



Cultivation of black poplar mushroom, *Cyclocybe aegerita*, on woody and non-woody lignocellulosic substrates with a high biological efficiency

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Abstract

The Black Poplar mushroom, *Cyclocybe aegerita*, is considered a high-quality mushroom due to its high nutritional value, delicious taste, and unique aroma. This fungus is commonly grown in East Asia and Europe. It has shown low biological efficiency (BE) on a majority of lignocellulosic substrates studied to date. This study aims to investigate the possibility of enhancing the BE of *C. aegerita* and to evaluate the effect of the carbon to nitrogen (C:N) ratio in the substrate. The performance of *C. aegerita* was determined using five woody and non-woody lignocellulosic substrates with a precisely determined C:N ratio. The result showed that the highest significant BE was 194%, obtained from a non-woody substrate composed of 78% wheat straw supplemented with 20% wheat bran ($p \leq 0.05$), followed by a woody substrate composed of 73% of wood chips, 10% of wheat bran, 10% of cottonseed, and 5% of wheat seed, which generated 123% BE. The remaining substrates were unable to produce more than 100% BE ($p \leq 0.05$). The mycelial growth of *C. aegerita* in the substrate as well as in the substrate-derived media was also significantly improved using a non-woody substrate consisting of 78% wheat straw supplemented with 20% wheat bran compared with other substrates ($p \leq 0.05$). Further analyses also revealed that an increased level of the C:N ratio resulted in a decline in the total fresh yield that was obtained from the substrates, so that the optimal C:N ratio was found to be within the range of 48–56%. These findings may have implications for improving the BE of *C. aegerita* and removing one of its most important barriers in commercial production.

Keywords – Black Poplar mushroom – C:N ratio – mycelial growth – wheat straw – yield performance

Introduction

Cyclocybe aegerita (V. Brig.) Vizzini (previously known as *Agrocybe aegerita*) is known commonly as the black poplar mushroom. This fungus has been reported to be grown in a variety of countries, including China, Europe, Japan, South Korea, and Taiwan (Frings et al. 2020). It is regarded as a high-quality culinary-medicinal mushroom due to its high nutritional value, deliciousness, and unique aroma (Jasińska & Siwulski 2021). The annual output of fresh products of this mushroom in China's Jiangxi province has exceeded 193,000 tons, with market size of one billion yuan per year (Wang et al. 2021). *Cyclocybe aegerita* has a curved and flattened cap, with a

diameter of 20 cm and is yellowish grey to greyish brown, while the gills are initially grey and turn grey as they mature. Mature fruiting bodies have a well-developed ring of membranes and are produced on the white stipe. Basidiospores usually turn chocolate brown when touched. *Cyclocybe aegerita* has a typical agaric morphology and passes through the entire life cycle under laboratory conditions to produce fruiting bodies (Orban et al. 2021). *Cyclocybe aegerita* has antibiotic, antioxidant, antitumor, antifungal, and cholesterol-lowering properties (Stránský et al. 1992, Wasser et al. 1999, Lo & Cheung 2005, Ngai et al. 2005, Chen et al. 2012, Petrović et al. 2015). Proteins, polyunsaturated fatty acids, dietary fibres, and tocopherols were the nutritional compositions (Petrović et al. 2015). Successful cultivation based on solid-state, and organic substrates using lignocellulosic wastes (wheat straw, millet, tea pulp, beechwood, oak wood, and alder wood) (Philippoussis et al. 2001, Uhart et al. 2008, Isikhuemhen et al. 2009, Jasińska et al. 2012, Kleofas et al. 2014). However, most of these substrates have been reported to generate biological efficiencies (BE) lower than 100%. The lower BE of *C. aegerita* has been indicated to be the main obstacle limiting its large-scale production (Isikhuemhen et al. 2009).

The proper formulation and adequate C:N ratio of a lignocellulosic substrate are among the most important factors controlling the outcome of solid-state cultivation of primary decomposer mushrooms (Attaran Dowom et al. 2019). However, there is scarce and conflicting data on the optimum range of C:N ratio of *C. aegerita* reporting the best C:N ratio of 72, 81, and 59 (Philippoussis et al. 2001, Isikhuemhen et al. 2009). Moreover, no proven significant correlation between the C:N ratio and the yield has been obtained. Thus, the optimum C:N ratio for solid-state cultivation of *C. aegerita* and whether it is possible to gain a higher yield than those previously reported for *C. aegerita* warrant further investigations.

Our previous studies with commercially cultivated and wild-grown strains of Enoki (*F. velutipes*) showed that various combinations of wheat straw, wood chips, and wheat bran provided optimal substrates for both the vegetative and reproductive stages of the mushroom (Rahman et al. 2012). Other studies have also found that wheat straw or rice straw supplemented with 10–30% wheat bran could lead to the highest BE for *Pleurotus* spp. (Rahman et al. 2012, Ganjikutna et al. 2020). Based on these findings, the current study was designed to test the hypothesis that *C. aegerita* may show increased levels of yield on wheat straw and wood chip substrates enriched with wheat bran compared to the substrates reported previously. In addition, the correlation between the C:N ratios of the formulated substrates and the yield performance of *C. aegerita* was investigated in this study. Further, the efficacy of the solid substrates was determined by measuring the growth rate of *C. aegerita* mycelia in agar media made from extracts of the substrates.

Materials & methods

Sample collection

The mother culture of a commercial strain of *Cyclocybe aegerita* (code number M4101) was purchased from the biotechnology company Mycelia (Deinze, Belgium). The leading edges of the *C. aegerita* mycelium were then subcultured on malt extract agar media (Merck, Darmstadt, Germany) and incubated at $25 \pm 2^\circ\text{C}$ in the dark for two weeks to obtain a pure mycelial culture. The subcultures were maintained at 4°C until they were used.

Preparation of lignocellulosic substrates

A range of woody and non-woody lignocellulosic wastes was utilized as ingredients of the substrates based on previous findings that showed wheat straw and wood chips enriched with wheat bran and soybean meal are effective formulations for the cultivation of Enoki and oyster mushrooms (Rahman et al. 2012, Rezaeian et al. 2017, Ganjikutna et al. 2020). A total of 20 substrates with different formulations were prepared using the above raw ingredients. In brief, the raw ingredients of each substrate were soaked for 18 hr to achieve 70% moisture content, and the pH was adjusted with 1% of gypsum to prevent the substrate from rising. One kilogram of each substrate was filled into polypropylene plastic bags and autoclaved at 15 psi, 121°C for 120 min. Further, the carbon content, nitrogen content, and carbon to nitrogen (C:N) ratios of all the

substrates were determined (Rezaeian et al. 2017). Finally, five different substrates with C:N ratios in the previously optimal reported range for *C. aegerita* (Ngai et al. 2005, Harith et al. 2014) were selected and designated as S1, S2, S3, S4, and S5 (Table 1).

Solid-state cultivation

The substrates were inoculated with wheat grain spawn of *C. aegerita* at a 5% inoculum rate on a dry weight basis and maintained at 23 –25°C and 45% humidity for 9 –14 days. The substrates were then transferred to the cropping room at 16 –20°C, 80 –85% humidity, 2,000 ppm CO₂, and 5,000 lux light until the first primordia appeared. The cropping period lasted two and a half months, during which the performance of *C. aegerita* was evaluated. Biological efficiency (BE) was determined using the following formula: BE (%) = weight of total mushrooms/weight of dry substrate × 100 (Tsegaye & Tefera 2017).

Evaluation of mycelial growth in substrate extract agar

Three hundred grams of each substrate were boiled in one liter of distilled water for one hour, followed by centrifugation at 5,000 rpm for 5 min. The upper phase was filtered and used as the substrate extract (SE). The SE was mixed with 1.5% agar (Merck, Darmstadt, Germany), w/v, to serve as an agar medium. A commercial MEA was also used as the control. Following the inoculation with the *C. aegerita* mycelia, cultures were kept at 25°C, where radial growth rates of mycelia were examined (Masoumi et al. 2015). In brief, the mean longitudinal readings of mycelia extensions at four points on two perpendicular lines were recorded daily using a caliper. After 14 days, growth rates of mycelia were measured based on daily averages, expressed in mm per day.

Statistical analyses

Evaluation of the mycelial growth in petri dishes was carried out in triplicate, and each test was repeated three times independently. The solid-state cultivation of *C. aegerita* was performed based on a completely randomized design with three replications. Data analyses and ANOVA tests were conducted using SPSS v. 22 with a significance level of 0.05.

Results

A similar organic carbon (OC) content at 54% (dry weight) was found on the substrates S1, S2, S3, and S5, which was significantly higher than that of S4 ($p \leq 0.05$). Compared with OC, a significant difference was observed in the nitrogen (N) content, while the different C:N ratios among the substrates are shown in Table 1. The highest amount of N was observed in S4 with 1.37% dw ($p \leq 0.05$), followed by S1, S5, S2, and S3 (Table 1). Inversely, the highest C:N ratio was seen in S3 (99.61), followed by S2, S5, S1, and S4, respectively.

Yield performance of *Cyclocybe aegerita* on different substrates

Cyclocybe aegerita fruiting bodies appeared three weeks after the appearance of pinheads. The basidiocarps were 5 cm in diameter, cream or goldish color, darker in the center than around (Fig. 1). Total yield and BE were measured using the mature fruiting bodies (comprising caps and stipes) and compared statistically on the substrates tested (Table 2).

Total fresh yield

Substrate S1 and S4 produced the highest total fresh yield (251.47 g and 234.84 g), while the lowest mushroom yield obtained from S3 was 63.50 g ($p \leq 0.05$). There were no significant differences in the total fresh yield between S2 and S5 ($p \geq 0.05$) (Table 2).

Biological efficiency (BE)

The quantitative values revealed a significant difference in the BE among the substrates tested (Table 2). The highest BE was obtained from S4 (approximately 194%), followed by S1 (approximately 123%), while other substrates produced a BE of less than 100%. Unlike the total

yield, less similarity was observed between the mean values of BE obtained from the substrates tested, and the significant differences between them were more obvious ($p \leq 0.05$).

Table 1 Composition and chemical analysis of lignocellulosic substrates utilized for growing *Cyclocybe aegerita*.

Substrates	Substrate composition	N (%)	OC (%)	C:N
S1	73% wood chips, 10% wheat bran, 10% cotton seed, 5% wheat seed, 1% + 1% gypsum and lime	0.97	54.90	56.77
S2	70% wood chips, 18% wheat straw, 5% soybean meal, 1% + 1% gypsum and lime	0.81	54.18	66.43
S3	60% wood chips, 23% wheat straw, 5% wheat seed, 10% rice bran, 1% + 1% gypsum and lime	0.55	54.54	99.61
S4	78% wheat straw, 20% wheat bran, 1% + 1% gypsum and lime	1.37	49.69	48.32
S5	78% wood chips, 10% wheat seed, 10% wheat bran, 1% + 1% gypsum and lime	0.84	54.36	64.80

N: nitrogen; OC: Organic carbon; C:N: the ratio of OC (%) to N (%) in the dried substrate. Values represent the average of three replicates obtained from each substrate.

Time required for mycelia to fully colonize substrates

There were no major differences in the time taken for *C. aegerita* mycelia to fill the substrate between most of the tested substrates (Table 2). On average, the mycelia of *C. aegerita* completely colonized the substrates within 9-11 days, with the exception of S5, which took 14 days to colonize the mycelia.



Fig. 1 – Mature fruiting bodies of *Cyclocybe aegerita* produced on a suitable substrate (composed of 78% of wheat straw + 20% of wheat bran + 1% of gypsum + 1% of lime).

Table 2 Effects of substrate composition on yield performance of *Cyclocybe aegerita*.

Substrate	Growth parameters*		
	FY* (g)	BE (%)	T (days)
S1	251.4783 ± 10.0727 ^a	123.8017 ± 8.5931 ^b	9
S2	120.7183 ± 7.1448 ^b	64.5898 ± 12.4053 ^c	11
S3	63.5025 ± 7.6191 ^c	30.8370 ± 2.7980 ^d	10
S4	234.8494 ± 32.1639 ^a	194.3635 ± 14.6406 ^a	9
S5	153.9767 ± 20.8298 ^b	61.5734 ± 6.1321 ^c	14

Values shown are the means of triplicates ± standard deviation. Statistical comparisons have been made between substrates within each column of the growth parameter. Values followed by the same superscript lower-case letters are not significantly different ($p \leq 0.05$). *FY; fresh yield (g/ kilogram of the fresh substrate), BE; biological efficiency (g/dried substrate per g × 100), T; Time (days) required to completely fill the substrate with mycelium

Correlation between *Cyclocybe aegerita* yield and C:N ratio of substrate

As shown in Fig. 2, the C:N ratios of the substrates were decreased, while the total fresh yield of the substrate was increased. There was also an inverse relationship between the C:N ratio of the substrate and the BE values (data not shown). Within the range of C:N ratios tested in this study, a 15% reduction in the C:N ratio could lead to an approximately 2-fold increase in total yield and BE of *C. aegerita*. Accordingly, two substrates, S1 and S4 are clearly distinguished from the others, which is consistent with the performance of the substrates shown in Table 2.

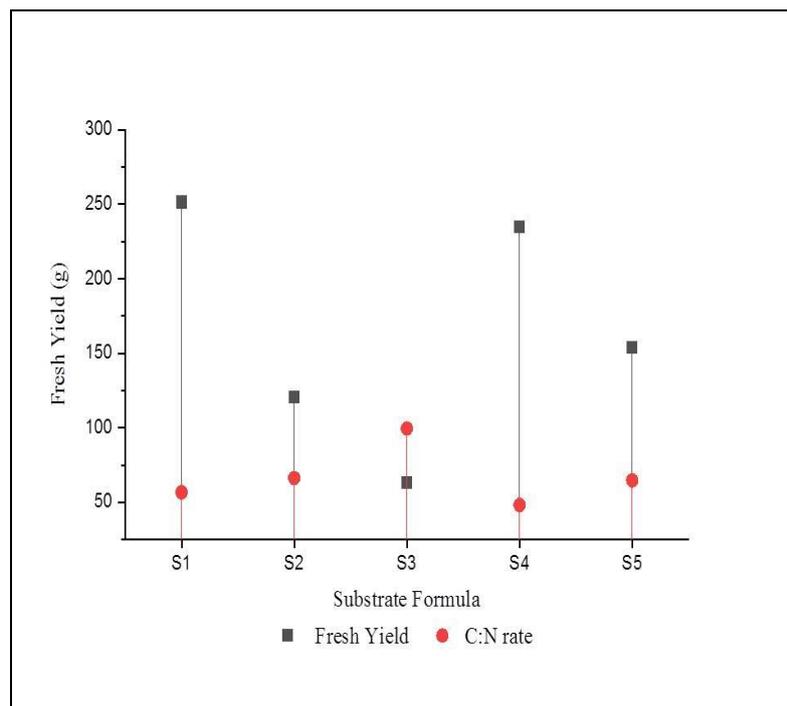


Fig. 2 – Correlation between the C:N ratio and the yield of *Cyclocybe aegerita* (g) across different substrates.

Mycelial growth of *Cyclocybe aegerita* in SE

All the agar media prepared from the substrate extracts were able to grow *C. aegerita* mycelia at higher rates than MEA ($p \leq 0.05$). As displayed in Fig. 3, the extract of S4 supported the fastest mycelial growth with 3.27 mm/day, while the lowest growth rate was observed in MEA with 1.76 mm/day ($p \leq 0.05$). Statistical comparisons also revealed a significant difference in mycelial growth rate between media obtained from S4 and those from S3 and S2, and no significant difference was observed between media derived from S4 and those from S5 and S1 ($p \geq 0.05$).

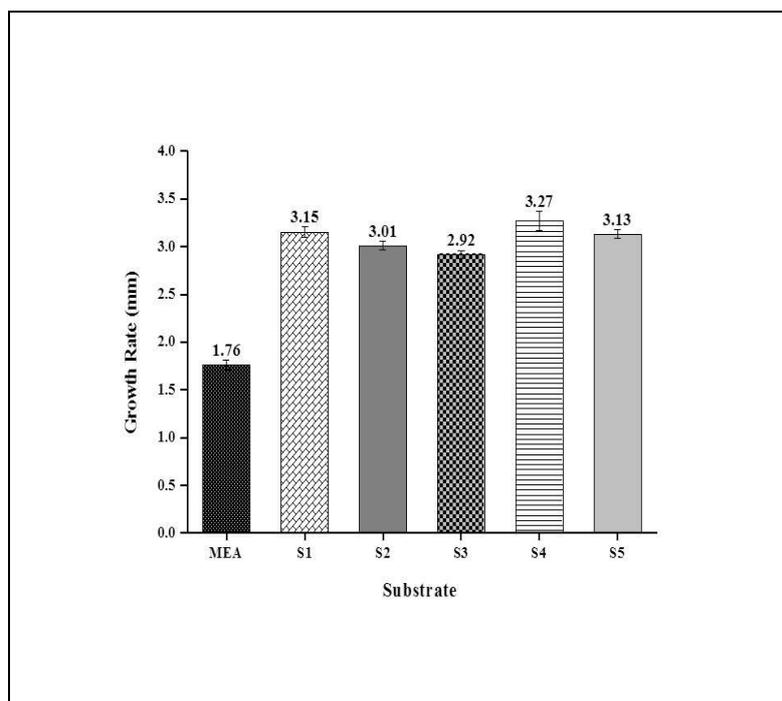


Fig. 3 – Mycelial growth rate of *Cyclocybe aegerita* on different agar media (mm/day).

Discussion

Among the yield performance characteristics of *Cyclocybe aegerita*, BE is the most influenced by the substrate composition (Uhart et al. 2008). This present study aimed to investigate whether the high BE producing substrates are consistent with those previously used to grow *Flammulina velutipes* (Rezaeian et al. 2017). The results confirmed the above hypothesis that the substrates (S4) composed of 78% of wheat straw and 20% wheat bran improved the yield of *C. aegerita* with a BE value of 194%. This value is higher than previously reported BEs (Philippoussis et al. 2001, Uhart et al. 2008, Isikhuemhen et al. 2009, Jasińska et al. 2012, Kleofas et al. 2014, Jasińska & Siwulsky 2021). Similarly, the use of wheat straw supplemented with soybean flour also increased the BE of *C. aegerita* up to 179% (Uhart et al. 2008). Other nitrogen sources such as black tea pomace, solid waste, millet, and cotton waste could not significantly improve the BE of *C. aegerita* (Philippoussis et al. 2001, Isikhuemhen et al. 2009, Jasińska et al. 2012, Kleofas et al. 2014). Therefore, it can be concluded that *C. aegerita* shows the best performance on non-woody substrates composed of 70–80% wheat straw enriched with nitrogen sources, namely wheat bran or soybean flour.

In this study, S1 was the only wood chip-based substrate that gave a BE greater than 100%, although the BE was significantly lower than that of non-woody S4. The substrate S1 consisted of 73% wood chips, 10% wheat bran, 10% cottonseed, and 5% wheat seed. Similar results were obtained with wood-based substrates consisting of sawdust, 20% wheat bran, 2.5% maize flour enriched with CaCO₃ at 8 g/100 g dry weight. This substrate significantly enhanced the BE of *C. aegerita* (Jasińska & Siwulsky 2021). However, the reported BE value was much lower than that obtained from S1 reported in the present study. Moreover, the use of non-woody wheat straw-based substrates for the efficient and sustainable cultivation of culinary-medicinal mushrooms (such as *C. aegerita*) might have implications in low forested countries such as Iran, where wood chips or sawdust obtained from trees may not be readily available due to growing concerns about the loss of wood and forest resources.

Wood chip-based substrates of S2, S3, and S5 exhibited BE values of 64.5, 30.8, and 61.5, respectively. These values are lower than the substrates S1 and S4. However, some of these substrates have been reported to generate high BE when used to grow mushrooms other than *C. aegerita*. For example, a wood-based substrate composed of 80% of sawdust and 16% of wood

straw (similar to substrate S2) increased the BE of Shiitake mushrooms by 18%, compared to a substrate consisting only of sawdust (Royse & Sanchez 2007). The substrate S5 produced BE and yielded performance with similar values to the substrate S3, although the composition of the two substrates was different. The difference between substrates S5 and S1 was mainly due to cottonseed, which increased the BE and yield of S1 compared to S5. These results are consistent with a previous study that found BE of the oyster mushroom (*Pleurotus ostreatus*) increased 52% to 61% with the addition of cotton waste to a wood chips-based substrate (Tsegaye & Tefera 2017). The substrate S3 (consisting of 60% of wood chips + 23% of wheat straw + 5% of wheat seed + 10% of rice bran) showed the lowest BE and yield performance. The main difference between this substrate and substrate S2 was that rice bran and the wheat seed were replaced by soybean meal. This substitution doubled the BE and yield performance of *C. aegerita* in substrate S2 compared to S3. This finding is consistent with earlier studies that showed the addition of 4% dried weight of soybean meal and corn improved the yield and BE of *P. eryngii* var. *eryngii* (Rodríguez Estrada et al. 2009).

The second objective of this study was to evaluate how the C:N ratio of the substrate affects the yield performance of *C. aegerita*. Inappropriate C:N ratios in the substrate have been shown to inhibit mycelium growth and yield performance in cultivated mushrooms (Cueva et al. 2017). Therefore, the low yield and BE of substrate S3 showed a higher C:N ratio compared to other substrates. While the carbon content of all the tested substrates is similar, the low level of nitrogen in substrate S3 (0.55%) correlates with low yield and BE levels of substrate. However, the relationship between the yield performance and growth parameters of mushrooms and the C:N ratio (or nitrogen content) of the substrate is an arguable issue. Although, the growth rate and fruiting body development of *P. eryngii*, *L. edodes*, and *C. aegerita* were found to be positively correlated with the C:N ratios of the tested substrates (Philippoussis et al. 2001), no significant associations were observed between the nitrogen content of the tested substrates and the growth parameters of the Enoki mushroom, *F. velutipes* (Harith et al. 2014, Rezaeian et al. 2017). Such statistical measures are not available for *C. aegerita*, so none of the related studies and the present study contained a statistically sufficient number of substrates to perform an accurate statistical analysis of the correlation coefficient between the C:N ratio and the yield. For example, a previous study on *C. aegerita* showed that some substrate formulations did not produce high yield or BE, even though they were within the recommended optimal C:N ratio of 81 (Isikhuemhen et al. 2009). Also, as demonstrated in this study, the lower association between the C:N ratio and BE than between the C:N ratio and fresh yields are most likely due to differences in dry weight of the tested substrates. Despite the conflicting data on the relationship between the C:N ratio and the yield performance of *C. aegerita*, it could still be concluded that the optimal range of the C:N ratio is 48 – 56 to achieve a BE greater than 100% in *C. aegerita*, and a C:N ratio greater than 65 would be considered inappropriate. These data are well compatible with the previous studies on *C. aegerita* (Sarker et al. 2008, Philippoussis et al. 2001) and *P. ostreatus* (Cueva et al. 2017).

In this study, the superiority of the S4 substrate was also confirmed by the high growth rate of *C. aegerita* mycelia in agar media. The significant differences in the radial growth rate of mycelia between S4 based media and those from S2 and S3 were well compatible with the significant differences in yield and BE between these substrates. However, the extracts obtained from all the substrates (S1-S5) supported the good growth of mycelia at significantly higher levels than the commercial media (MEA). Similar to these findings, early studies showed that the differences in the mycelial growth of *C. aegerita* between several agar media were not statistically significant (Philippoussis et al. 2001, Jasińska et al. 2012). In accordance with the findings of mycelial growth rate in agar media, it was observed that during the cropping experiments, the mycelia of *C. aegerita* filled the high-yielding substrates of S4 and S1 faster than the low-yielding substrates of S2, S3, and S5. However, the mycelia of *C. aegerita* could fill all the tested substrates within 14 days of inoculation. These results imply that *C. aegerita* might reproduce vegetatively on many types of lignocellulosic substrates but its fructification and BE are dependent on the composition and C:N ratio of the substrates. Such results have also been obtained with *Ganoderma lucidum* where it was

found that a high-yielding substrate should not be discarded for a long colonization time (Ozcariz-Fermoselle et al. 2018).

In conclusion, the data presented here suggest that substrate composition and proper nitrogen content should be considered as important factors in *C. aegerita* yield performance. Accordingly, an efficient and economical non-woody substrate composed of 78% of wheat straw and 20% of wheat bran could be recommended for solid-state cultivation of *C. aegerita* that has not been reported by others, and this formulation significantly increased the BE of *C. aegerita* more than previous studies. Furthermore, the extract of this substrate prompted a high rate of mycelial growth in agar media. This study also provides evidence that the most appropriate C:N ratio of lignocellulosic substrates to achieve a BE greater than 100% for *C. aegerita* would be 48–56. These findings may facilitate the efficient cultivation of *C. aegerita* as a culinary-medicinal mushroom in order to convert lignocellulosic wastes into valuable nutritional and medicinal compounds.

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