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Efficiency of Mycelial Biomass and Bioactive Compound Production by *Lentinus squarrosulus* Mycelia Grown in an Airlift Bioreactor as a New Source of Cosmeceutical Biological Substances

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Abstract

Cosmeceutical industry is continually searching for new substances or extracts from natural sources that are nontoxic and can delay the aging process. *Lentinus squarrosulus* is a wild edible mushroom valued by local communities in Asian countries as food as well as its medicinal benefits. However, the bioactive substances and cosmeceutical biological properties of *L. squarrosulus* mycelia grown in submerged culture have not been demonstrated. This study aims to investigate the feasibility of producing *L. squarrosulus* mycelial biomass and cosmeceutical bioactive compounds through large-scale submerged fermentation. *L. squarrosulus* mushroom mycelia grown in a 5-L airlift bioreactor submerged culture was investigated as a new source of cosmeceutical biological substances. An ethanolic extract derived from *L. squarrosulus* mycelia possesses potential antioxidant agents capable of inhibiting dermal enzymes such as collagenase, elastase, and tyrosinase for simultaneously treating skin aging and hyperpigmentation. The Molisch test, FTIR analysis, and β-glucan assays revealed that the main polysaccharide present in the *L. squarrosulus* mycelial extract was β-glucan (64.3 %), which acts as a major contributor to its cosmeceutical properties. The *L. squarrosulus* mycelial extract was non-toxic to human skin keratinocytes (HaCaT). Cosmeceutical formulations developed with the mycelial extract, especially in essence form, preserved the extract's bioactivities, presented a pale-yellow color and a pH of 6.0, which is considered suitable for cosmeceutical design. This study provides new information regarding submerged growth of *L. squarrosulus* mycelia in an airlift bioreactor, which has potential as a novel source of useful bioactive β-glucans for cosmeceutical applications. High mycelial biomass was produced over a short time in this airlift bioreactor that concomitantly had anti-aging and skin whitening properties. The results suggest that the mycelial extracts of *L. squarrosulus* could be further developed to produce safe natural ingredients for inclusion in cosmeceutical products.

Keywords: Airlift bioreactor, Anti-aging, β-glucan, Cosmeceutical, *Lentinus squarrosulus*, Mycelial extract, Submerged culture

Introduction

Edible and medicinal mushrooms, either in the form of fruiting bodies or mycelia, contain a variety of bioactive compounds such as polysaccharides, phenolics, terpenoids, and fatty acids. These compounds contribute valuable antioxidant, anti-aging, anti-inflammatory and photo-protective bioactivities that are useful for cosmeceutical products [1-3]. Mushroom extracts, such as those of *Ganoderma lucidum*, *Cordyceps sinensis*, *Grifola frondose*, and *Tremella fuciformis*, are now incorporated into commercial

skincare and cosmetic products [4-6]. However, there are still many other mushroom species with significant potential are currently unexplored in cosmeceutical research.

Solid-state and submerged cultures are recognized as the most effective techniques for large-scale production of fungal biomass and its metabolites. However, industrial use of bioactive compounds derived from mushrooms has obstacles. Most commercial mushroom products are obtained from the fruiting bodies of mushrooms grown in fields. Cultivating these fruiting bodies typically takes several months and demands substantial amounts of solid substrates and space. Furthermore, it is difficult to control the quality of the final product due to differences in the environmental conditions for cultivation [7,8]. This makes commercial development of high-quality standardized products such as health food and medicine difficult. Therefore, more standardized and cost-effective methods are required for these processes. Growing mushroom mycelial biomass under submerged culture is an excellent and promising method for producing bioactive metabolites. This technique offers several advantages, including consistent quality of mycelial biomass in a controlled environment, continuous availability of bioactive metabolites throughout the year, and a shorter cultivation period with reduced contamination risk. Recent research has shown that many edible mushrooms can be cultivated in submerged culture, producing high mycelial biomass with a variety of bioactive compounds [9-11]. However, there may be differences in the chemical composition of mycelia and fruiting bodies [12].

Skin aging results from both internal factors, such as age-related hormonal changes and external factors like UV exposure, which generates free radicals. Oxidative stress induced by reactive oxygen species (ROS), free radicals containing oxygen, significantly contributes to the aging process of human skin. ROS promotes upregulated expression of matrix metalloproteinases (MMPs) and downregulated collagen synthesis. This causes damage to skin integrity, stimulates collagen and elastin breakdown as well as inhibiting collagen production, leading to accelerated skin aging, wrinkles, sagging, hyperpigmentation, and skin cancer [13,14]. Treatment with antioxidants is a practical defense against oxidative stress to reduce skin aging. Various skincare products, including anti-aging ones, contain antioxidants to neutralize free radicals and protect the products from auto-oxidation.

Natural ingredients for skin care are becoming more popular due to their capability to protect against free radical formation and decrease dermal enzyme production, such as collagenase and elastase. These enzymes contribute to the breakdown of the skin's extracellular matrix (ECM), as well as tyrosinase, an enzyme crucial in melanin biosynthesis [15]. Therefore, identifying natural antioxidant sources that can block these enzymes and incorporating them into cosmetic formulations is a crucial focus. Additionally, consumers with concerns about certain chemicals in skincare products can opt for safer natural ingredients. To meet consumer demand, the cosmeceutical industry is continually searching for new substances or extracts from natural sources that are nontoxic and can delay the aging process [1,16].

Lentinus squarrosulus is a wild edible mushroom valued by local communities in Asian countries such as Thailand, Malaysia, and India as food as well as its medicinal benefits. Since it takes several months to obtain the fruiting bodies, submerged culture of *L. squarrosulus* is a promising alternative to produce mycelial biomass and associated bioactive substances. *L. squarrosulus* mycelia grown in submerged culture contains high protein and low-fat contents, similar to its fruiting bodies, which makes it suitable for functional food ingredients. Researchers have also indicated that mycelial extracts of *L. squarrosulus* promote antioxidant, antiproliferative, and antiulcer activities [17-19]. Consequently, due to its multifaceted biological effects, *L. squarrosulus* is gaining interest in both the pharmaceutical and food industries. Although research has primarily emphasized the fruiting bodies of *L. squarrosulus*, the submerged mycelia of this fungus should also be recognized as a valuable source of bioactive substances. Thus far, no reports have addressed *L. squarrosulus* mycelia grown in submerged culture as a natural source of cosmeceutical bioactive substances.

Many researchers have attempted to determine the optimal submerged culture conditions for mycelial growth and bioactive substance production of various

fungal strains. However, the bioactive substances and cosmeceutical biological properties of *L. squarrosulus* mycelia grown in submerged culture have not been demonstrated. Therefore, the current study aims to (a) investigate the feasibility of producing *L. squarrosulus* mycelial biomass and cosmeceutical bioactive compounds in flask culture and large scale airlift bioreactor submerged fermentation, (b) determine biological components of ethanolic extracts derived from *L. squarrosulus* mycelia grown in an airlift bioreactor, (c) assess the cosmeceutical potential of the mycelial extracts by examining antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase activities, (d) assess *in vitro* cytotoxic effects of a mycelial extract on HaCaT cells, and (e) develop a potential mycelial extract for cosmeceutical applications and create value-added products by incorporation into cosmeceutical base products. Furthermore, this is the first report on the *in vitro* collagenase, elastase, and tyrosinase inhibitory activities of *L. squarrosulus* mycelial extracts.

Materials and methods

Chemicals and microorganisms used in the current study

A pure culture of *L. squarrosulus* Mont. was obtained from the Thailand Mushroom Culture Collection Centre, Department of Agriculture, Ministry of Agriculture and Cooperatives. The stock culture was preserved on potato dextrose agar (PDA, Difco) slants and kept at 4 °C with a bimonthly subculture. All chemicals were of analytical grade and purchased from Sigma Chemical Co. unless otherwise indicated.

Production of *L. squarrosulus* mycelia

Submerged fermentation in flask culture

Flask culture procedures modified from Teerapatsakul and Chitradon [20] were used to cultivate *L. squarrosulus* under submerged fermentation. An active inoculum was prepared by growing the fungus on PDA plates at room temperature (RT, 30 ± 2 °C) for 4 days. Fifteen 5-mm diameter plugs taken from the mycelial growing edge were used as an inoculum in a 250 mL Erlenmeyer flask containing 50 mL of potato dextrose broth (PDB, Difco), initial pH 7.0, with pH uncontrolled during cultivation. The cultures were grown at RT for 7 days under aeration by agitating the flasks on a rotary shaker set at 0 (static culture), 100, and 120 rpm. Shaking conditions at 3 different levels were selected based on the results of our previous study in 50 mL PDB, which showed that mycelia biomass increased with shaker speed. Well-grown mycelia were filtered through Whatman No. 4 filter paper, followed by washing with large amounts of doubly distilled water, and drying at 60 °C to a constant dry weight of biomass. All experiments were conducted in triplicate to ensure reproducibility. The whole 50 mL culture in each flask was used for analysis of each replicate.

Batch fermentation in an airlift bioreactor

Batch fermentation was conducted using the nutrient medium described in the previous section, submerged fermentation in flask culture. Details of the 5-L airlift bioreactor designed and assembled by our research group are shown in **Figure 1**. We developed and refined a suitable procedure to promote growth of *L. squarrosulus* mycelia under a batch fermentation scaled up in a 5-L airlift bioreactor as follows. The 5-L stainless steel airlift bioreactor was comprised of a vessel 300 mm high and 170 mm in diameter. It had a working volume of 4 L and a 100 mm diameter air sparger. Air was supplied through a glass sparger using an air compressor. A 0.22 μm cellulose acetate syringe filter was positioned at the system inlet to maintain sterility.

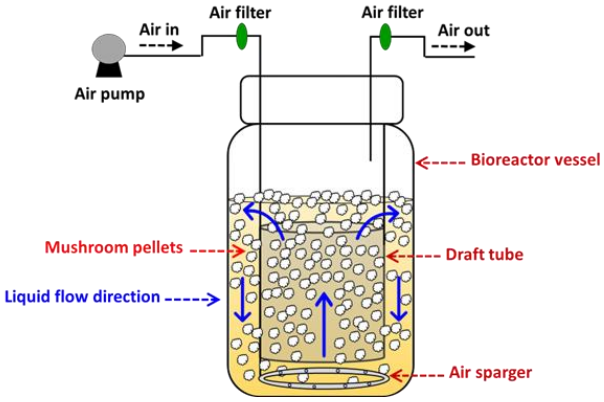

Figure 1 Schematic of the airlift bioreactor used for *L. squarrosulus* submerged fermentation.

A seed culture was prepared by growing the fungus on a PDA plate at RT for 4 days, and then fifteen 5-mm diameter plugs taken from the growing mycelia were transferred into 50 mL of PDB in a 250 mL flask. The seed flasks were incubated at RT for 3 days on a rotary shaker at 120 rpm, and the culture broth with mycelia was used as the seed. About 20 % (v/v) of the seed culture (800 mL) was transferred into 3.2 L of PDB in the 5 L bioreactor. Experiments were run in two replicates in the bioreactor at RT. The effect of aeration rate (1.0, 2.5 and 5.0 vvm) on the yield of mycelial biomass was evaluated. After 7 days of cultivation, the mycelial mass of *L. squarrosulus* obtained from PDB were filtered through double layers of nylon cloth, followed by washing with large amounts of doubly distilled water. The dry weight of mycelial biomass was obtained by drying at 60 °C to a constant weight. The mycelium of the whole 4 L culture in each bioreactor was used as one replicate.

Preparation of mycelial extract

Dried *L. squarrosulus* mycelia were ground to a fine powder with a mortar and pestle. The obtained mycelial powder was extracted by macerating with 60 % ethanol (1:1.5 w/v) under a dark condition at RT for 48 h and then filtered through double layers of nylon cloth. The obtained extract solution was concentrated in a rotary evaporator (Büchi R-210 Rotavapor) at 45 - 55 °C to remove ethanol. It was then lyophilized (ScanVac CoolSafe 100-9 Pro). The dried ethanolic extracts were stored in glass vials at −20 °C until further use. The extracts were dissolved in 10 % DMSO to yield concentrations suitable for assay.

Chemical analysis of mycelial extracts

Preliminary mycochemical screening

The presence of various mycochemicals in the mycelial extract was examined using standard protocols for basic biochemical screening for carbohydrate, phenol, flavonoid, and alkaloid compounds [21,22]. Molisch's test was used to determine the presence of carbohydrates in the extract. Briefly, 1 mL of a 5 mg/mL extract was added to Molisch's reagent (3 drops) with a few drops of H_2SO_4. Violet ring formation indicated a carbohydrate content. A ferric chloride test was used to determine the presence of phenolic compounds in the extracts. Ferric chloride reagent (3 drops) was added to a 5 mg/mL extract. Compounds with a phenol group exhibited a green color. An alkaline reagent test was done to determine the presence of flavonoids. A few drops of NaOH were added to the extract and yellow color was formed. Subsequently, dilute acid was added, and a colorless appearance confirmed the presence of flavonoids. Mayer's test was used to determine the presence of alkaloids. A few drops of Mayer's solution were added to the extract and formation of a milky precipitate indicated the presence of alkaloids.

Determination of β-glucan, phenolic, and protein contents

The β-glucan content of the mycelial extract was assessed using a Mushroom and Yeast β-glucan assay kit from Megazyme, Ireland. The assay was performed following the manufacturer's protocol. The β-glucan content was calculated from the difference between total glucan and α-glucan contents. All values were expressed as the percentage of the glucan content by weight of the extract. Yeast β-glucan (Megazyme) was used as a

reference. The total protein content of the extract was evaluated using the Bradford protein assay and bovine serum albumin (BSA) was employed as a standard. The total phenolic content of the extract was determined following the method of Kaewnarin *et al*. [23]. Gallic acid was used as a reference.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed to identify and analyze the biological components in the mycelial extract. The FTIR spectrum of the mycelial extract was analyzed employing a spectrum 400 FTIR spectrophotometer (PerkinElmer Inc., USA) using a potassium bromide pellet technique. The spectrum was recorded within the wavelength range of 4,000 to 400 cm^{-1}. The software used to analyze the spectrum was the Origin 2018 version.

Assays of antioxidant activity

Scavenging effect on 2,2-diphenyl-1-picrythydrazyl (DPPH) radicals

A DPPH radical scavenging assay was done following the modified method of Liu and Zhang [24]. The extract (20 µL) was added to a 96-well microplate, then mixed with 180 µL of 0.1 mM DPPH in methanol and incubated at RT for 30 min in the dark. Absorbance at 517 nm was measured using a microplate reader (Thermo Fisher Scientific Inc., USA). The DPPH radical scavenging ability was calculated as scavenging activity (%) = 100×[(A_{517} of blank – A_{517} of sample)/A_{517} of blank]. Ascorbic acid served as the positive control. The IC_{50} value corresponded to the extract concentration causing 50 % inhibition of DPPH radical formation.

Scavenging effect on H_2O_2

An H_2O_2 scavenging assay was done following the modified method of Liu and Zhang [24]. The reaction mixture containing 50 µL of the extract, 120 µL of 0.1 M phosphate buffer (pH 7.4), and 30 µL of a 40 mM H_2O_2 solution was incubated at RT for 10 min. Absorbance at 230 nm was measured with a microplate reader. The H_2O_2 scavenging ability was calculated as scavenging activity (%) = 100×[(A_{230} of blank – A_{230} of sample)/A_{230} of blank]. Ascorbic acid served as the positive control. The IC_{50} value corresponded to the extract concentration causing 50 % inhibition of H_2O_2 accumulation.

Reducing effect on ferrous ion (FRAP)

A FRAP assay was done following the method of Benzie and Strain [25]. Briefly, 30 µL of the extract were mixed with 900 µL of FRAP reagent and 900 µL of distilled water in a microplate before incubating at 37 °C for 10 min. Absorbance at 593 nm was measured using a microplate reader. Ascorbic acid served as the positive control. The IC_{50} value corresponded to the extract concentration which resulted in an absorbance of 0.5.

Assays of enzyme inhibition activity

Tyrosinase inhibition activity

Tyrosinase inhibition assays were done as previously described by Kozarski *et al*. [26]. Briefly, 20 µL of extract, 140 µL of 20 mM phosphate buffer (pH 6.8), and 20 µL of mushroom tyrosinase were mixed in a microplate and then pre-incubated for 10 min at 25 °C. After pre-incubation, 20 µL of 2.5 mM L-DOPA (3-(3,4-dihydroxyphenyl)-L-alanine) in a 20 mM phosphate buffer (pH 6.8) were added and the samples were incubated for an additional 20 min at 25 °C. The dopachrome level was measured at 492 nm using a microplate reader. Tyrosinase inhibition (%) was calculated as 100×[(A_{492} of blank – A_{492} of sample)/A_{492} of blank]. Kojic acid served as the positive control. The IC_{50} value corresponded to the extract concentration causing 50% inhibition of tyrosinase.

Collagenase inhibition activity

Collagenase inhibition assays were done following the method of Kozarski *et al*. [26]. Briefly, 40 µL of an extract dissolved in 50 mM of tricine buffer (0.4 M NaCl and 10 mM $CaCl_2$ at pH 7.5) were added to a microplate. Next, 20 µL of collagenase from *Clostridium histolyticum* and 70 µL of tricine buffer were added. The reaction mixtures were incubated at 37 °C for 20 min in the dark and then 40 µL of 0.8 mM FALGPA [N-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala] were added to the microplate and further incubated at 37 °C for 30 min in the dark. The absorbance was immediately and continuously measured at 340 nm for 20 min. Enzyme activity was analyzed by measuring

decreased absorbance over time. Collagenase inhibition (%) was calculated as $100\times[1 - (A_{340}$ of blank/A_{340} of the sample)]. Epigallocatechin (EGCG) served as the positive control. The IC_{50} value corresponded to the extract concentration causing 50 % inhibition of collagenase.

Elastase inhibition activity

Elastase inhibition assays were done following the modified method of Kozarski *et al*. [26]. Briefly, 50 µL of extract in 0.2 M tris-HCl buffer (pH 7.6) containing 5 % DMSO were incubated with 100 µL of tris-HCl buffer (pH 7.6) and 25 µL of elastase (0.3 units/mL) at RT for 20 min. Then, 25 µL of 10 mM N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN) were added and incubated for a further 40 min at 25 °C. Absorbance was measured at 405 nm with a microplate reader. EGCG served as the positive control. Elastase inhibition (%) was calculated as $100\times[(A_{405}$ of blank – A_{405} of sample)/A_{405} of blank]. The IC_{50} value corresponded to the extract concentration causing 50 % inhibition of elastase.

Cytotoxicity determination

Cytotoxicity was evaluated using HaCaT cells received from the CLS Cell Line Service, Germany, and kept in Dulbecco's modified Eagle Medium (DMEM) with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin, in a 5 % CO_2 humidified atmosphere at 37 °C. Then, the HaCaT cells were washed with Dulbecco's phosphate buffer saline (PBS) before resuspending them in 3 mL of 0.25 % trypsin/EDTA. The resuspended cells were centrifuged at 1,500 rpm at 4 °C for 5 min and then resuspended in DMEM [27].

Cytotoxicity was determined using a WST-1 assay as described by Yin *et al*. [27]. Extract solutions in DMEM at different concentrations (1.25 - 40 mg/mL) were added to separate wells with 200 µL of HaCaT cells (2×10^4 cells/mL) and then incubated for 24 h. Next, the treated cells were washed with PBS before adding 10 µL of a WST-1 solution and 100 µL DMEM in each well followed by further incubation for 30 min. Absorbance was measured at 450 nm using a microplate reader (Thermo Scientific Multiskan Go, Thermo Fisher Scientific). The cell viability (%) was calculated as (Absorbance of sample/Absorbance of control)×100.

Development of cosmeceutical formulations with mycelial extracts added

Ethanolic mycelial extracts of *L. squarrosulus* as an active ingredient were formulated into three cosmeceutical products in the form of an essence, gel, and cream. Commercial cosmetic bases were kindly supplied by the Asoke Skin Hospital (Thailand) for use in this study. The base cream presented a white color whereas the base gel and the base essence were free of colorant. All cosmetic base formulations were fragrance-free with a pH of 6.0. Furthermore, all cosmetic base formulations are certified as safe by the Thai Food and Drug Administration (FDA). To cover all the bioactivities evaluated, the base formulations were supplemented separately with the mycelial extracts at 1.0 % concentrations, which corresponds to 10 mg/g of base cream or base gel, and 10 mg/mL of base essence. The base formulations and the mycelial extracts were thoroughly mixed to ensure sample uniformity and analyzed immediately after incorporation to assess the mycelial extract's capability to retain its bioactivities in the cosmeceutical formulations. The base formulations with 10 % DMSO instead of mycelial extract were used as a negative control.

Statistical analysis

All experiments were performed in triplicate, with results presented as mean ± standard deviation (SD). The experimental data were analyzed using one-way analysis of variance (ANOVA). Tukey's post-hoc test at $p < 0.05$ was applied to detect significant differences between the mean values using SPSS version 26.0 for Windows (SPSS Inc., USA).

Results and discussion

Production of *L. squarrosulus* mycelia

The present study focused on the production of *L. squarrosulus* mycelial biomass and cosmeceutical bioactive substances formed in submerged fermentation. We also attempted to confirm the possibility of increasing the mycelial biomass and bioactive substance productivity in an airlift bioreactor. Producing mycelium from basidiomycetes with beneficial properties requires selecting the right strains, optimizing cultivation conditions, and implementing modern biotechnology. Furthermore, study on fungal morphology in submerged cultures plays a key role

since morphological changes can significantly influence product yield, affecting mass transfer and mixing [10]. Therefore, maintaining the appropriate morphological structure is crucial for optimizing the production of target metabolites.

Submerged fermentation in flask culture was performed to illustrate the initial hyphal growth of *L. squarrosulus* prior to fermentation in an airlift bioreactor. Aeration is one of the most important physical factors for production of mushroom mycelia as well as their metabolite biosynthesis in submerged fermentation [20]. Mycelial growth of *L. squarrosulus* under submerged cultivation was investigated in 250 mL flask culture prior to implementation in a 5-L airlift bioreactor. The effects of shake flask and static-flask conditions on the yield of mycelial biomass and fungal morphology were also investigated. The *L. squarrosulus* fermentation followed the normal fungal growth curve pattern, reaching the highest mycelial biomass after 7 days of submerged cultivation. Aeration of *L. squarrosulus* during shake-flask culture was crucial for production of mycelial biomass and its morphological development. As shown in **Table 1**, shaking significantly enhanced *L. squarrosulus* growth over that of static conditions. Increasing the aeration rate by adjusting the shaking speed from 100 to 120 rpm for a 50 mL culture in a 250 mL flask resulted in higher mycelial biomass yield. The highest *L. squarrosulus* mycelial biomass yield, 7.2 g/L, was recorded in a shake-flask culture at 120 rpm, which was 3 and 1.5 times greater than that obtained with a static culture and 100 rpm shake-flask culture, respectively. A noticeable difference in *L. squarrosulus* morphology under shaking and static conditions was observed. Under shake-flask conditions, *L. squarrosulus* formed 0.1 - 0.6 mm diameter spherical mycelial pellets. By contrast, poor growth was observed under the static condition, with free filaments forming and dispersing in the medium (**Figure 2**). Our results showed that *L. squarrosulus* exhibited a higher yield of mycelial biomass in shake-flask culture than static culture. These results are comparable with Anike *et al*. [28]. Mycelial biomass increased with the volume of culture medium. Similarly, shake-flask culture was favorable for efficiently producing mycelial *Coprinopsis cinerea*, *G. lucidum*, *Pleurotus albidus*, and *P. ostreatus* biomass [29]. However, static-flask culture was favorable for mycelial growth of *Calocybe indica*, *P. cystidiosus*, *Schizophyllum commune*, and *Volvariella volvacea* [3,29-32].

Table 1 Mycelial biomass and ethanolic extract yields of *L. squarrosulus* after 7 days of growth under various submerged culture conditions.

Cultivation	Mycelial dry weight (g/L)	Extraction yield (% w/w)
Shake-flask culture at		
0 rpm (static culture)	2.4 ± 1.2 [f]	35.2 ± 0.8 [f]
100 rpm	4.7 ± 0.8 [e]	40.3 ± 2.4 [e]
120 rpm	7.2 ± 1.4 [c]	50.2 ± 1.0 [c]
Airlift bioreactor with different airflow rates		
1.0 vvm	5.4 ± 1.2 [d]	47.3 ± 2.4 [d]
2.5 vvm	28.9 ± 1.1 [a]	78.5 ± 1.2 [a]
5.0 vvm	19.6 ± 1.8 [b]	60.3 ± 2.1 [b]

Data are presented as the mean ± SD, and n = 3 repetitions. Except in the 5-L airlift bioreactor, data is expressed as mean ± SD of 4 repetitions. In the same row, the means followed by different letters are significantly different (α = 0.05, ANOVA, Tukey's HSD test).

A 5-L airlift bioreactor was designed and constructed to investigate the possibility of enhancing the production of *L. squarrosulus* mycelial biomass and its cosmeceutically bioactive compounds. When the airflow rates were adjusted between 1.0 and 5.0 vvm, the morphology of *L. squarrosulus* grown in this bioreactor was similar. After 7 days in the airlift bioreactor, mycelial growth of *L. squarrosulus* was

observed as numerous uniform fluffy pellets with hairy surfaces. It was observed that the mycelial biomass yield varied in the airlift bioreactor system with the airflow rate. The highest mycelial biomass was obtained from *L. squarrosulus* grown in the airlift bioreactor under a 2 vvm airflow rate. After 7 days of growth in an airlift bioreactor with a 2.5 vvm airflow rate, its yield was 28.9 g/L (productivity 4.1 g/L/day), which is higher than the shake flask condition at 120 rpm, 7.2 g/L (productivity 1.0 g/L/day), representing a 4-fold improvement. Consequently, the airflow rate was set to 2.5 vvm to ensure a homogenous distribution of oxygen and nutrients throughout the entire reactor volume to ensure a high yield of *L. squarrosulus* mycelia.

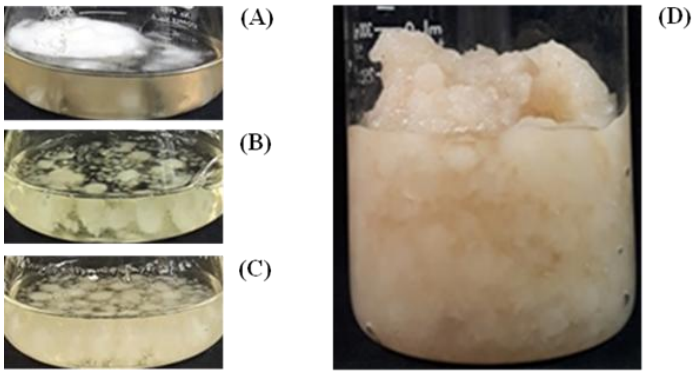

Figure 2 *L. squarrosulus* free-filament morphology under a static condition (A), pellets under shaking at 100 rpm (B), 120 rpm (C), and pellets under cultivation in an airlift bioreactor with a 2.5 vvm airflow rate (D) for 7 days.

Large-scale production of *L. squarrosulus* mycelia using our airlift bioreactor system was significantly enhanced compared to the shake-flask experiment. The primary reason for this notable rise in mycelial biomass production in an airlift bioreactor is likely the aeration process. By regulating the aeration rate in the airlift bioreactor, the overall productivity of submerged macrofungi cultivation is enhanced, as it improves the mass transfer of substrates, products, and oxygen [33]. The airlift bioreactor system applied in this study produced significantly higher *L. squarrosulus* mycelial biomass (25.9 g/L, productivity 3.7 g/L/day) compared with most mushroom strains cultivated in airlift and stirred-tank bioreactors, which typically produce 8.8 - 20.3 g/L (productivity 0.6 - 2.2 g/L/day) [34-36]. It is usually difficult to culture aerobic fungi in an airlift bioreactor due to mycelial morphology, an apparent increased viscosity due to mycelial growth, and unplanned mixing [37]. However, in this study, it was shown that the applied airlift bioreactor can be successfully used to cultivate *L. squarrosulus* with a high yield of mycelial biomass and numerous pellets with uniform size and shape.

The *L. squarrosulus* mycelia obtained from a 7-day submerged cultivation was collected and extracted with 60 % ethanol to evaluate its biological components and cosmeceutical properties. A 60 % (v/v) ethanol solution was chosen as the extraction solvent due to its capability to effectively and safely extract a wide range of bioactive compounds as well as its environmental friendliness. Additionally, our previous study noted that the ethanolic extract of *L. squarrosulus* mycelia displayed greater antioxidant activity than ethyl acetate and water extracts.

As shown in **Table 1**, high mycelial biomass led to greater yields of mycelial extract. All six extracts had similar characteristics. The mycelial extracts were viscous semisolids with a dark brown appearance. The highest extraction yield was obtained from *L. squarrosulus* mycelia grown in the airlift bioreactor with a 2.5 vvm airflow rate. Therefore, this bioreactor is suitable for large-scale cultivation of *L. squarrosulus* to yield significant mycelial biomass and extract yields in a short time. Enhancing productivity through effective bioreactor operating strategies will facilitate commercialization of these processes.

Biological components of mycelial extracts

The ethanolic mycelial extracts derived from *L. squarrosulus* grown in the airlift bioreactor were

qualitatively screened for mycochemicals and the presence of bioactive metabolites that may contribute to its cosmeceutical activities. Interestingly, only carbohydrate was detected in the mycelial extract while phenol, flavonoid, and alkaloid were absent. Since mushroom polysaccharides are present mostly as glucans, further experimentation was done to evaluate the glucan content in the mycelial extract. As shown in **Table 2**, the results revealed a high β-glucan content in the mycelial extract (64.3 %), which is comparatively greater compared to α-glucan (4.6 %). A similar result was found in a control yeast β-glucan. The main polysaccharide component present in *L. squarrosulus* mycelia was β-glucan.

Table 2 Evaluation of glucan contents of ethanolic extracts from *L. squarrosulus* mycelia grown in a 5-L airlift bioreactor and a reference β-glucan from yeast.

Sample	Glucan content (% w/w)		
	Total glucan	**α-glucan**	**β-glucan**
Control yeast β-glucan	99.6 ± 0.4 [a]	5.9 ± 0.2 [b]	93.7 ± 0.3 [a]
L. squarrosulus mycelial extract	68.9 ± 0.4 [b]	4.6 ± 0.3 [b]	64.3 ± 0.7 [b]

All data are presented as mean values ± SD (n = 3). In the same column, the means followed by different letters indicate significant differences (α = 0.05, ANOVA, Tukey's HSD test).

In the current study, the protein content of *L. squarrosulus* mycelia was 24 %, which is higher than that (7 - 19 %) from cultivated fruiting bodies of *L. squarrosulus* [19]. The mycelial extract of *L. squarrosulus* is a more abundant source of β-glucan and protein, potentially offering health benefits. Therefore, further investigation was done on the mycelial extracts to explore their potential use in cosmeceuticals. FTIR spectroscopy was performed to analyze glucans as well as the position and anomeric configuration of glycosidic linkages in the mycelial extracts of *L. squarrosulus*.

The FTIR spectrum of the mycelial extract recorded in the frequency range of 4000 to 400 cm^{-1} displayed a typical pattern of polysaccharides (**Figure 3**). The mycelial extracts displayed a broad band at 3500 to 3000 cm^{-1}, which corresponds to O-H stretching vibrations in the sugar residues. The bands at 1425 and 810 cm^{-1} were ascribed to carbohydrates. Higher band intensities at 1425 and 1078 cm^{-1} suggest a high polysaccharide content [38]. The strong absorbance peaks at 1155 and 1078 cm^{-1} indicated that the mycelial extracts contained β-glucan along with pyranose sugar, which confirmed the results of β-glucan content in the extracts shown in **Table 2**. The peak at 1155 cm^{-1} shows the stretching of C-O-C (glycosidic) bonds, and the peak at 1078 cm^{-1} shows the C-O stretching in β-glucans of carbohydrates. The band at 892 cm^{-1} indicates β-glycosidic linkages in the mycelial extracts. Weaker peaks around 920 and 850 cm^{-1} were attributed to α-glycosidic linkages. The absorbance band at 802 cm^{-1} is the characteristic adsorption peak of mannose. A peak at 2935 cm^{-1} corresponds to C-H stretching of the methylene group of aliphatic compounds from lipids and phospholipids. The absorption peak at 1743 cm^{-1} was ascribed to C=O stretching of lipids. Peaks around 1657 cm^{-1} show C-O and C=N stretching of amide I, indicating the presence of protein [39,40].

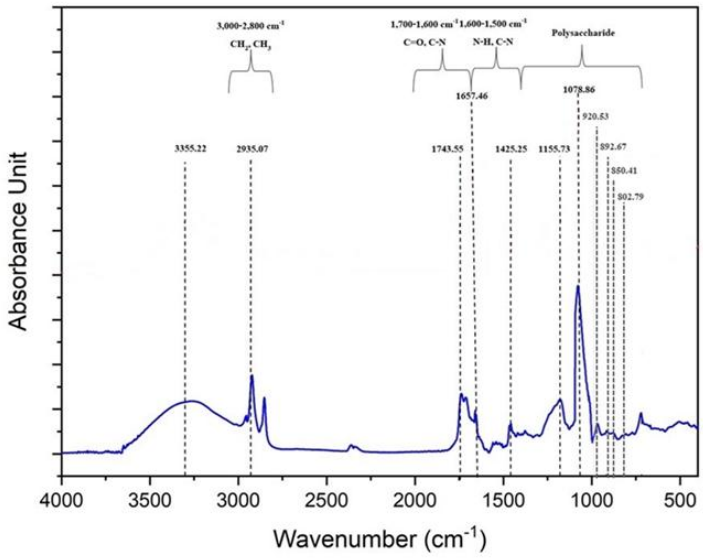

Figure 3 FTIR spectra of an ethanolic extract of *L. squarrosulus* mycelia.

These results are comparable to well-studied β-glucan of *L. squarrosulus* fruiting bodies [39]. Our findings revealed that the ethanolic extracts of *L. squarrosulus* mycelia were predominantly composed of β-glucan with pyranose sugar residues linked by β-glycosidic bonds, along with minor quantities of proteins and lipids. The extracts derived from *L. squarrosulus* mycelia produced in this bioreactor were shown to be a good source of bioactive β-glucan polysaccharide. Since it is a mycelial producer with a high yield β-glucan, uses an inexpensive culture medium, and a simple apparatus, the airlift bioreactor may lead to realization of large-scale mycelial and bioactive compound production.

Polysaccharides (80 - 90 %), consisting of α- or β-glucans and glycoprotein (a protein-polysaccharide complex) are the main components present in the cell wall structure of mushrooms. These polysaccharides affect biological activities. It has been reported that polysaccharides, especially β-glucan, and proteins have potential health-promoting attributes, such as antioxidation, anti-aging, anti-inflammatory, anti-tumor, and antimicrobial properties [41]. β-glucans penetrate skin and form a thin film to promote moisturization as well as stimulating collagen synthesis within the dermis leading to enhanced skin elasticity [42]. Proteins can offer a defense against ROS, as numerous amino acids exhibit strong antioxidant capabilities due to the presence of polar R groups. Bioactive peptides might function as potent inhibitors of oxidative enzymes engaged in EMC protein turnover and melanin synthesis [43].

The results obtained in this study reveal polysaccharides and proteins in the ethanolic extracts from *L. squarrosulus* mycelia grown in an airlift bioreactor, corroborating earlier observations of these compounds in fruiting bodies of various mushroom species, including *L. squarrosulus* [39]. Additionally, evaluation of the mycelial extracts using Molisch's test, FTIR analysis, and a β-glucan assay confirmed that β-glucan was the most abundant active polysaccharide (50.5 %) in the mycelial extract. However, several studies reported that phenolic compounds are a large group of bioactive metabolites in mushrooms that have various health-promoting properties [1]. Furthermore, high phenolic contents promote antioxidant capacities as observed for the ethanolic extracts obtained from *L. squarrosulus* fruiting bodies [44]. However, phenolic content, as revealed by the chemical identification methods employed in this study, was not detected in the ethanolic extracts from *L. squarrosulus* mycelia. Therefore, it is remarkable that the mycelial extracts contain a high yield of β-glucans and proteins which may have potential health benefits as well as correlating with the observed cosmeceutical activities.

Cosmeceutical properties of mycelial extracts

To assess potential cosmeceutical antioxidant and inhibitory actions on dermal-related enzymes including collagenase, elastase, and tyrosinase of *L. squarrosulus*,

mycelial extracts were evaluated and IC_{50} values determined, where a lower value corresponds to a stronger cosmeceutical potential. The mycelial extracts were evaluated with three different assays commonly used to determine the antioxidant activity of biological extracts. DPPH and H_2O_2 assays were used to evaluate the potential of the extracts for radical scavenging. The extract capability in reducing ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) was confirmed through the FRAP assay. As shown in **Table 3**, the mycelial extracts exhibited promising antioxidant properties through their capability to reduce ferric ions and scavenge DPPH radicals as well as H_2O_2. Interestingly, there was no significant difference ($p < 0.05$) in IC_{50} values for the reducing power of the mycelial extract and ascorbic acid, a common antioxidant added to cosmetic products. The results indicated that the mycelial extracts had potent reducing capacities as potential antioxidants.

Table 3 Cosmeceutical properties of an ethanolic extract derived from *L. squarrosulus* mycelia grown in a 5-L airlift bioreactor.

Property	IC_{50} (mg/mL)			
	Mycelial extract	**Ascorbic acid**	**Kojic acid**	**EGCG**
Anti-radical activity				
- DPPH scavenging activity	9.2 ± 0.0	0.1 ± 0.0	-	-
- H_2O_2 scavenging activity	8.6 ± 0.1	1.6 ± 0.0	-	-
- Ferric reducing power	0.6 ± 0.0	0.1 ± 0.0	-	-
Enzyme inhibition activity				
- Anti-tyrosinase	8.7 ± 0.0	-	0.1 ± 0.0	-
- Anti-collagenase	8.5 ± 0.0	-	-	0.1 ± 0.0
- Anti-elastase	9.5 ± 0.0	-	-	0.5 ± 0.0

All data are presented as mean values ± SD (n = 3). DPPH: 2,2-diphenyl-1-picrythydrazyl. EGCG: Epigallocatechin gallate.

The radical scavenging properties of the mycelial extracts and their IC_{50} values using the DPPH method were comparable to those obtained using the H_2O_2 assay. Ascorbic acid exhibited a lower IC_{50} value in the two methods and higher radical scavenging activity than the mycelial extracts. The results indicated that the mycelial extracts have a moderate radical scavenging capacity compared to the ascorbic acid standard. As described by Pai *et al*. [45], antioxidants help protect the skin by shielding cell membranes from oxidative stress, eliminating reactive oxygen species (ROS), and enhancing collagen production, among other mechanisms. Therefore, extracts or compounds with significant antioxidant properties are essential in effective cosmeceutical and skincare anti-aging and whitening products.

The antioxidant capabilities of mushroom extracts are linked to the presence of various bioactive compounds in the fruiting bodies or mycelia of mushrooms. Previous studies demonstrated that mushroom polysaccharides and polysaccharide-protein complexes exhibit antioxidant properties due to the ROS-scavenging activity of hydroxyl groups in their monosaccharide units [46,47]. In this study, it was shown that the *L. squarrosulus* mycelial extracts are comprised of bioactive compounds that exert antioxidant activity by reducing ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). This is done via an electron transfer reaction in the FRAP assay and acting as a hydrogen donor, reacting with free radicals of H_2O_2 and DPPH to convert them to stable substances relieving free radical-mediated oxidative stress. The radical scavenging activity of the mycelial extract was very similar to that of ascorbic acid, a widely used antioxidant. It is established that the hydroxyl group of glucose units in β-glucan can donate electrons and hydrogen to reduce free radicals. Considering the FTIR spectrum in **Figure 3**, the *L. squarrosulus* mycelial extract exhibited a strong absorbance band at 3355 cm^{-1} representing a high proportion of hydroxyl groups, which can result in strong antioxidant capacity. There has been increasing concern about synthetic antioxidants, including

butylated hydroxyanisole and butylated hydroxytoluene, due to their potential to cause liver damage and cancer in animals [48]. As a result, natural antioxidants, such as those derived from the *L. squarrosulus* mushrooms and its mycelia, which are less cytotoxic and potentially more effective, are emerging as promising alternatives.

The inhibitory activities for tyrosinase, collagenase, and elastase exhibited by the mycelial extracts are demonstrated in **Table 3**. The results show that the mycelial extracts had capabilities to inhibit these enzymes and exhibited a potential comparable to the standard inhibitors currently used in topical dermatological products. Owing to the antioxidant, anti-collagenase, anti-elastase and anti-tyrosinase capacities of the mycelial extracts of *L. squarrosulus*, their use is suggested as anti-aging and skin whitening agents. Collagen and elastin are the extracellular matrices that promote skin tightening and resilience. Their breakdown by collagenase and elastase leads to formation of wrinkles and other signs of skin aging. Suppression of tyrosinase activity may block melanin biosynthesis and decrease skin hyperpigmentation, contributing to prevention of premature aging and treatment of abnormal skin pigmentation [49]. Inhibiting these enzymes delays skin aging. Therefore, the anti-aging capacities of the *L. squarrosulus* mycelial extract were investigated by means of the inhibitory actions on dermal-related enzymes including collagenase, elastase and tyrosinase. Apart from their antioxidant activities, the *L. squarrosulus* mycelial extracts also possess anti-collagenase, anti-elastase and anti-tyrosinase effects. Degradation of skin fibrous proteins is primarily mediated by metalloproteinases, whose catalytic action involves metal, which includes collagenase, elastase, and tyrosinase. A recent study reported on β-glucan of *Ganoderma lucidum* fruiting bodies as sources of collagenase, elastase and tyrosinase inhibitors involving metal-chelation. Collagenase and elastase are zinc-dependent metalloproteinases. The hydroxyl group of β-glucan might interact with the Zn^{2+} ions in these enzymes, blocking their capability to bind with substrates. However, tyrosinase, which contains Cu^{2+}, can bind with the hydroxyl group of β-glucan [40]. Therefore, the anti-aging properties of *L. squarrosulus* mycelial extracts are likely related to the presence of β-glucan.

Cytotoxicity analysis

The effects of the mycelial extracts on the cell viability of the HaCaT cell line were assessed through WST-1 assays to evaluate the safety of *L. squarrosulus* mycelial extracts for further cosmeceutical application. This cell line has been previously established as a reliable *in vitro* model. From the results shown in **Figure 4**, the extracts exhibited non-toxicity to the HaCaT cell line at concentrations reaching 10 mg/mL, and more than 80 % cell viability was detected. The maximum tested concentration, 40 mg/mL, was found to inhibit cell viability by up to 60 %. The IC_{50} value was 30.2 mg/mL.

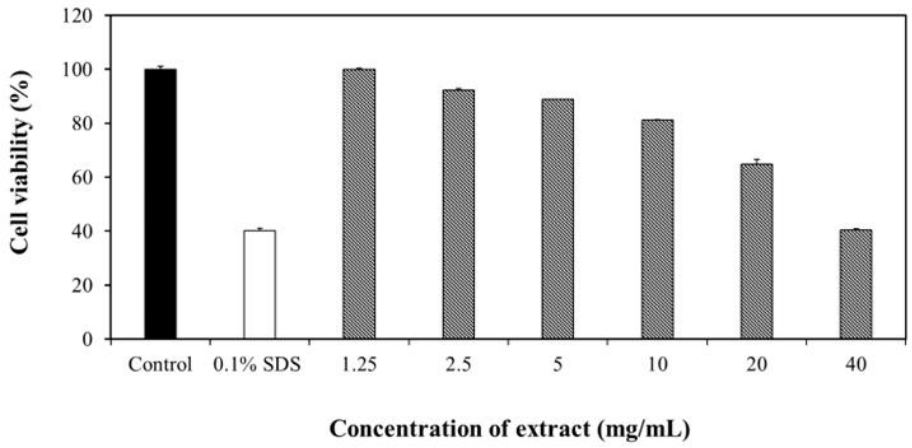

Figure 4 Viability of the keratinocyte (HaCaT) cell line after treatment with ethanolic extracts of *L. squarrosulus* mycelia at various concentrations. Data are expressed as mean values ± SD (n = 3).

Development of cosmeceutical formulations with mycelial extracts added

As a result of their multifaceted biological effects, *L. squarrosulus* mycelial extracts are promising candidates for creating topical formulations for cosmeceutical applications. The effects of cosmeceutical formulations in the form of an essence, gel, and cream on the cosmeceutical-related bioactivities were investigated. Base formulations of essence, gel, and cream incorporating 10 % DMSO instead of a mycelial extract were used as a negative control and exhibited a lack of antioxidation, anti-collagenase, anti-elastase and anti-tyrosinase activities. The bioactive properties of the produced cosmeceutical formulations were confirmed. All three formulated cosmeceutical products, especially in essence form, preserved all cosmeceutical-related bioactivities exhibited by the mycelial extracts (**Table 4**). When the mycelial extracts were incorporated into the cream and gel formulations, a decreased percent inhibition was noted. This decline may result from interference by the cream and gel bases, potentially limiting the availability of the mycelial extract to exhibit the same bioactivity.

Table 4 Cosmeceutical biological properties in different formulations based on an ethanolic extract of *L. squarrosulus* mycelia, 10 mg/mL.

Cosmeceutical formulation	Biological activity (% inhibition)			
	Antioxidation	**Anti-tyrosinase**	**Anti-collagenase**	**Anti-elastase**
Mycelial extract (ME)	76.9 ± 0.1 [a]	84.5 ± 0.2 [a]	77.6 ± 0.1 [a]	64.6 ± 0.2 [a]
Essence + ME	76.6 ± 2.7 [a]	83.7 ± 0.5 [a]	77.3 ± 2.3 [a]	63.2 ± 0.0 [a]
Gel + ME	40.4 ± 2.4 [c]	44.5 ± 2.9 [b]	75.1 ± 1.9 [a]	60.8 ± 0.2 [a]
Cream + ME	34.7 ± 1.2 [c]	18.3 ± 0.4 [c]	15.8 ± 2.0 [b]	14.6 ± 0.4 [b]
Essence + 10 % DMSO	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]
Gel + 10 % DMSO	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]
Cream + 10 % DMSO	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]	0.0 ± 0.0 [d]

Values are expressed as mean ± SD (n = 3). In the same column, the means followed by different letters indicate significant differences (α = 0.05, ANOVA, Tukey's HSD test).

The color attributes of the produced cosmeceutical formulations with mycelial extract showed a pale-yellow coloration with a pH of 6.0, which is considered within the suitable pH range for skin contact purposes. These achievements are of interest to the cosmetic industry for minimizing the use of synthetic ingredients. The results suggest that mycelial extracts of *L. squarrosulus* can be further developed as natural ingredients for use in cosmeceutical products. Most reports have suggested that cosmetic formulations with a pH range of 4.0 to 6.0 are often considered advantageous because most pathogenic bacteria grow optimally at neutral pH levels. Skin microflora and barrier homeostasis are maintained at these conditions [50,51]. Based on the antioxidant properties exhibited by the produced formulations, the mycelial extract can also scavenge free radicals and protect the formulations from auto-oxidation.

The growing demand for multifunctional products is driving innovation in the cosmeceutical industry, as budget-minded consumers seek items that offer both enhanced vitality and effective skin protection. The information obtained in the present study highlights the potential capacity of ethanolic *L. squarrosulus* mycelia extracts to serve as effective components in cosmeceuticals for blocking free radicals, potentially enhancing the skin barrier, providing skin-lightening effects by inhibiting tyrosinase, and offering anti-collagenase and anti-elastase benefits to improve skin elasticity and firmness. Moreover, no apparent toxicity was detected in HaCaT keratinocytes, which function as the primary barrier protecting the body from the external environment. This paper is the first report regarding

mycelial extracts of *L. squarrosulus* grown in an airlift bioreactor with potential development of cosmeceutical formulations that delay skin aging.

Conclusions

This study provides new information regarding submerged growth of *L. squarrosulus* mycelia in an airlift bioreactor, which has potential as a novel source of useful bioactive β-glucans for cosmeceutical applications. Bottlenecks in the production of mushroom-derived bioactive compounds can be overcome using the developed airlift bioreactor submerged fermentation. High mycelial biomass was produced over a short time in this airlift bioreactor that concomitantly had cosmeceutical properties yielding considerable cost and time savings thereby improving the potential for industrial-scale implementation. *L. squarrosulus* mycelial extracts are highly valuable as they possess no toxic effects and have multiple cosmeceutical functionalities. They are promising antioxidant agents that can inhibit dermal enzymes such as tyrosinase, collagenase, and elastase, providing a dual approach to simultaneously treating both skin aging and hyperpigmentation. These findings introduce a novel potential for application of *L. squarrosulus* mycelia as a safe natural ingredient for use in cosmeceutical products. Conduction of skin permeation studies with *in vitro* skin models and assessing the stability of formulations over various storage periods will further confirm the potential of *L. squarrosulus* mycelial extracts as topical ingredients.

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