (MS) growth media, carbon (glucose, fructose, galactose, sucrose, and xylose) and nitrogen (beef extract, yeast extract, peptone, ammonium nitrate,

ammonium sulphate, ammonium citrate, sodium nitrate, and potassium nitrate) sources were purchased from Sigma Aldrich, USA. All the other chemicals used were of analytical reagent grade.

Collection of *C. cibarius* **and preparation of pure culture**

The fruiting bodies of *C. cibarius* were collected from forests in Juva, Finland. Pure culture of the mycelia was obtained using Murashige and Skoog (MS) media (Pamire and Parawira [2020](#page-6-12)) (Table S1, supplementary data) and were incubated in the dark at 22 °C for 2 weeks.

Optimization of the nutritional requirements/ growth parameters

The optimal nutritional requirements for the growth of the mycelia were determined using agar plate technique (Nasim et al. [2001](#page-6-13)) by one-factor-at-a-time (OFAT) method. From the advancing (actively growing) margins of the subcultures, mycelial discs of 5 mm diameter were cut with a cork borer and carefully placed at the centre of new Petri plates (9 cm diameter) flled with sterile MS media. The inoculated Petri plates were sealed with a paraflm and incubated at room temperature (22 °C) for 2 weeks in the dark. The parameter showing the highest growth of the mycelia (in terms of diameter) was selected as optimum and kept constant for the rest of the study. Mycelial colony diameter (mm) was determined by measuring along two axes with a meter scale and an average was calculated.

Optimum pH requirement of the media was determined by growing the mycelium in the pH range of 5 to 8. The pH adjustments were made using 1 M NaOH and 1 M HCl. The optimisation of incubation temperature was carried out in the range of $15-30$ °C.

In order to investigate the effect of nitrogen sources on the mycelial growth, various nitrogen sources such as beef extract, yeast extract, peptone, ammonium nitrate, ammonium sulphate, ammonium citrate, sodium nitrate, and potassium nitrate were added to MS media at a concentration of 1% (w/v). After selecting the optimal nitrogen source, its optimal concentration was studied in the range of 0.5–5% (w/v). For the optimization of carbon source, MS media was supplemented with 5 diferent sugars viz. glucose, fructose, galactose, sucrose, and xylose. The sugars were flter-sterilized and then aseptically added at a concentration of 2% (w/v) to the MS medium. The medium without any carbon source served to be the control. After selecting the optimal carbon source, its optimal concentration was studied in the range of $0.5-5\%$ (w/v). Finally, the effect of carbon to nitrogen ratio was evaluated by varying the range from 2:0.25 to 2:2.

Growth characteristics such as colony diameter, and colony pigmentation were observed for each varying parameter. The colony diameter was a direct measure of the suitability of the respective parameter.

Statistical analysis

All the experiments were performed in triplicates, unless stated otherwise. The statistical analysis of the data obtained was performed using IBM® SPSS® version 20. The data was subjected to Tukey's HSD test $(p < 0.05)$.

Results and discussion

Efect of temperature on mycelial growth of *C. cibarius*

Factors such as temperature and pH play a signifcant role in mycelial growth. Several studies have reported the efect of temperature on the growth of edible mushrooms (Zervakis et al. [2001](#page-6-14); Hoa and Wang [2015](#page-6-15)). Thus, it was crucial to take into consideration the efect of temperature on mycelial growth of *C. cibarius*. The colony growth of *C. cibarius* was studied at a varying temperature range $(15-30 \degree C)$ (Fig. S1, supplementary data). Significantly low mycelial growth was observed at 15 °C, which may be attributed to lower metabolic activities of the fungus that are responsible for the absorption and assimilation of essential nutrients required for growth (Mensah-Attipoe and Toyinbo [2019](#page-6-16)). The colony diameter was observed to increase with an increase in the temperature from 15 to 22.5 °C. The growth was signifcantly highest at an incubation temperature of 22.5 °C. At a temperature of 30 °C no mycelial growth was detected, which may be attributed to denaturation of some essential enzymes catalysing the metabolic processes.

Kalyoncu et al. (Kalyoncu et al. [2009\)](#page-6-17) studied the effect of temperature on the mycelial growth of the edible fungi of *Morchella* species. The authors reported similar results where the highest growth was observed in the temperature range of 20–25 °C, which dropped signifcantly with a further increase up to 30 °C. A few other edible fungi such as *Pleurotus eryngii* and *Auricularia auricula-judae* have also shown a temperature optima ranging around 25 °C, which was infuenced by the nutrient medium used (Zervakis et al. [2001\)](#page-6-14). However, the temperature dependence of mycelial growth is species-specifc where some species might show optimal growth at temperature of 30–35 °C. For instance, mycelia of the edible fungi *Volvariella volvacea* grew faster at temperature as high as 35 °C (Zervakis et al. [2001\)](#page-6-14).

Efect of pH on mycelial growth of *C. cibarius*

The pH of the culture media also plays a signifcant role in the growth of the mycelia of basidiomycetes. Although fungi are capable of growing over a wide range of pH (Neto et al. [2009](#page-6-18)), the optimum pH value of the growth media is crucial to obtain maximum mycelial growth. The pH of the medium was found to substantially affect the mycelial growth and pigment production of *C. cibarius*. The mycelial growth significantly $(p < 0.05)$ increased with an increase in the pH from 5 to 6. The maximum growth was observed at a pH of 6. An increase in the pH beyond this, lead to a signifcant decrease in the mycelial growth. The lowest mycelial growth was observed at pH 8 (Fig. S2, supplementary data). These results are in accordance with a previous report on the pH optima for the mycelial growth of *C. cibarius*, where the highest growth was observed at a pH of 5.5 and 6, whereas the growth dropped at lower or higher pH values (Straatsma and van Griensven [1986](#page-6-19)). Similar study was performed by Kalyoncu et al. ([2009](#page-6-17)) on the growth of various species of the edible fungi of the genus *Morchella*. Alike the results obtained in this study, the authors reported a pH optima of 6 for the mycelial growth of *M. intermedia*, and the lowest growth was observed at a pH of 8.

Efect of carbon source and concentration on mycelial growth

The substrates that are used to cultivate mushrooms greatly afect their chemical, sensorial, and functional characteristics (Bellettini et al. [2019\)](#page-5-1). Considering this, it becomes crucial to study the efect of the carbon and nitrogen sources on the growth of mushrooms. The efect of carbon sources such as glucose, fructose, sucrose, galactose, and xylose was studied on the mycelial growth of *C. cibarius* (Fig. [1](#page-2-0))*.* The maximum growth was observed in case of sucrose as

Fig. 1 Effect of carbon source on mycelial growth of *C. cibarius*. Data is given as mean of triplicates and error bars represent standard deviation. Data with the same data labels does not vary signifcantly (*p*<0.05; Tukey's HSD test)

the carbon source, followed by fructose, glucose, xylose, and galactose, in that order. Sucrose showed growth twice as much as the control. In case of glucose and xylose, the growth was statistically similar. Utilization of sucrose could be due to the activity of invertase enzyme leading to maximum mycelial growth.

On the basis of visual inspection, cultures grown on sucrose showed the most intense yellow pigment production, followed by fructose and glucose (Fig. [2](#page-3-0)). Thus, the pigmentation could be correlated with the mycelial growth which is in turn dependent on the carbon source and its utilisation. Chen and Johns ([1994\)](#page-6-20) studied the efect of carbon sources such as glucose and maltose on the pigment production by *Monascus purpureus*. The authors observed the highest pigmentation using maltose as the carbon source, especially with peptone as the nitrogen source. Thus, the variation in the carbon source afects not only the mycelial growth, but also the pigmentation.

After choosing sucrose as the optimal carbon source, its concentration in the nutrient medium was studied in the range of 0.5–5% (Fig. S3, supplementary data). Signifcantly low growth was observed with 0.5% sucrose, and increased with an increase in the concentration up to 2%. Further increase in the sucrose concentration up to 5% lead to a decrease in the mycelial growth. Such a drop in the mycelial growth with an increase in the concentration of carbon source has been reported (Yuan et al. [2012](#page-6-21); Itoo and Reshi [2014\)](#page-6-22). Hence, 2% was selected as the optimal sucrose concentration.

Efect of nitrogen source on mycelial growth and pigmentation

Apart from the carbon source, the growth and pigmentation of fungi is also dependent on the nitrogen source. In the present study, the mycelial growth of *C. cibarius* was evaluated for various nitrogen sources such as beef extract, yeast extract, peptone, ammonium nitrate, ammonium sulphate, ammonium citrate, sodium nitrate, and potassium nitrate. As shown in Fig. [3,](#page-4-0) the highest mycelial growth was seen and was statistically similar $(p < 0.05)$ in case of ammonium sulphate, peptone, and sodium nitrate. The lowest growth was seen in case of yeast extract, followed by beef extract, ammonium citrate, ammonium nitrate, and potassium nitrate, in that order. Ammonium sulphate has been reported to show a positive effect ($p \le 0.05$) on the biomass and phenolic content gain (Schmidt and Furlong [2012\)](#page-6-23). Ammonium sulphate has also been reported as a better supplement for primordial initiation in case of *Pleurotus forida* cultivation (Naraian et al. [2009](#page-6-24)). Considering this ammonium sulphate was chosen as the suitable nitrogen source.

Researchers have shown that pigment production by mushrooms is afected by factors such as the nature of nitrogen source and pH of the growth media (Chen and Johns [1993\)](#page-6-25). In the present study, the nitrogen source afected not only the mycelial growth, but also the pigmentation. The pigmentation of the colonies did not vary with the use of peptone, ammonium nitrate, ammonium sulphate, ammonium citrate, sodium nitrate, and potassium nitrate as

Fig. 2 Comparison of the yellow/orange pigmentation observed in *C. cibarius* colonies with the use of fructose, sucrose, and glucose as carbon sources, as compared to control

Fig. 3 Efect of nitrogen source on mycelial growth of *C. cibarius*. Data is given as mean of triplicates and error bars represent standard deviation. Data with the same data labels does not vary significantly $(p < 0.05)$; Tukey's HSD test)

Nitrogen source

Table 1 Efect of nitrogen source on the pigmentation of *C. cibarius*

Nitrogen source	Colony pigmentation
Beef extract	Dark greyish
Yeast extract	Dark greyish
Peptone	Dark yellow
Ammonium nitrate	Yellowish brown
Ammonium sulphate	Yellowish brown
Ammonium citrate	Yellowish green
Sodium nitrate	Light yellow
Potassium nitrate	Yellow

the nitrogen sources. However, in case of beef and yeast extracts, the colour of the colonies was observed to be dark grey (Table [1](#page-4-1)). The efect of beef extract and yeast extract on pigmentation has already been reported in case of the fungi *Fusarium verticillioides* (Boonyapranai et al. [2008\)](#page-5-2). The change caused by complex nitrogen sources (beef extract and yeast extract) may be due to alteration in pH due to metabolites or bio-actives released by the mycelium (Fig. [4](#page-4-2)).

The effect of the concentration of ammonium sulphate was also studied on the mycelial growth of *C. cibarius* in the range of 0.5–5% (Fig. S4, supplementary data). The optimal

Fig. 4 Grey pigmentation observed in *C. cibarius* colonies with the use of yeast and beef extract

mycelial growth was observed at the lowest tested concentration i.e. 0.5%. Further increase in the concentration lead to a signifcant decrease in the mycelial growth. Such decrease in the mycelial colony diameter with an increase in the concentration of nitrogen sources has been reported in case of *Rhizoctonia solani* (Koehler and Miller [2017](#page-6-26)).

Efect of carbon to nitrogen (C:N) ratio on the mycelial growth

Apart from the source and concentrations of carbon and nitrogen sources, the carbon:nitrogen (C:N) ratio in the growth medium also acts as a signifcant factor in mycelial growth in anexic cultivation (Mantovani et al. [2007](#page-6-27)). The C:N ratio has also been reported to affect the exopolysaccharide production (Kim et al. [2002\)](#page-6-28). The presence of nitrogen in excess, or its lack leads to compromised fungal growth. Excessive nitrogen causes the reduction in the substrate compounds such as ammonium sulphate and urea, which are converted to ammonia (Mantovani et al. [2007](#page-6-27)). The toxicity of ammonia depends on its concentration, and hence excessive ammonia could substantially hinder the mycelial growth. On the other hand, excessive carbon is readily converted and stored as glycogen (Mantovani et al. [2007\)](#page-6-27). This explains the higher proportion of carbon in culture media used for fungi.

In the present study, the C:N ratio was studied ranging from 2:0.25 to 2:2 $(\%)$ (Fig. [5\)](#page-5-3). The optimal mycelial growth was observed for C:N ratios of 2:0.5 and 2:1. This is close to 20:1 which is the optimal C:N ratio for mycelial growth of most of the fungal species. For instance, for *A. bisporous*, the optimal C:N ratio ranges between 15:1 and 17:1 (Mantovani et al. [2007](#page-6-27)). In another study (Dong and Yao [2005\)](#page-6-29) a C:N ratio of 12:1 was reported for the optimal growth of the medicinal fungi *Cordyceps sinensis*. A ratio of 12:1 has also been reported as optimal for the growth of the fungi

Fig. 5 Effect of carbon to nitrogen (C:N) ratio on mycelial growth of *C. cibarius*. Data is given as mean of triplicates and error bars represent standard deviation. Data with the same data labels does not vary significantly $(p < 0.05$; Tukey's HSD test)

Paecilomyces sinclairii (Kim et al. [2002\)](#page-6-28). However, in case of certain fungi such as *Pleurotus tuber-regium*, an optimal C:N ratio as high as 24:1 has been reported (Wu et al. [2004](#page-6-30)). In the present study, increasing the C:N ratio from 2:1 to 2:2 lead to a significant $(p < 0.05)$ decrease in the mycelial growth. This could be due to generation of ammonia from excessive ammonium sulphate, leading to a compromised growth.

Conclusion

Although extensive research has been carried out on various edible fungi, *C. cibarius* remains less studied for its growth requirements. The present study focused on assessing the growth requirements of the edible mushroom *C. cibarius.* The growth was signifcantly afected with variations in the parameters. The use of yeast and beef extracts as nitrogen sources showed substantial variations in the pigmentation of the colonies, where the colour changed from yellow/orange to dark grey. Further investigation is required for understanding the change in metabolism of such complex organic nitrogen sources. This study could be considered as a base to lead to additional insights into optimum growth requirements. We emphasize on the fact that *C. cibarius* is a popular edible mushroom and has great economic value. Knowledge of the nutritional requirements of *C. cibarius* would be helpful in improving its cultivation technology. Further investigations in liquid media with varying carbon and nitrogen sources needs to be carried out to study pH variations and release of metabolites.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no confict of interest.

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