DEVELOPMENT OF FUNCTIONAL FOOD PRODUCT FROM SPLIT GILL MUSHROOM

NUTTAPONG SAETANG

DOCTOR OF PHILOSOPHY IN BIOTECHNOLOGY

MAEJO UNIVERSITY

2023

DEVELOPMENT OF FUNCTIONAL FOOD PRODUCT FROM SPLIT GILL MUSHROOM

NUTTAPONG SAETANG

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY
ACADEMIC ADMINISTRATION AND DEVELOPMENT MAEJO UNIVERSITY
2023

DEVELOPMENT OF FUNCTIONAL FOOD PRODUCT
FROM SPLIT GILL MUSHROOM

NUTTAPONG SAETANG

THIS DISSERTATION HAS BEEN APPROVED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY
IN BIOTECHNOLOGY

APPROVED BY **Advisory Committee**

Chair ----------
(Assistant Professor Dr. Yuwalee Unpaprom)
...../...../ .....

Committee ----------
(Associate Professor Dr. Doungporn Amornlerdpison)
...../...../ .....

Committee ----------
(Assistant Professor Dr. Paweena Pumisutapon)
...../...../ .....

Committee ----------
(Associate Professor Dr. Rameshprabu Ramaraj)
...../...../ .....

Program Chair, Doctor of Philosophy ----------
in Biotechnology (Assistant Professor Dr. Yuwalee Unpaprom)
...../...../ .....

CERTIFIED BY THE OFFICE OF ----------
ACADEMIC ADMINISTRATION (Associate Professor Dr. Yanin Opatpatanakit)
AND DEVELOPMENT Vice President
...../...../ .....

ชื่อเรื่อง การพัฒนาผลิตภัณฑ์อาหารเชิงหน้าที่จากเห็ดแครง
ชื่อผู้เขียน นายนัฐพงษ์ แซ่ตั้ง
ชื่อปริญญา ปรัชญาดุษฎีบัณฑิต สาขาวิชาเทคโนโลยีชีวภาพ
อาจารย์ที่ปรึกษาหลัก ผู้ช่วยศาสตราจารย์ ดร.ยุวลี อันพาพรม

บทคัดย่อ

เห็ดแครง (*Schizophyllum commune*) เป็นเห็ดที่สามารถรับประทานได้ อีกทั้งเป็นเห็ดทางเภสัชกรรมและมีคุณค่าทางโภชนาการต่างๆ เห็ดนี้เจริญแพร่กระจายทั่วไปในหลายประเทศแถบทวีปเอเชีย โดยบริเวณผนังเซลล์อุดมไปด้วยสารชิโซฟิลแลน (บีตา-กลูแคนหรือพอลิแซ็กคาไรด์จากเห็ดแครง) ที่มีฤทธิ์ทางชีวภาพโดดเด่นต่างๆ มากมาย ในงานวิจัยนี้จึงมีความสนใจในการศึกษาจำแนกสายพันธุ์ของเห็ด วิเคราะห์คุณค่าทางโภชนาการของเห็ดแครง ศึกษาสภาวะที่เหมาะสมของกระบวนการสกัดชิโซฟิลแลนด้วยน้ำร้อนและการตกตะกอนด้วยเอทานอลเพื่อแยกส่วนของพอลิแซ็กคาไรด์ โดยการออกแบบการทดลองแบบส่วนประสมกลาง (central composite design; CCD) ซึ่งใช้วิธีพื้นที่ผิวตอบสนองเพื่อหาสภาวะที่เหมาะสมของกระบวนการสกัดดังกล่าว รวมถึงศึกษาสภาวะที่เหมาะสมในการละลายสารสกัด นอกจากนี้วิเคราะห์คุณลักษณะทางเคมี (พอลิแซ็กคาไรด์ มอโนแซ็กคาไรด์ ออลิโกแซ็กคาไรด์ บีตา-กลูแคน และโปรตีน) และฤทธิ์ทางชีวภาพของสารสกัดนี้ (ฤทธิ์ต้านอนุมูลอิสระและสมบัติความเป็นพรีไบโอติก) รวมถึงพัฒนาสภาวะที่เหมาะสมในกระบวนการผลิตและสูตรที่เหมาะสมของผลิตภัณฑ์ซุปเห็ดแครงสกัดที่เสริมสารสกัดที่เป็นประโยชน์เชิงหน้าที่ เพื่อการยอมรับของผู้บริโภคด้วย

จากงานวิจัยนี้พบว่า เห็ดชนิดนี้ได้จำแนกสายพันธุ์เป็นเห็ดแครง (*S. commune*) เมื่อวิเคราะห์ความเหมือนของลำดับนิวคลีโอไทด์ด้วย BLAST analysis เห็ดนี้มีองค์ประกอบของคาร์โบไฮเดรต เยื่อใย โปรตีนในปริมาณสูง มีแคลอรี่ของไขมันที่ต่ำ ปราศจากการปนเปื้อนของโลหะหนัก ซึ่งได้รับการพิจารณาว่าเป็นเห็ดที่ปลอดภัย นอกจากนี้สภาวะที่เหมาะสมในการสกัดสารชิโซฟิลแลนเพื่อให้คุ้มค่าในการผลิต ควรใช้ปริมาณเห็ดแครงที่น้อยคือ 5 - 8% (w/v) ที่อุณหภูมิ 100 - 110°C ของการสกัดด้วยน้ำร้อนเป็นเวลา 2 - 3 ชั่วโมง แล้วตกตะกอนด้วยเอทานอลที่ความเข้มข้น 62 - 65% (v/v) ทำให้ได้ค่าระดับการเกิดพอลิเมอร์ที่สูง (DP เท่ากับ 5.40) ปริมาณน้ำตาลทั้งหมดสูง (total sugar เท่ากับ 447.98 mg/g ของสารสกัด) ปริมาณน้ำตาลรีดิวซ์ต่ำ (reducing sugar เท่ากับ 92.08 mg/g ของสารสกัด) ปริมาณโปรตีนที่เหมาะสมในสารสกัด (5.30 mg/g ของสารสกัด) และน้ำหนักผลผลิตของสารสกัด (4.03 g/100 g ของเห็ดอบแห้ง) ในส่วนสภาวะที่

เหมาะสมในการละลายสารสกัด พบว่าที่อุณหภูมิ 120°C ของการละลายสารสกัดเป็นเวลา 120 นาที ที่ความเข้มข้นของสารสกัด 5.00 mg/ml ทำให้ได้ปริมาณน้ำตาลทั้งหมดสูง น้ำตาลรีดิวซ์สูง ค่า DP ที่เหมาะสม และสารฟีนอลิกรวมสูงสุด

นอกจากนี้สารสกัดนี้ประกอบไปด้วยบีตา-กลูแคน 271.42 mg/g ของสารสกัด ทำให้สารสกัดดังกล่าวมีค่า DP สูงสุด อีกทั้งมีความเป็นไปได้ในการกระตุ้นภูมิคุ้มกัน ต้านการอักเสบ และต้านการเกิดมะเร็ง สารสกัดนี้ยังมีองค์ประกอบของบีตา-กลูโคออลิโกแซ็กคาไรด์ หรือบีตา-กลูแคนออลิโกแซ็กคาไรด์ ที่มีค่า DP เท่ากับ 2 - 3 กล่าวคือ laminaribiose, laminaritriose และบีตา-กลูแคนในสารสกัด ที่มีสมบัติความเป็นพรีไบโอติกเพื่อส่งเสริมการเจริญของแบคทีเรียกลุ่มโพรไบโอติก สารสกัดข้างต้นประกอบไปด้วยโปรตีนไฮโดรโฟบิน เลคติน และ purpurin (รงควัตถุสีแดง) ที่อาจมีฤทธิ์ทางชีวภาพต่างๆ มากมาย นอกจากนี้สารสกัดนี้ยังมีปริมาณสารฟีนอลิกรวมสูง (6.49 mg GAE/g ของสารสกัด) ที่มีฤทธิ์ต้านอนุมูลอิสระ (ค่าเปอร์เซ็นต์การยับยั้งอนุมูลอิสระ DPPH และ ABTS สูงถึง 83.23% ที่ความเข้มข้นของสารสกัด 80 mg/ml และ 98.77% ที่ความเข้มข้นของสารสกัด 20 mg/ml ตามลำดับ) อีกทั้งมีศักยภาพในการยับยั้งอนุมูลอิสระ DPPH และ ABTS ที่ความเข้มข้นต่ำของสารสกัด (ค่าความเข้มข้นของสารสกัดที่สามารถยับยั้งอนุมูลอิสระ DPPH และ ABTS ได้ร้อยละ 50 (IC_{50}) เท่ากับ 15.36 และ 7.08 mg ของสารสกัด/ml ตามลำดับ) ในการทดสอบความเป็นพิษของสารสกัดในระดับเซลล์ (cytotoxicity) ด้วยวิธี MTT assay โดยบ่มสารสกัดในเซลล์แมคโครฟาจ RAW 264.7 ที่ความเข้มข้น 250 - 1,000 μg/ml เป็นเวลา 1 - 3 ชั่วโมง ของเวลาในการบ่มสารสกัดที่แตกต่างกัน ทำให้เปอร์เซ็นต์การอยู่รอดของเซลล์สูง ไม่มีความแตกต่างอย่างมีนัยสำคัญ ดังนั้นทั้งเห็ดแครงและสารสกัดชิโซฟิลแลนจึงสามารถนำมาพัฒนาเป็นผลิตภัณฑ์ซุปเห็ดแครงสกัดมูลค่าสูงที่อุดมไปด้วยสารประกอบเชิงโภชนเภสัช เพื่อส่งเสริมสุขภาพที่ดีของผู้บริโภค โดยมีสภาวะเหมาะสมในกระบวนการผลิตซุปเห็ดแครงสกัดที่อุณหภูมิ 100°C เป็นเวลา 120 นาที รวมถึงสูตรที่เหมาะสมของซุปเห็ดแครงสกัด ดังนี้คือ เห็ดแครง (2.500% w/v) สารสกัดชิโซฟิลแลน (0.021% w/v) มะขามป้อม (0.017% w/v) และหญ้าหวาน (0.004% w/v) เป็นสารให้ความหวานทางธรรมชาติ

คำสำคัญ : เห็ดแครง, ชิโซฟิลแลน, การสกัดในสภาวะที่เหมาะสม, ฤทธิ์ทางชีวภาพ, อาหารเชิงหน้าที่

Title DEVELOPMENT OF FUNCTIONAL FOOD PRODUCT FROM SPLIT GILL MUSHROOM

Author Mr. Nuttapong Saetang

Degree Doctor of Philosophy in Biotechnology

Advisory Committee Chairperson Assistant Professor Dr. Yuwalee Unpaprom

ABSTRACT

The edible split gill mushroom (*Schizophyllum commune*) is considered as both nutritive and pharmaceutical mushroom. This mushroom is widely spread in many Asian nations. Its cell wall consists of abundant schizophyllan (β-glucan or polysaccharide from this mushroom) with various distinguished biological properties. Thus, this research was interested to investigate into the identification of mushroom strain, nutritional values of the mushroom, study on optimized hot water extraction and ethanol precipitation process of the schizophyllan using response surface methodology (RSM) on central composite design (CCD), including suitable solubility of this extract. Furthermore, this schizophyllan extract was characterized the chemical (polysaccharide, monosaccharide, oligosaccharide, β-glucan and protein compositions) and biological activities (antioxidant and prebiotic properties), as well as split gill mushroom essence was developed suitable mushroom essence production condition and formula supplemented with this functional extract for consumer acceptance.

From this research, this mushroom was specified as *S. commune* in comparison with the nucleotide sequence homology by BLAST analysis. The mushroom consisted of superior carbohydrate, crude fiber, protein, low calories of fat without any heavy metal contamination, considered as a safe mushroom. Moreover, the cost-effective optimization of schizophyllan extraction was the low mushroom content of 5 - 8% (w/v) at 100 - 110°C of hot water extraction for 2 - 3 hours and ethanol precipitation at 62 - 65% (v/v) ethanol concentration with the highest degree of polymerization (DP value of 5.40), the greatest total sugar

content (447.98 mg/g extract), minimized reducing sugar content (92.08 mg/g extract), suitable protein content (5.30 mg/g extract) and optimized extraction yield (4.03 g/100 g dry mushroom). Additionally, the optimized solubilization condition of this extract was 120°C for 120 minutes with 5.00 mg/ml extract concentration with superior total sugar content, highest reducing sugar content, optimal DP value and greatest total phenolic content.

Furthermore, this extract contained greater β-glucan content 271.42 mg/g extract. As a result, the optimized extract had highest DP value along with possibly potential immunomodulatory, anti-inflammatory and anticarcinogenic properties. The substance also consisted of β-glucooligosaccharide or β-glucan oligosaccharide with DP value about 2 - 3, namely laminaribiose, laminaritriose and β-glucan as prebiotic effect by promoting probiotic bacteria proliferation. This extract comprised of bioactive hydrophobin, lectin protein and purpurin (red pigment) along with probably various biological activities. Besides, the schizophyllan had total phenolic substance (6.49 mg GAE/g extract), as well as revealed antioxidant properties (83.23% DPPH inhibition at 80 mg extract/ml and 98.77% ABTS inhibition at 20 mg extract/ml, respectively), and exhibited more potential to inhibit DPPH and ABTS radical at inferior content (half-maximal inhibitory concentration (IC_{50} value) of 15.36 and 7.08 mg extract/ml, respectively). To study cytotoxicity of the extract by MTT assay, this extract were pretreated to RAW 264.7 cells (macrophages) at 250 - 1,000 μg/ml of extract concentration and 1 - 3 hours of various incubation times with non-significantly high cell viability. Therefore, both this mushroom and the schizophyllan would be developed as high value-added mushroom essence products with nutraceutical compounds for beneficial consumer health at the appropriate production condition of this mushroom essence (100°C, 120 minutes), including suitable mushroom essence formula, namely split gill mushroom (2.500% w/v), schizophyllan extract (0.021% w/v), Indian gooseberry (0.017% w/v) and stevia (0.004% w/v) as natural sweeteners.

Keywords : split gill mushroom, schizophyllan, optimized extraction, biological properties, functional food

ACKNOWLEDGEMENTS

I want to express my sincere gratitude to my advisor, Assistant Professor Dr. Yuwalee Unpaprom (Senate Council Member of Maejo University, Associate Director of Academic and Development Affairs Office, MJU, Head of Ph.D. Program in Biotechnology, Faculty of Science, MJU), for her invaluable support and guidance throughout my academic journey, from the moment she took me under her wing as her student until my graduation. Her unwavering belief in me has been truly inspiring. Furthermore, I would like to extend my heartfelt appreciation to my co-advisors, Associate Professor Dr. Doungporn Amornlerdpison, Associate Professor Dr. Rameshprabu Ramaraj, and Assistant Professor Dr. Paweena Pumisutapon, for their invaluable guidance, constructive suggestions, and kind assistance in enhancing the quality of my research. I am also grateful to the external examiner, Associate Professor Dr. Chutima Srimaroeng Vaddhanaphuti, and the internal examiner, Assistant Professor Dr. Kanjana Narkprasom, for their expertise and valuable input throughout the evaluation process. Their collective support has been instrumental in the ultimate improvement of my research.

I would like to express my gratitude to the Sustainable Resources and Sustainable Engineering Research Lab (SRSE), Food and Agro-Industry Lab, Program in Biotechnology (Faculty of Science), Center of Excellence in Agricultural Innovation for Graduate Entrepreneur, Program in Food Engineering (Faculty of Engineering and Agro-industry) at Maejo University, and the Department of Physiology (Faculty of Medicine) at Chiang Mai University. Their generous assistance and provision of necessary facilities during the experiments have been invaluable. Additionally, I extend my appreciation to Ampol Food Processing Company Limited and the National Research Council of Thailand (NRCT) for their support in funding this research.

Finally, I want to convey a special and heartfelt thanks to my parents and family. Their unwavering love, kindness, encouragement, and support throughout my life have been helpful in my achievements.

Nuttapong Saetang

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

1.1 Principles, theory, rationale and/or hypothesis

Numerous pandemics of various infections, diseases, and pollution issues nowadays exist. People must pay greater attention to their health and consume more healthy foods, particularly functional foods. Functional food is a food that supplements a functional component for preventing any diseases or promoting health (Commission, 2010).

Mushrooms are an essential source of both nutritional and medicinal foods. They can help you avoid problems including hypertension, hypercholesterolemia, and cancer (Yim et al., 2013), including their antioxidant, immune-enhancing and anti-tumor properties (Maity et al., 2015). According to the previous study, they are excellent antioxidant sources because they contain various phenolic compounds and secondary metabolites that act as highly effective scavengers of free radicals (Yim et al., 2013).

Split gill mushroom (*Schizophyllum commune*) is extensively spread in many countries in Asia, such as Thailand, Myanmar, Laos and Northeastern India (Yelithao et al., 2019). This mushroom grows on a broadleaved tree, dead, fallen, or standing wood. It is distinguished by light yellow to brown gills, dense white hairs, and the absence of stalks (Hobbs, 2005). The mushroom comprises β-glucans cross-linked with chitin in the cell wall (Leung et al., 2006). The conformation of schizophyllan or β-glucan is a triple-stranded helix. This β-glucan is a β-1,3-D-glucan backbone with β-1,6-D-branches (Lee and Ki, 2020).

Moreover, the split gill mushroom is medicinal (Klaus et al., 2011) with plentiful nutritional values, particularly high fiber, protein and low lipid (Basso et al., 2020). For decades, the mushroom has been a popular alternative protein source to animal proteins due to the high content of essential amino acids for vegetables (González et al., 2021). The production of healthy foods with a high protein content that does not contain meat is gaining interest worldwide, such as soy, wheat protein,

and mushroom protein (Kim et al., 2011). Additionally, this mushroom contains peptides formed during protein hydrolysis by a protease enzyme that exhibits antioxidant properties (Wongaem et al., 2021), hydrophobin protein with anticancer activity in S180 mouse sarcoma and B16-F10 mouse melanoma (Akanbi et al., 2013), lectin protein with inhibition of cancer cell growth and proliferation (Han et al., 2010), both hydrophobin and lectin protein via possibly immunomodulatory (Akanbi et al., 2013; Zhao et al., 2020), as well as schizolysin (hemolysin) with antiviral activity due to inhibition of HIV-1 reverse transcriptase (Han et al., 2010).

Furthermore, this mushroom is abundant schizophyllan, which is distinguished by a variety of biological properties (Klaus et al., 2011), for instance, antioxidant (Chandrawanshi et al., 2017; Yelithao et al., 2019), anti-inflammatory (Du et al., 2017; Lee and Ki, 2020), immunomodulatory in immune cells from whole human blood (Smirnou et al., 2017) and macrophage cells (Yelithao et al., 2019), anticancer activity in lung, gastric, cervical, breast carcinoma cells (Zhong et al., 2013) and tumor-bearing mouse (Zhong et al., 2015), antiviral, antifungal (Hobbs, 2005), along with prebiotic properties (Chaikliang et al., 2015). Additionally, β-glucan is crucial in preventing oxidative damage caused by free radicals (Yelithao et al., 2019). As a result, the mushroom is used in a wide variety of industrial applications, including pharmaceuticals (Lee and Ki, 2020), vaccines, cosmetics, and functional food (Smirnou et al., 2017; Zhong et al., 2013; Zhong et al., 2015) and traditional food widely in Southeast Asia and India (Hobbs, 2005).

In addition, β-glucan is a prebiotic oligosaccharide or polysaccharide indigestible in the human digestive system (Van Loo, 2006). It promotes the immune system and helps gastrointestinal tract health by promoting the growth of helpful probiotic bacteria, controlling the balance of intestinal flora due to acidic chemicals produced by these probiotic bacteria, and inhibiting harmful bacteria growth (Singdevsachan et al., 2016).

Therefore, this research aims to determine the mushroom strain's identity, and the nutritional value of *S. commune* and optimize the extraction process for schizophyllan and protein. Additionally, this research analyzed the polysaccharide,

monosaccharide, and protein compositions and the biological activities of these extracts, including antioxidant and prebiotic properties. Eventually, a high-value-added functional food product containing various bioactive compounds was developed from this mushroom to promote a healthy immune system in consumers.

1.2 Objectives of the research

1. To identify the mushroom strain and investigate the nutritional value of *S. commune*.
2. To study the optimized extraction process of schizophyllan and protein from *S. commune*.
3. To study chemical characterization and biological activity of schizophyllan and protein from the optimized extraction process.
4. To develop a functional food product from *S. commune.*

1.3 Scopes of research

1. This study used a split gill mushroom harvested from a mushroom farm in Thailand's southern region as a raw material.
2. The anti-inflammatory and immune-enhancing properties of the extract were investigated using macrophages.

1.4 Benefits of research

1. The extraction process was optimized to extract the maximum yield of functional ingredients from the mushroom.
2. The extract contained various bioactive compounds to develop a high-value-added functional food product with health benefits.

CHAPTER 2

LITERATURE REVIEW

2.1 Split gill mushroom

The split gill mushroom (*Schizophyllum commune*) is a therapeutic mushroom (Klaus et al., 2011) found in many Asian nations (Yelithao et al., 2019). For cell wall composition, it consists of rich β-glucans (schizophyllan) (Lee and Ki, 2020; Leung et al., 2006). Additionally, this mushroom possesses abundant nutritional value (Hobbs, 2005) and various biological properties (Klaus et al., 2011). As a result, the mushroom has a wide range of industrial applications.

Figure 1 Split gill mushroom.
Source: Concier (2019)

2.1.1 Taxonomic classification

Kingdom: Fungi

Phylum: Basidiomycota

Class: Agaricomycetes

Order: Agaricales

Family: Schizophyllaceae

Genus: *Schizophyllum*

Species: *S. commune*

(Du et al., 2017)

2.1.2 Physical characteristics

S. commune is known as split gill mushroom. This mushroom has dense white hairs, no stalks, and light yellow to brown gills that branch constantly; thus this mushroom name is called split gill (Hobbs, 2005) as illustrated in Figure 1. The fruiting body is small around 1 - 5 cm and fan-shaped with white spore. The life cycle completes within 10 days (Chandrawanshi et al., 2017). It grows on dead, fallen, or standing broadleaved tree logs. The species is called *commune*, which means widespread (Hobbs, 2005). As a result, the mushroom is found in various Asian countries, including Thailand, Laos, Myanmar, and Northeastern India (Yelithao et al., 2019).

2.1.3 Nutritional value

Split gill mushroom, fresh and dry from the forest or mushroom farming, is commonly eaten in many tropical nations. This mushroom has a lot of nutritional value, especially in terms of carbohydrate and fiber (68.0%), and protein (15.9%), however, it has a low lipid content (2.0%). The essential amino acid content is about 34% less than protein from the egg, with methionine as a limiting amino acid. The true digestibility of protein (TDP) is around 53%. Around 72 - 77% of the total fat in the mushroom is composed of linoleic and oleic acids. Additionally, it has a higher phosphorus, magnesium, calcium, iron, zinc, manganese, copper, and chromium concentration. Mineral content varies according to mushroom strain and habitat (Hobbs, 2005).

2.1.4 Schizophyllan

This mushroom's cell wall comprises glycoprotein, mannoprotein, β-glucan, chitin and plasma membrane (Figure 2) (Chan et al., 2009). When we consider the linkage of β-glucans, it is found that aldehyde groups of β-glucans are cross-linked covalently to amino groups of chitins to form C=N double bonds by Schiff reaction. Figure 3 shows cell wall assembly of this mushroom (Leung et al., 2006). The conformation of schizophyllan (β-glucan from *S. commune*) is a triple-stranded helix.

This water-soluble β-glucan is β-1,3-D-glucan backbone with β-1,6-D-branches (Lee and Ki, 2020), as shown in Figure 4. The molecular weight is about 450,000 Da (Singdevsachan et al., 2016). The conformational complexity of β-glucan is important in immune function (Chan et al., 2009). Triple helical β-glucan, with a higher degree of branching structural complexity, has a greater potential for immunomodulatory and anticancer effects (Khan et al., 2018) than linear or less branched β-glucan with low molecular weight significantly (Lee and Ki, 2020).

Furthermore, high-performance liquid chromatography (HPLC), liquid chromatography/mass spectrometry (LC/MS), atomic force microscopy, and X-ray crystallography can determine the composition or structure of β-glucan. Additionally, the β-glucan content can be determined using the aniline blue staining method, the phenol-sulfuric acid method, or an ELISA (enzyme-linked immunosorbent assay) (Chan et al., 2009).

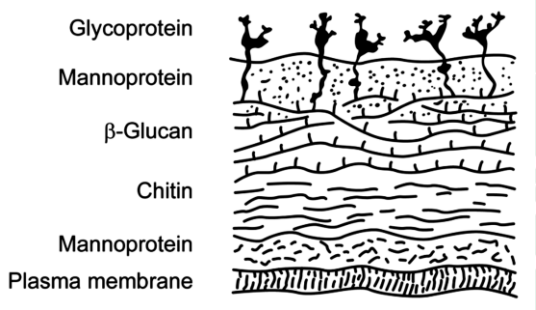


Figure 2 The key components of the mushroom cell wall.

Source: Chan et al. (2009)

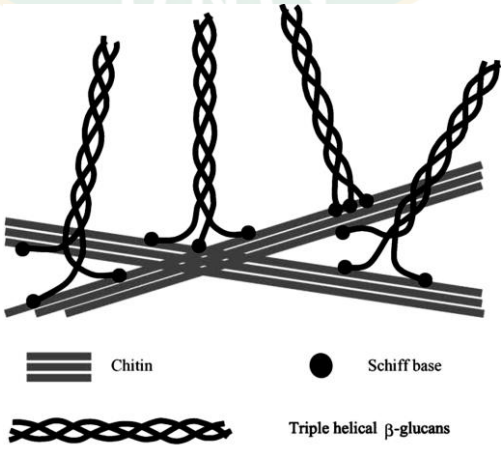


Figure 3 Cell wall assembly of split gill mushroom.

Source: Leung et al. (2006)

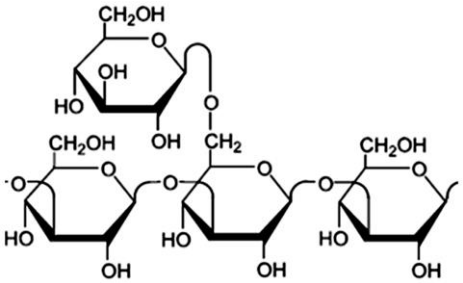


Figure 4 Structure of schizophyllan.

Source: Khan et al. (2018)

2.1.5 Extraction process of schizophyllan

In general, mushroom polysaccharides are obtained from mycelial biomass, fruit bodies, or exopolysaccharides (EPS) released during liquid culture broth fermentation (Ruthes et al., 2015). Several extraction methods are available to obtain schizophyllan or β-glucan. Extraction methods affect β-glucan's molecular weight and structure. Typically, β-glucan is extracted using hot water to solubilize it into a solution, as well as alkaline, acidic, enzyme-assisted, ultrasound-assisted, and microwave-assisted extraction (Zhu et al., 2016). Different factors affect extraction, including the temperature of the extraction (ambient, boiling, or other), the type of organic solvent used to separate the polysaccharide from other components (ethanol, acetone, chloroform: methanol ($CHCl_3$: MeOH)), and the presence of an alkaline solution (NaOH or KOH), as well as the use of ultrasonic or microwave technology to increase efficiency, yield, and reduce extraction time (Ruthes et al., 2015). The entire extraction and production process of β-glucan on a laboratory and industrial scale can be summarized as shown in Figure 5.

In addition, triple helical β-glucan is stable at ambient temperature and pH 7. Nonetheless, after β-glucan is effectively pretreated with an alkaline solution, hot water at high temperature, or solvent, the conformation of β-glucan will change from triple helix to single helix and random coil, respectively (Figure 6), because hydrogen bonds that are intermolecular forces for maintaining the single helical and triple-helical conformers are broken (Leung et al., 2006).

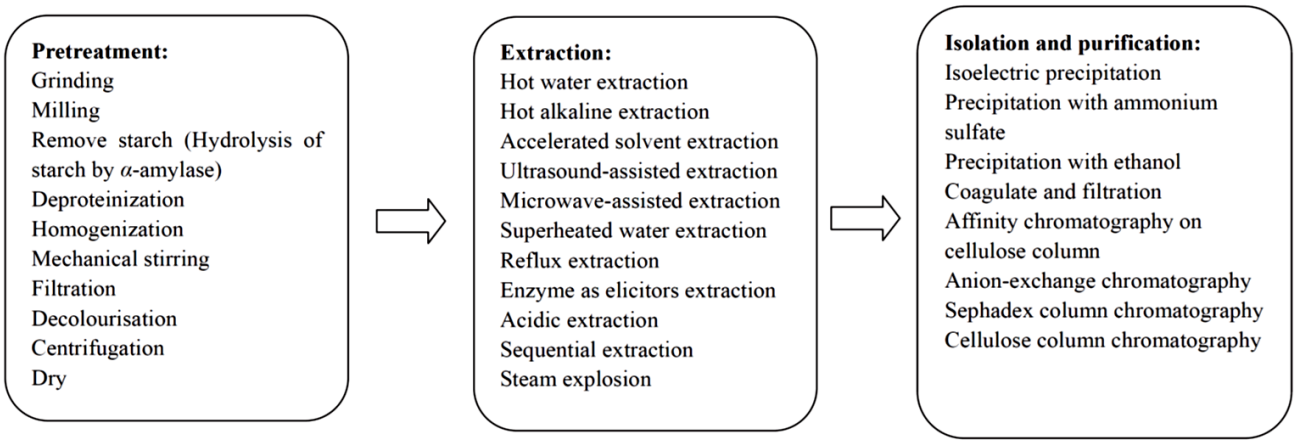


Figure 5 The extraction and production of β-glucan in lab and pilot plant scale.

Source: Zhu et al. (2016)

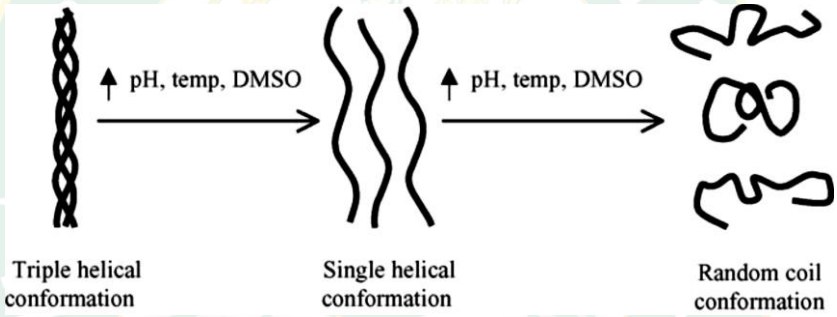


Figure 6 Schematic illustration of the transition of conformation of triple helical β-glucan in response to physical and chemical treatment.

Source: Leung et al. (2006)

Moreover, there is some research on β-glucan extraction process. Some examples are: Chandrawanshi et al. (2017) studied the appropriate extraction process of the extracts from *S. commune* on antioxidant capacity. It was noticed that the highest yield was obtained by hot water extraction (19.06 g/100 g dry mushroom) followed by ethanol and methanol extractions, respectively. The antioxidative capacity of mushroom extract was investigated using various assay methods in this study. DPPH radical scavenging activity, total phenolic determination, reducing power, and hydrogen peroxide scavenging method discovered that ethanolic extract had a higher antioxidant capacity than hot water and methanol extracts. For the chelating

effect of ferrous ions, hot water extract showed great scavenging activity compared to other extracts.

Furthermore, Klaus et al. (2011) reported antioxidant activities and chemical characterization of polysaccharides extracted from fruiting bodies of this mushroom, such as hot water extract (HWE), hot water extracted polysaccharides (HWP) and hot water alkali extracted polysaccharides (HWAE). All extracts consisted of glucose as the dominant monosaccharide. HWAE yielded the highest (22.6 g/100 g of dry mushroom). However, the total polysaccharide contents of HWP (41.7 g/100 g dry extract) and total glucan contents of HWE (26.9 g/100 g dry extract) were the greatest compared to another extract, respectively. HWP also exhibited higher possible median effective concentrations (EC_{50} values) of antioxidant and DPPH scavenging activities, but HWE had higher EC_{50} values of reducing power and ferrous ion chelating abilities.

Besides, Cheung et al. (2012) researched a comparison of polysaccharide-protein (PSP) complexes from three medicinal mushrooms, namely *Coriolus versicolor*, *Grifola frondosa* and *Lentinus edodes*, by ultrasound-assisted extraction (UAE) and conventional hot-water extraction (HWE) before precipitation of the extract with ethanol. When compared to HWE, UAE PSP yield was similar to *G. frondosa*, significantly superior to *L. edodes* but inferior to *C. versicolor*, and UAE extraction rate was significantly higher with *L. edodes* and *G. frondosa* but significantly lower with *C. versicolor*. UAE found that the protein content of PSPs from three mushrooms was higher, while the carbohydrate content was lower than that of HWE. The antioxidative properties of UAE PSPs were likewise more promising than HWE's.

Moreover, Li et al. (2012) studied a polysaccharide-peptide complex from an abalone mushroom (*Pleurotus abalonus*) obtained with hot water extraction combined with ethanol precipitation. After that, the extract was purified using DEAE-cellulose anion exchange chromatography and Sephadex G-200 column gel filtration. Glucose, galactose, rhamnose, and glucuronic acid were all present in this polysaccharide-peptide combination. This extract also showed increased antioxidant activity, anticancer activity against breast cancer MCF7 cells and hepatoma HepG2

cells, hypoglycaemic capability in diabetic mice, and antiviral activity by inhibiting HIV-1 reverse transcriptase function.

2.1.6 Biological properties

2.1.6.1 Antioxidant properties

Antioxidants are micro components present in edible green plants which can help to protect the body against oxidative stress induction by free radicals and prevent harmful reactive oxygen species (ROS). Many physiological and biochemical processes in the body may produce free radicals and other reactive oxygen species (Paudel et al., 2018). They are extremely unstable and react quickly with other compounds in the body, causing oxidative damage to cells and tissues (Li et al., 2018). Excessive free radicals and reactive oxygen species levels have been linked to various degenerative disorders, including aging, cancer, cardiovascular disease, Alzheimer's disease, and other chronic conditions (Ramalingam et al., 2017). To prevent these diseases, antioxidants play a vital role in controlling oxidative stress in the body (Li et al., 2018). Polyphenols, flavonoids, carotenoids, glutathione, and lipoic acid are antioxidants. They are primarily found in fruits and vegetables. Despite this, fruit and vegetables are in short supply in many parts of the world. As a result, researchers are looking for more antioxidants from different sources (Asghari et al., 2016).

Mushrooms are well-known for their nutritional value and as a source of therapeutic foods that can help avoid diseases, such as cancer, hypertension, and hypercholesterolemia (Yim et al., 2013). Moreover, they have drawn attention to their antioxidant, antitumor, and immunomodulatory properties (Maity et al., 2015). Some researchers have reported their excellent antioxidant source, while they consist of various secondary metabolites and phenolic compounds as greatly capable scavengers of free radicals (Yim et al., 2013). Additionally, the schizophyllan from the split gill mushroom has an antioxidant capacity to protect the human body from oxidative damage by scavenging free radicals (Chandrawanshi et al., 2017; Yelithao et al., 2019).

2.1.6.2 Anti-inflammatory and immune-enhancing properties

S. commune is a common schizophyllan with various biological activities and potential pharmaceutical applications (Klaus et al., 2011). For instance, antioxidant capacity (Chandrawanshi et al., 2017; Yelithao et al., 2019), anti-inflammatory activity via suppression of proinflammatory cytokines or stimulation of anti-inflammatory cytokines (Du et al., 2017; Lee and Ki, 2020), immunomodulatory capacity in macrophage proliferation (Smirnou et al., 2017; Yelithao et al., 2019), as well as anticancer activity in controlling growth of breast, lung, cervical, gastric, cancer cells (Zhong et al., 2013) and mouse tumor-bearing model (Zhong et al., 2015). Antiviral activity exhibits protection from viral infections in mice, including enhancing survival rates and phagocytic activity, antifungal activity by induction of candida static activity in mouse macrophage (Hobbs, 2005), as well as prebiotic property in the stimulation of probiotic bacteria growth and inhibition of pathogenic bacteria growth (Chaikliang et al., 2015).

In addition, Figure 7 depicts the mechanism by which β-glucan activates immune cells to eliminate pathogens. β-glucan is a potent immunomodulator that positively affects innate and adaptive immune systems. Human cells do not contain β-glucan. Innate immunity is quick to recall and responds to pathogens to minimize infection. On immune cells, a variety of β-glucan receptors are expressed. This may act exclusively on β-glucan or in combination with other ligands. Different signaling pathways are induced, and the downstream signaling molecules associated with them are identified. The reactor cells are neutrophils, monocytes, dendritic cells, macrophages, lymphocytes, and natural killer, it is mentioned Dectin-1 which is a protein receptor binding β-1,3 and β-1,6 glucan. This receptor can begin and conduct innate immunity. It results in active responses, phagocytosis, proinflammatory cytokine production and infectious agent elimination. Dectin-1 is responsible for the innate immune system and is also found in neutrophils, dendritic cells and macrophages (Chan et al., 2009).

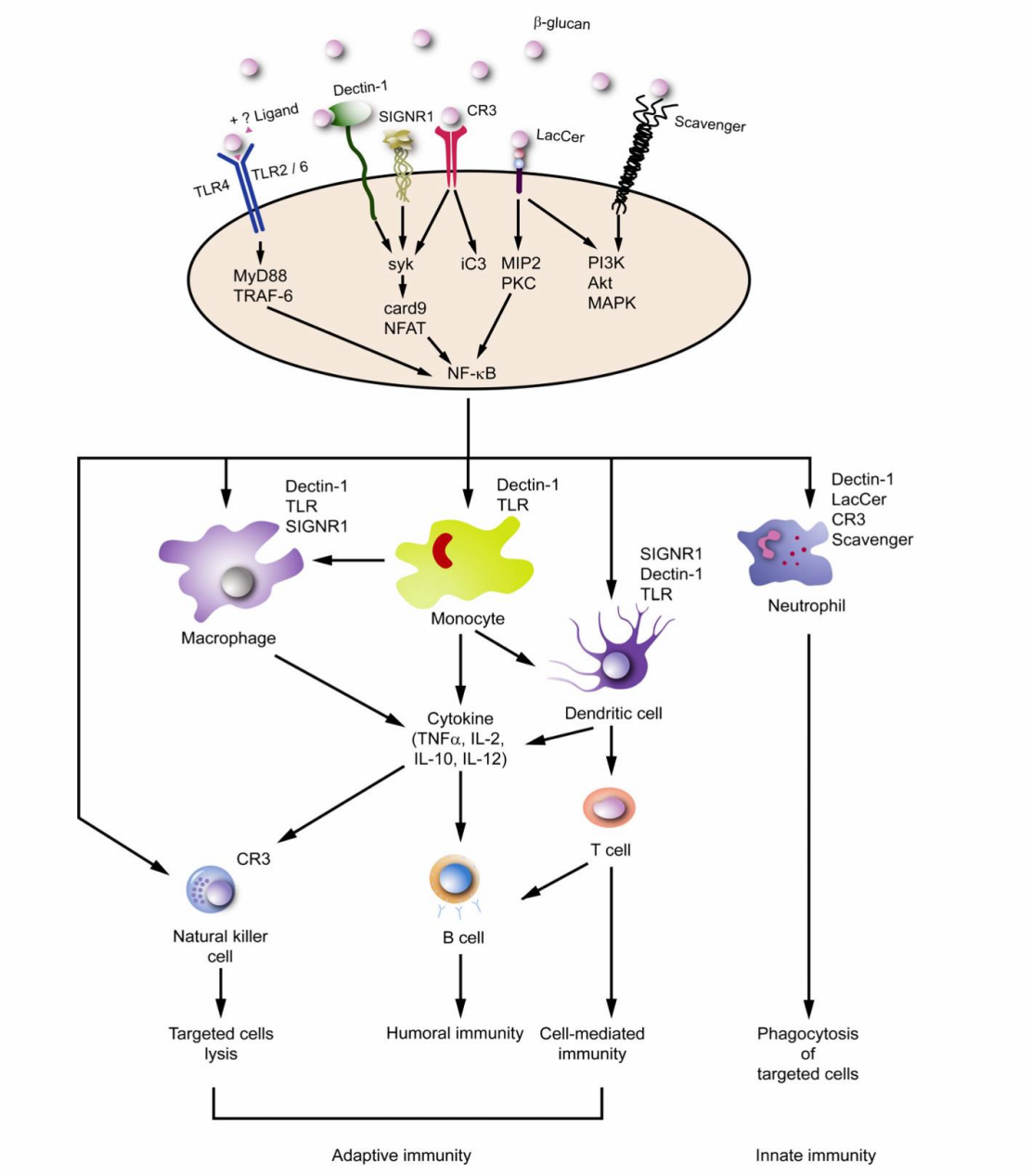


Figure 7 Diagram of immune activation induced by β-glucan.

Source: Chan et al. (2009)

The Dectin-1 cytoplasmic tail contains an immunoreceptor tyrosine based activation motif (ITAM) that signals through the tyrosine kinase in collaboration with Toll-like receptors 2 and 6 (TLR-2/6). Several signaling molecules have been reported to be involved. They are NF-**K**B (nuclear factor-kappa B) through Syk-mediate pathway (spleen tyrosine kinase), signaling adaptor protein CARD9 and nuclear factor

of activated T cells (NFAT). This will eventually lead to the release of cytokines including interleukin (IL)-12, IL-6, tumor necrosis factor (TNF)-α, and IL-10. Generally, bigger and more complex β-glucans have higher immunomodulating potency (Chan et al., 2009).

Furthermore, pro-inflammatory agents, such as lipopolysaccharide (LPS), can significantly increase nitric oxide (NO) production in macrophages through the activation of inducible nitric oxide synthase (iNOS). There are many pro-inflammatory cytokines, for example, TNF-α, nitric oxide (NO), IL-1β, IL-6, cyclooxygenase-2 (COX-2) and 5- lipoxygenase (5-LOX). Nitric oxide is recognized as a regulator in pathological reactions, especially in acute inflammatory responses. 5-LOX is a key enzyme in synthesizing leukotrienes, inflammatory mediators of arachidonic acid. Both inducible nitric oxide synthase (iNOS) and COX-2 are important enzyme mediators that mediate inflammatory processes. iNOS and COX2 synthesize inflammatory mediators, such as nitric oxides and prostaglandins. iNOS mRNA was measured by real-time PCR using specific primers (Du et al., 2017). Additionally, there are various anti-inflammatory cytokines, namely IL-10, IL-12, and transforming growth factor-beta (TGF-β).

Moreover, the prior research reported that β-glucan (from *S. commune*, SPG) interacts with dectin-1 as receptor and promotes the IL-10 expression induced by virulence factor from periodontal bacteria (*A.a*. LPS from *Aggregatibacter actinomycetemcomitans*) via enhancing NF-κB (nuclear factor-kappa B) and MSK1 phosphorylation (mitogen- and stress-activated protein kinase 1) including cAMP-responsive-element-binding protein (CREB) by Syk activation (spleen tyrosine kinase) in murine macrophages. In immune cells, dectin-1, an important β-glucan receptor, binds with β-glucans to initiate and regulate the immune response, such as the production of antimicrobial reactive oxygen species, inflammatory cytokines, and chemokines. Although dectin-1 activation induces the secretion of pro-inflammatory cytokines, it also strongly promotes the production of IL-10, a key anti-inflammatory cytokine that plays a critical role in controlling inflammation. LPS is a potent virulence factor of these periodontal pathogens, and the binding to TLR4 activates

the NF-**K**B pathway (via TIRAP and MyD88), further producing inflammatory cytokines. Dectin-1 is a lectin with a single lectin-like domain and an intracellular ITAM-like motif, which binds to β-glucan and initiates signaling events via Syk (Figure 8). Therefore, it is investigated the involvement of Syk with the dectin-1 signaling pathway is investigated. These observations indicate that SPG binding to the dectin-1 receptor activates Syk, followed by NF-**K**Bα activation and MSK1 phosphorylation, ultimately upregulating IL-10 expression. Although dectin-1 is the main receptor responsible for β-glucan activation, TLR and other receptors may also recognize components of β-glucans. This work suggests that SPG may help to prevent the exacerbation of infection from periodontitis and regulate inflammation, which occurs by host response against the periodontal pathogen. Thus, SPG is a potential therapeutic agent for periodontal disease (Thongsiri et al., 2021).

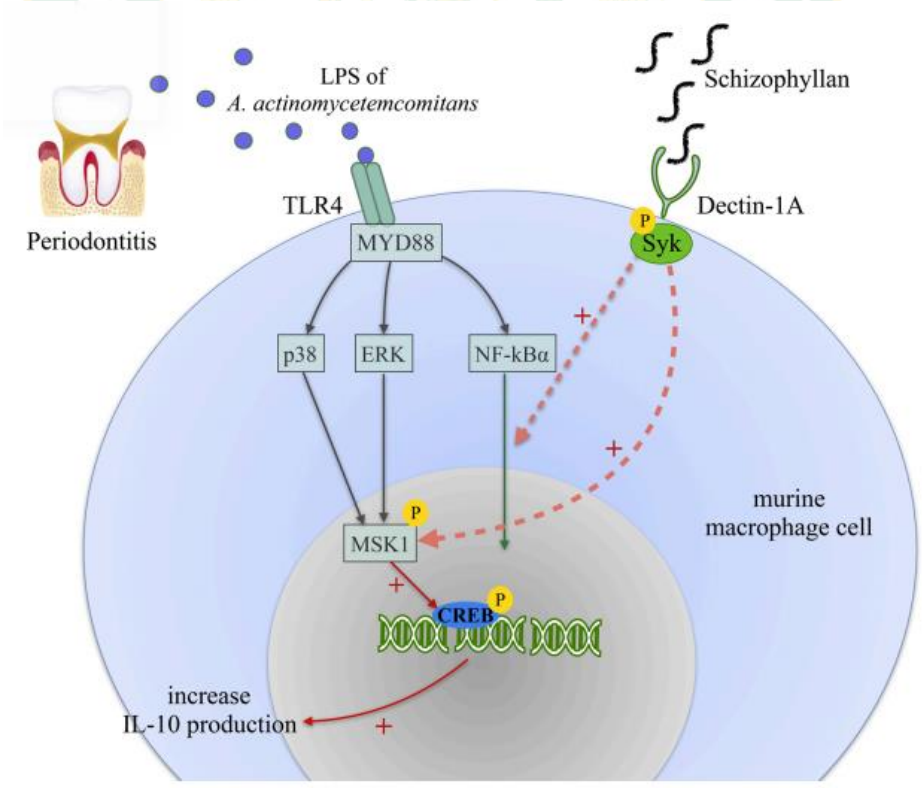


Figure 8 Cell signaling diagram of dectin-1 mediated macrophages induced by LPS (*A.a*).

Source: Thongsiri et al. (2021)

Adaptive immunity is another immune mechanism that works with antigen-presenting cells and T-lymphocytes. Particularly, peptides from intracellular pathogens for class I major histocompatibility complex (MHC-I) antigen-presenting cells present to cytotoxic T-cells (CD8+ T-cells). On the contrary, the class II MHC (MHC-II) antigen-presenting cells display only peptides from extracellular pathogens to T-helper cells (CD4+ T-cells). Hence, β-glucan could stimulate immune responses through T-lymphocytes activation (Chan et al., 2009). Antigen-presenting capacity, macrophage effector function, ability to modulate acquired immunity by antigen presentation, production of cell adhesion molecule, and cytokine release are all improved by the β-glucan. The proliferation of T-lymphocytes, B-lymphocytes, macrophages and natural killer cells would increase in response to stimulation of β-glucan when β-glucan binds with the receptor (Leung et al., 2006).

Besides, β-glucan functions with the activated complement receptor 3 (CR3; CD11b/CD18) on neutrophils, lymphocytes, and natural killer cells. When β-glucan binds to the lectin domain of CR3, resulting in binding to inactivated complement 3b (iC3b) on the reactor cell surface. This pathway is β-glucan opsonization. As a result, it happens phagocytosis and lysis of reactor cells. β-glucan's immunomodulatory effects affect both the innate and adaptive immune systems. β-glucan also enhance phagocytosis and cytokines secretion, such as tumor necrosis factor-α (TNF-α) and various types of interleukins (IL-6, IL-10, IL-12) (Chan et al., 2009; Lee and Ki, 2020).

2.1.7 Application of split gill mushroom

This mushroom is an essential nutrient source (Basso et al., 2020) and has plentiful pharmaceutical value with biologically active compounds (Klaus et al., 2011). In consequence, the mushroom is widely used in Southeast Asia and India for a variety of industrial applications, including traditional meals (Hobbs, 2005), functional food, bioactive cosmetic, immunomodulator, vaccine (Smirnou et al., 2017; Zhong et al., 2013; Zhong et al., 2015) and anticancer drug (Lee and Ki, 2020).

Furthermore, there are varied products of β-glucan from other sources for industrial applications. For example, β-glucan is utilized in foods (prebiotic sausage,

yogurt, dairy product, beverage, gluten-free bread, snack, cake), medicines (vaccine, wound dressing material, poly-membrane, bone-substituting material), cosmetics (skin and dermatological composition, film-forming moisturizer, eye drop), along with feeds (animal feed additive, fish feed additive) (Zhu et al., 2016).

2.2 Prebiotic

Prebiotics are non-digestible and non-absorbable oligosaccharides and polysaccharides found in the human upper gastrointestinal (GI) tract that promote the growth of helpful probiotic bacteria while inhibiting the proliferation of bad bacteria to improve host health. Prebiotics would boost the immune system, enhance digestion and feces elimination, minimize constipation and diarrhea, increase short-chain fatty acids (SCFAs) production, lower colon pH, limit pathogen growth, and lower blood cholesterol levels (Singdevsachan et al., 2016), including reducing cardiovascular disease, prevent obesity and inhibit cancer (Lam and Chi-Keung, 2013).

2.2.1 Concept of prebiotic

The important criteria for prebiotic compounds were described: (1) the prebiotic should be tolerant to low pH acid of the stomach and resistant to digestive enzymes in small intestines. (2) This bioactive compound should stimulate the beneficial probiotic bacteria growth (*Lactobacillus* sp., *Bifidobacterium* sp. and *Streptococcus* sp.) in the colon (3) to ferment SCFAs (such as acetic, propionic, and butyric acids) with low large intestine pH for suppression of pathogenic bacteria growth (*Clostridium* sp., *Salmonella* sp. and *Bacteroides* sp.), (4) leading to beneficial to GI tract health and well-being of host (Shi et al., 2018). Additionally, (5) it should stabilize to food processing condition without degradation or chemical alteration, while probiotic bacteria in the gut are available (Singdevsachan et al., 2016).

Recently, prebiotics is developed commercial applications for human health, for example chicory root extract, fructooligosaccharide (FOS), galactooligosaccharide (GOS), glucooligosaccharide (Shi et al., 2018), xylooligosaccharide (XOS) (Singdevsachan et al., 2016), isomaltooligosaccharide (IMO), polydextrose, β-glucan

(Lam and Chi-Keung, 2013), mannooligosaccharides (MOS), inulin and so on (Halsted, 2003; Saetang and Khanongnuch, 2014).

2.2.2 Production process of prebiotic

According to earlier studies, the mushroom is a viable alternative source of prebiotics because it contains β-glucan with prebiotic properties. This β-glucan is non-digestible and unable to hydrolyze the β-glycosidic bond of β-glucan because it is resistant to hydrolyzing by acid in the stomach and human digestive enzymes (Singdevsachan et al., 2016). The sources of β-glucans with prebiotic activity are laminarin from *Laminaria digitata* seaweed, curdlan from *Alcaligenes faecalis* bacteria, β-glucans from barley, oat, *Poria cocos* and *Polyporous rhinocerus* mushroom. These β-glucans promote the probiotic bacteria to ferment SCFAs and organic acids to inhibit pathogenic bacteria proliferation and benefit host health (Zhao and Cheung, 2013).

Afterward, β-glucan extract from the prior source would be hydrolyzed by the β-glucanase enzyme to produce β-glucan oligosaccharide (BGO). In β-glucan hydrolysis, β-glucanase would cleavage β-1,3 and β-1,6-glycosidic linkage of β-glucan into BGO. Their different glycosidic linkages and molecular weight depend on β-glucans utilization from various sources. Consequently, the received BGO has a short oligosaccharide with low molecular weight and prebiotic property that could be tolerant to acid and digestive enzymes in the live enzymes in GI tract (Lam and Chi-Keung, 2013). Smaller molecular oligosaccharides are more easily accessible to probiotic bacteria in the colon than polysaccharides with a high molecular weight for consumption and fermentation (Nowak et al., 2018). This bioactive sugar also demonstrates immune-potentiation (Otaka, 2006). The sources of this enzyme for hydrolysis of β-glucan are laminarinase (β-1,3-glucanase) from *Streptomyces matensis* bacteria (Guan et al., 2018), β-1,3-glucanase from *Rhizomucor miehei* fungi (Shi et al., 2018), *Trichoderma harzianum* fungi (Giese et al., 2011), *Bacillus circulans* bacteria (Kim et al., 2006), *Cellulosimicrobium cellulans* bacteria (Fu et al., 2015) and

lichenase (β-1,3-1,4-glucanase) from *Penicillium occitanis* fungi (Chaari et al., 2016) etc.

In addition, Chaikliang et al. (2015) studied β-glucans and oligo-β-glucans from *S. commune* Fr and *Auricularia auricula* Judae on prebiotic properties. These β-glucans were extracted under high pressure and temperature. Laminarinase was used to hydrolyze these oligo-β-glucans from β-glucans. Following that, fecal fermentation in batch culture *in vitro* was performed to compare fecal bacteria growth during fermentation of the β-glucans and oligo-β-glucans from these mushrooms and commercial yeast β-glucan, and the number of each probiotic bacteria and pathogenic bacteria strains were counted for the calculation of the prebiotic index of each extract. It was noticed that β-glucan from *A. auricula* Judae raised *Lactobacillus* sp. and *Bifidobacterium* sp. significantly. The prebiotic index of β-glucan from *A. auricula* Judae was superior to that of yeast β-glucan, β-glucan from *S. commune* Fr and oligo-β-glucan from both mushrooms, respectively. The probiotics also produced SCFAs, such as acetate, propionate, butyrate and lactate. Thus, the β-glucans from these mushrooms are prebiotic candidates.

Furthermore, Shi et al. (2018) evaluated curdlan-β-1,3-glucan oligosaccharide *in vitro* digestibility under simulated gastrointestinal settings, as well as stability of this oligosaccharide at low pH, high temperature, and Maillard reaction conditions during food processing. In the oligosaccharide production, curdlan (β-1,3-glucan) from *Alcaligenes faecalis* bacteria was degraded by β-1,3-glucanase from *Rhizomucor miehei* fungi. The results revealed that this oligosaccharide was stable to simulated GI digestion, including low pH, thermal, and tolerant to Maillard reaction conditions. It also enhanced *Lactobacillus* sp. and *Bifidobacterium* sp. populations that produced SCFAs of acetic, propionic and lactic acid, while this oligosaccharide inhibited *E. coli* growth. The short oligosaccharides with the degree of polymerization (DP) of 2 and 3 were easily metabolized by *L.* strains. They modulated large bowel environment pH to benefit the host's well-being and health. This might be potentially utilized as functional food materials.

Moreover, Nowak et al. (2018) reported the potential of polysaccharides from 53 strains of wild mushrooms to promote the proliferation of *L. rhamnosus* and *L. acidophilus*, as well as examined the digestibility of polysaccharide fractions. This study would use ethanol to extract polysaccharides from each mushroom's fruit bodies and sonication, heat, and the Savage reagent (chloroform: isoamyl alcohol) for deproteinization. The promotion of probiotic bacteria growth and *in vitro* digestion of these fungal polysaccharides were then in a simulated GI tract. It was noticed that mushroom polysaccharides stimulate *L.* strains' proliferation greater than commercial prebiotics (FOS or inulin). The polysaccharides were also resistant to artificial gastric juice and remained stable (undigested). The polysaccharides could pass through the upper GI tract, reach the large intestine, and promote beneficial probiotic bacteria growth. As a result, edible mushroom polysaccharides would be possibly utilized in functional foods and nutraceutical production.

2.2.3 Prebiotic and other biological properties

The mechanism of probiotics action was presented in Figure 9. These mechanisms comprise (1) the effect of prebiotics on the composition of dietary ingredients for substrate availability by gut microbe obviously; (2) the bioconversion of carbohydrates into fermentable products with inhibitory activities by microbiota; (3) the production of growth substrates, such as vitamins and exopolysaccharide to regulate the microorganism; (4) impact of probiotics bacteria on the normal flora possibly by direct antagonism with bacteriocins; (5) increasing competitive exclusion for binding sites of harmful bacteria; (6) enhancement of barrier action of epithelial cell; (7) decreasing inflammation, resulting in modifying intestinal activities for colonization and existence within; and (8) enhancement of innate immunity (Singdevsachan et al., 2016).

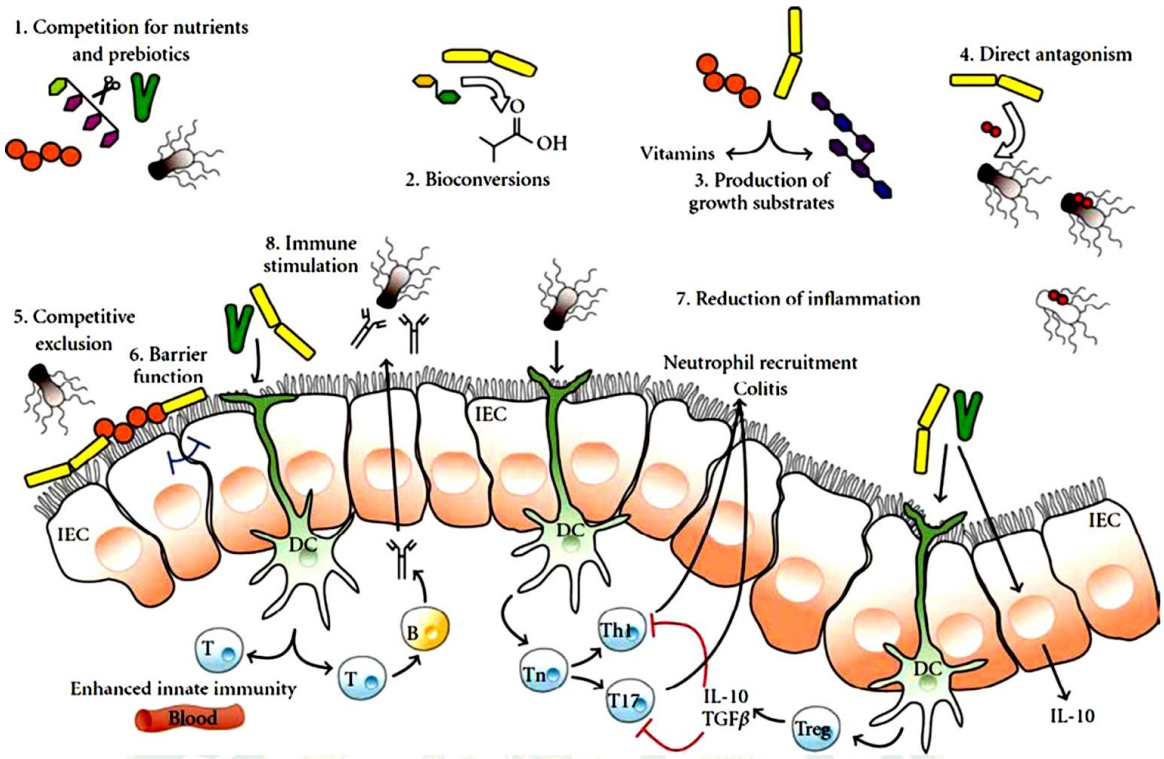


Figure 9 Mechanism illustration of probiotics function.

Source: Singdevsachan et al. (2016)

2.3 Alternative protein

The FAO (Food and Agriculture Organization of the United Nations) now forecasts that the world's population will be unable to consume enough protein without alternative proteins from other sources. Agriculture production must be raised by around 70% to meet the world food demand growing by 2050 (Stephan et al., 2018). The manufacture of high-protein, meat-free health meals has piqued international interest. Both vegetarians and non-vegetarians are concerned about paying a higher price for a food product that is high in nutrition, health, safe, and mentally friendly. As a result, it is expected that the market for alternative proteins is expected to isolate, mycoprotein (mushroom protein), and other alternative protein sources available (Kim et al., 2011).

Mycoprotein is recognized as the alternative of good quality protein to animal proteins (González et al., 2021). The mycoprotein also has a naturally meaty flavor since the mushroom comprises glutamic and aspartic acid (Poojary et al., 2017). Protein is also necessary for the growth and maintenance of the human body and

the crucial physiological functions of hormones and enzymes. Although animal proteins have a higher nutritional value, they increase human cardiovascular disease and colorectal cancer before, other alternative protein sources should be promoted to produce commercially and consume more (González et al., 2021).

2.3.1 Nutritional value

The split gill mushroom was a good alternative protein source and rich in essential amino acids compared to various vegetables (González et al., 2021). This mushroom consisted of plentiful protein (18.8% of dried mushroom). Essential amino acids, such as lysine, threonine, valine, leucine, and arginine, accounted for about 41% of the total amino acid composition (Ivanova et al., 2015). However, the chemical score of this mushroom was 28 lower than whole egg protein, while methionine was a limiting amino acid. The true digestibility of protein (TDP) was about 53% less than casein for the control diet (Hobbs, 2005).

2.3.2 Extraction process of protein

Various proteins from various sources are extracted using both traditional (water, acid, alkaline solution, salt, solvent, detergent) and modern green technologies for cell disruption in the protein extraction process (enzymes, sonication, pulsed electric field, microwave, high pressure, homogenization). Occasionally, conventional methods may result in a lower yield and quality of protein extract due to protein degradation. Numerous variables affect protein yield, including temperature, pH, extraction time, and solvents. As a result, research on non-thermal green technologies focuses on increasing extraction efficiency and minimizing protein degradation during extraction. These procedures affect the eco-friendly environment and the protein extracts are safe for intake without utilizing toxic solvents and chemicals. Novel protein extraction techniques could improve the yield of protein extract, and nutritional and functional properties. Exploiting conventional cooperating with non-conventional methods for protein extraction accomplishes greater protein yields (Kumar et al., 2021).

Additionally, extraction solvents, such as aqua, acids, alkalis, and organic solvents are used in chemical procedures. Chemical approaches are also used with other methods to improve protein recovery. Typically, the protein extraction process consists of three steps: fat removal, protein extraction, and protein precipitation. The following steps are: (1) fat and other compounds in the sample interfering with protein extraction are removed by solvents like n-pentane, n-hexane and petroleum ether. (2) Proteins are extracted by salts (NaCl), non-ionic detergents (Triton X100, NP-40) and ionic detergents (SDS), along with organic solvent, for example, alcohols (methanol, ethanol), and buffers/strong denaturing agents (Tris-HCl, urea, phenol). A lot of innovative techniques like enzymes, ultrasound and microwave could enhance the efficiency of protein extraction. (3) The isolated protein is precipitated at the isoelectric point and concentrated using solvents or chemicals like methanol, ethanol, acetone, ammonium sulfate, hydrochloric acid, citric acid and trichloroacetic acid (Kumar et al., 2021).

Organic solvents are important in protein extraction and precipitation, producing pure protein products. The water extraction is commonly used with various protein sources because the separated protein has great solubility and stability. Proteins can bind to lipids, have polar and/or non-polar side chains, and is made up of aromatic amino acids that are soluble in organic solvents (acetone, ethanol, butanol). Furthermore, alkali extraction is the most used method for protein extraction. Alkaline solutions, such as NaOH and KOH, are widely used to maintain a basic pH and achieve a higher extraction yield. Protein disulfide links are broken when the pH is too high for cell wall degradations, including augments protein recovery and yield. Protein solubility increases when the pH of alkali rises. Improving either temperature or time increase normally enhances protein diffusion into solvent and protein yield. Temperature plays also a key role that should be considered in alkali extraction, resulting in stable protein structure, folding and functionality (Kumar et al., 2021).

Enzyme-assisted extraction (EAE) is a viable technique for commercially recovering high-quality proteins in biochemical protein extraction procedures. Any cell's stiff cell wall acts as a barrier to protein extraction. This extraction particularly

assists to destroy cell walls through specifically degrading cell wall components (cellulose, hemicellulose, and pectin) with the enzyme. Each enzyme function to catalyze efficiently at the optimum acidic and/or alkaline environments (Kumar et al., 2021). Single or multiple enzymes, such as proteases and carbohydrates, are frequently used to degrade cell walls during extraction. Protease extracts the protein more effectively because proteolysis reduces the size of the protein, allowing it to dissolve more easily in solution. Carbohydrases can also break down cell walls and liberate intercellular components like protein. This enzyme can be used in conjunction with protease or alkali therapy. The major enzyme is used to improve protein recovery in acidic or somewhat alkaline circumstances. Nevertheless, protein content hydrolyzed by the mixture of Alcalase (protease) and carbohydrase was not much better than that of protease hydrolysis alone (Sari et al., 2015). Besides, these protein extracts demonstrate high protein yield, superior quality proteins, greater thermal stability, lower viscosity, inferior resistance to oxidative stress resistance, and functional and biological properties (antioxidant, free radical scavenging and metal chelation). Consequently, due to the environmentally favorable impact, enzyme-mediated extraction would be suitable for the large-scale manufacture of numerous functional foods, including high-quality protein. The reuse of immobilized enzyme decreases the cost of this protein extraction approach (Kumar et al., 2021).

Ultrasound-assisted extraction (UAE) is a physical technique for protein extraction that uses an ultrasonic wave to create gas bubbles and cavitation on the cell wall surface, causing cell wall damage and the liberation of intracellular protein into the extracting solvent with higher extraction yields. PEFAE (pulsed electric field-assisted extraction) is a technique in which samples are subjected to a higher electric field strength for a short period, usually a few micros to milliseconds. The electric field creates holes in the cell membrane, allowing internal proteins to escape. Microwave-assisted extraction (MAE) uses electromagnetic radiation to create a microwave that penetrates the cell, helps soluble proteins diffuse into the solvent, and increases protein recovery. High-pressure-assisted extraction (HPE) breaks down cells by applying pressure to their cell deformation, boosting solvent mass transfer into the cell wall, and improving protein extraction efficiency. Therefore, most of

these advanced green procedures have several benefits, while compared to conventional, i.e., higher yield, rapid extraction time, cost-efficient, lesser solvent consumption and user-friendly extraction technique. Enzymatic, ultrasonic, pulsed electric field, microwave, or high-pressure treatment approaches could be improve over and yield (Kumar et al., 2021). Figure 10 is an illustration of non-traditional green protein extraction processes.

Furthermore, Oli et al. (2020) researched the evaluation of the antimicrobial potentiality of protein extract and the determination of bioactive compounds from *A. auricula-judae* mushroom. The mushroom proteins were extracted by Tris buffer and warm aqueous solution before precipitating by cold acetone. The phytochemical analysis of these extracts showed similar protein contents (23.75 µg/100 g weight of dry extract), along with carbohydrates, flavonoids, alkaloids, saponin and tannin. These protein extracts revealed antimicrobial activity against bacterial and fungal pathogens (*Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Candida albicans*).

Moreover, Wongaem et al. (2021) reported peptide production from the split gill mushroom by hydrolysis of mushroom protein with Alcalase. The optimal conditions of peptide production were enzyme to substrate ratio of 2% (v/w) at 55°C for 161.41 minutes. The protein hydrolysate with a molecular weight of less than 0.65 kDa could demonstrate the highest radical-scavenging capability in the human intestinal cancer cell line (HT-29). The peptide with putative antioxidant properties fights cancer effectively.

Besides, Poojary et al. (2017) studied enzyme-assisted extraction of increasing peptides and umami taste amino acids from shiitake mushroom (*Lentinus edodes*) with β-glucanase and Flavourzyme (protease) for degradation of the cell wall and proteins, respectively. It was noticed that only β-glucanase mediated extraction did not raise the extraction efficiency. In contrast, a mixture of β-glucanase and Flavourzyme significantly improved the extraction efficiency approximately 20-fold compared to conventional assisted extraction with HCl. The optimized enzyme hydrolysis condition analysis by aqueous extraction was β-glucanase concentration

of 5% (v/w), Flavourzyme concentration of 5% (v/w) at initial pH 7.0, temperature 50°C for 1 hour. Conditions reached the greatest source of umami taste amino acids.

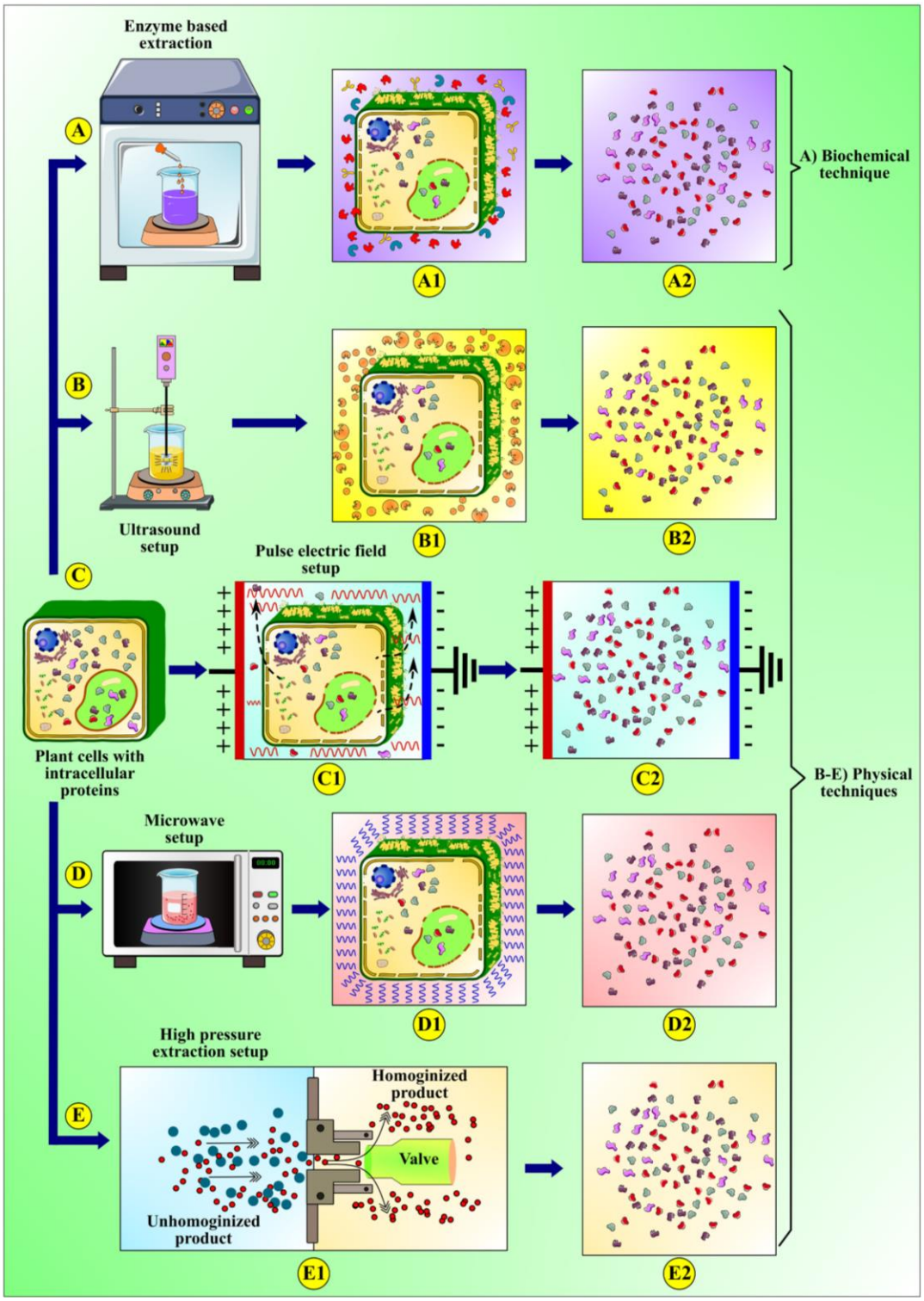


Figure 10 Advanced green methods for protein extraction.

Source: Kumar et al. (2021)

2.3.3 Chemical characterization and biological properties

The split gill mushroom comprised plentiful protein (Basso et al., 2020) with various biological properties, for instance, protein hydrolysate obtained from protein hydrolysis with Alcalase (protease enzyme) with radical-scavenging activity in the human colorectal adenocarcinoma cell line (HT-29) (Wongaem et al., 2021). Moreover, the hydrophobin protein demonstrated anticancer activities in each mouse model induced with S180 sarcoma and B16-F10 melanoma cell line, respectively (Akanbi et al., 2013). The lectin protein showed suppressive-carcinoma growth and proliferation (Han et al., 2010), both nontoxic hydrophobin and lectin protein through likely immune-enhancing as adjuvant associating with radiation and chemotherapy (Akanbi et al., 2013; Zhao et al., 2020). Schizolysin (hemolysin) protein exhibited antiviral properties due to the suppression of HIV-1 reverse transcriptase enzyme, inhibiting HIV-1 virus multiplication (Han et al., 2010).

Furthermore, this mushroom produces more hydrophobin proteins or the proteins may be released from other filamentous fungi. The short hydrophobins comprise eight cysteine amino acids linked by four disulfide bonds. The hydrophobic proteins could be classified into two types based on the solubility features of the assembled protein, namely assembled type I hydrophobins are very constant and soluble only in formic acid or trifluoroacetic acid (TFA). Another type is type II hydrophobins trouble with ethanol, SDS, or using pressure. The assembly of both proteins causes surface hydrophobicity. Hydrophobins are therefore used in medical applications, such as increasing the bioavailable hydrophobic medicines' bioavailability and improving implant surfaces' biocompatibility. This protein also affects immunomodulatory activity by raising mRNA levels of IL-10 and TNF-α cytokines in spleen cells (Akanbi et al., 2013).

Moreover, lectins from medicinal mushrooms have immune-boosting and cancer-fighting properties. The immunomodulatory processes of mushroom lectins vary, with some lectins helping to boost the immune system while others releasing potential cytokines that impact cytotoxic cells. Additionally, lectin proteins are unique proteins that can recognize and bind to various cell surface carbohydrates or

glycoproteins. For instance, these proteins can activate nitric oxide production, increase the expressions of interleukins and TNF-α to inhibit the proliferation of carcinoma cells, stimulate lymphocytes, enhance activation of macrophage to produce other components, and so on (Zhao et al., 2020).

CHAPTER 3
MATERIALS AND METHODS

3.1 Conceptual framework and methodology

The methodology for the experiments is depicted in Figure 11. The split gill mushroom was identified mushroom strain and investigated nutritional values. Then, schizophyllan and protein from this mushroom were studied for an optimized extraction process. After that, schizophyllan and protein from the optimized extraction processes were analyzed for chemical characterization and biological activity. Finally, the functional food product was developed for consumer acceptance.

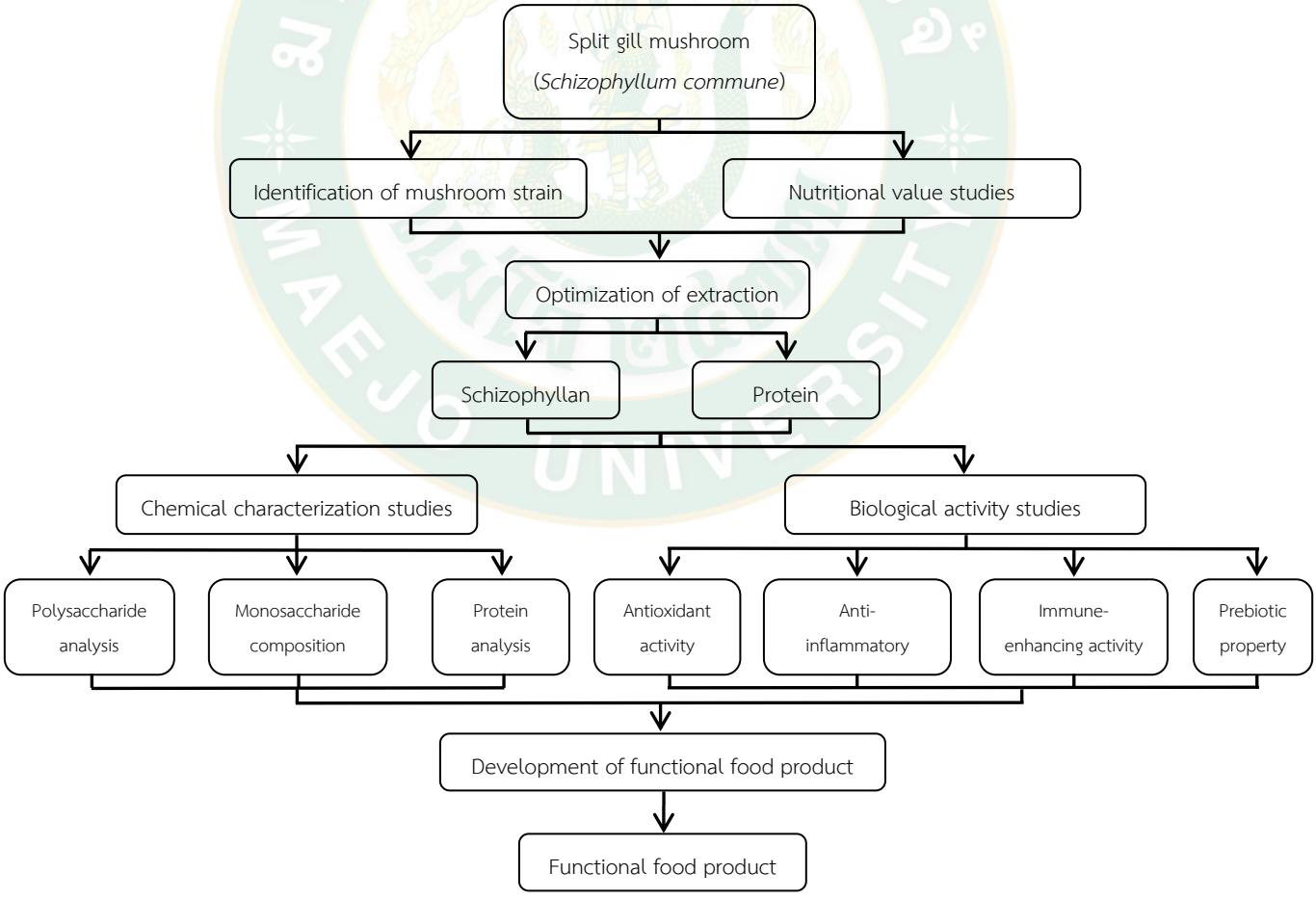


Figure 11 Conceptual framework and methodology.

3.2 Mushroom sample and maintenance

The dried split gill mushroom was purchased from Chaiyo Mushroom Farm in Surat Thani, Thailand. The dried mushroom was powdered finely before being in a vacuum bag and stored at room temperature until use in further experiments.

3.3 Mushroom strain identification

To begin with DNA extraction at the Institute of Product Quality and Standardization (IQS), Maejo University, Chiang Mai, Thailand, the cells of dried split gill mushroom powder were disrupted using buffer PL2 and proteinase K (incubated at 65°C for 30 minutes, added buffer PL3 and incubated on ice for 10 minutes), filtrated lysate using NucleoSpin® Filter, and bound DNA with NucleoSpin® Plant II Column (NucleoSpin® Plant II Protocols: Genomic DNA from Plant). Then, the DNA extract was washed using buffer PW1 and PW2 and dried silica membrane, eluted using buffer PE (incubated at 65°C, 5 minutes), including measured DNA and RNA concentration by Thermo Scientific NanoDrop 2000C Spectrophotometer.

After that, this target DNA extract was amplified using ITS5 forward primer and ITS4 reverse primer by polymerase chain reaction (PCR). The PCR conditions for DNA amplification showed in Table 1. Then, this PCR product was checked the molecular size on 1.5% (w/v) agarose gel containing ethidium bromide by agarose gel electrophoresis and visualized using ultraviolet light. Afterward, the purified PCR product was sent for DNA sequencing to Apical Scientific Sdn, Bhd (1st BASE Laboratories Sdn Bhd), Selangor, Malaysia. The DNA sequence analysis of the 18S rRNA gene was performed using an automated sequence analyzer and obtained these consensus sequences by aligning the forward ITS5 and reverse ITS4 sequences. At last, the nucleotide sequences of this mushroom were compared nucleotide sequence homology by Basic Local Alignment Search Tool (BLAST) analysis to other nucleotide sequences in a database of GenBank or National Center for Biotechnological Information (NCBI).

Table 1 Polymerase chain reaction (PCR) conditions for DNA amplification from the split gill mushroom.

PCR reaction	PCR conditions	
Pre-denaturation	94°C, 2 min	
Denaturation	94°C, 30 sec	25 – 40 cycles
Annealing	52°C, 30 sec	
Extension	68°C, 1 min	

3.4 Nutritional value investigation of the mushroom

Most nutritional values of this mushroom were determined by AOAC method (AOAC, 2012) at Institute of Product Quality and Standardization (IQS), Maejo University, Chiang Mai, Thailand, namely ash, moisture, protein, fat, carbohydrate, and crude fiber content, as following:

The ash content of dried split gill mushroom powder was estimated using AOAC protocol (AOAC, 2012). The powdered mushroom sample (10 g) were placed in a crucible and converted into ash in a muffle furnace at 525°C for 6 hours. Cooled crucibles were put in desiccators and weighed. The moisture content of the dried mushroom sample was determined using AOAC protocol (AOAC, 2012). The mushroom powder (5 - 10 g) was spread on metal dish, weighed, and dried in an oven at 70°C for 6 hours under 100 mm Hg (13.3 kPa). Upon drying the dish was transferred in a desiccator to cool. The heating and cooling process were repeated until constant weight was achieved. The loss in weight obtained after drying was regarded as the moisture content.

The Kjeldahl method determined the protein content (AOAC, 2012). A 0.7 - 2.2 g of the ground mushroom sample was digested in Kjeldahl flask using 98% (v/v) H_2SO_4, $CuSO_4.5H_2O$ as catalyst with K_2SO_4 as a boiling point elevator, before it was steam-distilled to release nitrogen from protein. The resulting distillate was titrated with 0.1 M HCl to first trace of pink or wine-red color, and the percentage of protein content was calculated as following equation (Eq. 1):

$$\text{Protein content (\%)} = \frac{(Vs - Vb) \times M \times 1.4007 \times 6.25}{W} \quad (1)$$

Where: V_s = volume (ml) of standard HCl acid used to titrate a sample;

V_b = volume of standard HCl acid used to titrate a blank;

M = molarity (0.1) of standard HCl acid;

W = weight (g) of dried sample used.

The fat content was determined according to the standard method Association of Official Agricultural Chemists (AOAC, 2012, 989.05). The content of the available carbohydrate was calculated by difference according to the following equation (Eq. 2):

Carbohydrate content (%) = 100 - [(ash + moisture + protein + fat) %] (2)

The crude fiber was estimated using AOAC protocol (AOAC, 2012). About 2 g of the mushroom sample was boiled in 1.25% (v/v) H_2SO_4 with antifoam solution for 30 minutes. The solution was filtered, and the residues were washed with boiling water 4 times. Crucibles containing the residues were dried at 130°C for 2 hours, cooled in a desiccator, and weighed. The crucibles with ash were then placed in a muffle furnace at 550°C for 2 hours, in a desiccator, and reweighed to find the fiber content percentage.

3.5 Heavy metal contamination of the mushroom

Heavy metal contamination, e.g., cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As) were determined by AOAC method (AOAC, 2019) at Institute of Product Quality and Standardization (IQS), Maejo University, Chiang Mai, Thailand. The dried mushroom sample (0.2 - 0.5 g) was digested with 3 M HNO_3 and 30% H_2O_2 under pressure in a closed digestion vessel heated by microwave. If test solution needed to be further diluted, it would be diluted with 3 M HNO_3 to the same acid concentration before detected metal contamination. Then, these heavy metals in

the test solution were determined by graphite furnace atomic absorption spectrophotometry (GFAAS), and expressed as parts per million (ppm) or mg/kg of the dried mushroom (AOAC, 2019).

3.6 Optimized hot water extraction of schizophyllan extract

The dried mushroom powder was weighed 10% (w/v) in deionized (DI) water and adjusted pH around 8 - 9 with natural limewater. The mushroom suspension was heated at various temperature (80 - 121°C) by water bath and autoclave, including time of hot water extraction (1 - 3 hours) before being filtrated through filter cloth. The filtrate was then precipitated with 95% ethanol in ratio of 1:2 at 4°C for 24 hours. After that, polysaccharide suspension was centrifuged at 3,000 rpm for 10 minutes to separate the polysaccharide from supernatant and dried at 60°C for 24 hours. The even of the supernatant was separated from ethanol solution by evaporation at 60°C before freeze-drying.

The hot water extraction process to produce polysaccharides was optimized using the central composite design (CCD) and response surface methodology (RSM). The CCD experiment was conducted under the optimal conditions of the significant factors, namely temperature (A) ranging from 80 to 121°C and extraction time (B) ranging from 1 to 3 hours. Table 2 provides the details of the experimental variables and their corresponding levels for the optimization study. To determine the code values of each variable in each treatment, Table 3 presents the central composite design code values. The yield of polysaccharide and supernatant extract was measured, and further analysis included the determination of total sugar, reducing sugar and protein content in the polysaccharide. The collected data was analyzed using the Design Expert program version 11, developed by Stat-Ease Inc. in Minneapolis, USA. This software facilitated the statistical analysis and optimization of the experimental results.

Table 2 Levels and ranges of operating parameters for optimization of hot water extraction.

Operating factors	Coding	Units	Levels and ranges		
			Low (-)	Center point (0)	High (+)
Temperature	A	°C	80	100	121
Time	B	hr	1	2	3

Table 3 Treatments generated based on the central composite design to hot water extraction conditions.

Treatments	A	B
1	-	-
2	-	-
3	+	-
4	+	-
5	-	+
6	-	+
7	+	+
8	+	+
9	-	0
10	-	0
11	+	0
12	+	0
13	0	-
14	0	-
15	0	+
16	0	+
17	0	0
18	0	0
19	0	0

A = temperature, B = time

3.6.1 Total sugar analysis

The total sugar content of the samples was determined using the phenol-sulfuric acid method described by Dubois et al. (1956). To perform this analysis, 0.5 ml of the appropriately diluted sample was mixed with 0.5 ml of 5% (w/v) phenol and 2.5 ml of 98% (v/v) sulfuric acid. The mixture was thoroughly mixed and then incubated at room temperature for 10 minutes. After the incubation period, the absorbance of the solution was measured spectrophotometrically at a wavelength of 490 nm. The total sugar content in each sample was quantified in micrograms per gram of dried extract. Glucose was used as a reference for this measurement.

3.6.2 Reducing sugar analysis

The reducing sugar content in the samples was determined using the modified 3,5-dinitrosalicylic acid (DNS) method developed by Miller (1959). For this analysis, 0.5 ml of the diluted sample was combined with 0.5 ml of DNS solution and then boiled in a water bath for 15 minutes under dark conditions. After the solution was cooled, 4 ml of distilled water was added and thoroughly mixed with the sample. The absorbance of the resulting mixture was measured using a spectrophotometer at a wavelength of 540 nm. The reducing sugar content in each sample was reported in micrograms per gram of dried extract, with glucose serving as the standard. Additionally, the degree of polymerization (DP) was calculated by dividing the total sugar content by the reducing sugar content.

3.6.3 Protein analysis

Protein content was analyzed by the dye-binding method (Bradford, 1976) using bovine serum albumin (BSA) as standard protein. The protein content was determined by mixing 0.060 ml of sample with 1.800 ml of Bradford reagent and then incubating at room temperature for 10 minutes. The sample absorbances were then measured spectrophotometrically at 595 nm. The protein content in each sample was expressed as micrograms per grams of dried extract.

3.7 Optimized ethanol precipitation of the schizophyllan

The extraction process for schizophyllan was carried out as follows: Firstly, a specific amount of dry mushroom powder was weighed, ranging from 5% to 15% (w/v), and suspended in deionized (DI) water. The pH of the suspension was then adjusted to approximately 8 - 9 using natural limewater. Subsequently, the mushroom suspension was heated at 100°C for 2 hours, based on the optimized conditions obtained from a previous study on hot water extraction conducted by Saetang et al. (2022b). The resulting mixture was then filtered through a filter cloth to remove any solid particles. The filtrate obtained was subjected to precipitation by adding 95% ethanol at varying final concentrations, ranging from 49% to 74% (v/v), and kept at 4°C for 24 hours. After the precipitation period, the polysaccharide pellet was separated from the solution through centrifugation at 3,000 rpm for 10 minutes. The pellet was then dried at 60°C for 24 hours to obtain the schizophyllan polysaccharide in a dry form.

The central composite design (CCD) was applied to optimize the significant variables for the ethanol precipitation of schizophyllan under response surface methodology (RSM). This experimental design had two numerical factors: split gill mushroom content (A: 5 - 15% w/v) and ethanol concentration (B: 49 - 74% v/v). The independent factors (A, B) with their coded (-1 = low, 0 = center point, 1 = high) and actual levels of optimization experiment were presented in Table 4. A total run on RSM by using CCD in this experimental design involved full factorial design, one block, had generated 19 runs including non-center points 16 runs, center points 3 runs, and axial point (α = 1) (Table 5). In further analysis, the polysaccharide extracts which were measured total sugar and reducing sugar and protein content, as well calculated DP value of these extracts were evaluated using the Design-Expert program version 11 from Stat-Ease, Inc., Minneapolis, USA.

Table 4 Levels and ranges of operating parameters for optimization of ethanol precipitation.

Operating factors	Coding	Units	Levels and ranges		
			Low (-)	Center point (0)	High (+)
Split gill mushroom content	A	% (w/v)	5	10	15
Ethanol concentration	B	% (v/v)	49.0	61.5	74.0

Table 5 Treatments generated based on the central composite design to ethanol precipitation conditions.

Treatments	A	B
1	-	-
2	-	-
3	+	-
4	+	-
5	-	+
6	-	+
7	+	+
8	+	+
9	-	0
10	-	0
11	+	0
12	+	0
13	0	-
14	0	-
15	0	+
16	0	+
17	0	0
18	0	0
19	0	0

A = split gill mushroom content, B = ethanol concentration

3.8 Chemical characterization studies of the schizophyllan

3.8.1 Solubility of the schizophyllan

In order to investigate the solubility of the extract, dry extract powder was weighed at different concentrations (mg/ml) and suspended in distilled water. The extract suspensions were then subjected to heating using a water bath and a hot air oven, with variations in temperature, time, and extract concentration. The specific factors and levels of the solubilization experiment are provided in Table 6. Following the heating process, the suspensions were separated by centrifugation at 3,000 rpm for 10 minutes. This step allowed for the separation of any insoluble particles or residues from the soluble extract. The supernatants were measured total sugar (phenol-sulfuric acid method) (Dubois et al., 1956), reducing sugar (modified 3,5-dinitrosalicylic acid (DNS) method) (Miller, 1959) and total phenolic content (modified method from Stankovic (2011)) in further analysis.

Table 6 Ranges of operating parameters for extract solubilization.

Operating factors	Units	Ranges	
		Low (-)	High (+)
Incubation temperature	°C	60	120
Incubation time	min	30	180
Extract concentration	mg/ml	5	10

Total phenolic content was determined according to modified method from Stankovic (2011). Briefly, reaction mixture was prepared by mixing 200 µl of diluted sample, 1,000 µl of 10% (v/v) Folin-Ciocalteu's reagent and 800 µl of 7.5% (w/v) sodium carbonate solution ($NaCO_3$). After that, reaction mixture was incubated at room temperature for 60 minutes, and absorbance was measured using spectrophotometer at 765 nm. Gallic acid (GA) was used as standard. Therefore, total

phenolic content in each extract was expressed as micrograms of gallic acid equivalent (GAE) per grams of dried extract.

3.8.2 Protein molecular mass determination of the schizophyllan

The determination of the protein molecular mass in the schizophyllan extract was conducted using SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) at Biosynthai Biotechnology Co., Ltd. in Angthong, Thailand, following the method described by Garfin (1990). The sample preparation began by mixing 20 µg of the extract with 10 µl of 5X sample loading buffer, which contained 0.4 M dithiothreitol (DTT). The mixture was then boiled for 10 minutes. A 15% polyacrylamide gel, known as the separating or resolving gel, was prepared. An electrophoresis chamber was assembled for the gel electrophoresis process. Next, a 1X SDS electrophoresis buffer, specifically Tris/glycine buffer, was added to both the inner and outer reservoirs of the electrophoresis chamber. The comb that was previously used to create wells in the stacking gel was carefully removed. The prepared sample was loaded into the well located in the stacking gel, positioned below the electrode buffer. Electrode plugs were attached to a power supply, and the power supply was turned on at 150 volts for a duration of 1.15 hours, or until the Bromophenol blue loading dye front reached the lower gel. After gel running, gel was stained in staining solution, Coomassie blue 15 ml, for 30 minutes, and de-stained 3 times for each 15 minutes, till the background was removed satisfactorily.

3.8.3 Bioactive compound identification in the schizophyllan by Raman confocal spectroscopy

The schizophyllan extract was identified bioactive compound by Raman confocal spectroscopy at Faculty of Engineering and Agro-Industry, Maejo University, Chiang Mai, Thailand. Raman spectra were acquired using the Raman confocal microscope. A spectrometer LabRAM Soleil (Horiba Scientific, Thailand) was used in this analysis with a diode laser's 785 nm excitation line (11 mW at the sample). This extract was observed under a microscope, and scattered light was collected long focus objectives 100×. Spectra were recorded with a SynapseEM device detector.

The spectral window ranges were from 50 to 3,500 cm^{-1}. Raman spectra of purpurin extract (red dye) from madder plant (UCL London) were used as references.

3.8.4 Monosaccharide composition of the schizophyllan

HPLC determined the extract's monosaccharide content at Central Laboratory (Thailand) Co., Ltd. (Bangkok Branch), Bangkok, Thailand. To begin with monosaccharide standard and sample dissolution, the sugar standard (3 g/100 ml) was dissolved in DI water: acetonitrile = 1:1, (v/v), and the sample (10 g/100 ml) was prepared in DI water : acetonitrile = 1:1, (v/v). Following the preparation of the standard and sample solutions, both were filtered through a 0.45 µm filter and subsequently diluted as required. The next step involved injecting the standard and sample solutions into an HPLC column (high-performance liquid chromatography column) to analyze the monosaccharide composition. This analysis involved comparing the results to a standard monosaccharide curve. The HPLC analysis was carried out using specific conditions outlined in Table 7, which included parameters, such as column type, mobile phase composition, flow rate, and detection wavelength. These conditions were carefully followed to ensure accurate and reliable monosaccharide composition analysis.

Table 7 The HPLC condition analysis of monosaccharide.

Column	Bondapak/Carbohydrate column (300 mm × 4 mm)
Mobile phase	Acetonitrile: DI water = 83 : 17
Flow rate	1.0 ml/min
Temperature	23°C
Detector	Refractive index detector
Injection volume for sample	10 µl
Reference method	In-house method TE-CH-164 based on AOAC (2019) 977.20 (AOAC, 2019)

3.8.5 Oligosaccharides analysis of the schizophyllan

Oligosaccharides of this extract were analyzed by TLC (thin layer chromatography) at Food and Ago-Industry Lab, Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand. To begin with, spotted samples and standard oligosaccharides on TLC plate, then soaked in TLC tank with mobile phase (butanol: ethanol: DI water = 5:3:2). Checked band of oligosaccharides by soaked TLC plate in 5% (v/v) H_2SO_4 in 99% (v/v) ethanol. Dried TLC plate at 150°C, 10 minutes.

3.8.6 β-glucan analysis of the schizophyllan

The schizophyllan extract from the split gill mushroom was analyzed β-glucan content by β-glucan assay kit (β-glucan assay procedure K-YBGL 02/21 (Megazyme Int.)) at Food and Ago-Industry Lab, Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand. The β-glucan content in the extract was expressed as micrograms per grams of dried extract, calculated from total glucan minus with α-glucan content in the extract.

3.9 Biological properties of the schizophyllan

3.9.1 Antioxidant activities of the schizophyllan

These extracts from extraction of mushroom content 5% (w/v) (M5 EtOH63.33) and 10% (w/v) with ethanol concentration 63.33% (v/v) (M10 EtOH63.33), respectively, were selected for analysis of antioxidant activities, compared to aqueous extract. Aqueous extract was prepared with 80 mg/ml of the mushroom in distilled water and stood at room temperature for 30 minutes. After that, the extract was filtrated through cotton and centrifuged at 3,500 rpm for 10 minutes. The supernatant was analyzed antioxidant activities by DPPH and ABTS radical scavenging activity, compared to both previous extracts.

DPPH assay (2,2–diphenyl–1–picrylhydrazyl radical scavenging activity) was carried out according to modified method of Mensor et al. (2001). DPPH radical key was prepared at 0.3 mM DPPH solution in 99.9% (v/v) methanol. The DPPH radical solution was diluted to give absorbance at 517 nm around 1.00 ± 0.02. After that,

400 µl of diluted sample were combined with 2,000 µl of DPPH radical solution and incubated at room temperature for 20 minutes in dark condition. If the sample had precipitant, it would be separated the supernatant by centrifugation at 3,500 rpm for 10 minutes. The samples were measured the absorbance at 517 nm. Percentage inhibition of DPPH radical scavenging activity was calculated according to Equation 3. In contrast, linear regression analysis evaluated half-maximal inhibitory concentration (IC_{50} value) for inhibition of free radicals from percentage inhibition with concentration plot.

$$\% \text{ DPPH inhibition} = [(AC - AS)/AC] \times 100 \quad (3)$$

AC and AS are absorbance at 517 nm of control (99.9% (v/v) methanol) and sample, respectively.

ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was carried out according to modified method of Re et al. (1999). ABTS radical cation ($ABTS^+$) was prepared by making 7 mM ABTS stock solution react with 2.45 mM $K_2S_2O_8$ (potassium persulfate) at ratio of 1:0.5 before standing in darkness at room temperature for 12 - 16 hours. Then, $ABTS^+$ solution was diluted with distilled water to obtain absorbance of 0.70 ± 0.05 at 734 nm. After that, 200 µl of diluted sample were combined with 1,800 µl of $ABTS^+$ solution and incubated for 6 minutes at room temperature. The absorbance was spectrophotometrically measured at 734 nm. Percentage inhibition of ABTS radical scavenging activity was calculated according to Equation 4. In contrast, linear regression analysis evaluated half-maximal inhibitory concentration (IC_{50} value) for inhibition of free radicals from percentage inhibition with concentration plot.

$$\% \text{ ABTS inhibition} = [(AC - AS)/AC] \times 100 \quad (4)$$

AC and AS are absorbance at 734 nm of control (distilled water) and sample, respectively.

3.9.2 Prebiotic activity of the schizophyllan

The schizophyllan extract was investigated prebiotic property by promoting probiotic bacteria growth in MRS medium (de Man, Rogosa and Sharpe). To begin with isolation of probiotic bacteria, this probiotic bacteria strain was isolate BG-NS02 from Bulgaria yogurt (CP-Meiji Co., Ltd., Saraburi, Thailand). This bacteria strain was activated to grow better in MRS broth and incubated at 37°C under anaerobic condition, before streaked on MRS agar supplemented with bromocresol blue to isolate single colony. Then, the isolate was tested Gram staining and observed under the light microscope (at 100× magnification).

Afterward, the starter of the bacteria strain was prepared by gaining their single colony cultivated in MRS broth for inoculum at 37°C under anaerobic condition. After that, the inoculum was measured optical density (OD) at 600 nm around 0.7 and inoculated of 10% (v/v) inoculum in each treatment, namely MRS broth without carbon source (negative control), MRS broth added 1% (w/v) glucose (positive control), and MRS broth supplemented with 1% (w/v) schizophyllan extract. Finally, these treatments were incubated at 37°C under anaerobic condition, and each sample was collected to determine OD at 600 nm every 6 hours for 30 hours.

3.9.3 Anti-inflammatory and immune-enhancing properties of the schizophyllan

To examine the cytotoxic effects of the schizophyllan extract (SC) obtained from *S. commune* on murine RAW 264.7 cells (macrophages), a study was conducted at the Department of Physiology, Faculty of Medicine, Chiang Mai University, Thailand. The cytotoxicity assessment was carried out using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay). To initiate the experiment, RAW 264.7 cells were cultured in DMEM at a temperature of 37°C for a duration of 3 days. The cells were grown in a humidified atmosphere containing 5% CO_2. For each well in a 96-well plate, 200 µl of cells at a concentration of 2.0×10^6 cells/ml were seeded. The cells were then incubated in complete DMEM at 37°C for a period of 72 hours.

After the incubation period, the DMEM medium was removed, and the cells were pretreated with 150 µl of the SC extract at various concentrations (ranging from 250 to 1,000 µg/ml) in incomplete DMEM. The cells were subsequently incubated for different time intervals (ranging from 1 to 3 hours) at 37°C. Following this, the DMEM medium was discarded, and the cells were washed with 200 µl of 1% (w/v) phosphate-buffered saline (PBS), and the PBS was then removed. Next, 100 µl of MTT reagent (0.5 mg/ml) was added to each well, and the cells were incubated at 37°C for 4 hours. Subsequently, the MTT solution was removed, and 100 µl of DMSO (dimethyl sulfoxide) was added to each well, followed by incubation at 37°C for 30 minutes. The viability of the cells, indicated by the formation of purple formazan, was measured at a wavelength of 570 nm using a microplate reader. A sample detected at a wavelength of 630 nm was used as a reference.

Cell viability (% control) was calculated using Equation 5. Further studies were conducted to explore the anti-inflammatory and immune-enhancing properties of the SC extract by investigating the supernatants of the medium and the cells that had been pretreated with this extract.

$$\text{Cell viability (\% control)} = [(AS_{570} - AS_{630})/(AC_{570} - AC_{630})] \times 100 \quad (5)$$

Where AC_{570} and AS_{570} are absorbance at 570 nm of control and sample, respectively.

AC_{630} and AS_{630} are absorbance at 630 nm of control and sample, respectively.

3.10 Development of the functional food product

3.10.1 Development of formula product

In the production process of split gill mushroom essence, which is used as a functional food product, the first step involved extracting the split gill mushroom using hot water extraction. This method was chosen to extract the beneficial components from the mushroom. Subsequently, the extracted essence was concentrated to preserve the nutritional value of the functional components present in the mushroom. In order to optimize the production process, various factors were

studied, including the temperature and duration of the hot water extraction. These factors were carefully examined to determine their impact on the extraction efficiency and the overall quality of the essence. Once the extraction and concentration processes were completed, the concentrated mushroom essence was used to develop an optimal formula for the final product. In this development phase, factors, such as the content of split gill mushroom and other ingredients were taken into consideration. The goal was to determine the ideal combination of ingredients that would complement the extracted content of the mushroom and enhance the functional properties of the final product.

To study suitable production process of this mushroom essence, dried mushroom (2 g), this extract (0.017 g) and other ingredients was weighed and added drinking water (100 ml). The mushroom suspension was boiled at 100°C (from optimized hot water extraction in the prior research of Saetang et al. (2022b)) with various time (45 - 120 minutes), then added other ingredients, such as Indian gooseberry (0.013 g) and stevia (0.003 g). After that, the mushroom suspension was heated at 60 - 70°C for 15 minutes before filtrated through filter cloth and sterilized at 121°C for 20 minutes. Afterward, the mushroom essence was cooled down and kept in product package at 4°C.

In order to determine the optimal formula for the mushroom essence, the dried mushroom and the extract were weighed according to the specifications outlined in Table 8. The weighed mushroom was then suspended in a liquid and subjected to boiling at a temperature of 100°C for a duration of 120 minutes. Following the boiling process, additional ingredients, such as Indian gooseberry and stevia were added to the mushroom suspension. Subsequently, the mushroom suspension was further heated at a temperature range of 60 - 70°C for 15 minutes. This heating step was performed to ensure proper mixing and integration of all the ingredients. The mixture was then filtered through a filter cloth to remove any solid particles, and the resulting liquid was sterilized at a temperature of 121°C for a period of 20 minutes to eliminate any potential contaminants during the sterilization process. The mushroom essence was allowed to cool down and then transferred into suitable packaging for the final product. To maintain its freshness and quality,

the packaged mushroom essence was stored at a temperature of 4°C. This controlled storage condition helps in preserving the nutritional and functional properties of the essence.

Table 8 Ingredient content in each split gill mushroom essence formulas.

Ingredients	Content (% w/v)		
	Formula 1	Formula 2	Formula 3
Split gill mushroom	2.000 g	2.500 g	3.333 g
Schizophyllan extract	0.017 g	0.021 g	0.021 g
Indian gooseberry	0.013 g	0.017 g	0.017 g
Stevia	0.003 g	0.004 g	0.004 g
Drinking water	100 ml	100 ml	100 ml

3.10.2 Consumer acceptance of the product

The split gill mushroom essences, derived from the previously developed optimized formula product, underwent a preference test conducted by a group of 30 consumer panelists. The evaluation focused on the attributes of color, aroma, taste, and overall acceptability of the product. A questionnaire was administered using a 5-point hedonic scale, where 1 indicated 'dislike very much' and 5 indicated 'like very much'. Following the preference test, the collected data was analyzed using descriptive statistics. This statistical analysis involved summarizing and examining the data in order to gain insights into the panelists' opinions and preferences. The descriptive statistics allowed for a comprehensive understanding of the consumer feedback, helping to assess the overall reception of the split gill mushroom essences based on their color, aroma, taste, and overall acceptability.

3.11 Statistical analysis

All experiments were carried out in triplicate. All data were performed as either the mean value ± standard deviation (SD) or error bars by Microsoft Excel 2016. Moreover, these results were statistically examined through one-way analysis

of variance (ANOVA). Tukey's test considered the significance of the difference between each sample at a confidence level of 95.0%

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Molecular characterization of the split gill mushroom

The genomic DNA of the split gill mushroom (from Chaiyo Mushroom Farm in Thailand) was extracted. ITS5 forward and ITS4 reverse primers were used for amplification, resulting in DNA segments ranging from 500 to 600 base pairs (bp), as confirmed by agarose gel electrophoresis (Figure 12). Nucleotide sequences obtained from the mushroom using ITS5 and ITS4 primers are shown in Figure 13a and Figure 13b, respectively. Molecular analysis of the ITS regions revealed that the DNA sequences of this mushroom (641 bp) exhibited high sequence similarity with various strains and isolates of *Schizophyllum commune*, as well as some strains of *Agaricaceae* sp., as evidenced by comparison with entries in the NCBI GenBank (Table 9).

Furthermore, extensive efforts have been made to explore the genetic diversity among *S. commune* isolates, with the ITS region proving to be a valuable marker for molecular testing in this mushroom. The ITS region, which is part of the ribosomal RNA coding gene, is widely utilized in fungal molecular systematic and population genetics research. It is a highly variable region within rRNA sequences, making it suitable for analyzing the genetic diversity of mushroom and fungal strains. ITS sequences serve as barcodes, facilitating the categorization of genetic distribution among strains in Asia and Europe (Choi et al., 2020; Singh et al., 2021).

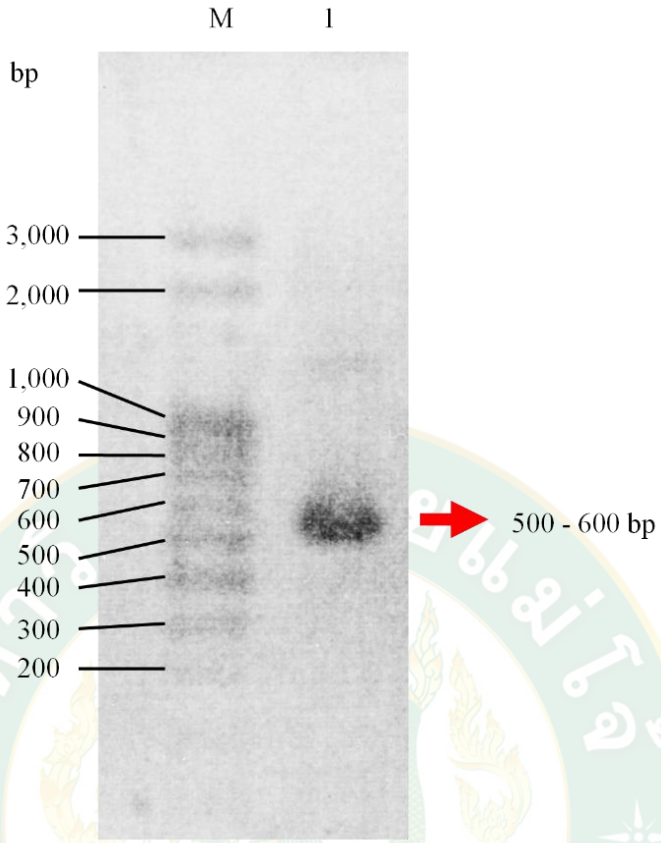


Figure 12 PCR amplification of split gill mushroom DNA profile with ITS5/ITS4 primer. Lane M, molecular size marker (M25 100 bp + 2 kb + 3 kb DNA ladder); lane 1, PCR product of this mushroom.

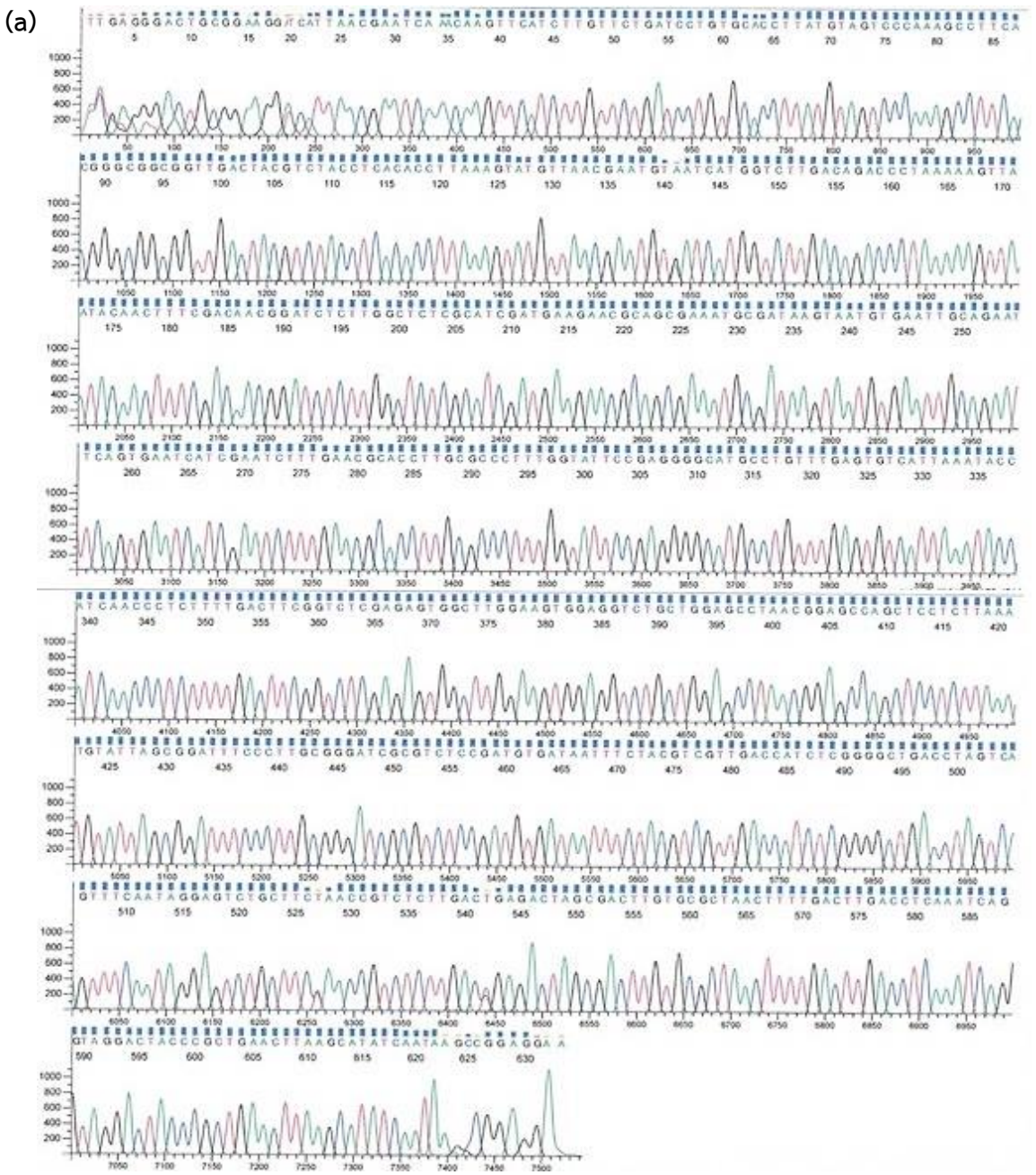


Figure 13 Nucleotide sequences of the split gill mushroom ITS regions.
(a) ITS5 forward primer and (b) ITS4 reverse primer.

(b)

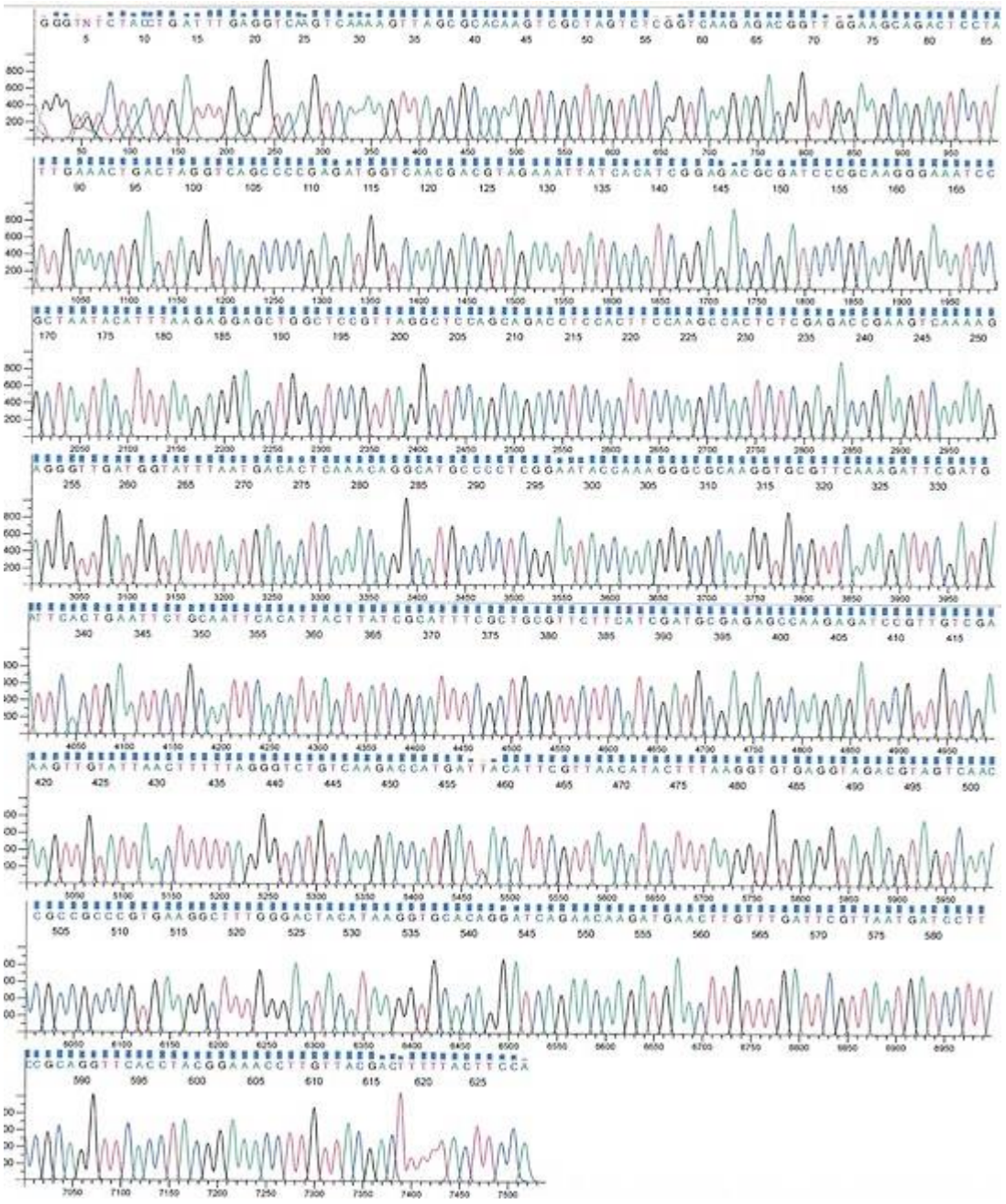


Figure 13 Nucleotide sequences of the split gill mushroom ITS regions.

(a) ITS5 forward primer and (b) ITS4 reverse primer (continued).

Table 9 Similarity of nucleotide sequence of the split gill mushroom after BLAST analysis with nucleotide sequence database of GenBank/ NCBI.

Mushroom species	Length of sequence (bp)	Sequence homology (%)	GenBank accession number
Schizophyllum commune strain BL1	653	100.00	MT466518.1
S. commune strain IFM 46097	657	100.00	AB369909.1
S. commune isolate Z-H-3A-2	647	100.00	OL471296.1
S. commune isolate H	650	100.00	MN547371.1
S. commune strain CBS579.83	634	100.00	MH861655.1
S. commune strain CBS405.96	643	99.84	MH862583.1
S. commune isolate Z-M-2A-1	695	99.84	OL471289.1
S. commune strainWB033_12	656	99.84	JX848644.1
S. commune isolate T28	674	99.84	JF439509.1
S. commune strain xsd08036	660	99.84	FJ478109.1
S. commune isolate Z3	674	99.84	EF155505.1
S. commune isolate HNO62	657	99.84	AF280750.1
S. commune strainUZ1552_14	651	99.84	KP326577.1
S. commune strain CBS124811	637	99.84	MH863418.1
S. commune isolate Z-E-4C-1	647	99.84	OL471293.1
Agaricaceae sp. BAB-4029	650	100.00	KM051395.1
Agaricaceae sp. GWY1(1)	662	99.84	KM268691.1

4.2 Nutritional composition and heavy metal contamination of the mushroom

Proximate analysis was performed to examine the nutrient composition of the split gill mushroom, including ash, moisture, protein, fat, carbohydrate, and crude fiber content (Table 10). It was obvious that this mushroom consisted of superior carbohydrate (64.06 ± 0.08%), high protein (20.99 ± 0.14%), crude fiber (4.87 ± 0.05%), low fat (0.75 ± 0.01%), ash (4.64 ± 0.01%), and moisture content (9.56 ± 0.04%). *S. commune* is highly regarded as a valuable mushroom with several health

benefits. It is known for being low in calories, free of fat and cholesterol. This mushroom serves as a rich source of various nutrients, particularly complex polysaccharides and proteins. Notably, schizophyllan, a polysaccharide, and proteins found in *S. commune* have been recognized for their potential anticancer properties (Singh et al., 2021). Carbohydrates in mushrooms play a vital role as an energy source. They exist in both digestible forms like glucose, mannitol, and glycogen, as well as non-digestible dietary fibers, such as chitin and β-D-glucans. The dietary fiber present in mushrooms contributes to several health benefits, including immune enhancement, anticarcinogenic properties, and regulation of blood glucose levels (Basso et al., 2020).

Table 10 Nutritional values of *S. commune*.

Analysis	Composition (g/100 g dried mushroom)	Test method
Ash	4.64 ± 0.01^{d}	AOAC (2012), 940.26
Moisture	9.56 ± 0.04^{c}	AOAC (2012), 934.06
Protein	20.99 ± 0.14^{b}	AOAC (2012), 991.20
Fat	0.75 ± 0.01^{e}	AOAC (2012), 989.05
Carbohydrate	64.06 ± 0.08^{a}	By difference
Crude fiber	4.87 ± 0.05^{d}	AOAC (2012), 978.10

Comparative analysis of the nutrient composition of this split gill mushroom in relation to other mushroom species is presented in Table 11. The results demonstrate that the protein content of this mushroom is significantly higher compared to *Auricularia polytricha* MFB, *Lentinus subnudus* YEB and *Pleurotus florida* YFB. However, the fiber content is similar to these mushrooms, and the fat content is lower in calories when compared to various mushroom species (Gbolagade et al., 2006). Furthermore, the protein and carbohydrate content of this mushroom are considerably greater than *Tuber aestivum* (Tejedor-Calvo et al., 2020).

Moreover, the protein and crude fiber content of this cultivated mushroom are higher than that of wild *S. commune*, whereas the carbohydrate and fat content are lower when compared to the wild mushroom (Okwulehie et al., 2007). However, it is important to note that in another study, the protein, fat, and crude fiber content of this cultivated mushroom were found to be lower than that of wild *S. commune*, possibly due to differences in geographical distribution and cultivation methods (Singh et al., 2021).

Table 11 Nutritional values of this dried mushroom compared with other mushrooms.

Mushroom species	Composition (g/100 g dried mushroom)						Reference
	Ash	Moisture	Protein	Fat	Carbohydrate	Crude fiber	
Auricularia polytricha MFB	5.20	4.40	8.50	5.20	NA	3.50	Gbolagade et al. (2006)
Lentinus subnudus YEB	5.90	7.20	6.50	3.60	NA	4.30	
Pleurotus florida YFB	9.70	5.80	15.30	0.90	NA	3.50	
Tuber aestivum	NA	NA	9.03	NA	31.02	NA	Tejedor-Calvo et al. (2020)
S. commune (wild)	7.46	NA	9.63	1.28	81.59	0.04	Okwulehie et al. (2007)
S. commune (wild)	4.40	4.03	24.51	1.32	45.86	19.88	Singh et al. (2021)
S. commune (farm)	4.64	9.56	20.99	0.75	64.06	4.87	This study

Remark: NA = not analyzed

Moreover, this mushroom was not detected heavy metal contamination, such as cadmium (Cd), lead (Pb), mercury (Hg) and arsenic (As), because these were less than the limit of detection (LOD), namely lower than 0.002 mg/kg dried mushroom (Table 12). These toxic heavy metals cause serious health at low content consumption, including acute and chronic effects on target organs. They would accumulate in the human skeleton, resulting in main nutrient depletion, central nervous system deficiency, immune, hematological, cardiac, gastric, hepatocellular, renal, and reproductive disorders (Munir et al., 2021). On the other hand, some researchers reported that heavy metal contamination in some raw materials, for instance, wheat bran, any mushroom (not specified), any fish, any liver and bovine muscle found lead (Pb) and cadmium (Cd) were more than 0.1 and 0.01 mg/kg dried raw material, respectively (AOAC, 2019). As a result, the split gill mushroom is confidently considered a safe mushroom without any heavy metal contamination and produces a variety of food, snack, beverage, drug and cosmetics supplemented with various beneficially bioactive compounds for consumer health.

Table 12 Heavy metal contamination of *S. commune*.

Heavy metal	Contamination (mg/kg dried mushroom)	Test method
Cadmium (Cd)	Not detected[1]	AOAC (2019), 999.10
Lead (Pb)	Not detected[1]	
Mercury (Hg)	Not detected[1]	
Arsenic (As)	Not detected[1]	

Remark: [1] Limit of detection (LOD) = 0.002 mg/kg

4.3 Optimized hot water extraction of schizophyllan extract

4.3.1 Yields of the schizophyllan

Two variables: the different temperatures and time of hot water extraction were studied for effects on extraction yields of polysaccharides and supernatants from the extraction of polysaccharide from *S. commune* as revealed in Table 13.

When the extraction temperature and time increased, polysaccharide and supernatant extracts reached superior yield. Consequently, the highest yield was obtained from polysaccharides (5.74 - 5.95 g/100 g of dried mushroom) and supernatant extracts (28.42 - 29.55 g/100 g of dried mushroom) at a temperature of this extraction 121°C for 2 - 3 hours, respectively.

Moreover, hot water extraction associated with alkaline solution treatment can be carried out to degrade exceedingly mushroom cell walls and release water-soluble intracellular components outside the cells. Thus, the result of polysaccharide yield is significantly higher than the only hot water extraction approach (Klaus et al., 2011). The greatest yield of the schizophyllan extract from hot water extraction combined with natural alkaline and ethanolic precipitation was 5.95 g/100 g of dried mushroom, including the yields of other mushroom species as presented in Table 14. It was observed that this polysaccharide extraction yield was superior to hot water extract associated with ethanol treatment of the same mushroom around 2.5-fold (Yelithao et al., 2019), hot water extract with ethanolic precipitation of *S. commune* at 4.30 g/100 g of dried mushroom, polysaccharide from the same extraction combined with polysaccharide purification of this mushroom about 12-fold (Klaus et al., 2011), polysaccharide extract from similar extraction of *Trametes hirsuta* about 2.5-fold, *Heterobasidion annosum* approximately fivefold and *Russula fragilis* at 14-fold (Nowak et al., 2018).

Besides, the greatest yield obtained from the supernatant extract of this mushroom may contain a lot of water-soluble components, for instance, a variety of phenolic compounds which are secondary metabolites as greatly potential scavengers of reactive oxygen species by oxidation action (Basso et al., 2020), including alkaloids, terpenes, steroids, free amines (Kabuyi et al., 2017), flavonoids (Arbaayah and Umi Kalsom, 2013), saponins and tannins. Other nutritional substances in the supernatant extract may also comprise various vitamins and minerals, such as vitamin B3 (niacin), vitamin C (ascorbic acid), vitamin B1 (thiamine), vitamin B2 (riboflavin), calcium, sodium, phosphorus, magnesium, and so on (Okwulehie et al., 2007).

Table 13 Effect of temperature and time of hot water extraction on extraction yields.

Extraction temperature (°C)	Extraction time (hr)	Extraction yield (g/100 g dried mushroom)	
		Polysaccharide	Supernatant
80	1	2.60 ± 0.77^{d}	25.37 ± 0.24^{bc}
80	2	2.90 ± 0.22^{cd}	26.93 ± 0.62^{abc}
80	3	3.02 ± 0.33^{cd}	26.46 ± 1.27^{abc}
100	1	4.00 ± 0.02^{bc}	26.14 ± 0.69^{abc}
100	2	4.03 ± 0.14^{bc}	27.30 ± 1.59^{abc}
100	3	3.74 ± 0.28^{cd}	27.26 ± 0.05^{abc}
121	1	5.07 ± 0.18^{ab}	24.23 ± 0.65^{c}
121	2	5.95 ± 0.04^{a}	29.55 ± 1.19^{a}
121	3	5.74 ± 0.04^{a}	28.42 ± 0.60^{ab}

Table 14 Polysaccharide extraction yields of different mushrooms.

Mushroom species	Extraction method	Yield (g/100 g dried mushroom)	Reference
S. commune	hot water extraction with ethanolic treatment (without purification)	2.39	Yelithao et al. (2019)
S. commune	hot water extraction with ethanolic treatment	4.30	Klaus et al. (2011)
	hot water extraction with ethanolic treatment and polysaccharide purification	0.50	
Trametes hirsuta	hot water extraction with ethanolic treatment	2.41	Nowak et al. (2018)
Heterobasidion annosum		1.15	
Russula fragilis		0.42	
S. commune	hot water extraction with alkaline and ethanolic treatment	5.95	This study

4.3.2 Total sugar content of the schizophyllan

The proper ranges of extraction time and temperature were optimized using the response surface methodology for maximal total sugar content, minimal reducing sugar content, and optimal degree of polymerization (DP) of the polysaccharide extracts from split gill mushroom. The effect of hot water extraction factors on polysaccharides' total sugar content was determined by variance analysis (ANOVA) as summarized in Table 15. A *p*-value less than 0.01 showed a significant effect on the total sugar content of polysaccharide extracts. The plus and minus values mean the positive and negative effects on total sugar content.

Table 15 ANOVA model for optimized hot water extraction of the polysaccharides' total sugar content.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	20870.23	7	2981.46	1069.13	< 0.0001
Temperature (A)	4944.82	1	4944.82	1773.18	< 0.0001
Time (B)	13.07	1	13.07	4.69	0.0533
AB	3106.45	1	3106.45	1113.95	< 0.0001
A^2	5621.47	1	5621.47	2015.82	< 0.0001
B^2	2580.70	1	2580.70	925.42	< 0.0001
A^2B	941.57	1	941.57	337.64	< 0.0001
AB^2	1234.59	1	1234.59	442.71	< 0.0001
Residual	30.68	11	2.79		
Lack of fit	0.3242	1	0.3242	0.1068	0.7505
Pure error	30.35	10	3.04		
Cor total	20900.90	18			
Std. dev.	1.67		R^2	0.9985	
Mean	416.13		Adjusted R^2	0.9976	
C.V.%	0.4013		Predicted R^2	0.9953	
			Adeq precision	76.7240	

In addition, the experimental data of the model to fit was proved by the lack of fit test as insignificant (*p*-value > 0.05). The higher regression coefficient in a model with a significant *p*-value demonstrated a more significant response of each factor (Yim et al., 2013). Both extraction temperature and time influenced total sugar content with a good regression coefficient (R^2 = 0.9985). The relationship between the total sugar content and the extraction variable is presented according to the following coded equation (Eq. 6):

Total sugar concentration (mg/g extract) = 454.09 + 35.16A - 1.81B + 19.71AB - $35.82A^2$ - $24.27B^2$ + $18.79A^2B$ - $21.52AB^2$ (6)

Moreover, extraction temperature was found to be the significant positive linear but negative quadratic factor (*p*-value < 0.01) affecting the total sugar content of polysaccharides. Extraction time did not have significant negative linear (*p*-value > 0.05) but had significant negative quadratic effects (*p*-value < 0.01). A significant positive interaction effect between extraction time and temperature was obtained for the total sugar content (Table 15). Figure 14 revealed the contour plot and response surface plot of extraction time and temperature on the total sugar content of polysaccharide extracts. The total sugar content was raised when the extraction temperature increased. While time enhanced, the total sugar content was not considerably higher. Thus, the maximum total sugar content predicted by the RSM design was 461.56 mg/g extract with an optimal extraction temperature of 106.5°C and time of 126.7 minutes.

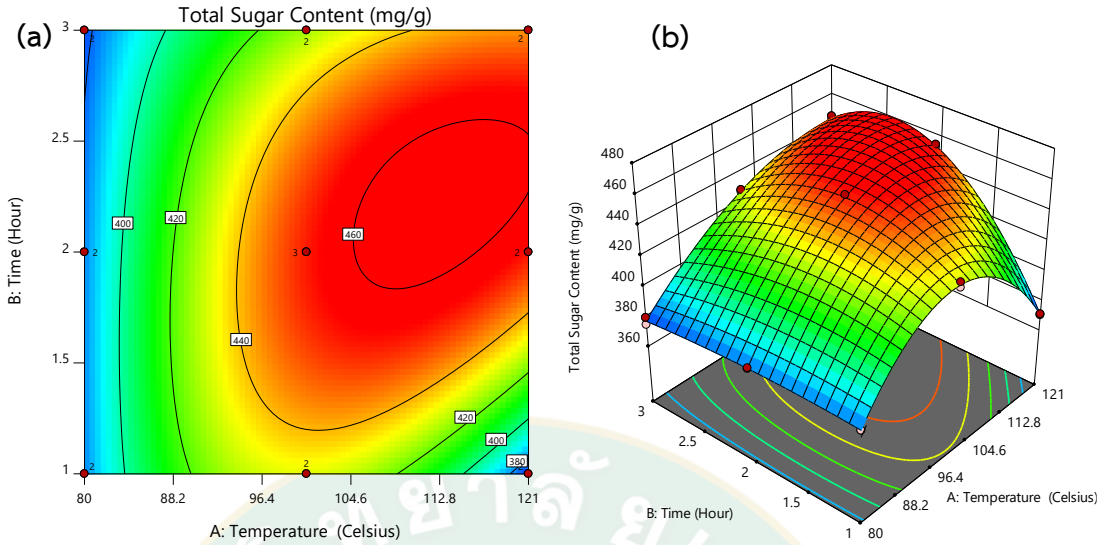


Figure 14 Contour plot (a) and response surface plot (b) of total sugar content of polysaccharides as a function of extraction temperature and time.

Furthermore, when alkaline, solvent, or high temperature of hot water is efficiently employed for pretreatment of β-glucan (polysaccharide of mushroom), hydrogen bonds (intermolecular forces for sustaining the triple-helical and single-helical β-glucan) are broken. As a result, it leads to the change of β-glucan conformation from triple helix to single helix and random coil, respectively (Leung et al., 2006). The total sugar content of schizophyllan by hot water extraction from split gill mushroom in comparison with other mushroom species was revealed in Table 16. It was also found that the maximal total sugar content of the schizophyllan was slightly higher than the polysaccharide-peptide complex of *Pleurotus abalonus* by hot water extraction with ethanol precipitation (Li et al., 2012), including polysaccharide-protein complexes of *Coriolus versicolor* by ultrasound-assisted extraction about twice (Cheung et al., 2012).

Table 16 Total sugar content of various mushrooms.

Mushroom species	Extraction method	Total sugar content (mg/g dried extract)	Reference
Pleurotus abalonus	hot water extraction with ethanolic treatment	372.10	Li et al. (2012)
Coriolus versicolor	ultrasound-assisted extraction	215.00	Cheung et al. (2012)
S. commune	hot water extraction with alkaline and ethanolic treatment	461.56	This study

4.3.3 Reducing sugar content of the schizophyllan

To investigate the effect of hot water extraction parameters on reducing sugar content, the suitable ranges of extraction time and temperature for reducing sugar content of polysaccharides were estimated by ANOVA (Table 17). The lack of fit of this experimental data was not significant (*p*-value > 0.05), indicating that the model was predictive. The relationship between reducing sugar content and extraction parameters is quadratic with a good regression coefficient (R^2 = 0.9999). The coded equation (Eq. 7) presented the relationship as follows:

$$\text{Reducing sugar concentration (mg/g extract)} = 84.06 - 5.61A - 4.12B + 0.1298AB + 18.80A^2 - 10.99B^2 + 10.03A^2B - 11.00AB^2 \quad (7)$$

Table 17 ANOVA model for optimized hot water extraction of the polysaccharides' reducing sugar content.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	4603.22	7	657.60	30863.14	< 0.0001
Temperature (A)	125.91	1	125.91	5909.21	< 0.0001
Time (B)	67.83	1	67.83	3183.57	< 0.0001
AB	0.1348	1	0.1348	6.33	0.0287
A^2	1547.87	1	1547.87	72645.99	< 0.0001
B^2	529.01	1	529.01	24827.69	< 0.0001
A^2B	268.37	1	268.37	12595.52	< 0.0001
AB^2	322.74	1	322.74	15147.23	< 0.0001
Residual	0.2344	11	0.0213		
Lack of fit	0.0005	1	0.0005	0.0222	0.8844
Pure error	0.2339	10	0.0234		
Cor total	4603.46	18			
Std. dev.	0.1460		R^2	0.9999	
Mean	88.99		Adjusted R^2	0.9999	
C.V.%	0.1640		Predicted R^2	0.9998	
			Adeq precision	478.3769	

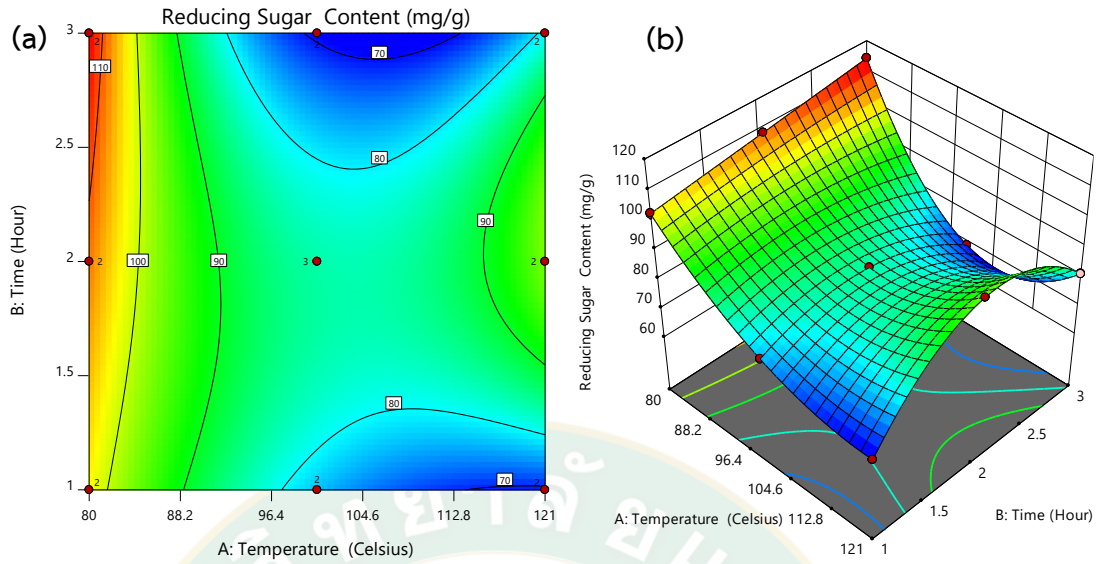


Figure 15 Contour plot (a) and response surface plot (b) of reducing sugar content of polysaccharides as a function of extraction temperature and time.

Furthermore, extraction temperature and time had significant negative linear effects (p-value < 0.01). Nevertheless, extraction temperature was found to be a significant positive quadratic factor. In contrast, time was found to be a significant negative quadratic factor (p-value < 0.01), as well as a significant positive interaction effect between extraction time and temperature was observed (p-value < 0.05) (Table 17). Figure 15 showed the contour plot and response surface plot of reducing sugar content of polysaccharide extracts as a function of extraction time and temperature. The reducing sugar content decreased when the extraction temperature increased. However, it was noticed that as time augmented, the reducing sugar content raised. The previous research reported that the extraction of carbohydrates at high temperatures and longer extraction times was affected by carbohydrate hydrolysis, leading to the presence of reducing sugar (Sawangwan et al., 2018). In this study, the optimal extraction conditions were predicted to be the extraction temperature of 103.1°C and time of 177.0 minutes for the minimum reducing sugar content of 68.74 mg/g extract.

4.3.4 Degree of polymerization (DP) of the schizophyllan

To evaluate the effect of these extraction variables on the degree of polymerization (DP), the appropriate ranges of extraction time and temperature for DP of polysaccharides were analyzed by ANOVA as concluded in Table 18. The model was to fit since the lack of fit predicted by the statistical program was not significant (*p*-value > 0.05). The relationship between DP and extraction factors is quadratic with a good regression coefficient (R^2 = 0.9998). The coded equation (Eq. 8) below showed the relationship:

$$\text{Degree of polymerization (DP)} = 5.45 + 0.5655A + 0.3023B + 0.1395AB - 1.39A^2 + 0.4191B^2 - 0.3815A^2B + 0.3825AB^2 \quad (8)$$

Besides, extraction temperature and time were the significant positive linear factors (*p*-value < 0.01). Nonetheless, extraction temperature had a significant negative quadratic effect. In contrast, time had a positive quadratic effect (*p*-value < 0.01), including a significant positive interaction effect between extraction time and temperature was obtained for the DP value (Table 18). Figure 16 revealed the contour plot and response surface plot of extraction time and temperature on the DP value of polysaccharide extracts. It could be observed that the DP value raised with increasing extraction temperature from 80 - 105°C. Likewise, it was obvious that increasing extraction time could promote higher DP value. The extractive DP value fell slightly while the extraction temperature was more than 105°C. The optimal extraction conditions were predicted to be the extraction temperature of 104.1°C and time of 175.5 minutes for the maximal DP value of 6.21.

Additionally, it is essential for the complex β-glucan conformation, mushroom polysaccharide, in the function of the immune system (Chan et al., 2009). It is revealed that the immunomodulatory and anticarcinogenic properties of these polysaccharides are also related to their molecular weights and configuration, particularly higher molecular weight polysaccharides (Zhong et al., 2013). The triple helical β-glucan, with a greater DP, higher molecular weight, and more degree of a

complex branching structure, has a superior potency for immunomodulatory and anticancer activities (Khan et al., 2018) to linear or less branched β-glucan with lower DP significantly (Lee and Ki, 2020).

Table 18 ANOVA model for optimized hot water extraction of the polysaccharides' degree of polymerization (DP).

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	17.86	7	2.55	9986.68	< 0.0001
Temperature (A)	1.28	1	1.28	5005.50	< 0.0001
Time (B)	0.3655	1	0.3655	1430.49	< 0.0001
AB	0.1557	1	0.1557	609.48	< 0.0001
A^2	8.46	1	8.46	33107.37	< 0.0001
B^2	0.7696	1	0.7696	3011.78	< 0.0001
A^2B	0.3880	1	0.3880	1518.60	< 0.0001
AB^2	0.3901	1	0.3901	1526.82	< 0.0001
Residual	0.0028	11	0.0003		
Lack of fit	0.0009	1	0.0009	4.54	0.0588
Pure error	0.0019	10	0.0002		
Cor total	17.87	18			
Std. dev.	0.0160		R^2	0.9998	
Mean	4.83		Adjusted R^2	0.9997	
C.V.%	0.3308		Predicted R^2	0.9996	
			Adeq precision	275.5905	

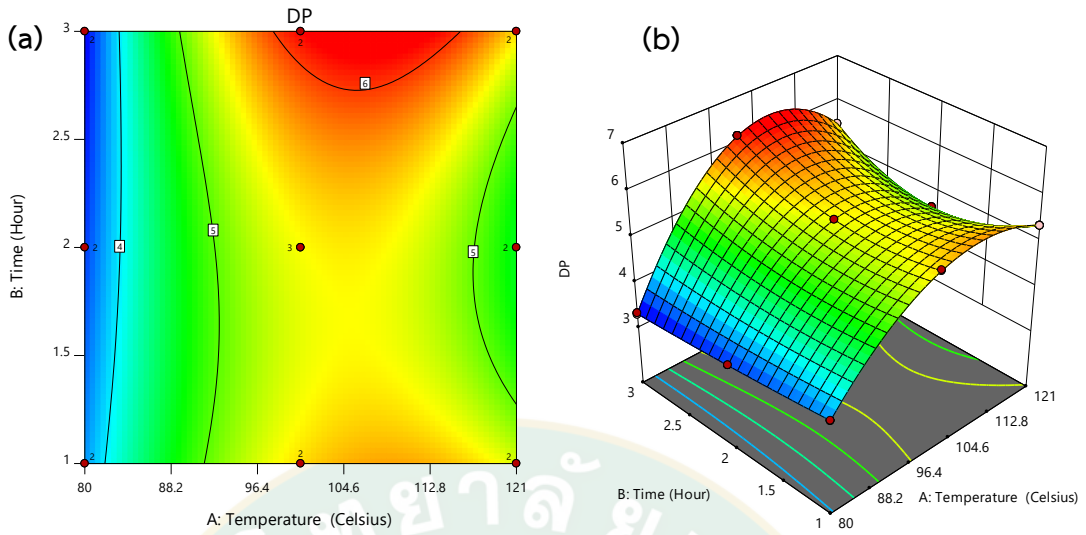


Figure 16 Contour plot (a) and response surface plot (b) of degree of polymerization (DP) of polysaccharides as a function of extraction temperature and time.

4.3.5 Protein content of the schizophyllan

To study the effect of the extraction parameters on protein content, the suitable ranges of the extraction factors on the protein content of polysaccharides were determined by ANOVA (Table 19). The lack of fit test proved the model's experimental data to fit as not significant (*p*-value > 0.05). Both temperature and time influenced the protein content with a good regression coefficient (R^2 = 0.9888). The relationship between the protein content and the extraction variable is presented according to the following coded equation (Eq. 9).

$$\text{Protein content (mg/g extract)} = 6.86 - 2.46A + 0.44B - 0.98AB + 1.43A^2 + 0.95B^2 - 1.48A^2B + 0.64AB^2 \quad (9)$$

Moreover, extraction temperature was found to be the significant negative linear but positive quadratic factor (*p*-value < 0.01) affecting the protein content of polysaccharides. Extraction time had both significant positive linear (*p*-value < 0.05) and quadratic effects (*p*-value < 0.01). A significant negative interaction effect between extraction time and temperature was obtained for the protein content (Table 19).

Table 19 ANOVA model for optimized hot water extraction of the polysaccharides' protein content.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	82.05	7	11.72	138.33	< 0.0001
Temperature (A)	24.22	1	24.22	285.90	< 0.0001
Time (B)	0.7656	1	0.7656	9.04	0.0120
AB	7.72	1	7.72	91.12	< 0.0001
A^2	8.96	1	8.96	105.71	< 0.0001
B^2	3.98	1	3.98	46.99	< 0.0001
A^2B	5.86	1	5.86	69.16	< 0.0001
AB^2	1.11	1	1.11	13.07	0.0041
Residual	0.9321	11	0.0847		
Lack of fit	0.0870	1	0.0870	1.03	0.3342
Pure error	0.8451	10	0.0845		
Cor total	82.98	18			
Std. dev.	0.2911		R^2	0.9888	
Mean	8.36		Adjusted R^2	0.9816	
C.V.%	3.48		Predicted R^2	0.9614	
			Adeq precision	30.2981	

Figure 17 revealed the contour plot and response surface plot of extraction time and temperature on protein content of polysaccharide extracts. The protein content decreased when extraction temperature increased. While time enhanced, the protein content was not significantly higher. Therefore, the maximum protein content predicted by RSM design was 11.12 mg/g extract with optimal extraction temperature of 80.0°C and time of 1 hour.

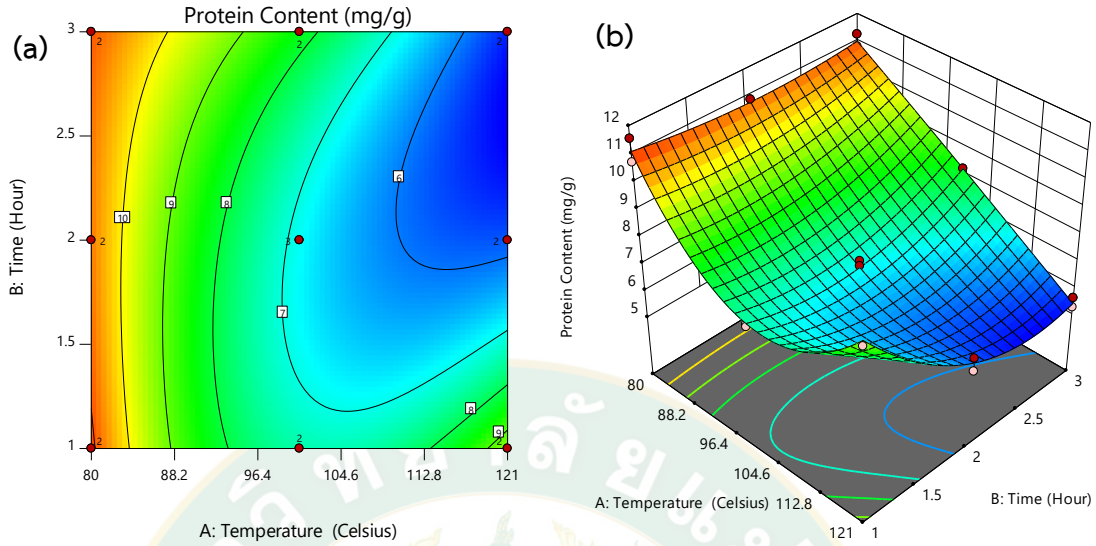


Figure 17 Contour plot (a) and response surface plot (b) of protein content of polysaccharides as a function of extraction temperature and time.

4.4 Optimized ethanol precipitation of the schizophyllan

4.4.1 Total sugar content of the schizophyllan

Two independent contributions the mushroom content (% w/v, A) and ethanol concentration (% v/v, B) were utilized in RSM (the response surface methodology) for maximized total sugar, optimized reducing sugar concentration and optimal DP value of the schizophyllan extracts from this mushroom. The trial data were calculated as the coefficients of the parameters to demonstrate the association between the total sugar concentration and the parameters according to the coded equation (Eq. 10) to predict the optimal response. Equation 10 showed the effect of each significant variable and the interactive effect of the variables on the response of total sugar concentrations.

Total sugar content (mg/g extract) = 432.76 - 31.48A - 3.02B - 23.32AB - 17.33A^2 - 96.43B^2 (10)

Table 20 ANOVA model analysis for optimized ethanol precipitation of the polysaccharides' total sugar concentration.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	60194.08	5	12038.82	602.91	< 0.0001
Split gill mushroom content (A)	11889.03	1	11889.03	595.41	< 0.0001
Ethanol concentration (B)	109.15	1	109.15	5.47	0.0360
AB	4351.13	1	4351.13	217.91	< 0.0001
A^2	1316.24	1	1316.24	65.92	< 0.0001
B^2	40736.09	1	40736.09	2040.10	< 0.0001
Residual	259.58	13	19.97		
Lack of fit	132.67	3	44.22	3.48	0.0581
Pure error	126.91	10	12.69		
Cor total	60453.67	18			
Std. dev.	4.47		R^2	0.9957	
Mean	360.91		Adjusted R^2	0.9941	
C.V.%	1.24		Predicted R^2	0.9912	
			Adeq precision	73.9591	

In addition, ANOVA evaluated the effect of the mushroom content and ethanol concentration on the total sugar concentration of the polysaccharides, as concluded in Table 20. The contour plot and response surface plot depicted the interactions of two independent variables and the coincident effect on the total sugar content of the polysaccharides (Figure 18). Total sugar concentration decreased when the mushroom content increased. Nevertheless, total sugar concentration increased with increasing ethanol concentration during 49.0 to 61.5% (v/v), and total sugar concentration fell with more than 61.5% (v/v) ethanol. The maximum total

sugar concentration predicted by the RSM design was 447.98 mg/g extract with optimal mushroom content of 5.11% (w/v) and precipitation ethanol concentration of 62.78% (v/v).

Besides, the total sugar concentration of the schizophyllan by hot water extraction with ethanolic precipitation from *S. commune*, compared to that from other mushroom species illustrated in Figure 19. It was noticed that the optimized total sugar concentration of this schizophyllan was superior to the polysaccharide extract of this mushroom using hot water extraction combined with ethanol precipitation around 1.5 folds (Klaus et al., 2011). Likewise, the total sugar concentration was slightly greater than polysaccharide-protein complexes of *Lentinus edodes* by ultrasonic extraction (Cheung et al., 2012), polysaccharide-peptide complex of *Pleurotus abalonus* using hot water extraction combined with ethanol treatment (Li et al., 2012), as well as the extract of *Pholiota nameko* by enzyme-assisted extraction with Alcalase (Rodrigues et al., 2017).

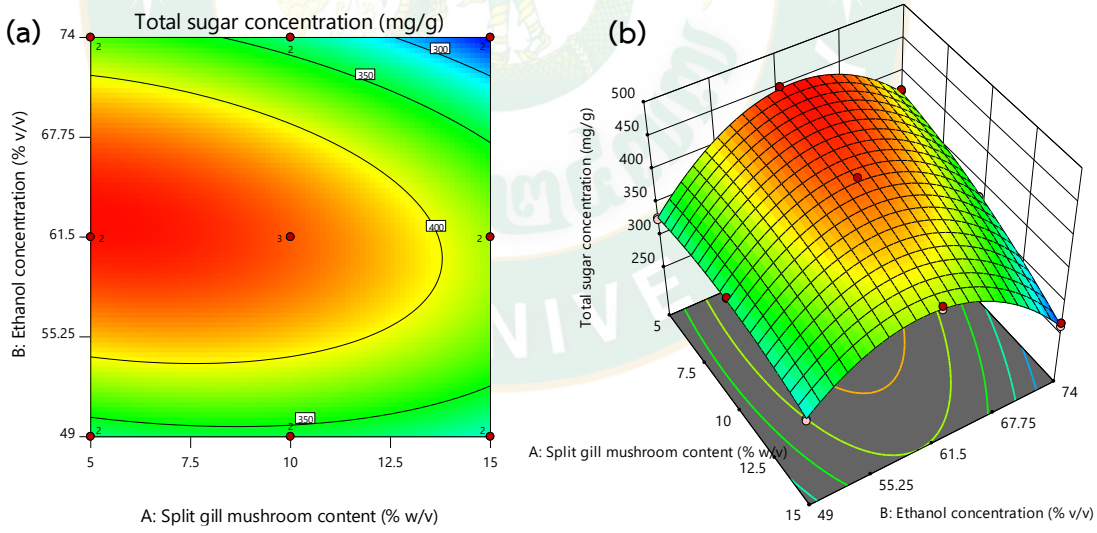


Figure 18 Contour plot (a) and 3D plot (b) obtained from total sugar concentration of the polysaccharides as a function of the mushroom content and precipitation ethanol concentration.

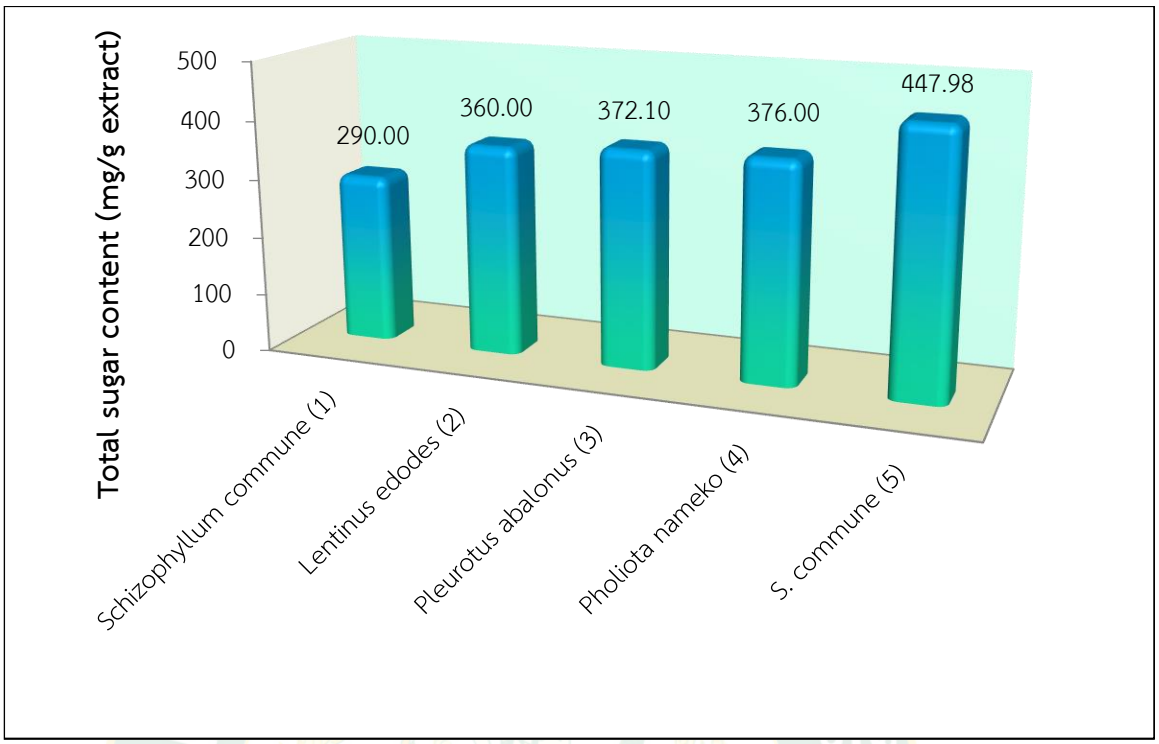


Figure 19 Total sugar concentration of the polysaccharide extracts from different mushrooms. * (1) from Klaus et al. (2011); (2) from Cheung et al. (2012); (3) from Li et al. (2012); (4) from Rodrigues et al. (2017); (5) from this study.

4.4.2 Reducing sugar content of the schizophyllan

To evaluate the effect of the mushroom content and ethanol concentration on reducing sugar concentration of the polysaccharides, the coded equation (Eq. 11) demonstrated the association between the reducing sugar concentration and the coefficients of the factors obtained from calculating the predicted data. Equation 11 presented the influence of each significant parameter and the interaction of the variables on reducing sugar concentrations.

$$\text{Reducing sugar content (mg/g extract)} = 81.83 - 4.96A - 12.65B - 0.91AB + 0.93A^2 + 0.74B^2 \quad (11)$$

Moreover, ANOVA analyzed the influence of the mushroom content and ethanol concentration on reducing sugar concentration of the polysaccharides, as summarized in Table 21. In addition, the contour plot and 3D plot portrayed the

interactions of two parameters and the concomitant effect on reducing sugar concentration of the polysaccharides (Figure 20). Reducing sugar concentration augmented when the mushroom content declined. While reducing sugar concentration dropped considerably, ethanol concentration raised. In this experimental design, the optimized extraction conditions were predicted to be the mushroom content of 15.00% (w/v) and ethanol concentration of 74.00% (v/v) for the minimized reducing sugar concentration of 64.99 mg/g extract.

Table 21 ANOVA model analysis for optimized ethanol precipitation of the polysaccharides' reducing sugar concentration.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	2227.43	5	445.49	1008.48	< 0.0001
Split gill mushroom content (A)	295.02	1	295.02	667.86	< 0.0001
Ethanol concentration (B)	1919.01	1	1919.01	4344.18	< 0.0001
AB	6.57	1	6.57	14.87	0.0020
A^2	3.78	1	3.78	8.57	0.0118
B^2	2.41	1	2.41	5.46	0.0361
Residual	5.74	13	0.4417		
Lack of fit	1.39	3	0.4628	1.06	0.4077
Pure error	4.35	10	0.4354		
Cor total	2233.17	18			
Std. dev.	0.6646		R^2	0.9974	
Mean	82.88		Adjusted R^2	0.9964	
C.V.%	0.8019		Predicted R^2	0.9950	
			Adeq precision	94.2676	

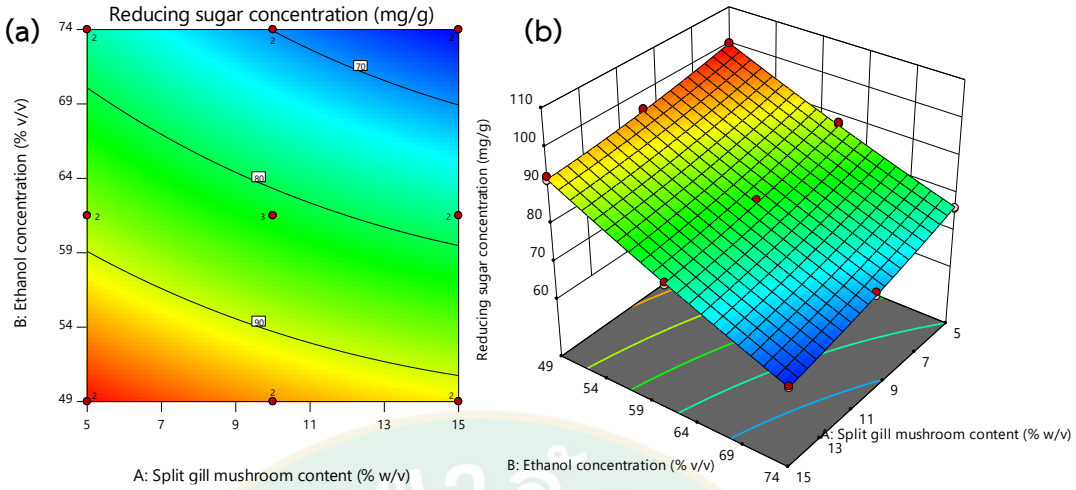


Figure 20 Contour plot (a) and 3D plot (b) obtained from reducing sugar concentration of the polysaccharides as a function of the mushroom content and precipitation ethanol concentration.

4.4.3 DP value of the schizophyllan

To investigate the influence of the mushroom content and ethanol concentration on the DP value of the polysaccharides, the coded equation (Eq. 12) revealed the relationship between the DP values and the coefficients of the variables from the calculation of the trial data. Equation 12 showed the effect of each significant factor and the interaction of the parameters on the DP values.

$$\text{Degree of polymerization (DP)} = 5.29 - 0.14A + 0.56B - 0.23AB - 0.27A^2 - 1.13B^2 \quad (12)$$

Furthermore, Table 22 summarized the evaluation of these parameters by ANOVA that affected on DP value of the polysaccharides. Besides, Figure 21 revealed the contour plot and response surface plot of the DP value of the polysaccharide extracts, including the interactions of two variables and the concurrent influence of the DP value. DP value went up slightly with increasing the mushroom content during 5 to 10% (w/v), and DP value fell insignificantly with greater than 10% (w/v) of the mushroom. This case meant that the mushroom content affected slightly on DP value of the polysaccharides. Nevertheless, the DP value climbed up exceedingly

with the raising of an ethanol concentration between 49.0 to 61.5% (v/v), and the DP value declined with higher than 61.5% (v/v) ethanol. These graphs could be summarized that the high mushroom content did not have efficient extraction of the polysaccharide with greater DP value. Hence, further scale up, the polysaccharide consisting of higher DP value will be extracted with the low mushroom content due to reduction of the production cost.

In this experiment, the optimal extraction conditions were predicted to be the mushroom content of 8.05% (w/v) and ethanol concentration of 65.10% (v/v) for the maximal DP value of 5.40.

Table 22 ANOVA model analysis for optimized ethanol precipitation of the polysaccharides' degree of polymerization (DP).

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	10.61	5	2.12	299.67	< 0.0001
Split gill mushroom content (A)	0.2410	1	0.2410	34.04	< 0.0001
Ethanol concentration (B)	3.74	1	3.74	528.69	< 0.0001
AB	0.4326	1	0.4326	61.11	< 0.0001
A^2	0.3138	1	0.3138	44.33	< 0.0001
B^2	5.57	1	5.57	786.68	< 0.0001
Residual	0.0920	13	0.0071		
Lack of fit	0.0463	3	0.0154	3.37	0.0627
Pure error	0.0457	10	0.0046		
Cor total	10.70	18			
Std. dev.	0.0841		R^2	0.9914	
Mean	4.41		Adjusted R^2	0.9881	
C.V.%	1.91		Predicted R^2	0.9828	
			Adeq precision	43.2390	

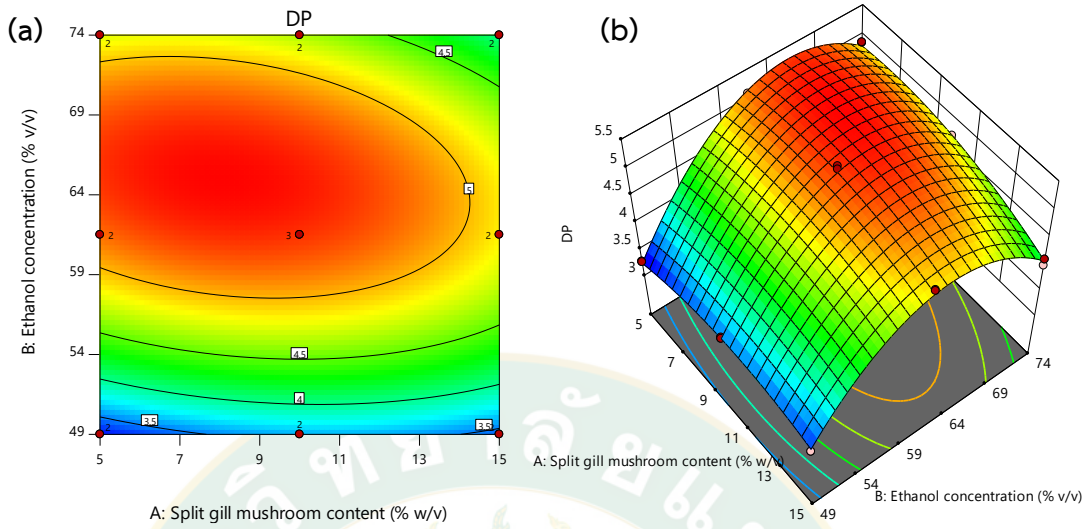


Figure 21 Contour plot (a) and 3D plot (b) obtained from degree of polymerization (DP) of the polysaccharides as a function of the mushroom content and precipitation ethanol concentration.

In addition, the conformational complexity of β-glucan, the mushroom polysaccharide, is essential in the immune system's function (Chan et al., 2009). The triple helical β-glucan, containing a higher DP value with more branching structural complexity, has a greater potency for immune-enhancing and anticarcinogenic properties (Khan et al., 2018) than linear or lower branched β-glucan consisting of low DP value obviously (Lee and Ki, 2020).

4.4.4 Protein content of the schizophyllan

To study the influence of the mushroom content and ethanol concentration on the protein concentration of the polysaccharides, the trial data were calculated the coefficients of the parameters to demonstrate the association between the protein concentration and the parameters according to the coded equation (Eq. 13) for prediction the optimal response. Equation 13 showed the effect of each significant variable and the interactive effect of the variables on the response of protein concentrations.

Protein content (mg/g extract) = $7.17 + 0.85A + 0.79B - 0.92AB - 0.58A^2 - 0.37B^2$ (13)

In addition, the effect of the mushroom content and ethanol concentration on protein concentration of the polysaccharides was evaluated by ANOVA, as concluded in Table 23. Moreover, the contour plot and response surface plot depicted the interactions of two independent variables and the coincident effect on the protein concentration of the polysaccharides (Figure 22). Protein concentration was enhanced by increasing the mushroom content and augmenting ethanol concentration. Thus, the maximized protein concentration predicted by the RSM design was 7.59 mg/g extract with optimal mushroom content of 9.73% (w/v) and ethanol concentration of 74.00% (v/v).

Table 23 ANOVA model for optimized ethanol precipitation of the polysaccharides' protein concentration.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	25.27	5	5.05	928.44	< 0.0001
Split gill mushroom content (A)	8.74	1	8.74	1606.26	< 0.0001
Ethanol concentration (B)	7.55	1	7.55	1387.67	< 0.0001
AB	6.71	1	6.71	1231.99	< 0.0001
A^2	1.47	1	1.47	270.01	< 0.0001
B^2	0.5973	1	0.5973	109.72	< 0.0001
Residual	0.0708	13	0.0054		
Lack of fit	0.0362	3	0.0121	3.48	0.0582
Pure error	0.0346	10	0.0035		
Cor total	25.34	18			
Std. dev.	0.0738		R^2	0.9972	
Mean	6.57		Adjusted R^2	0.9961	
C.V.%	1.12		Predicted R^2	0.9948	
			Adeq precision	94.9140	

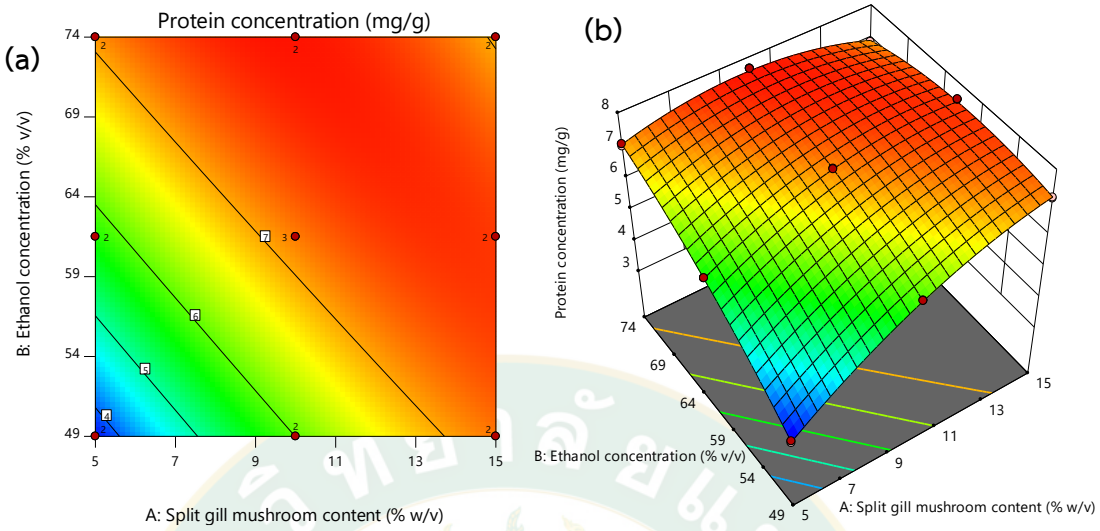


Figure 22 Contour plot (a) and response surface plot (b) of protein concentration of polysaccharides as a function of the mushroom content and precipitation ethanol concentration.

4.5 Chemical characterization studies of the schizophyllan

4.5.1 Solubility of the schizophyllan

Figure 23 presented the effect of extract concentration, temperature, and incubation time of extract solubility on total sugar content. It was found that total sugar content of this extract was the highest at solubilization at 120°C for 120 minutes with 5.00 mg/ml extract concentration. For the effect of extract concentration, temperature, and incubation time of extract solubility on reducing sugar content, it was noticed that reducing sugar content of this extract was the greatest at the same solubilization condition (Figure 24). However, the effect of extract concentration, temperature, and incubation time of extract solubility on degree of polymerization (DP value) showed a suitable DP value at the similar solubilization condition because this DP value from the optimized condition was not a too long chain and more chain number of polysaccharide to attach receptors of immune cells for stimulation of immune system (Figure 25). Additionally, the effect of extract concentration, temperature, and incubation time of extract solubility on total phenolic content depicted total phenolic content of this extract was superior at

solubilization at the same solubilization condition. Although the high temperature was utilized to solubilize the extract, the bioactive substance was not degraded, leading to the greater antioxidant candidate (Figure 26).

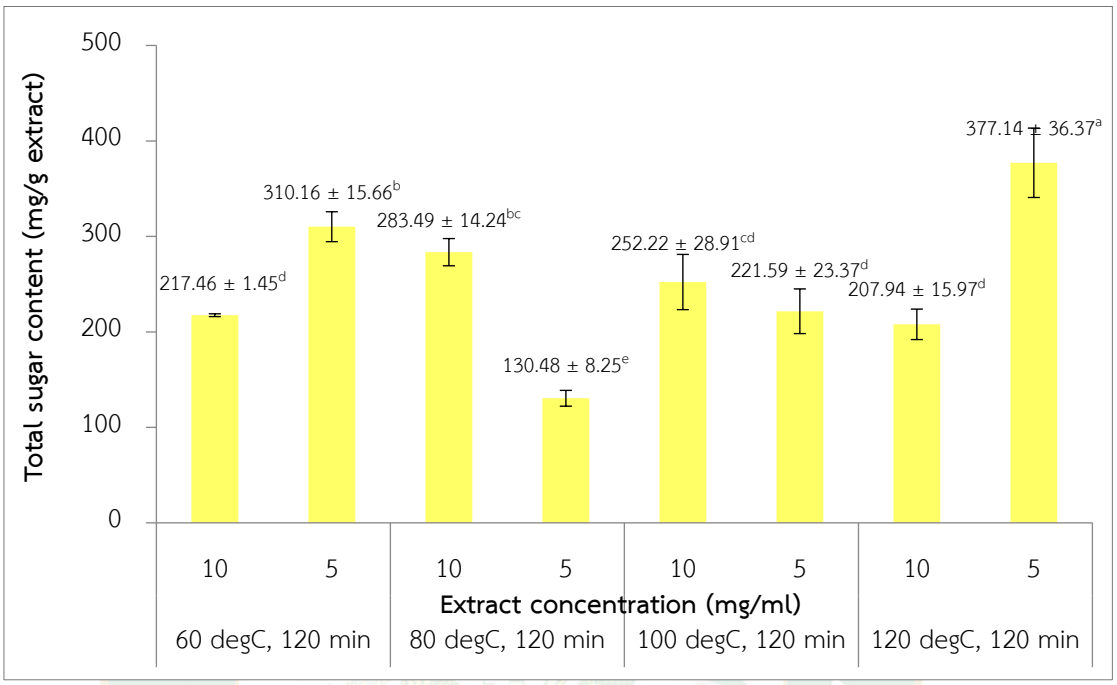


Figure 23 Effect of extract concentration, temperature and incubation time of extract solubility on total sugar content.

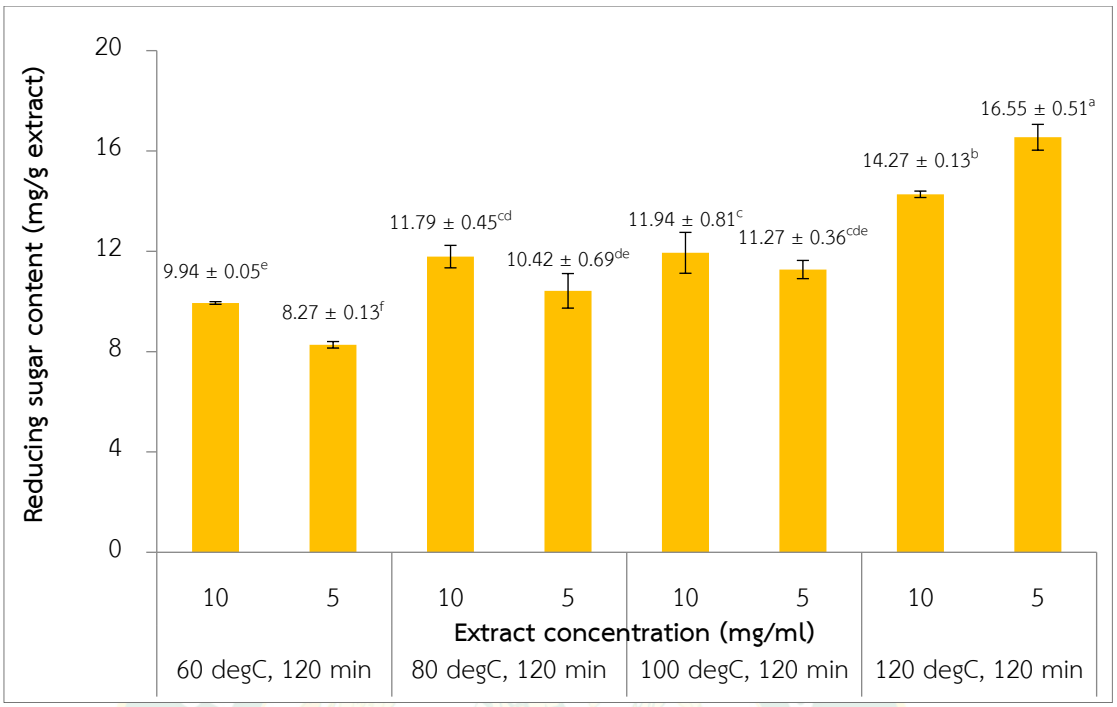


Figure 24 Effect of extract concentration, temperature and incubation time of extract solubility on reducing sugar content.

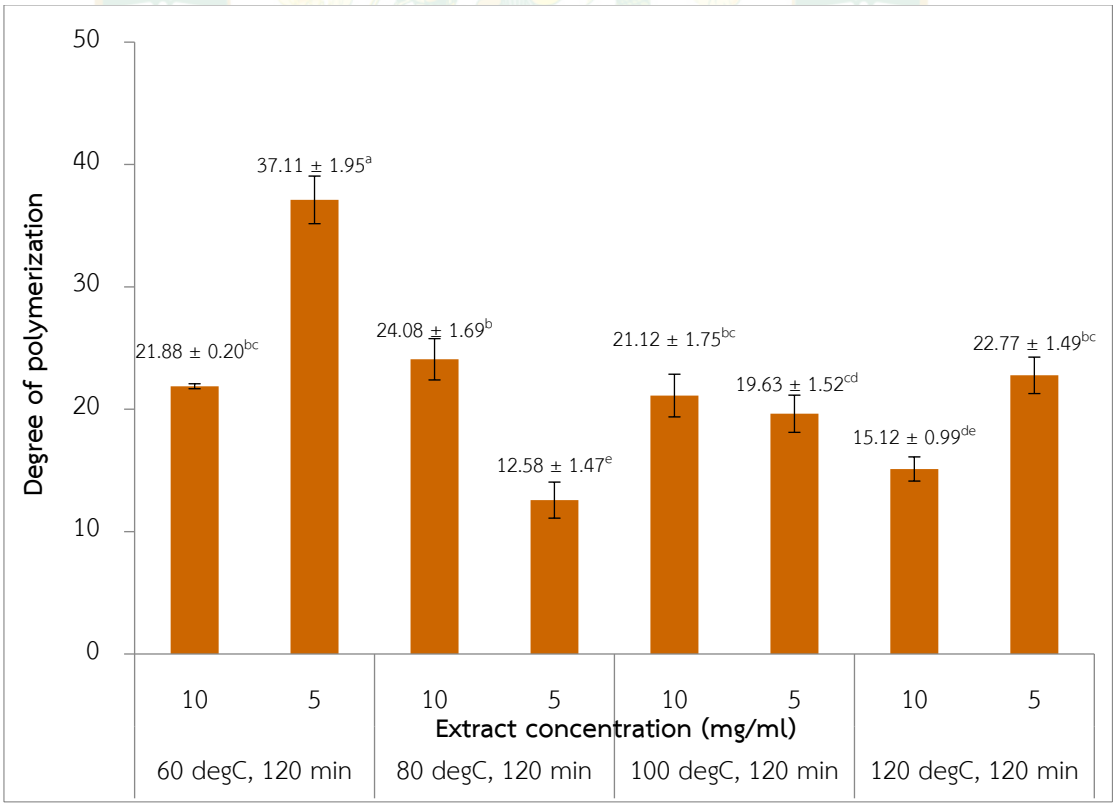


Figure 25 Effect of extract concentration, temperature and incubation time of extract solubility on degree of polymerization.

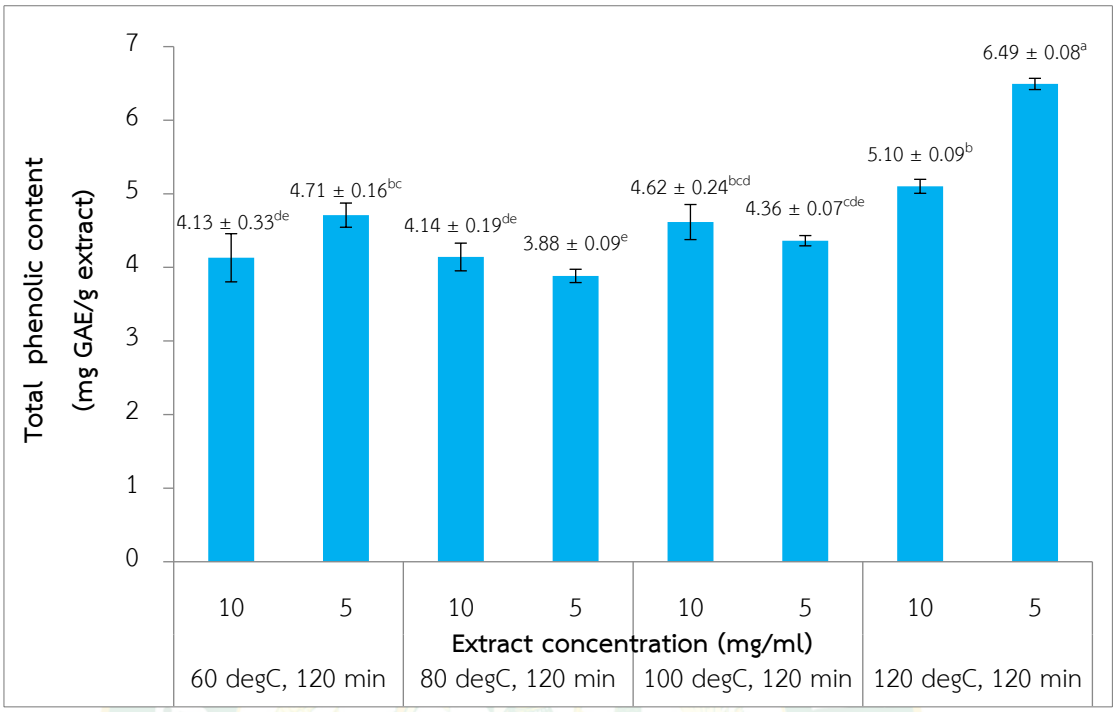


Figure 26 Effect of extract concentration, temperature and incubation time of extract solubility on total phenolic content.

Furthermore, total phenolic contents from *Schizophyllum commune* compared to other agricultural materials are revealed in Table 24. It was found that the entire phenolic content of this extract from the mushroom was remarkably higher than acetone extract of *Solanum melongena*'s peel approximately 22.4 times (Boulekbache-Makhlouf et al., 2013), methanolic extract of *Pleurotus ostreatus* cultivated with corncob as substrate about 5.4 folds (Jin et al., 2020), hot water extract of *Oryza sativa* var. glutinosa (Kum Doi Saket) around triple (Pasakawee et al., 2018), ultrasonic section combined with acetone of *P. citrinopileatus* about twice (Yin et al., 2019) as well as more than microwave extract associated with ethanol of *Coriolus versicolor* considerably (Maeng et al., 2017).

Table 24 Total phenolic contents of different agricultural materials.

Agricultural materials	Extraction method	Total phenolic content (mg GAE/g dry extract)	Reference
Solanum melongena peel	acetone extraction	0.29	Boulekbache-Makhlouf et al. (2013)
Pleurotus ostreatus (cultivated with corncob substrate)	methanolic extraction	1.21	Jin et al. (2020)
Oryza sativa var. glutinosa (Kum Doi Saket)	hot water extraction	2.25	Pasakawee et al. (2018)
P. citrinopileatus	ultrasonic extraction with acetone	2.68	Yin et al. (2019)
Coriolus versicolor	microwave-assisted extraction with ethanol	4.70	Maeng et al. (2017)
Schizophyllum commune	hot water extraction with alkaline and ethanolic treatment	6.49	This study

4.5.2 Protein molecular weight analysis

When analyzed by polyacrylamide gel electrophoresis, it was found that the extract from this mushroom was obtained in the range of protein molecular weight (MW) from 14 - 35 kDa (Figure 27). From the previous research, protein molecular masses with 14.0 - 18.5 kDa were indicated as hydrophobin protein, as well as their MWs with 21 - 35 kDa were specified as lectin protein (Table 25). Likewise, the protein molecular size of hydrophobin SC3 protein from the split gill mushroom was around 14 kDa (Akanbi et al., 2013), fruiting body protein SC1, SC3, SC6, hydrophobin 6 from *S. commune* about 11 - 18 kDa, including hydrophobin protein from *Pleurotus ostreatus* have an MW ranging from 10 - 20 kDa (Erjavec et al., 2012). Besides, the

presence of a purified N-acetyl-D-galactosamine (GalNAc)-specific lectin from *S. commune* revealed MW band of 31.5 kDa (Chumkhunthod et al., 2006), lectins (12 - 35 kDa) in *P. ostreatus* (González et al., 2021), a lectin HEA isolate (51 kDa) from dried fruiting bodies of *Hericium erinaceum*, a purified mannose-binding lectin NTL (26 kDa) from bulbs of Chinese daffodil *Narcissus tazetta*, a novel lectin RLL (32 kDa) from *Russula lepida*, along with a dimeric lectin isolate (60 kDa) from fresh fruiting bodies of wild mushroom *R. delica* (Xu et al., 2011).

In addition, the nontoxic fungal hydrophobin SC3 of *S. commune* possessed tumor-suppressive properties via immunomodulation in B16-F10 mouse melanoma and S180 mouse sarcoma, as well as may be beneficially applied as an adjuvant combined with radiation and chemotherapy (Akanbi et al., 2013). Additionally, *S. commune* lectin (SCL) showed greater selective cytotoxic activity against human epidermoid carcinoma cell lines (Chumkhunthod et al., 2006). Lectins from *Xylaria hypoxylon* exhibited antiproliferative effect on tumor cell lines and potential anti-mitogenic properties on mouse splenocytes, including lectin CNL isolate from the edible mushroom *Clitocybe nebularis* possessed immunomodulatory properties. A lectin isolate from *Pholiota adiposa*, a lectin HEA isolate from *H. erinaceum*, and a dimeric lectin isolate from of *Russula delica* revealed antiproliferative effects toward hepatoma HepG2 cells and breast cancer MCF7 cells, including HIV-1 reverse transcriptase inhibitory property. A purified mannose-binding lectin NTL from *Narcissus tazetta* also exhibited strong antiviral activities against several viruses, such as influenza A, influenza B viruses and human respiratory syncytial virus (RSV). Besides, a lectin ABL from *Agaricus bisporus* showed immunomodulatory activities, namely *in vitro* macrophage-stimulating functions in RAW264.7 cells by activating tumor necrosis factor-alpha (TNF-α) and nitric oxide (NO) production (Xu et al., 2011).

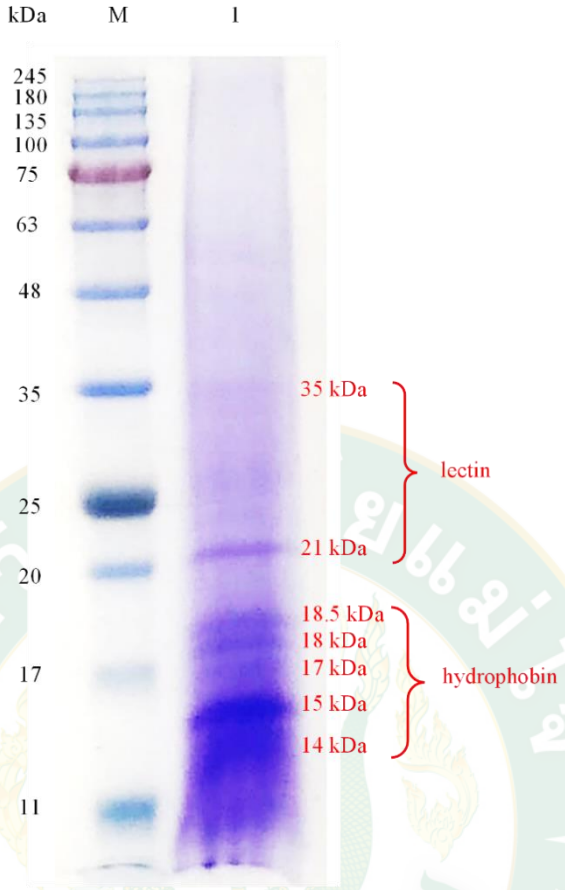


Figure 27 SDS-PAGE of the extract from *S. commune*.

Lane M, protein molecular weight marker; lane 1, the extract from *S. commune*.

Table 25 Protein molecular weight of hydrophobin and lectin protein in the extracts from different mushrooms.

Mushroom species	Protein molecular weight (kDa)		Reference
	Hydrophobin	Lectin	
Pleurotus ostreatus	10 - 20	12 - 190	Erjavec et al. (2012)
S. commune	11 - 18	NA	
S. commune	14	NA	Akanbi et al. (2013)
P. ostreatus	NA	12 - 35	González et al. (2021)
Hericium erinaceum	-	51	Xu et al. (2011)
Narcissus tazetta	-	26	
Russula lepida	-	32	
R. delica	-	60	
S. commune (purified lectin)	-	31.5	Chumkhunthod et al. (2006)
S. commune	14.0 - 18.5	21 - 35	This study

Remark: NA = not analyzed

4.5.3 Raman confocal spectroscopy

Raman spectroscopy serves as a valuable tool for identifying and characterizing the metabolites produced by fungi, providing a spectral fingerprint with extensive chemical data. Raman confocal spectroscopy enables direct imaging and mapping, combining Raman spectroscopy with light microscopy. This technique requires minimal sample preparation and does not require labeling applications. The spatial resolution of the microscope is in the range of the laser wavelength, allowing for chemical mapping of molecules at a resolution below 1 μm. Therefore, Raman confocal spectroscopy is an effective method for identifying compounds with low solubility in water and organic solvents, requiring less sample preparation and reduced measurement time (Menezes et al., 2015).

In this study, the colored metabolite produced by *S. commune* was identified using Raman spectroscopy. A Raman microscopic image was obtained from an

individual schizophyllan extract derived from this mushroom. The analysis revealed the presence of purpurin (red dye) in the extract, confirmed by comparing it with a purpurin reference and identifying the target component using Raman confocal spectroscopy (Figure 28). The spectral region between 952 and 1453 cm^{-1} exhibited characteristic peaks of purpurin, including bands at 1453, 1394, 1313, 1228, 1050, 1020, and 952 cm^{-1}. Thus, the identification of this colorant was successfully conducted using this equipment.

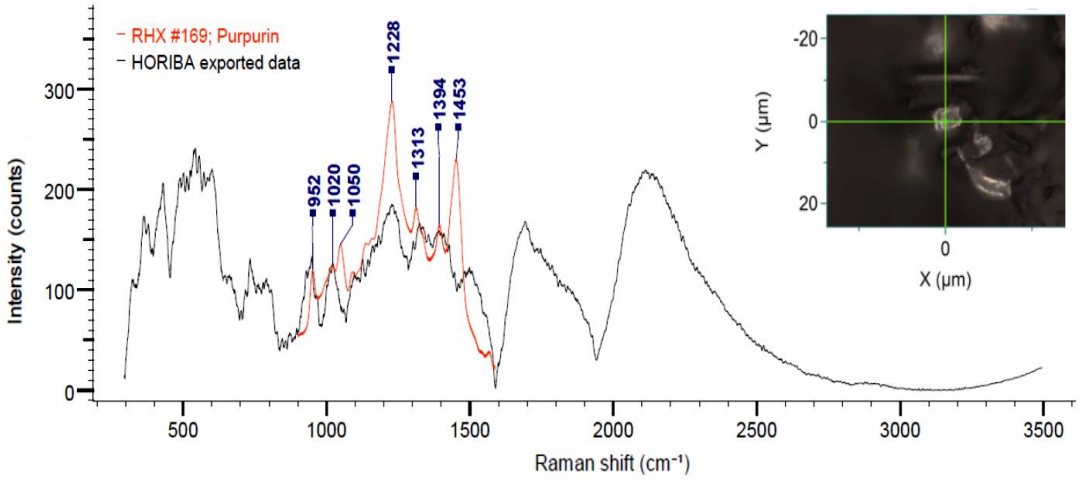


Figure 28 The results of bioactive compound identification in the extract from *S. commune* by Raman confocal spectroscopy.

Furthermore, purpurin, also known as 1,2,4-trihydroxy anthraquinone, is an alizarin-type anthraquinone compound and a component of the natural pigment, Madder Lake. Originally, it exists as a colorless glycoside in the roots of the madder plant, scientifically known as *Rubia tinctorum* L. The madder root has been historically used as a food colorant and in traditional medicine for various therapeutic purposes. In addition to its traditional uses, purpurin has been investigated for its health-promoting effects at the cellular and molecular levels, including both *in vitro* and *in vivo*. It has shown a range of biological activities, namely notable antioxidant, anti-inflammatory, immunomodulatory, anticarcinogenic, anti-adipogenic, anti-obesity, antifungal, and antibacterial properties (Nam et al.,

2017; Nam et al., 2019). These properties contribute to the reported health benefits associated with purpurin.

4.5.4 Monosaccharide composition

The HPLC chromatogram profiles of sugar analysis in this schizophyllan were confirmed by HPLC in a Bondapak/Carbohydrate column. This chromatogram showed the presence of fructose, glucose, and maltose in this extract at the retention time of 8.243, 10.132, and 17.533 minutes, respectively (Figure 29). On the other hand, Table 26 depicted this extract consisted of only fructose (0.57% w/w) and maltose (0.56% w/w), but low glucose concentration appeared in the extract (less than the limit of detection (LOD) = 0.30% w/w). Moreover, this extract was not detected sucrose and lactose. The extract obtained from this mushroom, which has low sugar content (mono- and disaccharides), offers significant health benefits due to its low-calorie content. Additionally, it contains water-soluble fiber in the form of β-glucan and β-glucooligosaccharide, which exhibit prebiotic activities by promoting the growth of probiotic bacteria in the human gastrointestinal tract. This, in turn, leads to the production of beneficial substances that contribute to improving the immune system and digestive system. Conversely, the supernatant obtained after the precipitation of polysaccharides with ethanol showed higher levels of reducing sugars (monosaccharides), making it less beneficial for human health (data not shown). Therefore, the schizophyllan extract was selected for further investigation, including optimized extraction processes, chemical characterization, assessment of biological properties, and development of functional food products.

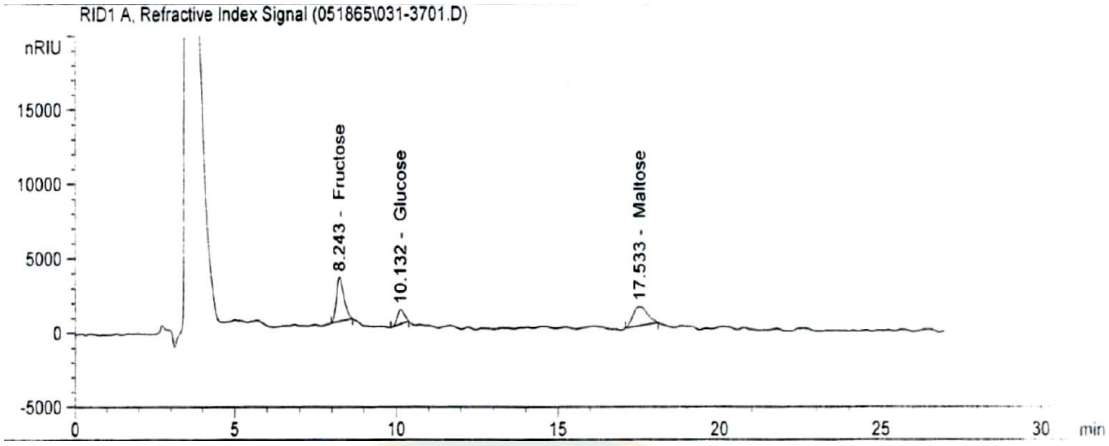


Figure 29 The HPLC chromatogram of sugar analysis in the extract from *S. commune*.

Table 26 Sugar composition of the extract from *S. commune* by HPLC.

Test item	Composition (g/100 g dried extract)	Reference method
Fructose	0.57	In-house method TE-CH-164 based on AOAC (2019) 977.20 (AOAC, 2019)
Glucose	Not detected[1]	
Sucrose	Not detected[1]	
Maltose	0.56	
Lactose	Not detected[1]	
Total reducing sugar	1.13	

Remark: [1] Limit of detection (LOD) = 0.30 g/100 g dried extract.

4.5.5 Oligosaccharides analysis

To analyze oligosaccharides of the extract by TLC (thin layer chromatography). Figure 30 presented that the extract consisted of β-glucooligosaccharide or β-glucan oligosaccharide with the degree of polymerization (DP value) around 2 - 3, namely laminaribiose and laminaritriose, compared to standard oligosaccharide, including most of the polysaccharides in this extract from that below spot. Moreover, prior research reported that these oligosaccharides had potential prebiotic properties to promote probiotic bacteria growth in the human gut tract, following further prebiotic effect of the extract.

In addition, Chaikliang et al. (2015) studied β-glucans and oligo-β-glucans from *S. commune* Fr. and *Auricularia auricula* Judae on prebiotic properties. These β-glucans were extracted under high pressure and temperature. Laminarinase was used to hydrolyze these oligo-β-glucans from β-glucans. Following that, fecal fermentation in batch culture *in vitro* was performed to compare fecal bacteria growth during fermentation of the β-glucans and oligo-β-glucans from these mushrooms and commercial yeast β-glucan, and the number of each probiotic bacteria and pathogenic bacteria strains were counted for the calculation of the prebiotic index of each extract. It was noticed that β-glucan from *A. auricula* Judae raised the proliferation of *Lactobacillus* sp. and *Bifidobacterium* sp. significantly. The prebiotic index of β-glucan from *A. auricula* Judae was superior to that of yeast β-glucan, β-glucan from *S. commune* Fr. and oligo-β-glucan from both mushrooms, respectively. The probiotics also produced short-chain fatty acids (SCFAs), such as acetate, propionate, butyrate, and lactate. Thus, the β-glucans from these mushrooms were prebiotic candidates.

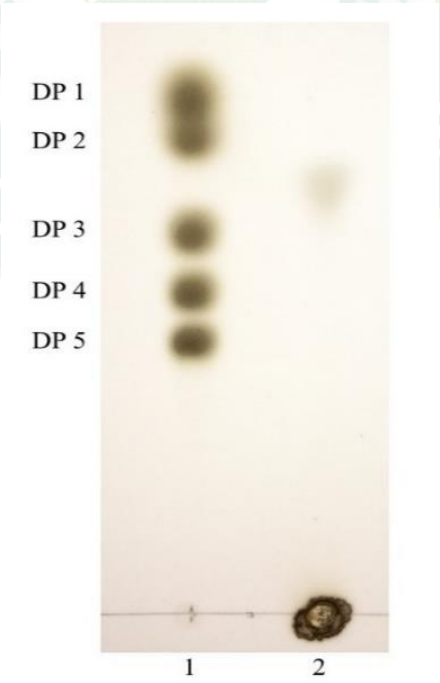


Figure 30 The TLC chromatogram of oligosaccharide analysis in the schizophyllan.

4.5.6 β-glucan analysis

The schizophyllan extract from the split gill mushroom was analyzed β-glucan content by β-glucan assay procedure K-YBGL 02/21 (Megazyme Int.) because this procedure is the specific kit using many specific enzymes and chemicals to hydrolyze the interested glucan in the extract for β-glucan analysis. It was noticed that the extract was comprised of β-glucan content 271.42 ± 27.61 mg/g extract, when calculated from total glucan and α-glucan content in the extract by this procedure around 280.40 ± 24.33 and 8.98 ± 3.30 mg/g extract, respectively.

Table 27 β-glucan content in the extract from various mushrooms.

Mushroom species	β-glucan content (mg/ g extract)	Reference
Agaricus bisporus	63.9	Nitschke et al. (2011)
Lentinus edodes	138.0	
Pleurotus eryngii	167.9	
Trametes versicolor	67.7	
Inonotus obliqus (H_2SO_4 procedure)	79.0	Megazyme (2021)
A. blazei (HCl procedure)	89.0	
Tremella fuciformis (H_2SO_4 procedure)	149.0	
Flammulina velutipes (HCl procedure)	210.0	
S. commune	271.4	This research

Furthermore, a comparison of β-glucan content in this extract with different mushroom extracts is depicted in Table 27. This schizophyllan extract showed superior β-glucan content than *Agaricus bisporus* quadruple, *Lentinus edodes* twice,

Pleurotus eryngii 1.6 times and *Trametes versicolor* 4 folds (Nitschke et al., 2011). Likewise, this extract had greater β-glucan content than from *Inonotus obliqus* (H_2SO_4 procedure) 3.4 times, *A. blazei* (HCl procedure) triple, *Tremella fuciformis* (H_2SO_4 procedure) 1.8 folds and also higher than from *Flammulina velutipes* (HCl procedure) (Megazyme, 2021).

4.6 Biological properties of the schizophyllan

4.6.1 Antioxidant activities

The different extracts obtained from the split gill mushroom displayed varying patterns of free radical inhibitions, as illustrated in Figure 31. The DPPH radical inhibitions of the aqueous extract, the polysaccharide from the extraction of the mushroom content 5% (w/v) with ethanol concentration 63.33% (v/v) (M5 EtOH63.33), and from the extraction of the mushroom content 10% (w/v) with same ethanol concentration (M10 EtOH63.33) increased in proportion to their concentrations. These mushroom extracts exhibited strong antioxidant properties, with approximately 86% DPPH inhibition observed at a concentration of 80 mg/ml. Analysis of the half-maximal inhibitory concentrations (IC_{50} values) for free radical inhibition (Table 28) revealed that the aqueous extract demonstrated the highest potential for inhibiting DPPH radicals at a low concentration of 7.00 mg of dry extract/ml, surpassing the inhibitory potential of the M5 EtOH63.33 extract (15.36 mg of dry extract/ml) and the M10 EtOH63.33 extract (20.08 mg of dry extract/ml).

Moreover, the results of all extracts from this mushroom presented different patterns of free radical inhibitions (Figure 32). ABTS radical inhibitions of aqueous extract, M5 EtOH63.33 polysaccharide extract, and M10 EtOH63.33 polysaccharide extract enhanced, while their concentrations augmented too. These mushroom extracts showed high antioxidant candidates with around 95 - 99% ABTS inhibition at 20 mg/ml. According to the half-maximal inhibitory concentrations (IC_{50} values) for inhibition of free radicals (Table 29), it was noticed that M10 EtOH63.33 extract exhibited more potential for inhibition of ABTS radicals at low concentration 5.69 mg of dry extract/ml, less than the M5 EtOH63.33 extract (7.08 mg of dry extract/ml) and treatment control extract (8.10 mg of dry extract/ml). Thus, M5 EtOH63.33 extract

would be chosen to produce the schizophyllan extract on a large scale, because it had great potential antioxidant by DPPH and ABTS radical scavenging assays (similar to M10 EtOH63.33 extract), including cost-effective extraction of schizophyllan with low content of this raw material.

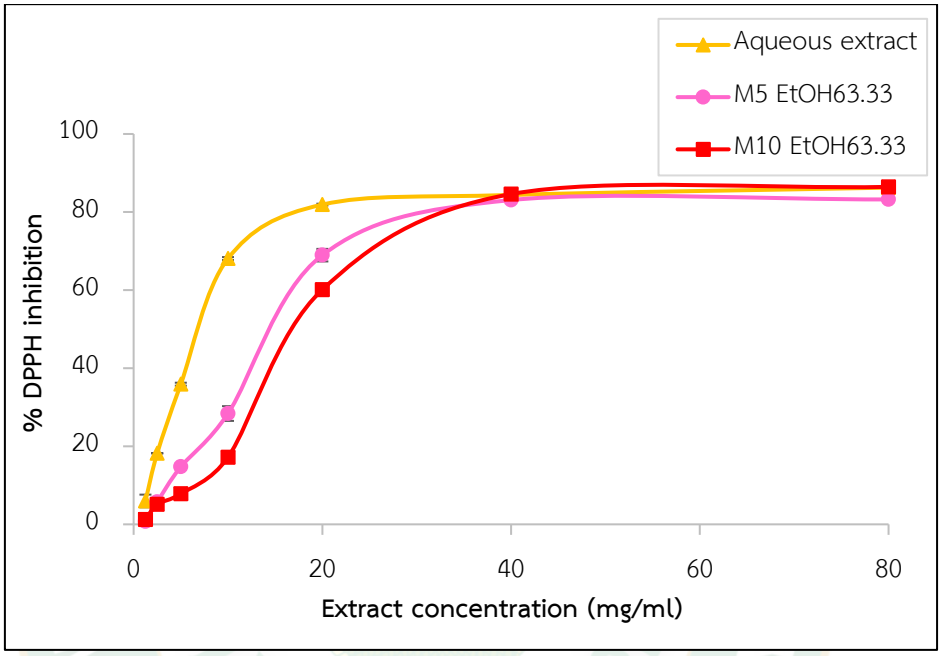


Figure 31 DPPH radical inhibition by different extracts from *S. commune*.

** M5 EtOH63.33 and M10 EtOH63.33 = the polysaccharide from the extraction of the mushroom content 5 and 10% (w/v) with ethanol concentration 63.33% (v/v), respectively.

Table 28 Inhibitory activities (IC_{50}) on DPPH assay of various extracts from *S. commune*.

Samples	IC_{50} values (mg extract/ml)
Aqueous extract	7.00 ± 0.04^{c}
M5 EtOH63.33	15.36 ± 0.52^{b}
M10 EtOH63.33	20.08 ± 0.21^{a}

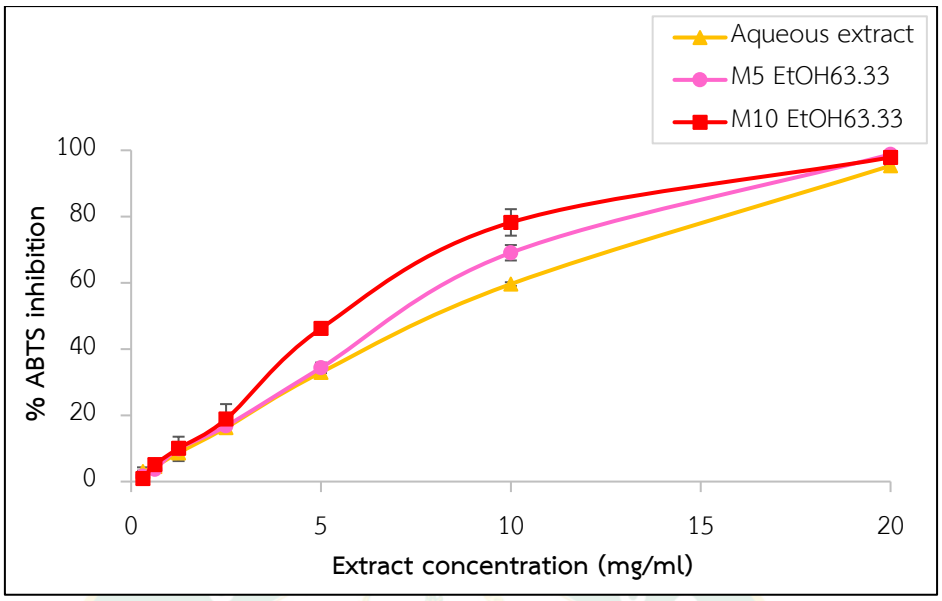


Figure 32 ABTS radical inhibition by different extracts from *S. commune*.

** M5 EtOH63.33 and M10 EtOH63.33 = the polysaccharide from the extraction of the mushroom content 5 and 10% (w/v) with ethanol concentration 63.33% (v/v), respectively.

Table 29 Inhibitory activities (IC_{50}) on ABTS assay of various extracts from *S. commune*.

Samples	IC_{50} values (mg extract/ml)
Aqueous extract	8.10 ± 0.07^{a}
M5 EtOH63.33	7.08 ± 0.34^{b}
M10 EtOH63.33	5.69 ± 0.21^{c}

Besides, some researches evaluated antioxidant activity from other agricultural materials. The IC_{50} value of the schizophyllan extract from the split gill mushroom (M5 EtOH63.33) was more efficient to inhibit free radicals on DPPH assay using a lower concentration (15.36 mg of dry extract/ml) than the aqueous extract of *Solanum melongena* (eggplant) stem (26.20 mg of dry extract/ml) nearly twice (Jung et al., 2011), as well as superior to inhibit free radicals on ABTS assay (7.08 mg of dry extract/ml) to *Pinus yunnanensis* Franch. (pine nut) protein hydrolysate (8.83 mg of dry extract/ml) considerably (Liu et al., 2021). However, the IC_{50} value of the schizophyllan from this mushroom on ABTS assay was inferior to this mushroom

essence using an electric pressure cooker significantly (0.73 mg of dry extract/ml) in the latest research (Saetang et al., 2022a) because the mushroom essence (similar to the supernatant from the separation of schizophyllan extraction) comprised of more water-soluble components of many phenolic compounds as greatly effective scavengers of free radicals of reactive oxygen species by oxidative reaction, including flavonoids, tannins, saponins, steroids, alkaloids, terpenes and free amines after the hot water extraction. Hence, the split gill mushroom essence was antioxidative potential candidate to develop as nutraceutical product along with beneficially bioactive substances to prevent oxidative diseases.

4.6.2 Prebiotic property

Isolate BG-NS02 from Bulgaria yogurt, which is a probiotic bacterium, was streaked on MRS agar supplemented with bromocresol blue to isolate single colonies. The appearance of yellow zones around the bacterial colonies on the agar (Figure 33) indicated the production of lactic acid by the bacteria, causing the pH indicator bromocresol blue to change from purple to yellow. Gram staining of the probiotic bacteria, observed under a light microscope (Figure 34), confirmed that isolate BG-NS02 is a Gram-positive cocci bacterium, exhibiting a purple stain with crystal violet. This bacterium belongs to the *Streptococcus* sp. genus and is considered a lactic acid bacteria commonly used in various fermentation processes.

Additionally, Figure 35 displayed the growth curves of this probiotic bacterium cultivated in MRS broth with different carbon sources. It was observed that the bacterial strain grew rapidly during the early log phase (approximately 6 hours) and then exhibited slower growth before reaching a steady state in the stationary phase. Comparatively, the growth of the bacteria in MRS broth supplemented with the schizophyllan extract was considerably inferior to that in MRS broth with glucose (used as a positive control), but slightly superior to their growth in MRS broth without a carbon source (used as a negative control). This suggests that the probiotic bacteria found it more challenging to utilize the schizophyllan, a polysaccharide with a long molecular weight, compared to glucose, which is a monosaccharide. In such cases, there are alternative solutions to enhance utilization of the extract by the bacteria in

the colon, such as enzymatic hydrolysis of the extract into shorter oligosaccharides or using natural acidic or alkaline solutions. These approaches can facilitate easier utilization by the bacteria, as polysaccharides with high molecular weights are less readily metabolized (Nowak et al., 2018). Nonetheless, the schizophyllan extract shows potential for prebiotic development.

In the case of long-chain polysaccharides, β-glucan extract from *S. commune* could be hydrolyzed by the β-glucanase enzyme for the production of β-glucan oligosaccharide (BGO). In β-glucan hydrolysis, β-glucanase would cleavage β-1,3 and β-1,6-glycosidic linkage of β-glucan into BGO. Consequently, the received BGO has a short oligosaccharide with low molecular weight and prebiotic property to tolerate acid and digestive enzymes in the enzymes in GI tract (Lam and Chi-Keung, 2013). Smaller molecular oligosaccharides are more easily accessible to probiotic bacteria in the colon than polysaccharides with a high molecular weight for consumption and fermentation (Nowak et al., 2018).

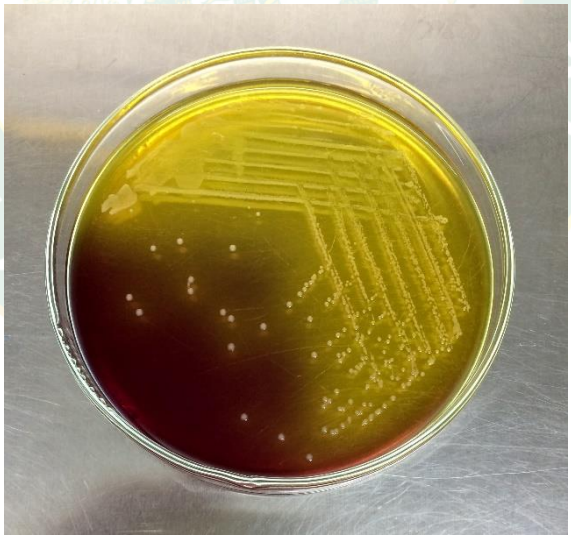


Figure 33 Single colony of probiotic bacteria, isolate BG-NS02 from Bulgaria yogurt, streaked on MRS agar.

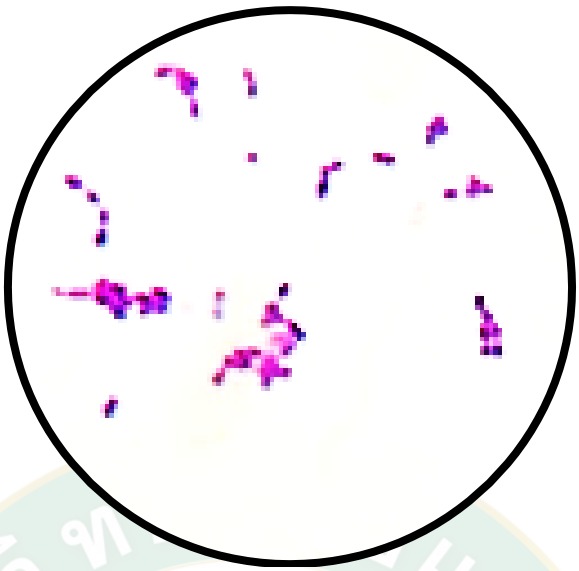


Figure 34 Gram staining of probiotic bacteria, isolate BG-NS02 from Bulgaria yogurt, under the light microscope at 100× magnification.

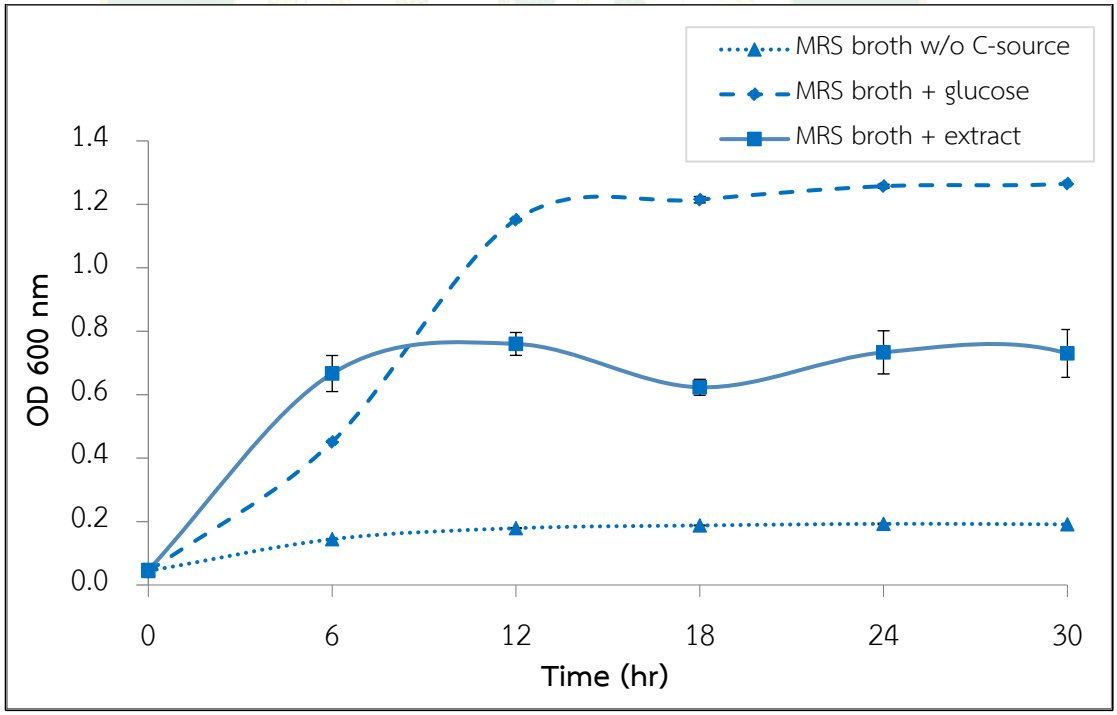


Figure 35 Growth curves of probiotic bacteria, isolate BG-NS02 from Bulgaria yogurt, cultivated in MRS broth with different carbon source.

Moreover, the probiotic proliferation of isolate BG-NS02 from the Bulgaria yogurt compared with other probiotic growth in different prebiotic agricultural materials is depicted in Table 30. It was noticed that this probiotic isolate's stimulating proliferation on the schizophyllan extract from this split gill mushroom was superior to *Bifidobacterium longum* BB536 and *Lactobacillus plantarum* TISTR 1854 on XOS (CbXyn10C) [xylooligosaccharides synthesis by bacterial recombinant GH10 from *Caldicellulosiruptor bescii* xylanase], and XOS (Xyn11A) [xylooligosaccharides synthesis by GH11 from *Bacillus firmus* K-1 xylanase] from sugarcane bagasse xylans around 3.7 and 1.7 folds, respectively. This probiotic growth on this extract also was more significant than *L. plantarum* TISTR 1854 and *B. longum* BB536 on XOS (CbXyn10C) and XOS (Xyn11A) from rice straw xylans considerably (Gufe et al., 2022). Additionally, it was greater than *L. brevis* NRRL B-4527 and *L. delbrueckii* subsp. *bulgaricus* NRRL B-548 on BGO [curdlan (1⟶3)-β-D-glucan oligosaccharides synthesis by a GH family 64 β-(1⟶3)-glucanase (*Rm*Lam81A)] from curdlan of *Alcaligenes faecalis* about triple and 1.3 times, respectively. It also was remarkably higher than *L. casei* subsp. *casei* NRRL B-1922 and *L. rhamnosus* AS 1.2466 on inulin from chicory root nearly twice and 1.5 folds, respectively (Shi et al., 2018).

Shi et al. (2018) evaluated of the *in vitro* digestibility and stability of curdlan-β-1,3-glucan oligosaccharide under simulated gastrointestinal conditions. They utilized curdlan (β-1,3-glucan) derived from *Alcaligenes faecalis* bacteria and degraded it using β-1,3-glucanase from *Rhizomucor miehei* fungi to produce the oligosaccharide. The results demonstrated that this oligosaccharide remained stable during simulated gastrointestinal digestion, including exposure to low pH, high temperature, and Maillard reaction conditions commonly encountered during food processing. Furthermore, the oligosaccharide promoted the growth of beneficial bacteria, such as *Lactobacillus* sp. and *Bifidobacterium* sp., which produced short-chain fatty acids (SCFAs), such as acetic, propionic, and lactic acid. Additionally, the oligosaccharide inhibited the growth of *E. coli*. Short oligosaccharides with a degree of polymerization (DP) of 2 and 3 were readily metabolized by *Lactobacillus* strains,

modulating the pH of the large intestine and benefiting the overall well-being and health of the host. These findings suggest the potential utilization of this oligosaccharide as functional food material.

Table 30 Growth results of probiotic bacteria in various prebiotic agricultural materials.

Agricultural materials	Prebiotic type	Probiotic bacteria species	Growth results	Reference
Sugarcane bagasse	XOS (CbXyn10C)	*Bifidobacterium longum* BB536	OD 600 nm = 0.200, 72 hr	Gufe et al. (2022)
	XOS (Xyn11A)	*Lactobacillus plantarum* TISTR 1854	OD 600 nm = 0.440, 72 hr	
Rice straw	XOS (CbXyn10C)	*L. plantarum* TISTR 1854	OD 600 nm = 0.550, 72 hr	
	XOS (Xyn11A)	*B. longum* BB536	OD 600 nm = 0560., 72 hr	
Curdlan from *Alcaligenes faecalis*	BGO	*L. brevis* NRRL B-4527	OD 595 nm = 0.217, 48hr	Shi et al. (2018)
		L. delbrueckii subsp. *bulgaricus* NRRL B-548	OD 595 nm = 0.560, 48 hr	
Chicory root	Inulin	*L. casei* subsp. *casei* NRRL B-1922	OD 595 nm = 0.400, 48 hr	
		L. rhamnosus AS 1.2466	OD 595 nm = 0.500, 48 hr	
Split gill mushroom	Schizophyllan (β-glucan)	Isolate BG-NS02 from Bulgaria yogurt	OD 600 nm = 0.730, 30 hr	This study

Remark: XOS (CbXyn10C) = xylooligosaccharides synthesis by bacterial recombinant GH10 from *Caldicellulosiruptor bescii* (CbXyn10C) xylanase; XOS (Xyn11A) = xylooligosaccharides synthesis by GH11 from *Bacillus firmus* K-1 (Xyn11A) xylanase; BGO = curdlan (1⟶3)-β-D-glucan oligosaccharides synthesis by a GH family 64 β-(1⟶3)-glucanase (*Rm*Lam81A).

Similarly, Nowak et al. (2018) explored the potential of polysaccharides extracted from 53 strains of wild mushrooms to promote the proliferation of *Lactobacillus rhamnosus* and *L. acidophilus*. They employed ethanol extraction from the fruit bodies of each mushroom and deproteinization techniques involving sonication, heat treatment, and the Savage reagent (chloroform: isoamyl alcohol). The polysaccharide fractions were then tested for their ability to promote the growth of probiotic bacteria and subjected to *in vitro* digestion simulating the gastrointestinal tract. The study revealed that mushroom polysaccharides stimulated the proliferation of *Lactobacillus* strains to a greater extent compared to commercial prebiotics, such as FOS or inulin. Furthermore, the polysaccharides exhibited resistance to artificial gastric juice, remaining stable and undigested until reaching the large intestine, where they could promote the growth of beneficial probiotic bacteria. Consequently, these edible mushroom polysaccharides hold potential for utilization in functional foods and nutraceuticals production.

4.6.3 Anti-inflammatory and immune-enhancing properties

The cytotoxicity of the extract (SC) to murine RAW 264.7 cells was evaluated using the MTT assay. The results indicated that a suitable range of extract concentrations for pretreating RAW 264.7 cells was 250 - 1,000 µg/ml, with incubation times of 1 - 3 hours. At these concentrations and incubation times, the cells exhibited high viability, and there was no significant difference observed at a 95% confidence level (Figure 36). These findings suggested that the extract concentrations and incubation times used were non-toxic to the cells. Similarly, a study by Du et al. (2017) found that an exopolysaccharide concentration of 200 µg/ml from submerged mycelial culture of *S. commune* did not affect RAW 264.7 cell viability. In the MTT assay, viable cells with active mitochondria produce mitochondrial dehydrogenase. When the yellow MTT solution is added, it is hydrolyzed by mitochondrial dehydrogenase in the cells, forming purple formazan crystals. These crystals are then solubilized with a DMSO solution to produce a formazan solution (Wasunan et al., 2022).

Furthermore, the prior research reported that β-glucan from the split gill mushroom (50 μg/ml) enhanced the IL-10 mRNA expression induced with LPS from *Aggregatibacter actinomycetemcomitans* in murine RAW 264.7 cells using real-time PCR. Dectin-1 in immune cells was a greater affinity to the β-glucan receptor to activate immune-modulatory activities due to the triple helix structure of β-1,3-1,6-glucan, namely releasing key anti-inflammatory cytokines like IL-10 expression to control inflammation and enhance immune responses (Thongsiri et al., 2021). Additionally, β-glucan could directly boost immunity through T-lymphocytes stimulation in the adaptive immune system. The proliferation of macrophages, including T-lymphocytes, B-lymphocytes and natural killer cells would enhance in response to β-glucan induction when β-glucan attached to the receptor, including secreted antibody for pathogen suppression in humoral immunity (Chan et al., 2009; Leung et al., 2006).

Moreover, Du et al. (2017) studied the effect of the exopolysaccharide on LPS-induced nitric oxide (NO), 5-lipoxygenase (5-LOX) production and inducible nitric oxide synthase (iNOS) mRNA expression levels in the macrophages (RAW 264.7 cells). Nitric oxide was recognized as a regulator in pathological reactions, especially acute inflammatory responses. Pro-inflammatory agents like LPS could considerably enhance nitric oxide production in the macrophages through activation of iNOS. Additionally, 5-LOX was a key enzyme in the synthesizing inflammatory mediators of arachidonic acid. The results exhibited that the polysaccharide decreased the production of 5-LOX. Both iNOS and cyclooxygenase-2 (COX-2) were the important enzyme mediators that mediated inflammatory processes. It was noticed that the polysaccharide also inhibited LPS-induced iNOS mRNA expression levels in a dose-dependent manner.

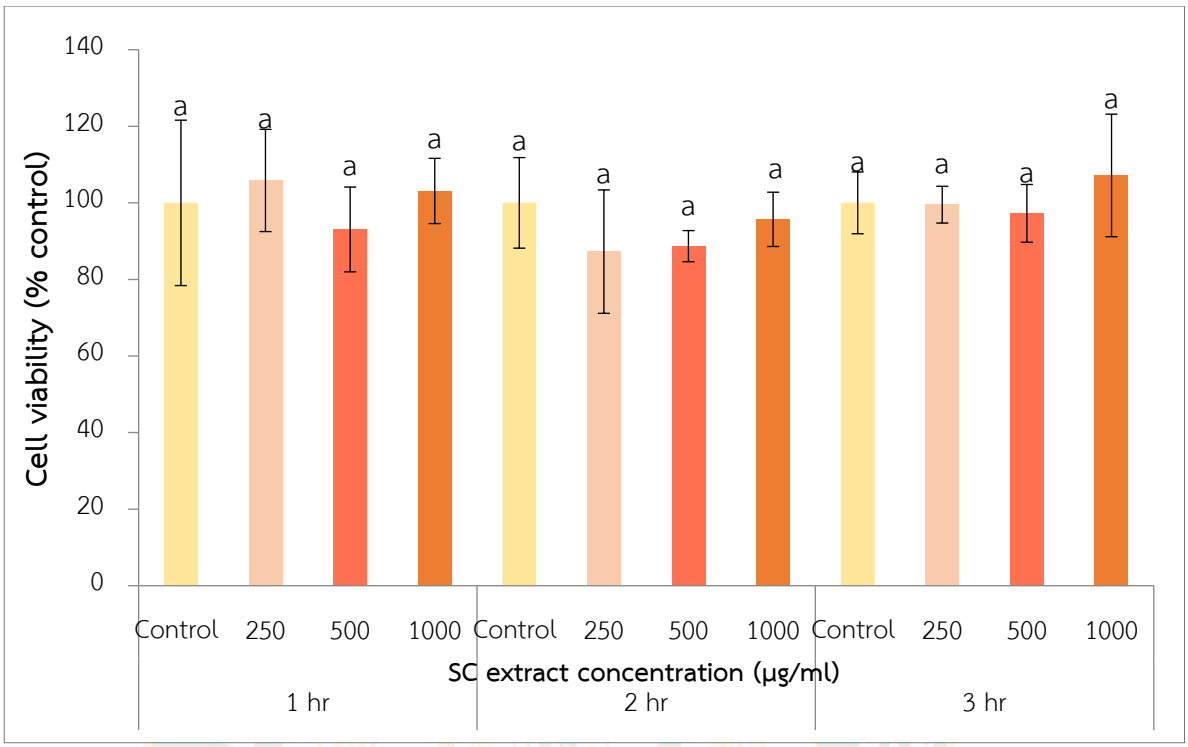


Figure 36 Cytotoxicity of schizophyllan extract (SC) from *Schizophyllum commune* to murine RAW 264.7 cells by MTT assay.

Besides, Lee andKi (2020) investigated the influence of ultrasonicated schizophyllan (uSPG) on LPS-induced mRNA expression of RAW 264.7 cells grown in two-dimensional (2D) and three-dimensional (3D) culture conditions. For 2D cell culture, the macrophages were maintained in high glucose DMEM medium (*in vitro*). For 3D cell culture, the cells were encapsulated in poly(ethylene glycol) (PEG) hydrogel (*in vivo*). The experimental results revealed that encapsulated cells grown in 3D were less sensitive to LPS than those grown in 2D. In the uSPG- and LPS-treated groups, expression levels of iNOS, COX-2, and TNF-**α** reduced, compared to the expression levels of cells treated with LPS. Thus, the uSPG would suppress the overproduction of pro-inflammatory mediators, resulting in an anti-inflammatory effect.

In the case of immune-boosting properties, the macrophages played an important role in host protection against attacking cells by releasing various cytokines. Yelithao et al. (2019) evaluated the immunomodulatory effect of the

polysaccharides from this mushroom on RAW 264.7 cells by determining levels of cytokines production. It was found that the proliferation activity of crude and fractions of polysaccharides using DEAE–Sepharose fast flow column against the macrophages. Both crude and fractions of polysaccharides were significantly greater than 100% of control and improved their proliferation activity. It was indicated that crude and fractions were nontoxic to the cells. On the other hand, excess amounts of pro-inflammatory cytokines (iNOS and TNF-α) produced could cause several inflammatory diseases. To overcome this problem, anti-inflammatory cytokines (IL-10 and IL-12) were produced. In this research, F1 polysaccharide significantly decreased iNOS and TNF-α expression levels, while IL-10 and IL-12 expressions were cleared after treatment with crude or F2 polysaccharides. Hence, these cytokines could also act as an excellent immune stimulant by modifying immune function (Yelithao et al., 2019).

4.7 Development of the split gill mushroom essence product

The split gill mushroom essence, enriched with herbs, offers a delightful and healthy twist to one's schizophyllan (β-glucan) consumption. This functional food product combines the potent ingredients of split gill mushroom, Indian gooseberry, and stevia. Abundant in this mushroom essence are schizophyllan (β-glucan), phenols, phyllanthin and chebulagic acid from Indian gooseberry, and stevioside from stevia, serving as natural sweeteners. Notably, the mushroom essence is renowned for its immune-boosting, anti-inflammatory, anticancer, antioxidant, and prebiotic properties, while also being low in calories to support blood sugar control. Furthermore, this product prides itself on containing only natural, chemical-free ingredients.

Moreover, the Indian gooseberry (*Phyllanthus* sp.) contributes to the function of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells in the immune system. This herb contains phyllanthin, chebulagic acid, and exhibits anti-inflammatory activity through the NF-**K**B signaling pathway. Previous research has demonstrated its ability to enhance immune responses, including both innate and adaptive immune

systems, making it a potential candidate for immunomodulation and medicinal applications (Jantan et al., 2019).

Table 31 Sensory evaluation of consumer acceptance of the split gill mushroom essence in different time of production process.

Evaluation	Score level		
	45 min	**90 min**	**120 min**
Color of mushroom essence	3.60 ± 0.55^{b}	3.67 ± 0.58^{b}	5.00 ± 0.00^{a}
Appearance	4.00 ± 0.71^{a}	3.67 ± 0.58^{a}	4.80 ± 0.45^{a}
Sourness	3.75 ± 0.96^{a}	4.33 ± 0.58^{a}	4.60 ± 0.55^{a}
Sweetness	4.00 ± 0.82^{a}	4.67 ± 0.58^{a}	4.60 ± 0.89^{a}
Saltiness	3.20 ± 1.79^{a}	46.7 ± 0.58^{a}	4.20 ± 0.84^{a}
Overall preference about aroma	3.40 ± 1.52^{a}	3.67 ± 0.58^{a}	4.60 ± 0.55^{a}
Overall preference about taste	2.80 ± 1.30^{a}	3.33 ± 0.58^{a}	4.40 ± 0.55^{a}

In previous research, the extraction of schizophyllan was optimized to determine a suitable production process for the mushroom essence. The study identified an optimal extraction temperature of 100°C, which was selected for the production of the mushroom essence (Saetang et al., 2022b). Table 31 presented the sensory evaluation of consumer acceptance of the mushroom essence at different stages of the production process. It was observed that a 120-minute extraction time yielded favorable results in terms of overall taste acceptability, color, and appearance of the mushroom essence (p-value < 0.10). Based on these findings, suitable mushroom essence formulas were developed.

Table 32 showcased the sensory evaluation of consumer acceptance for each formula of the mushroom essence. Formula 2, which contained functional schizophyllan with a β-glucan content of 507.55 mg/40 ml of mushroom essence, was selected as the appropriate formula for producing the mushroom essence on a

large scale. This formula demonstrated significant overall taste preference, particularly in terms of sweetness and saltiness (p-value < 0.05). The resulting mushroom essence products were described as dark brown and packaged as premium products (Figure 37). Table 11 presents a comparative analysis of the nutrient composition between this split gill mushroom and other mushroom species. The findings reveal that this mushroom has significantly higher protein content than *Auricularia polytricha* MFB, *Lentinus subnudus* YEB, and *Pleurotus florida* YFB. Its fiber content is similar to these mushrooms, while its fat content is lower in calories compared to various mushroom species (Gbolagade et al., 2006). Additionally, this mushroom exhibits considerably higher protein and carbohydrate content compared to *Tuber aestivum* (Tejedor-Calvo et al., 2020). Furthermore, when comparing the cultivated mushroom to wild *S. commune*, it is observed that the cultivated mushroom has higher protein and crude fiber content, but lower carbohydrate and fat content. These differences may be attributed to variations in geographical distribution and cultivation methods (Okwulehie et al., 2007; Singh et al., 2021).

Table 32 Sensory evaluation of consumer acceptance of each formula's split gill mushroom essence.

Evaluation	Score level		
	Formula 1	Formula 2	Formula 3
Color of mushroom essence	5.00 ± 0.00^{a}	4.75 ± 0.50^{a}	4.71 ± 0.49^{a}
Appearance	4.80 ± 0.45^{a}	4.75 ± 0.50^{a}	4.57 ± 0.53^{a}
Sourness	4.60 ± 0.55^{a}	4.75 ± 0.50^{a}	3.57 ± 1.13^{a}
Sweetness	4.60 ± 0.89^{a}	4.75 ± 0.50^{a}	3.00 ± 1.29^{b}
Saltiness	4.20 ± 0.84^{ab}	5.00 ± 0.00^{a}	3.14 ± 1.21^{b}
Overall preference about aroma	4.60 ± 0.55^{a}	4.25 ± 0.96^{a}	3.86 ± 0.90^{a}
Overall preference about taste	4.40 ± 0.55^{a}	4.75 ± 0.50^{a}	3.14 ± 0.90^{b}

Figure 37 The split gill mushroom essence product.

In a study by Li et al. (2011), the impact of different cooking methods on the flavor compounds of button mushroom (*Agaricus bisporus* (*Lange*)*Sing*) soup was investigated. It is known that various mushroom species possess unique taste profiles attributed to water-soluble substances, including free amino acids, 5'-nucleotides, and reducing sugars. When the mushrooms were boiled, the levels of certain amino acids increased, indicating hydrolysis through proteolysis reactions during the boiling process. The results demonstrated that both boiled and autoclaved mushroom soups had higher levels of free amino acids and 5'-nucleotides, compared to other cooking methods. There were main nonvolatile taste-active compounds in this mushroom, such as alanine (Ala), aspartic acid (Asp), glutamic acids (Glu), glycine (Gly), threonine (Thr), 5'-guanosine monophosphate (5'-GMP), 5'-inosine monophosphate (5'-IMP), and 5'-xanthosine monophosphate (5'-XMP). Glu and Ala are identified as key amino acids contributing to the umami and sweet tastes in various mushroom species. The levels of 5'-GMP, which is associated with a meaty

flavor and umami taste, can be influenced by cooking processes. Additionally, 5'-AMP and Gly have been reported to contribute to sweet and umami tastes, respectively.

Furthermore, the boiled mushroom soup exhibited higher levels of volatile compounds compared to the autoclaved mushroom soup. Key aroma-active substances in the mushroom soup included 3-octen-2-one (cooked mushroom-like), 1-octen-3-one (mushroom-like), and dihydro-5-methyl-2(3H)-furanone (sweet), among others. These flavor components of mushrooms undergo chemical reactions, particularly the Maillard reaction between free amino acids and soluble sugars during cooking processes. The sweeteners which are used such as glucose and fructose, serve as precursors for the Maillard reaction, resulting in the formation of flavor compounds during heating (Li et al., 2011).

In another study, Sun et al. (2019) focused on mushroom soup made from *Hypsizygus marmoreus*. The various cooking methods (autoclaving, microwaving, stewing, and sous vide) were evaluated for their impact on nutrient, phytochemical, and flavor content. The results showed that all cooking processes led to an increase in macro- and micronutrients (polysaccharides, free amino acids, and polyphenols) compared to uncooked soup. Autoclaving, in particular, resulted in the highest levels of polysaccharides due to the high temperature and pressure, causing significant cell and internal cellular damage. Autoclaving also led to increased phenolic content in the mushroom soup.

Moreover, autoclaving significantly increased the levels of free amino acids in the mushroom soup. Umami amino acids (Glu and Asp) and sweet-tasting amino acids (Ala, Gly, Pro, Ser, and Thr) were found in higher quantities in the autoclaved soup. These free amino acids may have been generated through mushroom degradation and protein hydrolysis. The autoclaved soup also exhibited higher total nucleotide content, contributing to its flavor profile. Notably, 5'-GMP, associated with meat flavor, and 5'-AMP, known for its sweet flavor, were present in the soup, enhancing its taste (Sun et al., 2019). Therefore, the split gill mushroom essence product developed in this research offers a promising taste profile and supports consumer health. It also encourages the utilization of Thai herbs and the creation of

high-value-added products. This mushroom essence represents a novel nutraceutical food enriched with beneficially active substances for consumer health.

CHAPTER 5
CONCLUSION

This research successfully extracted the genomic DNA of the split gill mushroom (641 bp) using ITS5 forward and ITS4 reverse primers. The homology comparison using BLAST analysis against the GenBank or NCBI database identified the mushroom as *Schizophyllum commune* based on its nucleotide sequence. Furthermore, this mushroom exhibited significantly superior nutritive values compared to other mushroom species, boasting high levels of carbohydrates (64.06%), crude fiber (4.87%), and alternative mycoprotein (20.99%), while maintaining low levels of fat (0.75%). Importantly, no harmful heavy metals were detected, making this mushroom a safe and reliable choice. These findings highlight the potential of *S. commune* as a nutritious and uncontaminated mushroom option. Moreover, the hot water extraction and ethanol precipitation process for schizophyllan, a low-cost commercial optimization, was conducted. The extraction involved using a mushroom content of 5 - 8% (w/v) at temperatures ranging from 100 to 110°C for 2 - 3 hours. The subsequent precipitation step utilized ethanol with a concentration of 62 - 65% (v/v) to isolate schizophyllan, resulting in a schizophyllan sample with the highest degree of polymerization (DP value of 5.40), the highest total sugar concentration (447.98 mg/g extract), minimal reducing sugar concentration (92.08 mg/g extract), optimized protein concentration (5.30 mg/g extract), and the highest extraction yield (4.03 g/100 g dry mushroom).

Additionally, the solubilization of this extract was optimized at a temperature of 120°C for 120 minutes, with an extract concentration of 5.00 mg/ml. This optimized solubilization process led to the highest total sugar content, excellent reducing sugar content, suitable DP value, and superior total phenolic content. These findings highlight the effectiveness of the optimized extraction and solubilization processes for obtaining high-quality schizophyllan with desirable properties. Moreover, the schizophyllan extract exhibited a higher β-glucan content (271.42 mg/g extract) than other extracts from different mushrooms, as determined by the

β-glucan assay procedure. This indicates its possible potential for more significant immune-enhancing and anticancer activities. The schizophyllan extract also contained β-glucooligosaccharides (β-glucan oligosaccharides) with a degree of polymerization value ranging from 2 to 3, specifically laminaribiose and laminaritriose. These oligosaccharides show promise as potential prebiotics for further study, as analyzed through TLC. Also, the HPLC analysis revealed lower sugar content, specifically fructose and maltose, in the schizophyllan extract. This makes it a lower-calorie option that is beneficial for human health. Furthermore, when analyzed through SDS-PAGE, the extract contained hydrophobin (14.0 - 18.5 kDa) and lectin proteins (21.0 - 35.0 kDa), suggesting probably potential immunomodulatory and anticarcinogenic properties. The extract also comprised bioactive purpurin, as identified by Raman confocal spectroscopy. These findings highlight the exceptional characteristics of the schizophyllan extract, including its high β-glucan content, β-glucooligosaccharide composition, lower sugar content, presence of hydrophobin and lectin proteins, and the bioactive purpurin compound. These properties suggest that the schizophyllan extract might hold excellent potential for immune-enhancing, prebiotic, and anticarcinogenic activities, making it a valuable subject for further exploration and potential applications.

Moreover, the schizophyllan, which consists of total phenolic compounds (6.49 mg GAE/g extract), displays remarkable antioxidant properties. It exhibits a significant capacity to inhibit DPPH radicals (83.23% inhibition at 80 mg extract/ml) and ABTS radicals (98.77% inhibition at 20 mg extract/ml). Furthermore, it demonstrates potent inhibition of DPPH and ABTS radicals at low concentrations, with IC_{50} values of 15.36 and 7.08 mg extract/ml, respectively. This makes it attractive for antioxidant applications, especially considering its cost-effective extraction process and low mushroom content.

However, the mushroom essence outperforms the extract with a significantly lower IC_{50} value (0.73 mg extract/ml) in the ABTS assay. Consequently, the mushroom essence exhibits substantial potential as an antioxidant candidate for developing nutraceutical products. In addition to its antioxidant properties,

schizophyllan also possesses prebiotic effects due to the presence of oligosaccharides, namely laminaribiose and laminaritriose, as well as β-glucan. These components promote the growth of probiotic bacteria, particularly the BG-NS02 strain isolated from Bulgaria yogurt, within the human gastrointestinal tract. Comparatively, they exhibit superior probiotic growth stimulation compared to other prebiotic agricultural materials. Moreover, this substance may be promising in boosting immune function, reducing inflammation, and displaying potential anti-cancer properties. To evaluate the cytotoxicity of the extract on murine RAW 264.7 cells (macrophages), MTT assay was conducted using extract concentrations ranging from 250 to 1,000 µg/ml and incubation times of 1 to 3 hours. The results reveal high cell viability and no significant differences (p-value < 0.05) among the different incubation times, indicating the extract's safety. Previous studies have reported that β-glucan derived from this mushroom (at a concentration of 50 µg/ml) enhances the production of IL-10 in RAW 264.7 cells induced with LPS. IL-10 is a crucial anti-inflammatory cytokine known for its ability to control inflammation and enhance immune-modulatory activities.

Consequently, both the mushroom itself and the schizophyllan substance were employed to create a functional mushroom essence enriched with herbs. The optimal conditions for producing the mushroom essence were determined to be a temperature of 100°C for 120 minutes. The resulting mushroom essence formulation consists of potent ingredients, including 2.500% (w/v) split gill mushroom, 0.021% (w/v) schizophyllan extract, 0.017% (w/v) Indian gooseberry, and 0.004% (w/v) stevia, which serves as a natural sweetener. This novel mushroom essence represents a high-value-added functional product containing a remarkable array of biologically active compounds that promote optimal human health.

Recommendations and suggestions:

1. To optimize the commercial extraction of schizophyllan from this mushroom on an industrial scale, cost-effective methods, such as the utilization of natural alkaline or acidic solutions, enzymes, and pulsed electric field-assisted extraction

(PEFAE) can be considered for cell disruption and improved extraction efficiency. It is important to choose extraction techniques that are low-cost, readily available, and proven to be effective.

2. The β-glucan extract can hydrolyze with an enzyme (β-glucanase) to short β-glucooligosaccharides, which exhibit more potential prebiotic properties to promote probiotic bacteria proliferation better.
3. Further research will investigate the schizophyllan extract for immune-boosting and anti-inflammatory properties in murine RAW 264.7 cells.
4. The split gill mushroom essence product should be sterilized by retort for the extended shelf life of this product without microbial contamination when kept at room temperature.
5. The mushroom essence product should be studied for certificates of analysis (COA), to verify the quality, purity, and performance of this product before distribution in the markets.
6. Obtaining ethical certifications, like the Thailand ethical certificate and FDA certificate, is crucial for marketability. These certifications indicate compliance with standards and regulations, building consumer confidence for successful market penetration.

APPENDIX A

QUESTIONNAIRE OF SPLIT GILL MUSHROOM ESSENCE PRODUCT

Sensory evaluation of consumer acceptance of
the split gill mushroom essence with herb

Explanation:

- This product consists of split gill mushroom, Indian gooseberry, and stevia.
- Please mark score in each level of product properties as following table.

When 1 = dislike very much, 2 = dislike slightly, 3 = neither like nor dislike,
4 = like slightly, 5 = like very much

Evaluation	Formula					Formula					Formula				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
Color of this mushroom essence															
Appearance															
Sourness															
Sweetness															
Saltiness															
Overall preference about aroma															
Overall preference about taste															

Comments: (e.g. aroma of split gill mushroom, Indian gooseberry, and stevia etc.)

.....

.....

.....

แบบสอบถามความพึงพอใจของผู้บริโภคต่อผลิตภัณฑ์ซุปเห็ดแครงสกัดเสริมสมุนไพร

คำชี้แจง :

- ในผลิตภัณฑ์นี้มีส่วนผสมของเห็ดแครง มะขามป้อม และหญ้าหวาน
- กรุณาให้คะแนนระดับที่ท่านมีต่อคุณลักษณะของผลิตภัณฑ์ในแต่ละด้านดังตาราง

โดยกำหนดให้ 1 = ไม่ชอบมาก 2 = ไม่ชอบเล็กน้อย 3 = เฉยๆ 4 = ชอบเล็กน้อย 5 = ชอบมาก

การประเมิน	สูตรที่					สูตรที่					สูตรที่				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
สีของซุปเห็ด															
ลักษณะปรากฏ															
รสเปรี้ยว															
รสหวาน															
รสเค็ม															
ความชอบโดยรวมด้านกลิ่น															
ความชอบโดยรวมด้านรสชาติ															

ข้อเสนอแนะ : (เช่น กลิ่นของเห็ดแครง มะขามป้อม และหญ้าหวาน เป็นต้น)

.....

.....

.....

APPENDIX B

RAW DATA OF OPTIMIZED SCHIZOPHYLLAN EXTRACTION

Table 33 Actual and predicted values for optimized hot water extraction on total sugar content of polysaccharides.

Treatments	Factor 1 A: temperature (°C)	Factor 2 B: time (hr)	Response: total sugar content (mg/g extract)		
			Actual value	Predicted value	Residual
1	80	1	383.00	383.07	-0.0727
2	80	1	383.00	383.07	-0.0727
3	121	1	370.50	370.95	-0.4477
4	121	1	371.25	370.95	0.3023
5	80	3	375.11	377.63	-2.52
6	80	3	380.00	377.63	2.37
7	121	3	445.75	444.33	1.43
8	121	3	442.75	444.33	-1.57
9	80	2	383.00	383.10	-0.1046
10	80	2	383.50	383.10	0.3954
11	121	2	454.25	453.42	0.8260
12	121	2	452.89	453.42	-0.5351
13	100	1	433.50	431.62	1.88
14	100	1	430.04	431.62	-1.59
15	100	3	428.75	428.01	0.7426
16	100	3	427.56	428.01	-0.4518
17	100	2	455.85	454.09	1.76
18	100	2	452.69	454.09	-1.40
19	100	2	453.13	454.09	-0.9506

Table 34 Actual and predicted values for optimized hot water extraction on reducing sugar content of polysaccharides.

Treatments	Factor 1 A: temperature (°C)	Factor 2 B: time (hr)	Response: reducing sugar content (mg/g extract)		
			Actual value	Predicted value	Residual
1	80	1	102.89	102.69	0.1963
2	80	1	102.50	102.69	-0.1905
3	121	1	69.25	69.21	0.0404
4	121	1	69.17	69.21	-0.0346
5	80	3	114.17	114.26	-0.0943
6	80	3	114.36	114.26	0.1001
7	121	3	81.40	81.30	0.1029
8	121	3	81.20	81.30	-0.0971
9	80	2	108.50	108.46	0.0358
10	80	2	108.42	108.46	-0.0475
11	121	2	97.25	97.24	0.0067
12	121	2	97.22	97.24	-0.0183
13	100	1	77.31	77.19	0.1192
14	100	1	77.06	77.19	-0.1308
15	100	3	69.00	68.95	0.0497
16	100	3	68.89	68.95	-0.0614
17	100	2	84.25	84.06	0.1930
18	100	2	83.86	84.06	-0.1960
19	100	2	84.08	84.06	0.0263

Table 35 Actual and predicted values for optimized hot water extraction on DP value of polysaccharides.

Treatments	Factor 1 A: temperature (°C)	Factor 2 B: time (hr)	Response: DP value		
			Actual value	Predicted value	Residual
1	80	1	3.75	3.75	0.0018
2	80	1	3.74	3.75	-0.0094
3	121	1	5.36	5.36	-0.0013
4	121	1	5.36	5.36	-0.0063
5	80	3	3.29	3.31	-0.0224
6	80	3	3.32	3.31	0.0148
7	121	3	5.48	5.48	-0.0069
8	121	3	5.48	5.48	-0.0007
9	80	2	3.50	3.49	0.0076
10	80	2	3.50	3.49	0.0076
11	121	2	4.63	4.62	0.0076
12	121	2	4.63	4.62	0.0076
13	100	1	5.56	5.56	-0.0037
14	100	1	5.58	5.56	0.0189
15	100	3	6.17	6.17	0.0076
16	100	3	6.17	6.17	0.0076
17	100	2	5.41	5.45	-0.0345
18	100	2	5.45	5.45	0.0021
19	100	2	5.45	5.45	0.0021

Table 36 Actual and predicted values for optimized hot water extraction on protein content of polysaccharides.

Treatments	Factor 1 A: temperature (°C)	Factor 2 B: time (hr)	Response: protein content (mg/g extract)		
			Actual value	Predicted value	Residual
1	80	1	11.56	11.12	0.4439
2	80	1	10.75	11.12	-0.3686
3	121	1	9.73	9.45	0.2758
4	121	1	9.25	9.45	-0.2005
5	80	3	10.81	10.99	-0.1811
6	80	3	11.25	10.99	0.2564
7	121	3	5.62	5.40	0.2211
8	121	3	5.25	5.40	-0.1458
9	80	2	10.84	10.75	0.0965
10	80	2	10.50	10.75	-0.2472
11	121	2	6.00	5.83	0.1747
12	121	2	5.50	5.83	-0.3253
13	100	1	7.34	7.37	-0.0285
14	100	1	7.25	7.37	-0.1222
15	100	3	8.25	8.25	0.0028
16	100	3	8.09	8.25	-0.1535
17	100	2	7.11	6.86	0.2537
18	100	2	6.84	6.86	-0.0126
19	100	2	6.92	6.86	0.0602

Table 37 Actual and predicted values for optimized ethanol precipitation on total sugar content of polysaccharides.

Treatments	Factor 1 A: mushroom content (% w/v)	Factor 2 B: ethanol concentration (% v/v)	Response: total sugar content (mg/g extract)		
			Actual value	Predicted value	Residual
1	5	49.0	329.05	330.17	-1.12
2	5	49.0	326.19	330.17	-3.98
3	15	49.0	312.86	313.86	-1.00
4	15	49.0	309.67	313.86	-4.19
5	5	74.0	371.90	370.78	1.12
6	5	74.0	373.81	370.78	3.03
7	15	74.0	260.48	261.19	-0.7102
8	15	74.0	265.95	261.19	4.77
9	5	61.5	449.52	446.90	2.62
10	5	61.5	445.24	446.90	-1.67
11	15	61.5	387.14	383.95	3.19
12	15	61.5	381.90	383.95	-2.05
13	10	49.0	343.95	339.35	4.60
14	10	49.0	345.05	339.35	5.70
15	10	74.0	329.52	333.32	-3.79
16	10	74.0	328.90	333.32	-4.41
17	10	61.5	438.57	432.76	5.81
18	10	61.5	426.19	432.76	-6.57
19	10	61.5	431.43	432.76	-1.33

Table 38 Actual and predicted values for optimized ethanol precipitation on reducing sugar content of polysaccharides.

Treatments	Factor 1 A: mushroom content (% w/v)	Factor 2 B: ethanol concentration (% v/v)	Response: reducing sugar content (mg/g extract)		
			Actual value	Predicted value	Residual
1	5	49.0	100.25	100.20	0.0548
2	5	49.0	99.75	100.20	-0.4452
3	15	49.0	92.75	92.09	0.6590
4	15	49.0	91.75	92.09	-0.3410
5	5	74.0	76.25	76.72	-0.4660
6	5	74.0	76.50	76.72	-0.2160
7	15	74.0	65.25	64.99	0.2631
8	15	74.0	64.75	64.99	-0.2369
9	5	61.5	88.00	87.71	0.2862
10	5	61.5	88.50	87.71	0.7862
11	15	61.5	78.00	77.80	0.2029
12	15	61.5	77.25	77.80	-0.5471
13	10	49.0	94.75	95.21	-0.4638
14	10	49.0	95.75	95.21	0.5362
15	10	74.0	70.75	69.92	0.8279
16	10	74.0	69.75	69.92	-0.1721
17	10	61.5	81.25	81.83	-0.5761
18	10	61.5	82.75	81.83	0.9239
19	10	61.5	80.75	81.83	-1.08

Table 39 Actual and predicted values for optimized ethanol precipitation on DP value of polysaccharides.

Treatments	Factor 1 A: mushroom content (% w/v)	Factor 2 B: ethanol concentration (% v/v)	Response: DP value		
			Actual value	Predicted value	Residual
1	5	49.0	3.28	3.24	0.0399
2	5	49.0	3.27	3.24	0.0277
3	15	49.0	3.37	3.42	-0.0509
4	15	49.0	3.38	3.42	-0.0489
5	5	74.0	4.88	4.82	0.0530
6	5	74.0	4.89	4.82	0.0620
7	15	74.0	3.99	4.08	-0.0839
8	15	74.0	4.11	4.08	0.0315
9	5	61.5	5.11	5.16	-0.0527
10	5	61.5	5.03	5.16	-0.1300
11	15	61.5	4.96	4.88	0.0859
12	15	61.5	4.94	4.88	0.0663
13	10	49.0	3.63	3.60	0.0293
14	10	49.0	3.60	3.60	0.0028
15	10	74.0	4.66	4.72	-0.0602
16	10	74.0	4.72	4.72	-0.0023
17	10	61.5	5.40	5.29	0.1110
18	10	61.5	5.15	5.29	-0.1365
19	10	61.5	5.34	5.29	0.0560

Table 40 Actual and predicted values for optimized ethanol precipitation on the protein content of polysaccharides.

Treatments	Factor 1 A: mushroom content (% w/v)	Factor 2 B: ethanol concentration (% v/v)	Response: protein content (mg/g extract)		
			Actual value	Predicted value	Residual
1	5	49.0	3.63	3.66	-0.0314
2	5	49.0	3.69	3.66	0.0311
3	15	49.0	7.16	7.19	-0.0387
4	15	49.0	7.19	7.19	-0.0074
5	5	74.0	7.02	7.07	-0.0558
6	5	74.0	7.09	7.07	0.0130
7	15	74.0	6.91	6.95	-0.0443
8	15	74.0	6.91	6.95	-0.0443
9	5	61.5	5.76	5.73	0.0215
10	5	61.5	5.76	5.73	0.0215
11	15	61.5	7.50	7.44	0.0580
12	15	61.5	7.52	7.44	0.0767
13	10	49.0	6.04	6.00	0.0326
14	10	49.0	6.02	6.00	0.0138
15	10	74.0	7.66	7.59	0.0707
16	10	74.0	7.65	7.59	0.0607
17	10	61.5	6.97	7.17	-0.1989
18	10	61.5	7.19	7.17	0.0199
19	10	61.5	7.17	7.17	0.0011

APPENDIX C

PROCEEDING PAPERS

The 3rd International Conference on Renewable Energy, Sustainable Environmental & Agri-Technological and Innovation
Virtual mode conference, 22nd – 23rd, December 2021
Venue: Maejo University, Chiang Mai, Thailand

Influence of hot water treatment on alternative mycoprotein from *Schizophyllum commune*

Nuttapong Saetang[1], Rameshprabu Ramaraj[2], Paweena Pumisutapon[1], Yuwalee Unpaprom[1,*]

[1]Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand
[2] School of Renewable Energy, Maejo University, Chiang Mai 50290, Thailand
[*]Corresponding author E-mail: yuwaleeun@gmail.com ; yuwalee@mju.ac.th

Abstract. *Schizophyllum commune* (split gill mushroom) is the alternative protein source. This mushroom comprises of nutritive mycoprotein (mushroom protein) with essential amino acids, including pharmaceutical schizophyllan (β-glucan), and so on. It also has plentiful nutritional values and various biological properties. This research focused on studying the influence of hot water treatment on this alternative mycoprotein. The factorial experiment in complete randomized design (CRD) was applied to investigate these response variables, namely temperature (80 - 121°C) and time (1 - 3 hr) of hot water extraction of mycoprotein. This experiment noticed that the maximum protein content (10.47 mg/g extract) was obtained from 80°C and 1 hr extraction temperatures. However, in order to extract the schizophyllan with biologically pharmaceutical activities, the optimized extraction conditions of the polysaccharide extracts were around 100°C for 2 hr with the highest degree of polymerization (DP value about 6), along with immunomodulatory and anticancer properties. However, the protein content of this extract was not greater (7.10 mg/g extract) due to the denaturation of some protein at a higher extraction temperature. Therefore, the actual commercial extraction of bioactive mycoprotein should consider both lower extraction temperature to sustain this mycoprotein's nutritional and pharmaceutical values, including the optimal yield of the protein extract. Additionally, the mycoprotein extract from *S. commune* is probably utilized as an alternative protein ingredient in functional food, snack or beverage products in further study.

Keywords: Split gill mushroom, Mycoprotein, Hot water extraction, Factorial design

1. Introduction

Schizophyllum commune is known as split gill mushroom. This mushroom is distributed extensively in many Asian nations, such as Thailand, Laos, Myanmar, and Northeastern India (Yelithao et al., 2019). It grows on the broadleaved trees, standing, dead, or fallen woods. Its characteristic has pale yellow to brown gills, dense white hairs, and an absence of stalks (Hobbs, 2005). Additionally, this mushroom is a medicinal mushroom (Klaus et al., 2011) with abundant nutritional values, especially in terms of high carbohydrate, protein, and low lipid (Basso et al., 2020). It is also composed of plentiful schizophyllan with various biological activities (Klaus et al., 2011), for example, immunomodulatory, anti-inflammatory, antioxidative, anticancer activity, antiviral, antifungal, including prebiotic properties (Zhong et al., 2013; Chaikliang et al., 2015; Zhong et al., 2015; Chandrawanshi et al., 2017; Du et al., 2017; Smirnou et al., 2017; Lee and Ki, 2020).

Moreover, the FAO (Food and Agriculture Organization of the United Nations) now forecasts that without alternative proteins from other sources, the world's population will not consume enough protein (Stephan et al., 2018). Therefore, the manufacture of high-protein, meat-free health meals has attracted international interest. Soy, wheat protein isolates, mycoprotein (mushroom protein), and other alternative protein sources are available (Kim et al., 2011). Mycoprotein is an alternative of good quality protein to animal proteins (Gonzalez et al., 2021). The mycoprotein is also a naturally meaty flavor since the mushroom comprises glutamic acid and aspartic acid (Poojary et al., 2017). Compared to various vegetables, this mushroom is rich in essential amino acids (Gonzalez et al., 2021), such as lysine, threonine,

valine, leucine, and arginine, which accounted for about 41% of the total amino acid composition (Ivanova et al., 2014). In addition, it consists of hydrophobin and lectin protein with anticancer and immune-boosting activities (Akanbi et al., 2013; Zhao et al., 2020). As a result, these proteins are utilized in functional food, drug, vaccine and cosmetics (Akanbi et al., 2013; Zhong et al., 2013; Zhong et al., 2015). In addition, a variety of proteins from different sources are extracted using both traditional (such as water, acid, alkaline solution, salt, solvent, detergent) and modern green technologies for cell disruption in the protein extraction process (for example, enzymes, sonication, pulsed electric field, microwave, high pressure, homogenization). Many variables affect protein yield, including temperature, pH, time, and solvents. Using conventional methods with non-conventional methods for protein extraction accomplishes greater protein yields and nutritional and functional properties (Kumar et al., 2021).

Furthermore, the factorial design is a statistical procedure employed to screen various factors in experiments. The most efficient technique is to estimate the main variables to enhance extraction effectiveness. However, this statistical approach also considerably declines the resources, is time-consuming, and attempts to work out solutions. Thus, the factorial experiments are examined as an effective procedure for analyzing results and gaining valid responses (Qamar et al., 2022). In consequence, this research aims to study maximized alternative mycoprotein extraction from *S. commune* by hot water treatment and investigate the significance of extraction temperature and time using the factorial experiment in CRD. The bioactive mycoprotein extract will be developed as the functional food product for promoting human health in a further study.

2. Materials and methods

2.1 Mushroom sample and maintenance

The dried split gill mushroom was obtained from Chaiyo Mushroom Farm, Surat Thani, Thailand. The mushroom sample was prepared by drying at 60°C in a hot air oven for 48 hours before being pulverized into powder, packed in a vacuum bag, and stored at ambient temperature until used in the further experiment.

2.2 Optimized mycoprotein extraction by hot water treatment

The 20.0-gram dried mushroom powder was suspended in 200.0 ml of deionized (DI) water before the adjusted pH range to 5.8 - 6.0 by natural calcium hydroxide. First, the mushroom suspension was heated by water bath and autoclave at different extraction temperatures (80 - 121°C) and time (1 - 3 hr), then filtrated through a filter cloth. After that, the filtrate was precipitated with 95 % (v/v) ethanol (ratio of 1:2) at 4°C for 24 hr. Next, the mycoprotein pellet was separated by centrifugation at 3,000 rpm for 10 min before being dried in a hot air oven at 60°C for 24 hours.

Table 1 Variables and their values applied for factorial design of hot water treatment

Coding	Variables	Units	Levels		
			Low (-)	Center point (0)	High (+)
A	Temperature	°C	80	100	121
B	Time	hr	1	2	3

The 3 × 3 factorial experiments in complete randomized design (CRD) were applied to investigate the significant parameters for the hot water treatment. In this experimental approach, there are two variables: extraction temperature (A: 80 - 121°C) and time (B: 1 - 3 hr). The independent factors were listed in Table 1 together with their coded and actual levels of the experiment. The extracts were weighed to estimate extraction yield and the protein content was assessed for further examination. The Design Expert application version 11 from Stat-Ease, Inc., Minneapolis, USA, evaluated the outcomes. Figure 1 shows a schematic diagram of the experimental method and extraction operation.

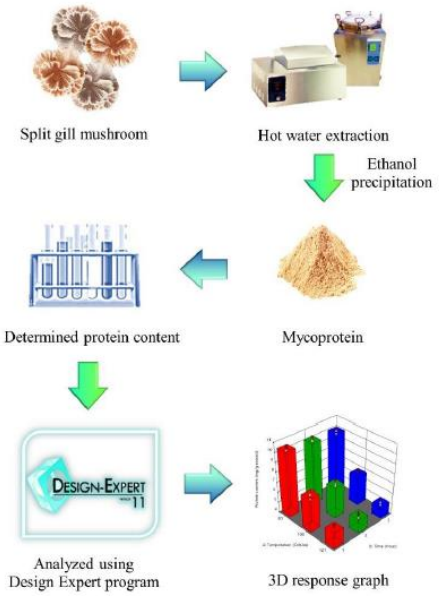


Figure 1 Schematic chart of the experimental process and the extraction procedure

2.3 Protein content analysis

Protein content was analyzed by the dye-binding method (Bradford, 1976) using bovine serum albumin (BSA) as standard protein. The protein content was determined by mixing 0.060 ml of sample with 1.800 ml of Bradford reagent and then incubating at room temperature for 10 min. The sample absorbances were then measured spectrophotometrically at 595 nm. The protein content in each sample was expressed as micrograms per grams of dried extract.

2.4 Statistical analysis and model fitting

All data will be carried out in three replicates. All extraction yield results were reported as mean value ± standard deviation (SD) using Microsoft Excel 2016. Additionally, the data were analyzed statistically through one-way analysis of variance (ANOVA), as well as the significance of the difference between each extract were evaluated by Tukey's test at a 95.0% confidence level. Furthermore, the statistical model analysis was applied for the experimental approach with the numerical results, including speculated from ANOVA. The normal graph, actual versus predicted data graph, and 3D response graph at the suitable condition was estimated by the Design Expert program version 11 from Stat-Ease, Inc., Minneapolis, USA. The graphs were evaluated the factors on the protein content of mycoprotein treatments. While *F*-value and *p*-value less than 0.05 revealed the significance and model fitting, non-significance of lack of fit tests indicated the good model to fit. The R^2, adjusted R^2 and predicted R^2 values were also analyzed.

3. Results and discussion

3.1 Mushroom extraction yields

Effect of the various extraction temperature and times of hot water treatment on extraction yields of extracts from the split gill mushroom presented in Table 2. The extracts achieved a higher yield when enhancing both extraction temperature and time. As a result, the greatest yield was obtained from the extract (5.74 - 5.95 g/100 g of dried mushroom) at 121°C for 2 -3 hr.

Table 2 Influence of temperature and time of hot water treatment on extraction yields.

Extraction temperature (°C)	Extraction time (hr)	Extraction yield (g/100 g dried mushroom)
80	1	2.60 ± 0.77^{d}
80	2	2.90 ± 0.22^{cd}
80	3	3.02 ± 0.33^{cd}
100	1	4.00 ± 0.02^{bc}
100	2	4.03 ± 0.14^{bc}
100	3	3.74 ± 0.28^{cd}
121	1	5.07 ± 0.18^{ab}
121	2	5.95 ± 0.04^{a}
121	3	5.74 ± 0.04^{a}

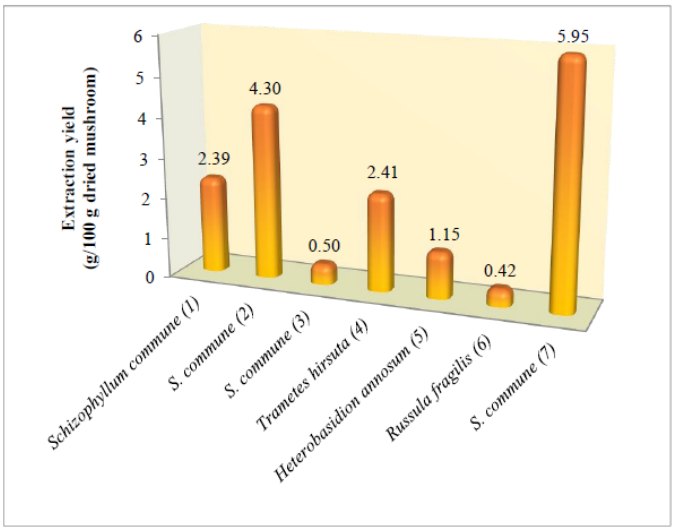


Figure 2 Extraction yields of different mushroom [* (1) hot water extract with ethanolic treatment without purification (Yelithao et al., 2019); (2) extract from the same extraction without purification, (3) with purification (Klaus et al., 2011); (4, 5, 6) extract from similar extraction (Nowak et al., 2018); (7) hot water extract with alkaline and ethanolic treatment (this study)].

Besides, hot water treatment combined with the alkaline solution could be conducted to degrade greatly mushroom's cell wall and liberate water-soluble intracellular components to external the cells.

Therefore, the extraction yield is considerably superior to the only hot water treatment (Klaus et al., 2011). The highest yield of this extract from hot water extraction associated with natural calcium hydroxide and ethanolic treatment was 5.95 g/100 g of dried mushroom, along with the extraction yields of other mushroom species as shown in Figure 2. It was noticed that this extraction yield was more than hot water extract with ethanol precipitation without purification of *S. commune* about 2.5-fold (Yelithao et al., 2019), extract from the similar extraction without purification of the same mushroom at 4.30 g/100 g of dried mushroom, with purification of this mushroom around 12 times (Klaus et al., 2011), extract from same extraction of *Trametes hirsuta* approximately 2.5 times, *Heterobasidion annosum* about 5-fold and *Russula fragilis* around 14-fold (Nowak et al., 2018).

3.2 Protein content of the mycoprotein extracts

Table 3 shows responses on the protein content of extracts to examine the effect of extraction parameters on protein content. Figure 3a portrayed the normal plot of residual's the distribution from the extracts. The points were close to a primary line. The chart was allowed errors' be the normal distribution with a mean zero. Figure 3b depicted the comparison of the predicted versus the actual data from the protein content of the extracts. This graph expressed great agreement cooperating between the predicted and actual results. The adjusted R^2 (0.9849) of protein content was adjacent to 1.0 and accepted the data in the chart (Manmai et al., 2020).

Table 3 Experimental design responses in protein content of extracts.

Std	Run	Factor 1 A: Temperature (°C)	Factor 2 B: Time (hr)	Protein content (mg/g extract)	
				Actual	Predicted
5	1	100	2	7.41	7.10
3	2	121	1	5.88	5.59
12	3	121	1	5.78	5.59
16	4	80	3	10.01	10.08
21	5	121	1	5.67	5.59
8	6	100	3	7.19	6.97
9	7	121	3	5.09	5.20
25	8	80	3	10.25	10.08
13	9	80	2	10.04	10.20
27	10	121	3	4.88	5.20
26	11	100	3	7.31	6.97
4	12	80	2	10.13	10.20
20	13	100	1	7.34	7.37
17	14	100	3	7.09	6.97
10	15	80	1	10.75	10.47
24	16	121	2	5.50	5.32
15	17	121	2	5.38	5.32
2	18	100	1	6.81	7.37
11	19	100	1	7.25	7.37
19	20	80	1	10.25	10.47
23	21	100	2	7.06	7.10
6	22	121	2	5.00	5.32
7	23	80	3	9.75	10.08
18	24	121	3	5.19	5.20
1	25	80	1	10.56	10.47
14	26	100	2	6.84	7.10
22	27	80	2	10.50	10.20

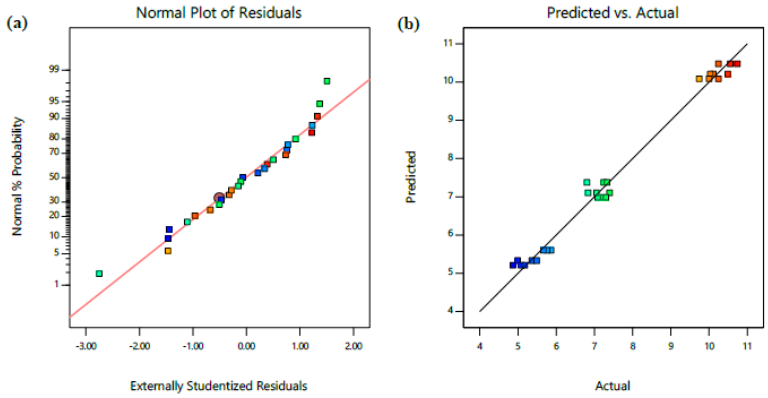


Figure 3 Protein content analytical graphs of (a) normal graph of residuals; (b) actual versus predicted graph from the extracts.

Furthermore, the suitable ranges of extraction factors on the protein content of extracts were evaluated by analysis of variance (ANOVA) (Table 4). The experimental results of the model fitting were demonstrated by non-significance of the lack of fit (*p*-value more than 0.05). Both extraction temperature and time affected the protein content with a good regression coefficient (R^2=0.9872). The model was significant at *F*-value of 425.06 with a 0.01% chance that this large could happen because of noise. The ANOVA model showed the highest *F*-value of 844.54 at model A (temperature), while model B (time) has the fewest influence on the result with an *F*-value of 5.59. The lack of fit was not significant relative to the pure error at *F*-value of 2.46 with an 8.27% chance which is a lack of fit *F*-value; this large could occur due to noise, resulting in the model to fit. The predicted R^2 of 0.9808 was the rational arrangement with the adjusted R^2 of 0.9849, i.e., the difference lower than 0.2. Therefore, the ANOVA model of protein content could guide the design space.

Table 4 ANOVA model analysis for the factorial design of protein content of extracts.

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*p*-value
Model	110.39	4	27.60	425.06	< 0.0001
Temperature (A)	109.66	2	54.83	844.54	< 0.0001
Time (B)	0.7261	2	0.3631	5.59	0.0109
Residual	1.43	22	0.0649		
Lack of fit	0.5047	4	0.1262	2.46	0.0827
Pure error	0.9237	18	0.0513		
Cor total	82.98	18			
Std. dev.	0.2548		R^2	0.9872	
Mean	7.59		Adjusted R^2	0.9849	
C.V. %	3.36		Predicted R^2	0.9808	
			Adeq precision	48.0507	

Moreover, Figure 4 presented the 3D response plot of extraction parameters on the protein content of extracts. When investigating the chart shape of separation on one factor of protein content models from Figure 5, Figure 5a was the negative non-linear relationship, i.e., protein content decreased when temperature increased. On the other hand, while Figure 5b was a negative linear relationship, namely time

enhanced, protein content fell insignificantly. Thus, the maximum protein content was 10.47 mg/g extract with a suitable extraction temperature of 80°C and time of 1 hr.

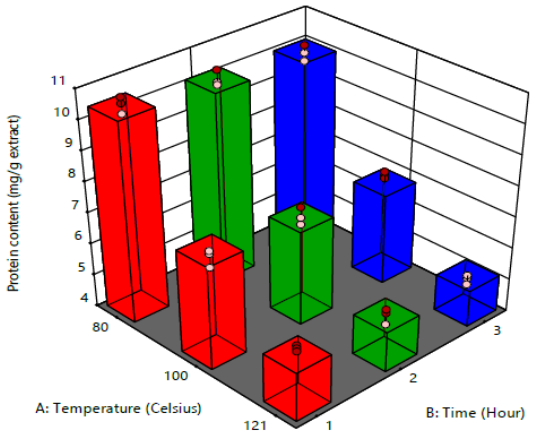


Figure 4 The 3D response graph obtained from protein content of extracts as a function of extraction temperature and time.

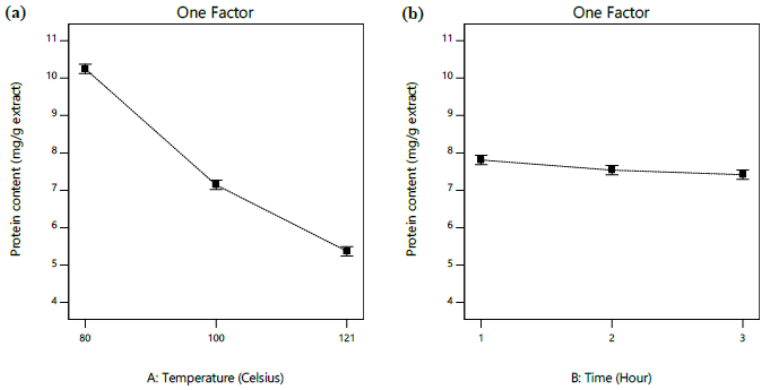


Figure 5 Protein content of extracts in each factor (a) the extraction temperature and (b) extraction time.

4. Conclusion

From this factorial experiment of hot water treatment of the mycoprotein from the split gill mushroom, it could be summarized that the appropriate condition of this extraction was a lower temperature of 80°C for 1 hr with cost-effective extraction. Furthermore, this mushroom consists of plentifully functional schizophyllan, more than mycoprotein. Therefore, when considering the pharmaceutical properties of the extract, the schizophyllan was extracted by hot water treatment at a higher temperature

with a superior degree of polymerization. However, on the other hand, some protein would denature at higher extraction temperature and the protein content of this extract would be inferior to the extract at a lower temperature. Hence, in order to commercially extract this mycoprotein with nutritive values and biological effects, it should consider both lower extraction temperature and optimized protein extraction yield. Hence, this bioactive alternative mycoprotein extract will be applied to produce innovative nutraceutical and pharmaceutical products to promote health.

Acknowledgements

The authors are sincerely grateful to the Program in Biotechnology, Faculty of Science, including Center of Excellence in Agricultural Innovation for Graduate Entrepreneur, Maejo University, Chiang Mai, Thailand, for supporting necessary facilities during this experiment.

References

Akanbi, M. H. J., Post, E., van Putten, S. M., de Vries, L., Smisterova, J., Meter-Arkema, A. H., Rink, R. & Scholtmeijer, K. (2013). The antitumor activity of hydrophobin SC3, a fungal protein. Applied Microbiology and Biotechnology, 97(10), 4385-4392.

Basso, V., Schiavenin, C., Mendonça, S., de Siqueira, F. G., Salvador, M., & Camassola, M. (2020). Chemical features and antioxidant profile by *Schizophyllum commune* produced on different agroindustrial wastes and byproducts of biodiesel production. Food Chemistry, 329, 127089.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2), 248-254.

Chaikliang, C., Wichienchot, S., Youravoug, W., & Graidist, P. (2015). Evaluation on prebiotic properties of β-glucan and oligo-β-glucan from mushrooms by human fecal microbiota in fecal batch culture. Functional Foods in Health and Disease, 5(11), 395-405.

Chandrawanshi, N. K., Tandia, D. K., & Jadhav, S. K. (2017). Nutraceutical properties evaluation of *Schizophyllum commune*. Indian Journal of Scientific Research, 13(2), 57-62.

Du, B., Yang, Y., Bian, Z., & Xu, B. (2017). Characterization and anti-inflammatory potential of an exopolysaccharide from submerged mycelial culture of *Schizophyllum commune*. Frontiers in Pharmacology, 8, 252.

González, A., Nobre, C., Simões, L. S., Cruz, M., Loredo, A., Rodríguez-Jasso, R. M., Contreras, J., Texeira, J., & Belmares, R. (2021). Evaluation of functional and nutritional potential of a protein concentrate from Pleurotus ostreatus mushroom. Food Chemistry, 346, 128884.

Hobbs, C. (2005). The chemistry, nutritional value, immunopharmacology, and safety of the traditional food of medicinal split-gill fugus *Schizophyllum commune* Fr.:Fr. (Schizophyllaceae). A literature review. International Journal of Medicinal Mushrooms, 7(1-2), 127-139.

Ivanova, T. S., Bisko, N. A., Krupodorova, T. A., & Barshteyn, V. Y. (2014). Breadcrumb as a new substrate for Trametes versicolor and *Schizophyllum commune* submerged cultivation. Microbiology and Biotechnology Letters, 42(1), 67-72.

Kim, K., Choi, B., Lee, I., Lee, H., Kwon, S., Oh, K., & Kim, A. Y. (2011). Bioproduction of mushroom mycelium of Agaricus bisporus by commercial submerged fermentation for the production of meat analogue. Journal of the Science of Food and Agriculture, 91(9), 1561-1568.

Klaus, A., Kozarski, M., Niksic, M., Jakovljevic, D., Todorovic, N., & Van Griensven, L. J. (2011). Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. LWT-Food Science and Technology, 44(10), 2005-2011.

Kumar, M., Tomar, M., Potkule, J., Verma, R., Punia, S., Mahapatra, A., Belwal, T., Dahuja, A., Joshi, S., Berwal, M.K., Satankar, V., Bhoite, A.G., Amarowicz, R., Kaur, C., & Kennedy, J. F. (2021). Advances in the plant protein extraction: Mechanism and recommendations. Food Hydrocolloids, 106595.

Lee, S., & Ki, C. S. (2020). Inflammatory responses of macrophage-like RAW264. 7 cells in a 3D hydrogel matrix to ultrasonicated schizophyllan. Carbohydrate polymers, 229, 115555.

Manmai, N., Unpaprom, Y., Ponnusamy, V. K., & Ramaraj, R. (2020). Bioethanol production from the comparison between optimization of sorghum stalk and sugarcane leaf for sugar production by chemical pretreatment and enzymatic degradation. Fuel, 278, 118262.

Nowak, R., Nowacka-Jechalke, N., Juda, M., & Malm, A. (2018). The preliminary study of prebiotic potential of Polish wild mushroom polysaccharides: The stimulation effect on *Lactobacillus* strains growth. European Journal of Nutrition, 57(4), 1511-1521.

Poojary, M. M., Orlien, V., Passamonti, P., & Olsen, K. (2017). Enzyme-assisted extraction enhancing the umami taste amino acids recovery from several cultivated mushrooms. Food Chemistry, 234, 236-244.

Qamar, S., Torres, Y. J. M., Parekh, H. S., & Falconer, J. R. (2022). Fractional Factorial Design study for the extraction of cannabinoids from CBD-Dominant cannabis flowers by supercritical carbon dioxide. Processes, 10(1), 93.

Saetang, N., Rattanapot, T., Manmai, N., Amornlerdpison, D., Ramaraj, R., & Unpaprom, Y. (2022). Effect of hot water extraction process on schizophyllan from split gill mushroom. Biomass Conversion and Biorefinery, 1-10. https://doi.org/10.1007/s13399-021-02286-z

Smirnou, D., Knotek, P., Nesporova, K., Smejkalova, D., Pavlik, V., Franke, L., & Velebny, V. (2017). Ultrasound-assisted production of highly-purified β-glucan schizophyllan and characterization of its immune properties. Process Biochemistry, 58, 313-319.

Stephan, A., Ahlborn, J., Zajul, M., & Zorn, H. (2018). Edible mushroom mycelia of *Pleurotus sapidus* as novel protein sources in a vegan boiled sausage analog system: functionality and sensory tests in comparison to commercial proteins and meat sausages. European Food Research and Technology, 244(5), 913-924.

Yelithao, K., Surayot, U., Lee, C., Palanisamy, S., Prabhu, N. M., Lee, J., & You, S. (2019). Studies on structural properties and immune-enhancing activities of glycomannans from *Schizophyllum commune*. Carbohydrate polymers, 218, 37-45.

Zhao, S., Gao, Q., Rong, C., Wang, S., Zhao, Z., Liu, Y., & Xu, J. (2020). Immunomodulatory effects of edible and medicinal mushrooms and their bioactive immunoregulatory products. Journal of Fungi, 6(4), 269.

Zhong, K., Liu, L., Tong, L., Zhong, X., Wang, Q., & Zhou, S. (2013). Rheological properties and antitumor activity of schizophyllan produced with solid-state fermentation. International journal of biological macromolecules, 62, 13-17.

Zhong, K., Tong, L., Liu, L., Zhou, X., Liu, X., Zhang, Q., & Zhou, S. (2015). Immunoregulatory and antitumor activity of schizophyllan under ultrasonic treatment. International journal of biological macromolecules, 80, 302-308.

2022

ICPPCT

2nd International Conference on Pollution Prevention and Clean Technologies

4th International Conference on Renewable Energy, Sustainable Environmental and Agricultural Technologies

i-RESEAT

Conference Proceedings

1st – 2nd December 2022

Warsaw University of Technology
Warsaw, Poland

Faculty of Building Services, Hydro and Environmental Engineering

GPCHEM

MUNICIPAL WATER SUPPLY AND SEWERAGE COMPANY IN WARSAW JOINT STOCK COMPANY

biowatt

https://icppct.com/

Paper No: ICPPCT-2022-P63

Characterization of split gill mushroom, chemical feature, and prebiotic property of schizophyllan extract stimulating probiotic bacteria

Nuttapong Saetang[1,2], Rameshprabu Ramaraj[2,3], Ruenkaew Praphruet[4],Yuwalee Unpaprom[1,2,*]

[1]Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand.

[2]Sustainable Resources and Sustainable Engineering Research Lab, Maejo University, Chiang Mai 50290, Thailand.

[3]School of Renewable Energy, Maejo University, Chiang Mai 50290, Thailand.

[4]Institute of Product Quality and Standardization, Maejo University, Chiang Mai 50290, Thailand.

*Corresponding author, E-mail: yuwalee@mju.ac.th ; yuwaleeun@gmail.com

Abstract

The edible split gill mushroom is considered as both nutritive and therapeutic superfood, as well as rich in schizophyllan and protein. The schizophyllan (β-glucan) is distinguished by prebiotic properties and other various biological effects. Thus, this research is an investigation into the identity of mushroom strain, nutritional composition of this mushroom, and the schizophyllan extract for further analysis, including its prebiotic activity, and so on. The experimental results revealed that this mushroom was identified as *Schizophyllum commune*, comprising of greater carbohydrate, protein, crude fiber, lower fat, along with not detected any heavy metal. Moreover, this extract consisted of pharmaceutical hydrophobin (14 -18.5 kDa), lectin protein (21 - 35 kDa), bioactive purpurin or red pigment, including the prebiotic β-glucan stimulating the proliferation of probiotic bacteria isolate from yogurt. Hence, both *S. commune* and the schizophyllan extract have probable potential to be utilized as prebiotic candidate, functional food, and nutraceutical products.

Keywords: *Split gill mushroom, Schizophyllan, Prebiotic, Protein, Nutritional value*

2nd International Conference on Pollution Prevention and Clean Technologies | 1st – 2nd December 2022 | Warsaw University of Technology

Characterization of Split Gill Mushroom, Chemical Feature, and Prebiotic Property of Schizophyllan Extract Stimulating Probiotic Bacteria

Nuttapong Saetang[1,2], Rameshprabu Ramaraj[2,3], Ruenkaew Praphruet[4], Yuwalee Unpaprom[1,2,*]

[1]Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand.
[2]Sustainable Resources and Sustainable Engineering Research Lab, Maejo University, Chiang Mai 50290, Thailand.
[3]School of Renewable Energy, Maejo University, Chiang Mai 50290, Thailand.
[4]Institute of Product Quality and Standardization, Maejo University, Chiang Mai 50290, Thailand.
*Corresponding author, E-mail: yuwalee@mju.ac.th; yuwaleeun@gmail.com

ABSTRACT

The edible split gill mushroom is considered as both nutritive and therapeutic superfood, as well as rich in schizophyllan and protein. The schizophyllan (β-glucan) is distinguished by prebiotic properties and other various biological effects. Thus, this research is an investigation into the identity of mushroom strain, nutritional composition of this mushroom, and the schizophyllan extract for further analysis, including its prebiotic activity, and so on. The experimental results revealed that this mushroom was identified as *Schizophyllum commune*, comprising of greater carbohydrate, protein, crude fiber, lower fat, along with not detected any heavy metal. Moreover, this extract consisted of pharmaceutical hydrophobin (14 -18.5 kDa), lectin protein (21 - 35 kDa), bioactive purpurin or red pigment, including the prebiotic β-glucan stimulating the proliferation of probiotic bacteria isolate from yogurt. Hence, both *S. commune* and the schizophyllan extract have probable potential to be utilized as prebiotic candidate, functional food, and nutraceutical products.

INTRODUCTION

METHODOLOGY

Identification of mushroom strain - DNA extraction, DNA amplification by polymerase chain reaction (PCR) & DNA sequence analysis

Nutritional value studies of split gill mushroom - Ash, moisture, protein, fat, carbohydrate & crude fiber

Heavy metal contamination of split gill mushroom - Cadmium (Cd), lead (Pb), mercury (Hg) & arsenic (As)

Extraction of optimized schizophyllan extract in large scale

Protein molecular mass determination of the extract by SDS-PAGE

Bioactive compound identification in the extract by Raman confocal spectroscopy

Prebiotic property of the extract

ACKNOWLEDGEMENT

The authors sincerely thank the Program in Biotechnology, Faculty of Science, Institute of Product Quality and Standardization (IQS), including Center of Excellence in Agricultural Innovation for Graduate Entrepreneur, Maejo University, Chiang Mai, Thailand, for supporting necessary facilities to accomplish this research. The authors also gratefully acknowledge BNK Bioscience Limited, Nonthaburi, including Biosynthai Biotechnology Co., Ltd, Angthong, and Faculty of Engineering and Agro-Industry, Maejo University, Chiang Mai, Thailand for their analysis services.

REFERENCES

Saetang, N., Amornlerdpison, D., Ramaraj, T., Ramaraj, R., & Unpaprom, Y. 2022a Processing of split gill mushroom as a biogenic material for functional food purpose. *Biocatalysis and Agricultural Biotechnology*, 41(1), 102314.
Saetang, N., Ramaraj, T., Mezuni, N., Amornlerdpison, D., Ramaraj, R., & Unpaprom, Y. 2022b. Effect of hot water extraction process on schizophyllan from split gill mushroom. *Biomass Conversion and Biorefinery*, https://doi.org/10.1007/s13399-021-02286-z.
Saetang, N., Ramaraj, R., & Unpaprom, Y. 2022c. Optimization of ethanol precipitation of schizophyllan from *Schizophyllum commune* by applied statistical modelling. *Biomass Conversion and Biorefinery*, https://doi.org/10.1007/s13399-022-02384-6
Saetang, N., Ramaraj, R., Pumsuwan, P. & Unpaprom, Y. 2022d. Influence of hot water treatment on alternative mycoprotein from *Schizophyllum commune*. In Proceeding of the 3rd International Conference on Renewable Energy, Sustainable Environmental & Agri-Technological and Innovation (I-RESEAT 2021). Maejo University, Chiang Mai, pp. 1-9.

RESULTS AND DISCUSSION

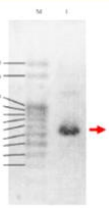


Figure 1 PCR amplification of DNA profile of the split gill mushroom with ITS5/ITS4 primer. Lane M, molecular size marker (M25 100 bp + 2 kb + 3 kb DNA ladder); lane 1, PCR product of this mushroom.

Table 1 Similarity of nucleotide sequence of the split gill mushroom after BLAST analysis with nucleotide sequence database of GenBank/ NCBI.

Mushroom species	Length of sequence (bp)	Sequence homology (%)	GenBank accession number
S. commune strain BL1	653	100.00	MT466518.1
S. commune strain IFM 46097	657	100.00	AB369909.1
S. commune isolate Z-H-3A-2	647	100.00	OL471296.1
S. commune isolate H	650	100.00	MN547371.1
S. commune strain CBS579.83	634	100.00	MH861655.1
S. commune strain CBS405.96	643	99.84	MH862583.1
S. commune isolate Z-M-2A-1	695	99.84	OL471289.1
S. commune strainWB033_12	656	99.84	JX848644.1
S. commune isolate T28	674	99.84	JF439509.1
S. commune strain xsd08036	660	99.84	FJ478109.1
S. commune isolate Z3	674	99.84	EF155505.1
S. commune isolate H9O62	657	99.84	AF280750.1
S. commune strainUZ1552_14	651	99.84	KP326577.1
S. commune strain CBS124811	637	99.84	MH863418.1
S. commune isolate Z-E-4C-1	647	99.84	OL471293.1
Agaricaceae sp. BAB-4029	650	100.00	KM051395.1
Agaricaceae sp. GWY1(1)	662	99.84	KM268691.1

Table 2 Nutritional values of *S. commune*.

Analysis	Composition (g/100 g dried mushroom)	Test method
Ash	4.64 ± 0.01[d]	AOAC (2012), 940.26
Moisture	9.56 ± 0.04[c]	AOAC (2012), 934.06
Protein	20.99 ± 0.14[b]	AOAC (2012), 991.20
Fat	0.75 ± 0.01[e]	AOAC (2012), 989.05
Carbohydrate	64.06 ± 0.08[a]	By difference
Crude fiber	4.87 ± 0.05[d]	AOAC (2012), 978.10

Table 3 Heavy metal contamination of *S. commune*.

Heavy metal	Contamination (mg/kg dried mushroom)	Test method
Cadmium (Cd)	Not Detected[1]	AOAC (2019), 999.10
Lead (Pb)	Not Detected[1]	
Mercury (Hg)	Not Detected[1]	
Arsenic (As)	Not Detected[1]	

Remark: [1]Limit of Detection (LOD) = 0.002 mg/kg

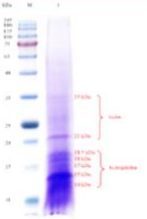


Figure 2 SDS-PAGE of the extract from *S. commune*. Lane M, protein molecular weight marker; lane 1, the extract from *S. commune*.

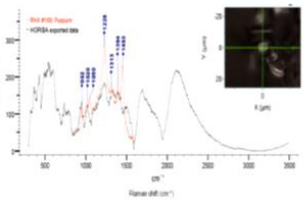


Figure 3 The results of compounds analysis in the extract from *S. commune* by Raman confocal spectroscopy.

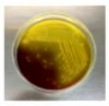


Figure 4 Single colony of probiotic bacteria, isolate BG-N502 from Bulgaria yogurt, streaked on MRS agar.

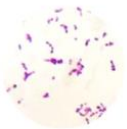


Figure 5 Gram staining of probiotic bacteria, isolate BG-N502 from Bulgaria yogurt, under the light macroscope at 100× magnification.

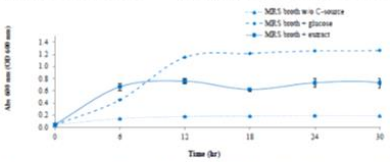


Figure 6 Growth curves of probiotic bacteria, isolate BG-N502 from Bulgaria yogurt, cultivated in MRS broth with different carbon sources.

CONCLUSION

1. The split gill mushroom was specified as *Schizophyllum commune*.
2. This mushroom had plentiful nutritive values, including abundant carbohydrate, alternative mycoprotein, crude fiber, inferior calories of fat, and without any harmful heavy metal.
3. The split gill mushroom was rich in potential schizophyllan substance comprising of hydrophobin and lectin protein with immunomodulatory via anticancer activities, along with biologically active purpurin.
4. This substance was also prebiotic β-glucan demonstrating stimulation of probiotic bacteria proliferation in human gastrointestinal tract and boosting immune system.
5. Both this mushroom and the schizophyllan would be possibly to be applied in nutraceutical and pharmaceutical development.


Southeast Asian University Consortium
for Graduate Education in Agriculture and Natural Resources
Agricultural Adaptation Strategies for Coping with Climate Change
2021 University Consortium Graduate Forum
National Taiwan University, Taipei, Taiwan
December 2nd & 3rd, 2021
NTU
INTERNATIONAL
COLLEGE
SEARCA

Optimization of ethanol precipitation of schizophyllan from *Schizophyllum commune*

Nuttapong Saetang
Program in Biotechnology, Faculty of Science, Maejo University, Thailand.
*E-mail: nutsaet@live.com; MJU6304502001@mju.ac.th

ABSTRACT

The split gill mushroom, also known as *Schizophyllum commune*, is a medicinal fungus. Schizophyllan (β-glucan or polysaccharide from *S. commune*) is abundant in its cell wall and has a variety of biological functions. Using response surface methodology (RSM) on a central composite design, this experiment looked at how to improve the schizophyllan's ethanol precipitation process for highest degree of polymerization (DP), largest total sugar, and best reducing sugar concentration. The split gill mushroom content (5-15 percent w/v) and ethanol concentration (49-74 percent v/v) were both investigated in the experimental design. Total sugar, reducing sugar concentration, and DP value models all had good determination coefficients (R^2) of 0.9957, 0.9974, and 0.9914, respectively, and were statistically significant (*P*-value 0.05). Furthermore, the optimal mushroom content and polysaccharide ethanol concentration were: 5.11 percent (w/v) and 62.78 percent (v/v) (total sugar concentration); 15.00 percent (w/v) and 74.00 percent (v/v) (reducing sugar concentration); and 8.05 percent (w/v) and 65.10 percent (v/v) (DP value). The optimal conditions for this cost-effective extraction were a low mushroom content of 5 to 8% (w/v) and an optimal ethanol concentration of 62 to 65 percent (v/v), with the highest DP value around 5, as well as efficient immunomodulatory and anticancer effects, according to the results of this experiment. As a result, future study will focus on developing pharmaceutical schizophyllan extract as nutraceutical goods or medicines.

Keywords: split gill mushroom, schizophyllan, ethanol precipitation, degree of polymerization

APPENDIX D

PUBLICATIONS

Biocatalysis and Agricultural Biotechnology 41 (2022) 102314

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Processing of split gill mushroom as a biogenic material for functional food purpose

Nuttapong Saetang [a,b], Doungporn Amornlerdpison [c], Thiravat Rattanapot [c], Rameshprabu Ramaraj [b,d], Yuwalee Unpaprom [a,b,*]

[a] Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand
[b] Sustainable Resources and Sustainable Engineering Research Lab, Maejo University, Chiang Mai, Thailand
[c] Center of Excellence in Agricultural Innovation for Graduate Entrepreneur, Maejo University, Chiang Mai, Thailand
[d] School of Renewable Energy, Maejo University, Chiang Mai, Thailand

ARTICLE INFO

Keywords:
Schizophyllum commune
Split gill mushroom essence
Schizophyllan
Antioxidant properties
Functional food

ABSTRACT

The nutritional value of routinely used synthetic antioxidants is being reemphasized in today's preventative medicine and food sector. The split gill mushroom is a pharmaceutical mushroom distinguished by its high nutritional content and biological activity. It contains schizophyllan and a polysaccharide made up of β-glucan. The β-glucan has a significant level of antioxidant activity. Therefore, this is an investigation into producing split gill mushroom essence rich in bioactive components. This study examined the total phenolic content and antioxidant capacity (radical scavenging activity) of the schizophyllan (the hot water combined with ethanolic extraction of polysaccharide), supernatant from the separation of the extraction of the polysaccharide, and the mushroom essence (the production of split gill mushroom essence steamed in an electric pressure cooker). From this research, split gill mushroom essence (using an electric pressure cooker) showed more great total phenolic content (14.07 mg of gallic acid equivalent [GAE]/g of dry extract) compared to the supernatant and ethanolic schizophyllan (12.85 and 6.22 mg of GAE/g of dry extract). Furthermore, this mushroom essence revealed more potential for antioxidant activity (IC_{50} value) for inhibition of free radical (0.73 mg of dry extract/ml), compared to other extracts (IC_{50} values of the supernatant and the schizophyllan extract as 0.76 and 2.64 mg of dry extract/ml). Therefore, split gill mushroom essence is probably developed as an innovative antioxidant functional food product with beneficial compounds to human health.

1. Introduction

Antioxidants are micro components present in the edible green plants which can help to protect the body against oxidative stress induction by free radicals and prevent harmful reactive oxygen species (ROS). Many physiological and biochemical processes in the body may produce free radicals and other reactive oxygen species (Paudel et al., 2018). They are extremely unstable and react quickly with other compounds in the body, causing oxidative damage to cells and tissues (Li et al., 2018). Excessive levels of free radicals and reactive oxygen species have been linked to various degenerative disorders, including aging, cancer, cardiovascular disease, Alzheimer's disease, and other chronic conditions (Ramalingam et al., 2017). To prevent these diseases, antioxidants play a vital role in controlling oxidative stress in the body (Li et al., 2018). Polyphenols, flavonoids, carotenoids, glutathione, and lipoic acid are examples

* Corresponding author. Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand..
E-mail addresses: yuwalee@mju.ac.th, yuwaleeun@gmail.com (Y. Unpaprom).

https://doi.org/10.1016/j.bcab.2022.102314
Received 17 October 2021; Received in revised form 19 February 2022; Accepted 20 February 2022
Available online 22 February 2022
1878-8181/

of antioxidants. They are primarily found in fruits and vegetables (Saetang et al., 2022a). Despite this, fruit and vegetables are in short supply in many parts of the world. As a result, researchers are looking for more antioxidants from different sources (Asghari et al., 2016).

Mushrooms are well-known for their nutritional worth and as a source of therapeutic foods that can help avoid diseases including cancer, hypertension, and hypercholesterolemia (Yim et al., 2013; Saetang et al., 2022b). Moreover, they have drawn attention for their antioxidant, antitumor, and immunomodulatory properties (Maity et al., 2015). Some researchers have reported their excellent antioxidant source, while they consist of various secondary metabolites and phenolic compounds as greatly capable scavengers of free radicals (Yim et al., 2013).

Split gill mushroom (*Schizophyllum commune*) is distributed widely in many countries in Asia, including North-East India and similarly South-East Asia, such as Thailand, Laos, and Myanmar (Yelithao et al., 2019; Saetang et al., 2022c). It grows on the dead, fallen, or standing wood of broadleaved trees. Its characteristic is dense white hairs, no stalks, and pale yellow to brown gills (Hobbs, 2005). Cell wall composition is composed of β-glucans cross-linked with chitin (Leung et al., 2006), and the conformation of β-glucans in the mushroom (schizophyllan) is a triple-stranded helix. Thus, schizophyllan is a β-1,3-D-glucan polymer with β-1,6-D-branches (Lee and Ki, 2020).

Furthermore, there are significant nutritional values, incredibly high fiber, protein, and low lipid (Hobbs, 2005), including phenolic compounds in its ethanolic extract (Yelithao et al., 2019; Saetang et al., 2022c). It is a medicinal mushroom with various biological properties (Klaus et al., 2011), for instance, antioxidant, anti-inflammatory, immune-enhancing, anticancer (Chandrawanshi et al., 2017; Du et al., 2017; Smirnou et al., 2017; Yelithao et al., 2019; Lee and Ki, 2020), antiviral and antifungal properties (Hobbs, 2005). In addition, the schizophyllan plays the leading role in preventing oxidative damage in the human body against free radicals (Yelithao et al., 2019). Thus, the mushroom has many industrial applications in traditional food extensively in Southeast Asia and India (Hobbs, 2005), functional food (Smirnou et al., 2017), drug (Lee and Ki, 2020), vaccine, and cosmetics (Smirnou et al., 2017).

However, the research on antioxidant activity and phenolic content of *S. commune* found in Thailand is scarce. Therefore, the present study aims to extract the schizophyllan, the supernatant, from the separation of the extraction of the schizophyllan and produce split gill mushroom essence, as well as determine the total phenolic content and antioxidant property of these extracts for evaluation potentiality of this mushroom essence functional food with bioactive antioxidant substances.

2. Materials and methods

2.1. Mushroom sample and maintenance

The dried split gill mushroom was purchased from Chaiyo Mushroom Farm, Surat Thani, Thailand. The sample was packed in a vacuum bag and stored at room temperature until the further experiment.

2.2. Extraction of schizophyllan

The dried mushroom (50 g) was soaked in deionized (DI) water for 15 min and filtered through stainless steel fine mesh. In a blender, the sample was mixed with 2.5 L of water and ground finely. Next, the suspension was boiled for 2 h before filtration through a filter cloth to separate from the residual mushroom. After that, the filtrate was precipitated with 95% ethanol in a ratio of 1:1 at 4 °C for 16 h. Next, the schizophyllan pellet was separated from the supernatant by centrifugation at 3500 rpm for 15 min and dried at 60 °C for 24 h. In the case of the supernatant, it was evaporated at 60 °C for separation from ethanol solution before freeze-drying. Finally, the schizophyllan and supernatant were weighed for each sample's estimation yield, including total phenolic content and antioxidant activity.

2.3. Production of split gill mushroom essence

Preparation of the mushroom (50 g) suspension was similar to the previous study before the sample was ground finely in DI water (2.5 L). After that, the suspension was steamed in an electric pressure cooker for 35 min and filtrated through a filter cloth to collect the mushroom essence. Then, the essence was concentrated by a rotary evaporator at 60 °C and freeze-dried. After that, the extract was weighed to evaluate its yield, analysis of total phenolic content, and ABTS radical scavenging activity, as described below.

2.4. Total phenolic content

Total phenolic content was determined according to the modified method from Stankovic (2011). Briefly, the reaction mixture was prepared by mixing 200 μL of a diluted sample, 1000 μL of 10% (v/v) Folin-Ciocalteu's reagent, and 800 μL of 7.5% (w/v) sodium carbonate solution. After that, the reaction mixture was incubated at room temperature for 60 min, and the absorbance was measured using a spectrophotometer at 765 nm. Gallic acid (GA) was used as the standard. Therefore, the total phenolic content in each extract was expressed as micrograms of gallic acid equivalent (GAE) per grams of dried extract.

2.5. Antioxidant activity

ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity was carried out according to the modified method of Re et al. (1999). The ABTS radical cation ($ABTS^{+}$) was prepared by making 7 mM ABTS stock solution react with 2.45 mM $K_2S_2O_8$ at a ratio of 1:0.5 before standing in darkness at room temperature for 12–16 h. Then, the $ABTS^{+}$ solution was diluted with distilled water to obtain an absorbance of 0.70 ± 0.05 at 734 nm. After that, 200 μL of the diluted sample were combined with 1800 μL of $ABTS^{+}$ solution and incubated for 6 min at room temperature. The absorbance was spectrophotometrically measured at 734 nm.

Table 1
Extraction yields of each sample from S. commune.

Samples	Yield (g/100 g dried mushroom)
Schizophyllan	1.01
Supernatant from the separation of extraction of schizophyllan	15.8
Split gill mushroom essence (using electric pressure cooker)	25.0

Table 2
Extraction yields of various mushrooms.

Mushroom species	Extraction method	Yield (g/100 g dried mushroom)	Reference
Auricularia polytricha	ethanolic extraction	2.00	Teoh et al. (2018)
Cantharellus cibarius	acetone extraction	4.83	Özcan and Ertan (2018)
***Trametes* sp. (TOD)**	ethanolic extraction	7.34	Doris (2018)
Pleurotus ostreatus	ethanolic extraction	7.88	Egra et al. (2019)
P. pulmonarius	ethanolic extraction	10.1	Arbaayah and Umi Kalsom (2013)
***Coriolus versicolor* (enriched with selenourea)**	methanolic extraction	11.98	Miletić et al. (2019)
Lentinus edodes	ultrasound-assisted extraction	12.4	Cheung et al. (2012)
S. commune	hot water extraction	19.6	Chandrawanshi et al. (2017)
S. commune	hot water extraction (using electric pressure cooker)	25.0	This study

Percentage inhibition of ABTS radical scavenging activity was calculated according to equation (1). In contrast, the half-maximal inhibitory concentration (IC_{50} value) for inhibition of free radical was evaluated from the percentage inhibition with concentration plot using linear regression analysis.

$$\% \text{ inhibition} = [(AC-AS)/AC] \times 100 \quad (1)$$

Where AC and AS is the absorbance of the control (distilled water) and sample, respectively.

2.6. Statistical analysis

All experiments were performed in triplicate. All results were reported as the mean value ± standard error (SE) or error bars using Microsoft Excel version-2016. Furthermore, these data were statistically analysed through a one-way analysis of variance (ANOVA). Tukey's test considered the significance of the difference between each sample at a 95.0% confidence level.

3. Results and discussion

3.1. Yields of mushroom extracts

The yields of schizophyllan, supernatant from the separation of the extraction of schizophyllan, and split gill mushroom essence are revealed in Table 1. The highest yield was obtained from split gill mushroom essence (25.0 g/100 g of dried mushroom) using an electric pressure cooker, compared to the supernatant from hot water combined with ethanolic extraction of schizophyllan, and only the schizophyllan separated by ethanol precipitation, respectively. In this case, this mushroom essence reached the most significant yield due to higher extraction temperature (>100 °C because of more significant pressure from steaming by an electric pressure cooker) than the temperature of schizophyllan extraction (by boiling around 100 °C). Likewise, the previous research of Saetang et al. (2022a) studied the effect of temperature and time of hot water extraction. It was observed that when enhancing both extraction temperature and time, the extracts gained superior extraction yields. To explain the physical basis of these extraction processes, hot water treatment may damage the mushroom's extremely rigid cell walls, allowing internal components to escape into solution outside the cells. Hence, the extraction yield value by hot water extraction at a higher temperature is greater than the extract from a lower temperature (Saetang et al., 2022b).

Additionally, it might comprise of many water-soluble components after hot water extraction, such as abundant schizophyllan (Klaus et al., 2011), water-soluble fiber, protein, along with various phenolic compounds as highly effective scavengers of free radicals by oxidative reaction (Basso et al., 2020), flavonoids (Arbaayah and Umi Kalsom, 2013), steroids, alkaloids, terpenes, free amines (Kabuyi et al., 2017), tannins and saponins. Other bioactive substances in the mushroom extract might also consist of a variety of vitamins and minerals, for instance, thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), ascorbic acid (vitamin C), calcium, magnesium, phosphorus, and sodium, etc. (Okwulehie et al., 2007).

Furthermore, the yields of this split gill mushroom essence, including other mushroom species, are shown in Table 2. It was noticed that yield of this mushroom's hot water essence using an electric pressure cooker was superior to ethanolic extract of *Auricularia polytricha* around 12.5 folds (Teoh et al., 2018), acetone extract of *Cantharellus cibarius* about five times (Özcan and Ertan, 2018), ethanolic extract of *Trametes* sp. (TOD) approximately triple (Doris, 2018), and ethanolic extract of *Pleurotus ostreatus* around threefold (Egra et al., 2019). Additionally, this mushroom essence's yield was more significant than ethanolic extract of *P. pulmonarius* at 2.5

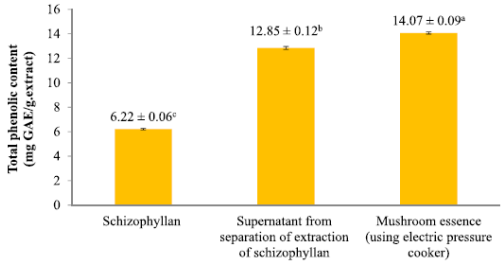


Fig. 1. Total phenolic contents of each extract from *S. commune*.

Table 3
Total phenolic contents of different agricultural materials.

Agricultural materials	Extraction method	**Total phenolic content (mg GAE/ g dry extract)**	Reference
Solanum melongena (peel)	acetone extraction	0.29	Boulekbache-Makhlouf et al. (2013)
Pleurotus ostreatus (cultivated with corncob substrate)	methanolic extraction	1.21	Jin et al. (2020)
Oryza sativa var. glutinosa (Kum Doi Saket)	hot water extraction	2.25	Pasakawee et al. (2018)
P. citrinopileatus	ultrasonic extraction with acetone	2.68	Yin et al. (2019)
Coriolus versicolor	microwave-assisted extraction with ethanol	4.70	Maeng et al. (2017)
Lentinula edodes	ethanolic extraction	5.66	Bach et al. (2019)
Termitomyces fuliginosus	ultrasonic extraction with methanol	6.31	Butkhup et al. (2018)
Auricularia polytricha	ethanolic extraction	11.38	Teoh et al. (2018)
Schizophyllum commune	hot water extraction (using electric pressure cooker)	14.07	This study

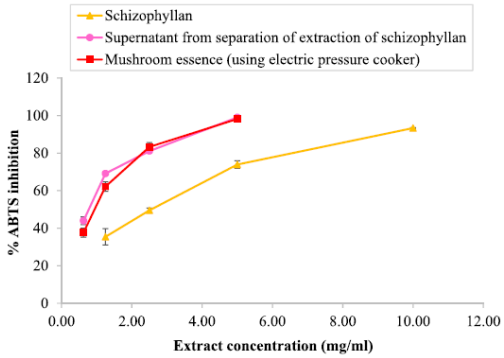


Fig. 2. ABTS radical inhibition by different extracts from *S. commune*.

times (Arbaayah and Umi, 2013), methanolic extract of *Coriolus versicolor* enriched with selenourea about double (Mileti'c et al., 2019), ultrasonic extract of *Lentinus edodes* around twice (Cheung et al., 2012) and hot water extract of *S. commune* at 19.6 g/100 g of dried mushroom (Chandrawanshi et al., 2017).

3.2. Total phenolic contents of mushroom extracts

Total phenolic contents of different extracts are presented in Fig. 1. Among all experimental extracts, split gill mushroom essence (using an electric pressure cooker) showed significantly highest total phenolic content at 14.07 ± 0.09 mg of gallic acid equivalent [GAE]/g of dry extract), followed by the supernatant from the separation of the extraction of the schizophyllan and the schizophyllan at 12.85 ± 0.12 and 6.22 ± 0.06 mg GAE/g of dry extract, respectively. Contents of total phenolics from split gill mushroom essence compared to other agricultural materials are revealed in Table 3. It was found that the entire phenolic content of this mushroom's hot

Table 4
Inhibitory activities (IC_{50}) on ABTS assay of various extracts from *S. commune*.

Samples	IC_{50} (mg extract/ml)
Schizophyllan	2.64 ± 0.23^{a}
Supernatant from the separation of the extraction of schizophyllan	0.76 ± 0.18^{b}
Split gill mushroom essence (using electric pressure cooker)	0.73 ± 0.02^{b}

Table 5
Antioxidant activity on ABTS assay of this mushroom compared with other agricultural materials.

Agricultural materials	IC_{50} (mg extract/ml)	% ABTS inhibition (at 0.1 mg extract/ml)	Reference
Albumin from coconut cake	N/A[a]	5.04	Li et al. (2018)
Guava leaves (methanolic extract)	N/A[a]	5.60	Gaber et al. (2021)
Pomegranate peels (ethanolic extract)		9.60	
Spirulina protein (ethanolic extract)	N/A[a]	25.96	Afify et al. (2017)
Pine nut protein hydrolysate	8.83	N/A[a]	Liu et al. (2021)
Phlebopus portentosus	1.78	N/A[a]	Kumla et al. (2021)
Selaginella tamariscina leaf (ethanolic extract)	1.02	N/A[a]	Kim and Lee (2021)
Split gill mushroom (essence using electric pressure cooker)	0.73	41.94	This study

[a] N/A = not available.

water essence was remarkably higher than acetone extract of *Solanum melongena*'s peel approximately 48 times, methanolic extract of *Pleurotus ostreatus* cultivated with corncob as substrate about 11.6 folds, hot water extract of *Oryza sativa* var. glutinosa (Kum Doi Saket) around sixfold as well as ultrasonic section combined with acetone of *P. citrinopileatus* about five times (Boulekbache-Makhlouf et al., 2013; Pasakawee et al., 2018; Yin et al., 2019; Jin et al., 2020).

Moreover, this mushroom essence (using an electric pressure cooker) 's total phenolic content was more than microwave extract associated with ethanol of *Coriolus versicolor* nearly triple (Maeng et al., 2017), ethanolic extract of *Lentinula edodes* around 2.5 folds (Bach et al., 2019), ultrasonic extract combined with methanol of *Termitomyces fuliginosus* about twice (Butkhup et al., 2018) and ethanolic extract of *Auricularia polytricha* approximately 1.24 times (Teoh et al., 2018).

3.3. ABTS radical scavenging activity

The results of all extracts from split gill mushrooms revealed different patterns of free radical inhibitions (Fig. 2). ABTS radical inhibitions of the schizophyllan, the supernatant from the separation of the extraction, and the mushroom essence (using an electric pressure cooker) increased while their concentrations increased. This mushroom essence showed a high antioxidant candidate with 98.18% ABTS inhibition at 5.00 mg/ml. According to the half-maximal inhibitory concentrations (IC_{50} values) for inhibition of free radical (Table 4), it was noticed that the split gill mushroom essence showed more potential for inhibition of ABTS radical at low concentration 0.73 ± 0.02 mg of dry extract/ml, less than the supernatant (0.76 ± 0.18 mg of dry extract/ml) and the schizophyllan (2.64 ± 0.23 mg of dry extract/ml).

In addition, some researchers studied antioxidant activity on ABTS assay from other agricultural materials, as revealed in Table 5. It was found that the percentage of ABTS inhibition from this mushroom essence at 0.1 mg of extract/ml (41.94% ABTS inhibition) was superior to albumin from coconut cake about eightfold (Li et al., 2018), methanolic extract of guava leaves around 7.5 times (Gaber et al., 2021), ethanolic extract of pomegranate peels approximately quadruple (Gaber et al., 2021) and *Spirulina* protein from ethanolic extract significantly (Afify et al., 2017). Besides, the IC_{50} value of this mushroom extract was necessary to just use a lower concentration for more efficient inhibition of free radical (0.73 mg of dry extract/ml) than pine nut protein hydrolysate at 12 times (Liu et al., 2021), methanolic extract of *Phlebopus portentous* around twice (Kumla et al., 2021), as well as ethanolic extract of *Selaginella tamariscina* leaf considerably (Kim and Lee, 2021).

4. Conclusion

From this study results, the split gill mushroom comprises abundant schizophyllan with high phenolic content, including strong scavenging activity against free radical at low IC_{50} value. Therefore, this mushroom is a more potential candidate for the split gill essence as high value-added functional food rich in bioactive compounds with antioxidant properties through suitable extraction, as well as might be beneficial for human health to prevent some associated diseases with oxidative damage.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are sincerely grateful to the Center of Excellence in Agricultural Innovation for Graduate Entrepreneur and Energy Research Center, Maejo University, Chiang Mai, Thailand, for providing necessary facilities during the research.

References

Afify, A., Abd El Baky, H.H., El Baroty, G.S., El Baz, F.K., Murad, S.A., 2017. Antioxidant activity of protein hydrolysates derived from blue-green algae *Spirulina platensis* extracted with three different methods and treated with enzymes. Biosci. Res. 14 (3), 485–497.

Arbaayah, H.H., Umi Kalsom, Y., 2013. Antioxidant properties in the oyster mushrooms (*Pleurotus* spp.) and split gill mushroom (*Schizophyllum commune*) ethanolic extracts. Mycosphere 4 (4), 661–673.

Asghari, A., Fazilati, M., Latifi, A.M., Salavati, H., Choopani, A., 2016. A review on antioxidant properties of *Spirulina*. J. Appl. Biotechnol. Rep. 3 (1), 345–351.

Bach, F., Zielinski, A.A.F., Helm, C.V., Maciel, G.M., Pedro, A.C., Stafussa, A.P., Ávila, S., Haminiuk, C.W.I., 2019. Bio compounds of edible mushrooms: *in vitro* antioxidant and antimicrobial activities. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 107 (1), 214–220.

Basso, V., Schiavenin, C., Mendonca, S., de Siqueira, F.G., Salvador, M., Camassola, M., 2020. Chemical features and antioxidant profile by *Schizophyllum commune* produced on different agroindustrial wastes and byproducts of biodiesel production. Food Chem. 329 (1), 127089.

Boulekbache-Makhlouf, L., Medouni, L., Medouni-Adrar, S., Arkoub, L., Madani, K., 2013. Effect of solvents extraction on phenolic content and antioxidant activity of the byproduct of eggplant. Ind. Crop. Prod. 49 (1), 668–674.

Butkhup, L., Samappito, W., Jorjong, S., 2018. Evaluation of bioactivities and phenolic contents of wild edible mushrooms from northeastern Thailand. Food Sci. Biotechnol. 27 (1), 193–202.

Chandrawanshi, N.K., Tandia, D.K., Jadhav, S., 2017. Nutraceutical properties evaluation of *Schizophyllum commune*. Indian J. Sci. Res. 13 (2), 57–62.

Cheung, Y.-C., Siu, K.-C., Liu, Y.-S., Wu, J.-Y., 2012. Molecular properties and antioxidant activities of polysaccharide–protein complexes from selected mushrooms by ultrasound-assisted extraction. Process Biochem. 47 (5), 892–895.

Doris, O.Y., 2018. Comparative phytochemical screening of *Trametes* species: a wild mushroom collected from Ondo State Nigeria. S.F. J. Mycology. 1 (4), 1–6.

Du, B., Yang, Y., Bian, Z., Xu, B., 2017. Characterization and anti-inflammatory potential of an exopolysaccharide from submerged mycelial culture of *Schizophyllum commune*. Front. Pharmacol. 8 (1), 252.

Egra, S., Irawan Wijaya, K., Enos Tangke, A., Harlinda, K., 2019. The potential of white-oyster mushroom (*Pleurotus ostreatus*) as antimicrobial and natural antioxidant. Biofarmasi J. Nat. Prod. Biochem. 17 (1), 17–23.

Gaber, N.B., El-Dahy, S.I., Shalaby, E.A., 2021. Comparison of ABTS, DPPH, permanganate, and methylene blue assays for determining antioxidant potential of successive extracts from pomegranate and guava residues. Biomass Convers. Biorefin. 1–10.

Hobbs, C., 2005. The chemistry, nutritional value, immunopharmacology, and safety of the traditional food of medicinal split-gill fugus *Schizophyllum commune* Fr.:Fr. (Schizophyllaceae). A literature review. Int. J. Med. Mushrooms 7 (1–2), 127–139.

Jin, Z., Hou, Q., Niu, T., 2020. Effect of cultivating *Pleurotus ostreatus* on substrates supplemented with herb residues on yield characteristics, substrates degradation, and fruiting bodies' properties. J. Sci. Food Agric. 100 (13), 4901–4910.

Kabuyi, M.K., Kapepula, P.M., Kabengele, J.K., Moni, B., Makengo, G., Mungitshi, P.M., Mukuta, A.T., Mutunda, C.M., Mpulusu, S.D., Ngbolua, J.P.K.-T.-N., Kabamba, N.N., Fundu, T.M., 2017. Selenium content and antioxidant potential of some edible wild mushrooms from Bandundu area, DR Congo. Nat. Resour. 8 (2), 103–113.

Kim, Y.-J., Lee, J.-A., 2021. Antioxidant, anti-inflammatory, and wound healing activities of *Selaginella tamariscina* leaf extract. J. Converg. Inf. Technol. 11 (4), 194–202.

Klaus, A., Kozarski, M., Niksic, M., Jakovljevic, D., Todorovic, N., Van Griensven, L.J.L.D., 2011. Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. LWT - Food Sci. Technol. (Lebensmittel-Wissenschaft -Technol.) 44 (10), 2005–2011.

Kumla, J., Suwannarach, N., Tanruean, K., Lumyong, S., 2021. Comparative evaluation of chemical composition, phenolic compounds, and antioxidant and antimicrobial activities of tropical black bolete mushroom using different preservation methods. Foods 10 (4), 781.

Lee, S., Ki, C.S., 2020. Inflammatory responses of macrophage-like RAW264. 7 cells in a 3D hydrogel matrix to ultrasonicated schizophyllan. Carbohydr. Polym. 229 (1), 115555.

Leung, M.Y., Liu, C., Koon, J.C., Fung, K.P., 2006. Polysaccharide biological response modifiers. Immunol. Lett. 105 (2), 101–114.

Li, Y., Zheng, Y., Zhang, Y., Xu, J., Gao, G., 2018. Antioxidant activity of coconut (*Cocos nucifera* L.) protein fractions. Molecules 23 (3), 707.

Liu, Z., Shi, Y., Liu, H., Jia, Q., Liu, Q., Tu, J., 2021. Purification and identification of pine nut (*Pinus yunnanensis* Franch.) protein hydrolysate and its antioxidant activity *in vitro* and *in vivo*. Chem. Biodivers. 18 (1), e2000710.

Maeng, J.-H., Muhammad Shahbaz, H., Ameer, K., Jo, Y., Kwon, J.-H., 2017. Optimization of microwave-assisted extraction of bioactive compounds from *Coriolus versicolor* mushroom using response surface methodology. J. Food Process. Eng. 40 (2), e12421.

Maity, P., Sen, I.K., Maji, P.K., Paloi, S., Devi, K.S., Acharya, K., Maiti, T.K., Islam, S.S., 2015. Structural, immunological, and antioxidant studies of beta-glucan from edible mushroom *Entoloma lividoalbum*. Carbohydr. Polym. 123 (1), 350–358.

Miletić, D., Turło, J., Podsadni, P., Pantić, M., Nedović, V., Lević, S., Nikšić, M., 2019. Selenium-enriched *Coriolus versicolor* mushroom biomass: potential novel food supplement with improved selenium bioavailability. J. Sci. Food Agric. 99 (11), 5122–5130.

Okwulehie, I.C., Nwosu, C., Okoroafor, C.J., 2007. Pharmaceutical and nutritional prospects of two wild macro-fungi found in Nigeria. Res. J. Appl. Sci. 2 (6), 715–120.

Özcan, Ö., Ertan, F., 2018. Beta-glucan content, antioxidant and antimicrobial activities of some edible mushroom species. Food Sci. Technol. 6 (2), 47–55.

Pasakawee, K., Laokuldilok, T., Srichairatanakool, S., Utama-ang, N., 2018. Relationship among starch digestibility, antioxidant, and physicochemical properties of several rice varieties using principal component analysis. Curr. Appl. Sci. Tech. 18 (3), 133–144.

Paudel, M.R., Chand, M.B., Pant, B., Pant, B., 2018. Antioxidant and cytotoxic activities of *Dendrobium moniliforme* extracts and the detection of related compounds by GC-MS. BMC Compl. Alternative Med. 18 (1), 1–9.

Ramalingam, L., Menikdiwela, K., LeMieux, M., Dufour, J.M., Kaur, G., Kalupahana, N., Moustaid-Moussa, N., 2017. The renin angiotensin system, oxidative stress and mitochondrial function in obesity and insulin resistance. Biochim. Biophys. Acta (BBA) - Mol. Basis Dis. 1863 (5), 1106–1114.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 26 (9–10), 1231–1237.

Saetang, N., Rattanapot, T., Manmai, N., Amornlerdpison, D., Ramaraj, R., Unpaprom, Y., 2022a. Effect of Hot Water Extraction Process on Schizophyllan from Split Gill Mushroom. Biomass Conv. Bioref. https://doi.org/10.1007/s13399-021-02286-z.

Saetang, N., Ramaraj, R., Unpaprom, Y., 2022b. Optimization of Ethanol Precipitation of Schizophyllan from Schizophyllum Commune by Applied Statistical Modelling. Biomass Conv. Bioref. https://doi.org/10.1007/s13399-022-02384-6.

Saetang, N., Ramaraj, R., Pumisutapon, P., Unpaprom, Y., 2022c. Influence of hot water treatment on alternative mycoprotein from Schizophyllum commune. In: Proceeding of the 3rd International Conference on Renewable Energy, Sustainable Environmental & Agri-Technological and Innovation (I-RESEAT 2021). Maejo University, Chiang Mai, pp. 1–9.

Smirnou, D., Knotek, P., Nesporova, K., Smejkalova, D., Pavlik, V., Franke, L., Velebny, V., 2017. Ultrasound-assisted production of highly-purified β-glucan schizophyllan and characterization of its immune properties. Process Biochem. 58 (1), 313–319.

Stankovic, M.S., 2011. Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. Kragujev. J. Sci. 33 (1), 63–72.

Teoh, H.L., Ahmad, I.S., Johari, N.M.K., Aminudin, N., Abdullah, N., 2018. Antioxidant properties and yield of wood ear mushroom, *Auricularia polytricha* (agaricomycetes), cultivated on rubberwood sawdust. Int. J. Med. Mushrooms 20 (4), 369–380.

Yelithao, K., Surayot, U., Lee, C., Palanisamy, S., Prabhu, N.M., Lee, J., You, S., 2019. Studies on structural properties and immune-enhancing activities of glycomannans from *Schizophyllum commune*. Carbohydr. Polym. 218 (1), 37–45.

Yim, H.S., Chye, F.Y., Rao, V., Low, J.Y., Matanjun, P., How, S.E., Ho, C.W., 2013. Optimization of extraction time and temperature on antioxidant activity of *Schizophyllum commune* aqueous extract using response surface methodology. J. Food Sci. Technol. 50 (2), 275–283.

Yin, C., Fan, X., Liu, C., Fan, Z., Shi, D., Yao, F., Cheng, W., Gao, H., 2019. The antioxidant properties, tyrosinase and α-glucosidase inhibitory activities of phenolic compounds in different extracts from the golden oyster mushroom, *Pleurotus citrinopileatus* (Agaricomycetes). Int. J. Med. Mushrooms 21 (9), 865–874.

Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-021-02286-z

ORIGINAL ARTICLE

Effect of hot water extraction process on schizophyllan from split gill mushroom

Nuttapong Saetang[1] · Thiravat Rattanapot[2] · Numchok Manmai[3] · Doungporn Amornlerdpison[2] · Rameshprabu Ramaraj[4] · Yuwalee Unpaprom[1]

Received: 31 October 2021 / Revised: 16 December 2021 / Accepted: 23 December 2021

Abstract
The split gill mushroom or *Schizophyllum commune* (*S. commune*) is a therapeutic mushroom. For cell wall composition, it consists of rich β-glucans (schizophyllan). This mushroom is high in nutritional content and has a wide range of biological properties. In this research, it is interesting to study hot water extraction of this functional schizophyllan. Central composite design (CCD) of response surface methodology (RSM) was applied to optimize these extraction parameters, namely, temperature (80–121 °C) and time (1–3 h) of schizophyllan extract (polysaccharide from *S. commune*) with a greater degree of polymerization (DP), maximum total sugar content, minimum reducing sugar content, and appropriate extraction yield. The results revealed the highest yield obtained from polysaccharide and supernatant extract (from the separation of polysaccharide extraction) at an extraction temperature of 121 °C for 2–3 h. Moreover, the optimal extraction temperature and time of polysaccharide extracts were 106.5 °C and 126.7 min (total sugar content); 103.1 °C and 177.0 min (reducing sugar content); and 104.1 °C and 175.5 min (DP value). This research shows that the optimum condition of schizophyllan extraction was around 100–110 °C for 2–3 h with the highest DP of about 6, including potential immune-enhancing and anticancer properties. As a result, the schizophyllan from the split gill mushroom is probably utilized as a functional ingredient with nutraceutical compounds and developed as other high value-added functional food products in further study.

Keywords Split gill mushroom · Schizophyllan · Hot water extraction · Optimization · Degree of polymerization

1 Introduction

Mushrooms are an essential source of both nutritional and medicinal foods. They can help you avoid problems including hypertension, hypercholesterolemia, and cancer [1], including their antioxidant, immune-enhancing, and antitumor properties [2]. According to some studies, they are excellent antioxidant sources because they contain different phenolic compounds and secondary metabolites that act as highly effective scavengers of free radicals [1]. The split gill mushroom, *Schizophyllum commune* (*S. commune*), is found in many Asian nations [3]. This mushroom grows on a broadleaved tree, dead, fallen, or standing wood. It is distinguished by light yellow to brown gills, dense white hairs, and the absence of stalks [4]. The fruiting body is small and fan-shaped with a white spore. Its life cycle completes within 10 days [5]. The mushroom cell wall comprises polysaccharides, which are β-glucans cross-linked with chitin [6]. The conformation of schizophyllan or β-glucan is a triple-stranded helix. This β-glucan is a β-1,3-D-glucan backbone with β-1,6-D-branches [7].

Furthermore, the split gill mushroom is a medicinal mushroom [8] with a variety of nutritional values, including higher phosphorus, magnesium, calcium, iron, zinc, manganese, copper, and chromium, as well as high carbohydrate, fiber, protein, and low lipid, as well as various mineral components, such as higher phosphorus, magnesium, calcium, iron, zinc, manganese, copper, and chromium [4, 9]. This mushroom is abundant in schizophyllan, which

✉ Yuwalee Unpaprom
yuwalee@mju.ac.th; yuwaleeun@gmail.com

[1] Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

[2] Center of Excellence in Agricultural Innovation for Graduate Entrepreneur, Maejo University, Chiang Mai 50290, Thailand

[3] Department of Forestry, National Chung Hsing University, Taichung 402, Taiwan

[4] School of Renewable Energy, Maejo University, Chiang Mai 50290, Thailand

Published online: 04 January 2022

is distinguished by a variety of biological properties [8], for instance, antioxidant [3, 5], anti-inflammatory [7, 10], immunomodulatory [3, 11], anticancer activity [12, 13], antiviral, and antifungal [4], along with prebiotic properties [14]. As a result, the mushroom is used in a wide variety of industrial applications, including pharmaceuticals [7], vaccines, cosmetic, functional food [11–13], and traditional food widely in Southeast Asia and India [4].

Furthermore, numerous extraction methods for schizophyllan or β-glucan are available. The molecular weight and structure of β-glucan are affected by extraction methods. Typically, β-glucan is extracted using hot water to solubilize it into solution and alkaline, acidic, enzyme-assisted, ultrasound-assisted, and microwave-assisted extraction [15]. Additionally, triple-helical β-glucan is stable at ambient temperature and pH levels about 7. Nonetheless, after β-glucan is effectively pretreated by alkaline solution, hot water at high temperature, or solvent, the conformation of β-glucan will change from triple helix to single helix and random coil, respectively [6]. Besides, polysaccharides can form from the linkages of monosaccharides, minutest carbohydrate, to complex carbohydrate structures, such as complex sugars, starches, and fibers. The classification of carbohydrates can be categorized with considering of degree of polymerization (DP). The DP value associates with the number of monomers in each structure. The types of carbohydrates are sorted of four groups: sugars (DP = 1–2; monosaccharides, disaccharides), oligosaccharides (DP = 3–9), polysaccharides (DP > 9; starch, non-starch polysaccharides), and polyols (DP 1 to > 9; xylitol, mannitol, sorbitol, etc.). The DP value of each sample could be evaluated from total sugar concentration divided by reducing sugar concentration, while total sugar and reducing sugar contents were analyzed from each sample [16]. Additionally, the conformational complexity of polysaccharides is important in immune function [17]. The polysaccharide with a higher DP and branching structural complexity has a greater potential for biological properties, especially immunomodulatory and anticancer effects [18], than linear or less branched polysaccharides with low DP considerably [7].

In addition, response surface methodology (RSM) is an efficient statistical approach for the organization of the model to estimate a methodology to study the association among the independent variables, whether it is alone or in combination in the process [16]. This statistical design is a beneficial technique for optimization of the chemical and biological procedure with decreasing time and cost-effectiveness in the process [1]. Therefore, this research aims to optimize the hot water extraction process for schizophyllan from *S. commune*, including efficiently studying the optimal temperature and time of this extraction with a response surface methodology approach. The schizophyllan extract will lead to the trend in developing the innovative functional food product with various bioactive substances for promoting consumer health in further research.

2 Materials and methods

2.1 Mushroom material and maintenance

The dried split gill mushroom from Chaiyo Mushroom Farm in Surat Thani Province, Thailand, was prepared by drying hot air oven at 60 °C for 48 h. After that, the mushroom material was ground into a fine powder before being packed in a vacuum bag and kept at room temperature until utilized in further research.

2.2 Optimized hot water extraction process for polysaccharide production

The dried mushroom powder was weighed 10% (w/v) in deionized (DI) water and adjusted pH around 5.8–6.0 with natural limewater. The mushroom suspension was heated at various temperatures (80–121 °C) and time of hot water extraction (1–3 h) before being filtrated through a filter cloth. The filtrate was then precipitated with 95% ethanol in a ratio of 1:2 at 4 °C for 24 h. After that, polysaccharide suspension was centrifugated at 3000 rpm for 10 min to separate the polysaccharide from the supernatant and dried at 60 °C for 24 h. Before freeze-drying, the supernatant was removed from the ethanol solution by evaporation at 60 °C.

The central composite design (CCD) was conducted in the optimum condition of the significant factors for the hot water extraction process under response surface methodology (RSM). In this experimental design, optimization is solved by varying two factors: temperature (A: 80–121 °C) and time of this extraction (B: 1–3 h). Table 1 shows the experimental variables and levels of the optimization experiment. The central composite design code values of each variable in each treatment are presented in Table 2. The polysaccharide and supernatant extract was weighed for evaluation yield of each extract, as well as the polysaccharide determined total sugar and reducing sugar content in the further assay. The data were also analyzed using the Design-Expert program version 11 from Stat-Ease, Inc., Minneapolis, USA.

Table 1 Levels and ranges of operating parameters for optimization

Operating factors	Coding	Units	Levels and ranges		
			Low (-)	Center point (0)	High (+)
Temperature	A	°C	80	100.5	121
Time	B	hr	1	2	3

Table 2 Treatments generated based on the central composite design to extraction conditions

Treatments	A	B
1	-	-
2	-	-
3	+	-
4	+	-
5	-	+
6	-	+
7	+	+
8	+	+
9	-	0
10	-	0
11	+	0
12	+	0
13	0	-
14	0	-
15	0	+
16	0	+
17	0	0
18	0	0
19	0	0

A temperature, *B* time

The schematic diagram of the experimental procedure and optimization of the extraction method is presented in Fig. 1.

2.3 Total sugar analysis

According to the phenol–sulfuric acid method from Dubois et al. [19], glucose's total sugar content was carried out as a reference. Of the appropriately diluted sample, 0.5 ml was combined with 0.5 ml of 5% (w/v) phenol and 2.5 ml of 98% (v/v) sulfuric acid. After that, the mixture was mixed well and incubated at room temperature for 10 min. The absorbance was spectrophotometrically measured at 490 nm. The total sugar content in each sample was expressed as micrograms per grams of dried extract.

2.4 Reducing sugar analysis

Reducing sugar content was determined according to the modified 3,5-dinitrosalicylic acid (DNS) method [20] using glucose as a standard. First, 0.5 ml of the diluted sample was mixed with 0.5 ml of DNS solution and boiled in a water bath for 15 min in dark conditions. After cooling, 4 ml of distilled water was added and mixed the sample well. The samples have measured the absorbance using a spectrophotometer at 540 nm. The reducing sugar content in each sample was expressed as micrograms per grams of dried extract. Then, the degree of polymerization (DP) is calculated from total sugar content divided by reducing sugar content.

2.5 Statistical analysis

All data will be carried out in three replicates. All results will be performed as either mean value ± standard deviation (SD) by Microsoft Excel 2016. Moreover, these results will be examined statistically through one-way analysis of variance (ANOVA). Tukey's test will evaluate the significance of the difference between each extract based on a confidence level of 95.0%.

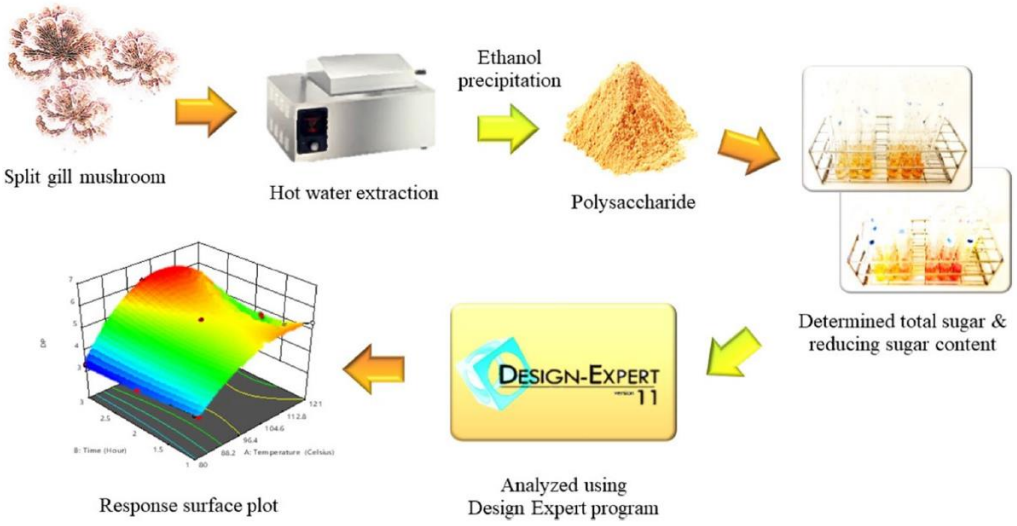


Fig. 1 Schematic diagram of experimental procedure and optimization of extraction method

3 Results and discussion

3.1 Edible split gill mushroom

Nowadays, split gill mushrooms are popular valuable foods because they are low in calories, carbohydrates, fat, and sodium: also; they are also cholesterol-free. Split gill mushrooms are antimicrobial, immune system boosters, cholesterol-lowering agents, and essential bioactive chemical sources [3, 4]. Some mushroom extracts are used to benefit human health and are available as dietary supplements as a result of these qualities. Mushrooms are also high in riboflavin (vitamin B2), niacin, folates, and traces of vitamin C, B1, B12, D, and E, making them a good source of vitamins. Mushrooms are the only natural vitamin D ingredients for vegetarians because they are the sole non-animal food source of vitamins. Moreover, the split gill mushroom is a medicinal mushroom [8] that has plentiful nutritional values, particularly high carbohydrate, fiber, protein, and low lipid, including various mineral components, such as higher phosphorus, magnesium, calcium, iron, zinc, manganese, copper, and chromium [4, 9].

Table 3 Effect of temperature and time of hot water extraction on extraction yields

Extraction temperature (°C)	Extraction time (hr)	Extraction yield (g/100 g dried mushroom)	
		Polysaccharide	Supernatant
80.0	1	2.60 ± 0.77^{d}	25.37 ± 0.24^{bc}
80.0	2	2.90 ± 0.22^{cd}	26.93 ± 0.62^{abc}
80.0	3	3.02 ± 0.33^{cd}	26.46 ± 1.27^{abc}
100.5	1	4.00 ± 0.02^{bc}	26.14 ± 0.69^{abc}
100.5	2	4.03 ± 0.14^{bc}	27.30 ± 1.59^{abc}
100.5	3	3.74 ± 0.28^{cd}	27.26 ± 0.05^{abc}
121.0	1	5.07 ± 0.18^{ab}	24.23 ± 0.65^{c}
121.0	2	5.95 ± 0.04^{a}	29.55 ± 1.19^{a}
121.0	3	5.74 ± 0.04^{a}	28.42 ± 0.60^{ab}

3.2 Yields of split gill mushroom extracts

Two variables, the different temperature and time of hot water extraction, were studied for effecting on extraction yields of polysaccharide and supernatant from extraction of polysaccharide from *S. commune* as revealed in Table 3. When increasing both temperature and time of the extraction, both polysaccharide and supernatant extracts reached superior yield. Consequently, the highest yield was obtained from polysaccharide (5.74–5.95 g/100 g of dried mushroom) and supernatant extract (28.42–29.55 g/100 g of dried mushroom) at the temperature of this extraction 121 °C for 2–3 h, respectively.

Moreover, hot water extraction associated with alkaline solution treatment can be carried out to degrade exceedingly mushroom cell walls and release water-soluble intracellular components outside the cells. Thus, the result of polysaccharide yield is significantly higher than the only hot water extraction approach [8]. The greatest yield of the schizophyllan extract from hot water extraction combined with natural alkaline and ethanolic precipitation was 5.95 g/100 g of dried mushroom, including the yields of other mushroom species as presented in Table 4. It was observed that this polysaccharide extraction yield was superior hot water extract associated with ethanol treatment of the same mushroom around 2.5-fold [3], hot water extract with ethanolic precipitation of *S. commune* at 4.30 g/100 g of dried mushroom, a polysaccharide from the same extraction combined with polysaccharide purification of this mushroom about 12-fold [8], polysaccharide extract from similar extraction of *Trametes hirsuta* about 2.5-fold, and *Heterobasidion annosum* approximately fivefold and *Russula fragilis* at 14-fold [21]. Besides, the greatest yield obtained from the supernatant extract of this mushroom may contain a lot of water-soluble components, for instance, a variety of phenolic compounds, which are secondary metabolites as greatly potential scavengers of reactive oxygen species by oxidation action [9], including alkaloids, terpenes, steroids, free amines [22], flavonoids [23], saponins, and tannins. Other nutritional

Table 4 Polysaccharide extraction yields of different mushroom

Mushroom species	Extraction method	Yield (g/100 g dried mushroom)	Reference
S. commune	Hot water extraction with ethanolic treatment (without purification)	2.39	[3]
S. commune	Hot water extraction with ethanolic treatment	4.30	[8]
	Hot water extraction with ethanolic treatment and polysaccharide purification	0.50	
Trametes hirsuta	Hot water extraction with ethanolic treatment	2.41	[21]
Heterobasidion annosum		1.15	
Russula fragilis		0.42	
S. commune	Hot water extraction with alkaline and ethanolic treatment	5.95	This study

substances in the supernatant extract may also comprise various vitamins and minerals [24].

3.3 Total sugar concentration of polysaccharide extracts from split gill mushroom

The proper extraction time and temperature ranges were optimized using the response surface methodology for maximal total sugar content, minimal reducing sugar content, and optimal degree of polymerization (DP) of the polysaccharide extracts from split gill mushroom. The effect of hot water extraction factors on the total sugar content of polysaccharides was determined by analysis of variance (ANOVA) as summarized in Table 5. The p value less than 0.01 showed a significant effect on the total sugar content of polysaccharide extracts. The plus and minus values mean the positive and negative effects on total sugar content, respectively.

In addition, the experimental data of the model to fit was proved by the lack of fit test as not significant (p value > 0.05). The higher regression coefficient in a model with a significant p value demonstrated a more significant response of each factor [1]. Both extraction temperature and time influenced the total sugar content with a good regression coefficient ($R^2 = 0.9985$). The relationship between the total sugar content and the extraction variable is presented according to the following coded equation (Eq. (1)):

$$\begin{aligned}\text{Total sugar content (mg/g extract)} = {} & 454.09 + 35.16A - 1.81B \\ & + 19.71AB - 35.82A^2 - 24.27B^2 \\ & + 18.79A^2B - 21.52AB^2\end{aligned} \quad (1)$$

Moreover, extraction temperature was found to be the significant positive linear but negative quadratic factors (p value < 0.01) affecting the total sugar content of polysaccharides. Extraction time did not have significant negative linear (p value > 0.05) but had significant negative quadratic effects (p value < 0.01). A significant positive interaction effect between extraction time and the temperature was obtained for the total sugar content (Table 5). Figure 2 revealed the contour plot and response surface plot of extraction time and temperature on the total sugar content of polysaccharide extracts. The total sugar content raised when the extraction temperature increased. While time enhanced, the total sugar content was not considerably higher. Thus, the maximum total sugar content predicted by RSM design was 461.56 mg/g extract with an optimal extraction temperature of 106.5 °C and time of 126.7 min.

Furthermore, when alkaline, solvent, or high temperature of hot water are efficiently employed for pretreatment of β-glucan (polysaccharide of mushroom), hydrogen bonds (intermolecular forces for sustaining the triple-helical and single-helical β-glucan) are broken. As a result, it will lead to the change of β-glucan conformation from triple helix to single helix and random coil, respectively [6]. The total sugar content of schizophyllan by hot water extraction from split gill mushroom in comparison with other mushroom species was revealed in Table 6. It was also found that maximal total sugar content of the schizophyllan was slightly higher than the polysaccharide-peptide complex of *Pleurotus abalonus* by hot water extraction with ethanol precipitation [25], including polysaccharide-protein complexes of *Coriolus versicolor* by ultrasound-assisted extraction about twice [26].

Table 5 ANOVA model for optimization of total sugar content of polysaccharide

Source	Sum of squares	Degrees of freedom	Mean square	F value	p value
Model	20,870.23	7	2981.46	1069.13	< 0.0001
Temperature (A)	4944.82	1	4944.82	1773.18	< 0.0001
Time (B)	13.07	1	13.07	4.69	0.0533
AB	3106.45	1	3106.45	1113.95	< 0.0001
A^2	5621.47	1	5621.47	2015.82	< 0.0001
B^2	2580.70	1	2580.70	925.42	< 0.0001
A^2B	941.57	1	941.57	337.64	< 0.0001
AB^2	1234.59	1	1234.59	442.71	< 0.0001
Residual	30.68	11	2.79		
Lack of fit	0.3242	1	0.3242	0.1068	0.7505
Pure error	30.35	10	3.04		
Cor total	20,900.90	18			
Std. dev	1.67		R^2	0.9985	
Mean	416.13		Adjusted R^2	0.9976	
C.V. %	0.4013		Predicted R^2	0.9953	
			Adeq precision	76.7240	

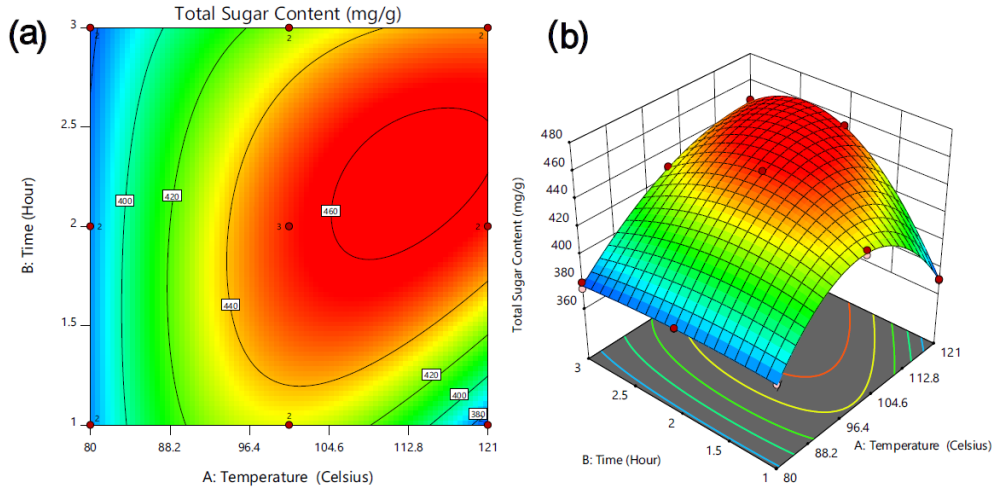


Fig. 2 Contour plot (**a**) and response surface plot (**b**) of total sugar content of polysaccharide as a function of extraction temperature and time

Table 6 Total sugar content of various mushroom

Mushroom species	Extraction method	Total sugar content (mg/g dried extract)	Reference
Pleurotus abalonus	Hot water extraction with ethanolic treatment	372.10	[25]
Coriolus versicolor	Ultrasound-assisted extraction	215.00	[26]
S. commune	Hot water extraction with alkaline and ethanolic treatment	461.56	This study

3.4 Reducing sugar concentration of polysaccharide extracts from split gill mushroom

To investigate the effect of hot water extraction parameters on reducing sugar content, the suitable ranges of extraction time and temperature for reducing sugar content of polysaccharides were estimated by ANOVA (Table 7). The lack of fit of this experimental data was not significant (p value > 0.05), indicating that the model was predictive. The relationship between reducing sugar content and extraction parameters is quadratic with a good regression coefficient ($R^2 = 0.9999$). The coded equation (Eq. (2)) presented the relationship as follows:

$$\begin{aligned}\text{Reducing sugar content (mg/g extract)} = {} & 84.06 - 5.61A - 4.12B \\ & + 0.1298AB + 18.80A^2 - 10.99B^2 \\ & + 10.03A^2B - 11.00AB^2\end{aligned} \quad (2)$$

Furthermore, extraction temperature and time had significant negative linear effects (p value < 0.01). Nevertheless, extraction temperature was found to be a significant positive quadratic factor. In contrast, time was found to be a significant negative quadratic factor (p value < 0.01), as well as a significant positive interaction effect between extraction time and the temperature was observed (p value < 0.05) (Table 7). Figure 3 shows the contour plot and response surface plot of reducing sugar content of polysaccharide extracts as a function of extraction time and temperature. The reducing sugar content decreased when extraction temperature increased. However, it was noticed that time augmented, the reducing sugar content raised. The previous research reported that the extraction of carbohydrates at high temperatures and longer extraction time was affected by carbohydrate hydrolysis, leading to the presence of reducing sugar [27]. In this study, the optimal extraction conditions were predicted to be the extraction temperature of 103.1 °C and time of 177.0 min for the minimum reducing sugar content of 68.74 mg/g extract.

Table 7 ANOVA model for optimization of reducing sugar content of polysaccharide

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* value
Model	4603.22	7	657.60	30,863.14	< 0.0001
Temperature (A)	125.91	1	125.91	5909.21	< 0.0001
Time (B)	67.83	1	67.83	3183.57	< 0.0001
AB	0.1348	1	0.1348	6.33	0.0287
A^2	1547.87	1	1547.87	72,645.99	< 0.0001
B^2	529.01	1	529.01	24,827.69	< 0.0001
A^2B	268.37	1	268.37	12,595.52	< 0.0001
AB^2	322.74	1	322.74	15,147.23	< 0.0001
Residual	0.2344	11	0.0213		
Lack of fit	0.0005	1	0.0005	0.0222	0.8844
Pure error	0.2339	10	0.0234		
Cor total	4603.46	18			
Std. dev	0.1460		R^2	0.9999	
Mean	88.99		Adjusted R^2	0.9999	
C.V. %	0.1640		Predicted R^2	0.9998	
			Adeq precision	478.3769	

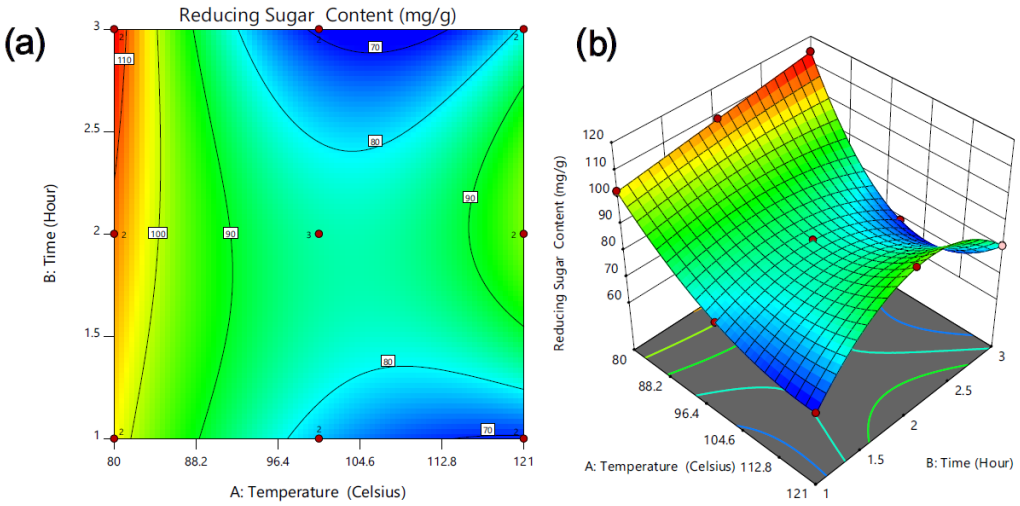


Fig. 3 Contour plot (**a**) and response surface plot (**b**) of reducing sugar content of polysaccharide as a function of extraction temperature and time

3.5 Degree of polymerization (DP) of polysaccharide extract from split gill mushroom

To evaluate the effect of these extraction variables on degree of polymerization (DP), the appropriate ranges of extraction time and temperature for DP of polysaccharides were analyzed by ANOVA as concluded in Table 8. The model was to fit, since the statistical program predicted the lack of fit was not significant (p value > 0.05). The relationship between DP and extraction factors is quadratic with a good regression coefficient ($R^2 = 0.9998$). The coded equation (Eq. (3)) below showed the relationship:

Table 8 ANOVA model for optimization of degree of polymerization (DP) of polysaccharide

Source	Sum of squares	Degrees of freedom	Mean square	*F* value	*p* value
Model	17.86	7	2.55	9986.68	<0.0001
Temperature (A)	1.28	1	1.28	5005.50	<0.0001
Time (B)	0.3655	1	0.3655	1430.49	<0.0001
AB	0.1557	1	0.1557	609.48	<0.0001
A^2	8.46	1	8.46	33,107.37	<0.0001
B^2	0.7696	1	0.7696	3011.78	<0.0001
A^2B	0.3880	1	0.3880	1518.60	<0.0001
AB^2	0.3901	1	0.3901	1526.82	<0.0001
Residual	0.0028	11	0.0003		
Lack of fit	0.0009	1	0.0009	4.54	0.0588
Pure error	0.0019	10	0.0002		
Cor total	17.87	18			
Std. dev	0.0160		R^2	0.9998	
Mean	4.83		Adjusted R^2	0.9997	
C.V. %	0.3308		Predicted R^2	0.9996	
			Adeq precision	275.5905	

$$\text{Degree of polymerization (DP)} = 5.45 + 0.5655A + 0.3023B + 0.1395AB - 1.39A^2 + 0.4191B^2 - 0.3815A^2B + 0.3825AB^2 \quad (3)$$

Besides, both extraction temperature and time were significant positive linear factors (p value < 0.01). Nonetheless, extraction temperature had a significant negative quadratic effect, while time had a significant positive quadratic effect (p value < 0.01), including a significant positive interaction effect between extraction time and the temperature, was obtained for DP value (Table 8). Figure 4 revealed the contour plot and response surface plot of extraction time and temperature on DP value of polysaccharide extracts. It could be observed that the DP value

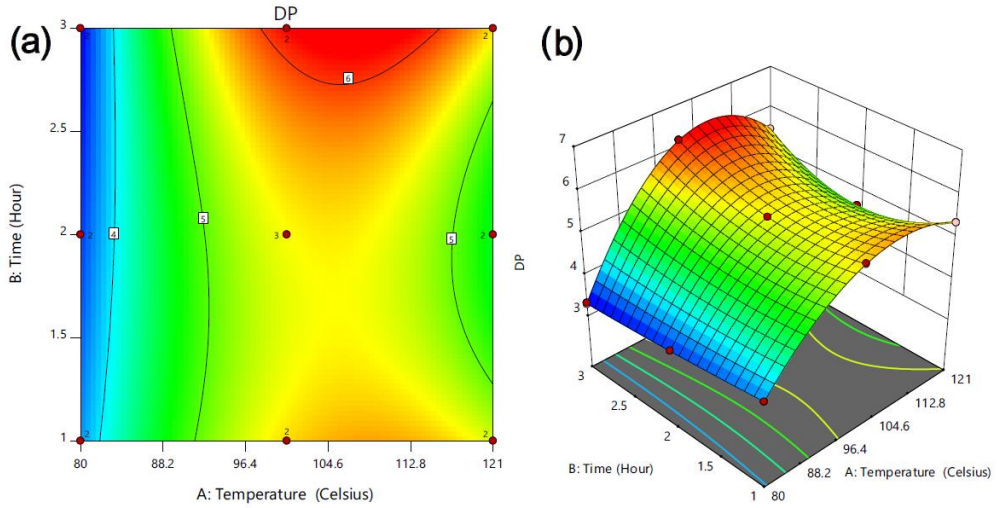


Fig. 4 Contour plot (**a**) and response surface plot (**b**) of degree of polymerization (DP) of polysaccharide as a function of extraction temperature and time

raised with an increase of extraction temperature from 80 to 105 °C. Likewise, it was obvious that increasing extraction time could promote a higher DP value. However, while the extraction temperature was more than 105 °C, the DP value fell slightly. Hence, the optimal extraction conditions were predicted to be the extraction temperature of 104.1 °C and time of 175.5 min for the maximal DP value of 6.21.

It is also required for the complex glucan structure, mushroom polysaccharide, to function properly in the immune system [17]. It has been discovered that the immunomodulatory and anticarcinogenic activities of polysaccharides, notably higher molecular weight polysaccharides, are connected to their molecular weights and structure [12]. The triple-helical β-glucan has a much better potency for immunomodulatory and anticancer activities [18] than linear or less branched β-glucan with lower DP [7]. This is because it has a higher DP, larger molecular weight, and a higher degree of the complex branching structure. As a result, schizophyllan is a possible functional material for manufacturing functional food products rich in pharmacological compounds that are beneficial to human health. The chemical characterization and biological activity of the extract, on the other hand, will require more investigation and biological activities will be necessary to study in further research.

4 Conclusion

From this study, it could be concluded that the optimal condition of the hot water extraction process for schizophyllan from *S. commune* was the temperature of this extraction at the ranges of 100–110 °C for 2–3 h with the highest degree of polymerization (DP ≈ 6), maximal total sugar content, minimal reducing sugar content, and suitable extraction yield. The schizophyllan was reported for its various biological properties, particularly a higher degree of polymerization and branching structure which had a greater potential for immunomodulatory and anticancer effects. Furthermore, other biological activities of this bioactive extract include antioxidant, anti-inflammatory, antiviral, antifungal, and prebiotic characteristics. As a result, this functional schizophyllan is being used to generate novel functional food and medicinal items, such as mushroom immunomodulators.

Acknowledgements The authors are sincerely grateful to the Center of Excellence in Agricultural Innovation for Graduate Entrepreneur and the Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai, Thailand, for providing the necessary facilities to accomplish the experimental study.

Author contribution Nuttapong Saetang: laboratory analysis, investigation, methodology, and writing (original draft preparation). Thiravat Rattanapot: literature review and validation. Numchok Manmai: validation and formal analysis. Doungporn Amornlerdpison: literature review and validation. Rameshprabu Ramaraj: methodology, resources, visualization, and writing (reviewing and editing). Yuwalee Unpaprom: data analysis, supervision, conceptualization, visualization, methodology, and writing (reviewing and editing).

Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

References

1. Yim HS, Chye FY, Rao V, Low JY, Matanjun P, How SE, Ho CW (2013) Optimization of extraction time and temperature on antioxidant activity of *Schizophyllum commune* aqueous extract using response surface methodology. J Food Sci Technol 50:275–283
2. Maity P, Sen IK, Maji PK, Paloi S, Devi KS, Acharya K, Maiti TK, Islam SS (2015) Structural, immunological, and antioxidant studies of beta-glucan from edible mushroom *Entoloma lividoalbum*. Carbohydr Polym 123:350–358
3. Yelithao K, Surayot U, Lee C, Palanisamy S, Prabhu NM, Lee J, You S (2019) Studies on structural properties and immune-enhancing activities of glycomannans from *Schizophyllum commune*. Carbohydr Polym 218:37–45
4. Hobbs C (2005) The chemistry, nutritional value, immunopharmacology, and safety of the traditional food of medicinal split-gill fugus *Schizophyllum commune* Fr.:Fr. (Schizophyllaceae). A literature review. Int J Med Mushrooms 7:127–139
5. Chandrawanshi NK, Tandia DK, Jadhav S (2017) Nutraceutical properties evaluation of *Schizophyllum commune*. Indian J Sci Res 13:57–62
6. Leung MY, Liu C, Koon JC, Fung KP (2006) Polysaccharide biological response modifiers. Immunol Lett 105:101–114
7. Lee S, Ki CS (2020) Inflammatory responses of macrophage-like RAW264. 7 cells in a 3D hydrogel matrix to ultrasonicated schizophyllan. Carbohydr Polym 229:115555
8. Klaus A, Kozarski M, Niksic M, Jakovljevic D, Todorovic N, Van Griensven LJLD (2011) Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. LWT - Food Sci Technol 44:2005–2011
9. Basso V, Schiavenin C, Mendonca S, de Siqueira FG, Salvador M, Camassola M (2020) Chemical features and antioxidant profile by *Schizophyllum commune* produced on different agroindustrial wastes and byproducts of biodiesel production. Food Chem 329:127089
10. Du B, Yang Y, Bian Z, Xu B (2017) Characterization and anti-inflammatory potential of an exopolysaccharide from submerged mycelial culture of *Schizophyllum commune*. Front Pharmacol 8:252
11. Smirnou D, Knotek P, Nesporova K, Smejkalova D, Pavlik V, Franke L, Velebny V (2017) Ultrasound-assisted production of highly-purified β-glucan schizophyllan and characterization of its immune properties. Process Biochem 58:313–319
12. Zhong K, Liu L, Tong L, Zhong X, Wang Q, Zhou S (2013) Rheological properties and antitumor activity of schizophyllan

produced with solid-state fermentation. Int J Biol Macromol 62:13–17
13. Zhong K, Tong L, Liu L, Zhou X, Liu X, Zhang Q, Zhou S (2015) Immunoregulatory and antitumor activity of schizophyllan under ultrasonic treatment. Int J Biol Macromol 80:302–308
14. Chaikliang C, Wichienchot S, Youravoug W, Graidist P (2015) Evaluation on prebiotic properties of β-glucan and oligo-β-glucan from mushrooms by human fecal microbiota in fecal batch culture. Funct Foods Health Dis 5:395–405
15. Zhu F, Du B, Xu B (2016) A critical review on production and industrial applications of beta-glucans. Food Hydrocoll 52:275–288
16. Manmai N, Saetang N, Unpaprom Y, Wu K (2019) Influential degree of polymerization of sugar extraction on alkali pretreatment from sunflower stalk wastes by applied statistical modelling. Maejo Int J Energ Environ Comm 1:37–43
17. Chan GC, Chan WK, Sze DM (2009) The effects of β-glucan on human immune and cancer cells. J Hematol Oncol 2:25
18. Khan AA, Gani A, Khanday FA, Masoodi FA (2018) Biological and pharmaceutical activities of mushroom β-glucan discussed as a potential functional food ingredient. Bioact Carbohydr Diet Fibre 16:1–13
19. Dubois M, Gilles KA, Hamilton JK, Pt R, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
20. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
21. Nowak R, Nowacka-Jechalke N, Juda M, Malm A (2018) The preliminary study of prebiotic potential of Polish wild mushroom polysaccharides: the stimulation effect on *Lactobacillus* strains growth. Eur J Nutr 57:1511–1521
22. Kabuyi MK, Kapepula PM, Kabengele JK, Moni B, Makengo G, Mungitshi PM, Mukuta AT, Mutunda CM, Mpulusu SD, Ngbolua JPK-T-N, Kabamba NN, Fundu TM (2017) Selenium content and antioxidant potential of some edible wild mushrooms from Bandundu area, DR Congo. Nat Resour 8:103–113
23. Arbaayah HH, Umi Kalsom Y (2013) Antioxidant properties in the oyster mushrooms (*Pleurotus* spp.) and split gill mushroom (*Schizophyllum commune*) ethanolic extracts. Mycosphere 4:661–673
24. Okwulehie IC, Nwosu C, Okoroafor CJ (2007) Pharmaceutical and nutritional prospects of two wild macro-fungi found in Nigeria. Res J Appl Sci 2:715–20
25. Li N, Li L, Fang JC, Wong JH, Ng TB, Jiang Y, Wang CR, Zhang NY, Wen TY, Qu LY, Lv PY, Zhao R, Shi B, Wang YP, Wang XY, Liu F (2012) Isolation and identification of a novel polysaccharide-peptide complex with antioxidant, anti-proliferative and hypoglycaemic activities from the abalone mushroom. Biosci Rep 32:221–228
26. Cheung Y-C, Siu K-C, Liu Y-S, Wu J-Y (2012) Molecular properties and antioxidant activities of polysaccharide–protein complexes from selected mushrooms by ultrasound-assisted extraction. Process Biochem 47:892–895
27. Sawangwan T, Wansanit W, Pattani L, Noysang C (2018) Study of prebiotic properties from edible mushroom extraction. Agric Nat Resour 52:519–524

Biomass Conversion and Biorefinery
https://doi.org/10.1007/s13399-022-02384-6

ORIGINAL ARTICLE

Optimization of ethanol precipitation of schizophyllan from *Schizophyllum commune* by applied statistical modelling

Nuttapong Saetang[1,2] · Rameshprabu Ramaraj[2,3] · Yuwalee Unpaprom[1,2]

Received: 1 December 2021 / Revised: 19 January 2022 / Accepted: 20 January 2022

Abstract
Schizophyllum commune or split gill mushroom is a pharmaceutical mushroom. Its cell wall contains abundant schizophyllan (β-glucan or polysaccharide from *S. commune*) distinguished by various biological activities. This experiment focused on studying the optimized ethanol precipitation process of the schizophyllan on the maximum degree of polymerization (DP), highest total sugar, and optimal reducing sugar concentration using response surface methodology (RSM) on central composite design (CCD). The experimental design examined the factors, including the split gill mushroom content (5–15% w/v) and ethanol concentration (49–74% v/v). From this experiment, it could be concluded that the optimized condition of this cost-effective extraction was the low mushroom content 5–8% (w/v) and optimal ethanol concentration 62–65% (v/v) with the highest DP value around 5, along with efficient immunomodulatory and anticancer effects. Thus, further research will develop the pharmaceutical schizophyllan extract as nutraceutical products or drugs.

Keywords Split gill mushroom · Schizophyllan · Ethanol precipitation · Degree of polymerization · Central composite design (CCD)

1 Introduction

Nowadays, there are numerous pandemics of various infections, diseases, and pollution issues. Therefore, people must pay greater attention to their health and consume more healthy foods, exceptionally functional foods. Functional food supplements are a functional component for preventing diseases or promoting health [1]. For example, mushrooms are an essential source of nutritional and medicinal foods. They can help you avoid problems including hypertension, hypercholesterolemia, and cancer [2], including their antioxidant, immune-enhancing, and anti-tumor properties [3]. According to some studies, they are excellent antioxidant sources because they contain various phenolic compounds and secondary metabolites that act as highly effective scavengers of free radicals [2].

Split gill mushroom (*Schizophyllum commune*) is extensively spread in many countries in Asia, such as Thailand, Myanmar, Laos, and Northeastern India [4]. This mushroom grows on a broadleaved tree, dead, fallen, or standing wood. It is distinguished by light yellow to brown gills, dense white hairs, and the absence of stalks [5]. The mushroom composes of β-glucans cross-linked with chitin in its cell wall [6]. The conformation of schizophyllan or β-glucan is a triple-stranded helix. This β-glucan is a β-1,3-D-glucan backbone with β-1,6-D-branches [7]. Moreover, the split gill mushroom is a medicinal mushroom [8] with plentiful nutritional values, particularly high fiber, protein, and low lipid [9]. For decades, mushroom has been a popular alternative protein source to animal proteins due to their high content of essential amino acids compared to vegetables [10]. Therefore, the production of healthy foods with a high protein content that does not contain meat is gaining interest worldwide, such as soy, wheat protein, and mycoprotein (mushroom protein) [11, 12].

Additionally, this mushroom contains peptides formed during protein hydrolysis by a protease enzyme that exhibits antioxidant properties [13], hydrophobin protein with

✉ Yuwalee Unpaprom
yuwalee@mju.ac.th; yuwaleeun@gmail.com

[1] Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

[2] Sustainable Resources and Sustainable Engineering Research Lab, Maejo University, Chiang Mai 50290, Thailand

[3] School of Renewable Energy, Maejo University, Chiang Mai 50290, Thailand

Published online: 01 February 2022

anticancer activity in S180 mouse sarcoma and B16-F10 mouse melanoma [14], lectin protein with inhibition of cancer cell growth and proliferation [15], both hydrophobin and lectin protein via possibly immunomodulatory [14, 16], and schizolysin (hemolysin) with antiviral activity due to inhibition of HIV-1 reverse transcriptase [15]. Besides, this mushroom is abundant schizophyllan, which is distinguished by a variety of biological properties [8], for instance, antioxidant [4, 17], anti-inflammatory [7, 18], immunomodulatory in immune cells from whole human blood [19] and macrophage cells [4], anticancer activity in lung, gastric, cervical, breast carcinoma cells [20] and tumor-bearing mouse [21], antiviral, antifungal [5], along with prebiotic properties [22].

Aside from that, β-glucan has a critical role in protecting the body from the oxidative damage caused by free radicals [4]. As a result, the mushroom is used in a wide variety of industrial applications, including pharmaceuticals [7], vaccines, cosmetics, functional food [19–21], and traditional food widely in Southeast Asia and India [5]. In addition, β-glucan is a prebiotic oligosaccharide or polysaccharide indigestible in the human digestive system [23]. Therefore, it promotes the immune system and helps gastrointestinal tract health by promoting the growth of helpful probiotic bacteria, controlling the balance of intestinal flora due to acidic chemicals produced by these probiotic bacteria, and inhibiting harmful bacteria growth [24]. Moreover, polysaccharides could shape from the links of monosaccharides (smallest carbohydrate) to the structural complexity of carbohydrates. Therefore, the sorting of carbohydrates could be classified by considering the degree of polymerization (DP). The DP value depends on the quantity of monosaccharides in each structure. So, the DP value of each treatment could be assessed from total sugar content divided by reducing sugar content; meanwhile, each treatment was determined total sugar and reducing sugar concentration [25].

In the extraction process of schizophyllan or β-glucan, various parameters are affecting on extraction, for example, the temperature of the extraction (ambient, boiling, or other); the type of organic solvent used to separate the polysaccharide from other components (ethanol, acetone, chloroform:methanol ($CHCl_3$:MeOH)); the presence of an alkaline solution (NaOH or KOH) and acidic solution; and the use of an enzyme, ultrasonic, or microwave technology to increase efficiency, yield, and reduce extraction time [26, 27]. Likewise, few research involving schizophyllan extraction from the split gill mushroom. For instance, Saetang et al. [25] researched the effect of hot water extraction of the schizophyllan using a response surface methodology (RSM) on DP value, total sugar, reducing sugar concentration, and extraction yield of schizophyllan extract.

In addition, Chandrawanshi et al. [17] studied the appropriate extraction process of the extracts from *S. commune* on antioxidant capacity. It was noticed that the highest yield was obtained by hot water extraction followed by ethanol and methanol extractions, respectively. Additionally, Klaus et al. [8] reported antioxidant activities and chemical characterization of polysaccharides extracted from fruiting bodies of this mushroom, such as hot water extract (HWE), hot water extracted polysaccharides (HWP), and hot water alkali extracted polysaccharides (HWAE). Thus, this research aims to optimize the ethanol precipitation process for schizophyllan of *S. commune* using a response surface methodology (RSM) on central composite design (CCD).

2 Materials and methods

2.1 Mushroom sample and maintenance

The dry split gill mushroom was obtained from Chaiyo Mushroom Farm, Surat Thani, Thailand. The mushroom sample was packed in a vacuum bag and stored at ambient temperature until used in further study.

2.2 Optimized ethanol precipitation of schizophyllan

The experimental procedure and optimization of the extraction process of schizophyllan are presented in Fig. 1. First, the schizophyllan extraction was described as follows: the dry mushroom powder was weighed various content (5–15% w/v) suspended in deionized (DI) water and adjusted pH about 5.7–6.0 with natural limewater. Next, the mushroom suspension was heated at 100 °C for 2 h (these conditions from optimization of hot water extraction in the previous study of Saetang et al. [25]) and filtrated through a filter cloth. After that, the filtrate was precipitated with 95% ethanol in different final concentrations (49–74% v/v) at 4 °C for 24 h. Then, the polysaccharide pellet was separated by centrifugation at 3000 rpm for 10 min before drying at 60 °C for 24 h.

The central composite design (CCD) was applied to optimize the significant variables for the ethanol precipitation of schizophyllan under response surface methodology (RSM). This experimental design has two numerical factors: split gill mushroom content (A: 5–15% w/v) and ethanol concentration (B: 49–74% v/v). The independent factors (A, B) with their coded (− 1 = low, 0 = center point, 1 = high) and actual levels of optimization experiment are presented in Table 1. A total run on RSM by using CCD in this experimental design involved full factorial design, one block, had generated 19 runs including non-center points 16 runs, center points 3 runs, and axial point ($\alpha = 1$). In further analysis, the total sugar and reducing sugar concentration were measured from polysaccharide extract. These results

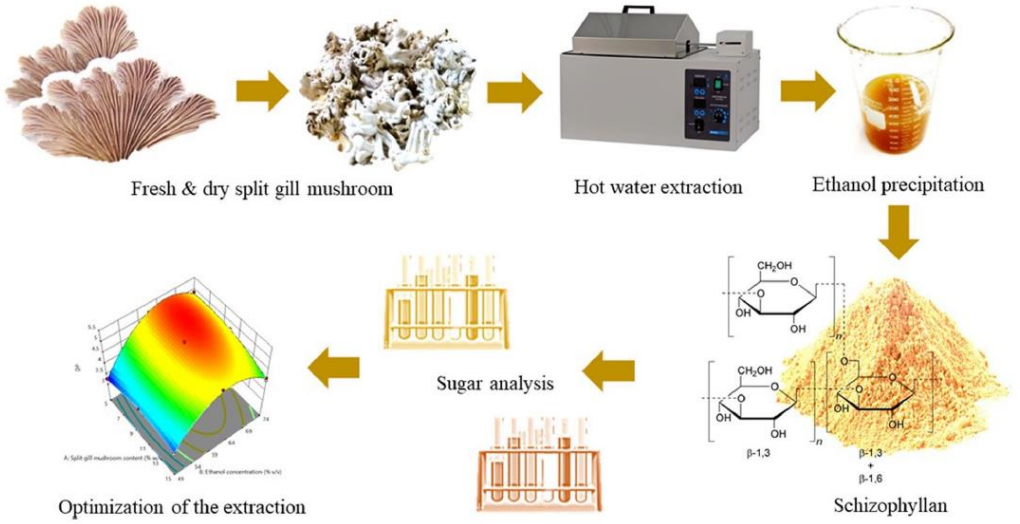


Fig. 1 The experimental procedure and optimization of the extraction process of schizophyllan

Table 1 Parameters and their values used for optimization experiment

Symbols	Parameters	Units	− 1	0	1
A	Split gill mushroom content	% (w/v)	5	10	15
B	Ethanol concentration	% (v/v)	49.0	61.5	74.0

were evaluated using the Design-Expert program version 11 from Stat-Ease, Inc., Minneapolis, USA.

2.3 Analytical methods

Total sugar concentration was analyzed by the phenol–sulfuric acid method [28], using glucose as a standard. First, 0.5 ml of the suitable diluted sample, 0.5 ml of 5% (w/v) phenol, and 2.5 ml of 98% (v/v) H_2SO_4 were dispensed in test tubes and mixed well. The mixture was then stood at ambient temperature for 10 min. The samples have measured the absorbance using a spectrophotometer at 490 nm. The total sugar concentration of each treatment was demonstrated as mg/g dry extract.

The modified 3,5-dinitrosalicylic acid (DNS) method [29] uses glucose as a reference. First, 0.5 ml of the diluted sample and 0.5 ml of DNS solution reagent were mixed well. Then, the mixture was boiled for 15 min in a water bath with dark conditions. It was cooled down before adding 4 ml of distilled water and mixed well. The absorbance was measured with a spectrophotometer at 540 nm. The reducing sugar concentration of each treatment was demonstrated as mg/g dry extract. After that, the degree of polymerization (DP) is evaluated from total sugar concentration divided by reducing sugar concentration.

2.4 Statistical analysis and model fitting

The statistical analysis of the model was utilized for the experimental design with the numerical responses and predicted from analysis of variance (ANOVA). The data of the normal plots, predicted versus actual values plots, residuals versus predicted values plots, residuals versus experimental run plots, contour plots, and response surface plots at the optimal condition were evaluated using the Design-Expert program version 11 from Stat-Ease, Inc., Minneapolis, USA. All the contours and graphs were estimated independent parameters on total sugar, reducing sugar concentration, and DP value of polysaccharide treatments. While F-test and P-value < 0.05 showed the significance and fit of the model, not significant lack of fit tests explained the good model to fit. The R^2, predicted R^2, and adjusted R^2 values were also determined.

3 Results and discussion

3.1 Total sugar concentration of schizophyllan extracts

Two independent contributions, the split gill mushroom content (% w/v, A) and ethanol concentration (% v/v, B), were utilized in the response surface methodology for maximized total sugar concentration, optimized reducing sugar concentration, and optimal degree of polymerization (DP) of the schizophyllan extracts from *S. commune*. The responses on total sugar concentration of the polysaccharide are exposed in Table 2. The trial data have calculated the coefficients of the parameters to demonstrate the association between the total sugar concentration and the parameters according to the coded equation (Eq. (1)) to predict the optimal response. Equation 1 shows the effect of each significant variable and the interactive effect of the variables on the response of total sugar concentrations. The plus and minus values indicated the positive and negative influences on total sugar concentration. The maximum total sugar concentration predicted by RSM design was 447.98 mg/g extract with optimal split gill mushroom content 5.11% (w/v) and precipitation ethanol concentration 62.78% (v/v).

Table 2 Experimental design results in total sugar concentration of the polysaccharide

Std	Run	Factor 1 A: Split gill mushroom content (% w/v)	Factor 2 B: Ethanol concentration (% v/v)	Total sugar concentration (mg/g)	
				Actual	Predicted
4	1	15	49.0	309.67	313.86
14	2	10	49.0	345.05	339.35
5	3	5	74.0	371.90	370.78
19	4	10	61.5	431.43	432.76
18	5	10	61.5	426.19	432.76
12	6	15	61.5	381.90	383.95
3	7	15	49.0	312.86	313.86
7	8	15	74.0	260.48	261.19
6	9	5	74.0	373.81	370.78
17	10	10	61.5	438.57	432.76
15	11	10	74.0	329.52	333.32
1	12	5	49.0	329.05	330.17
10	13	5	61.5	445.24	446.90
8	14	15	74.0	265.95	261.19
13	15	10	49.0	343.95	339.35
11	16	15	61.5	387.14	383.95
9	17	5	61.5	449.52	446.90
2	18	5	49.0	326.19	330.17
16	19	10	74.0	328.90	333.32

$$\text{Total sugar concentration (mg/g extract)} = 432.76 - 31.48A - 3.02B - 23.32AB - 17.33A2 - 96.43B^2 \quad (1)$$

Figure 2a presents the distribution of the normal residual plot from the polysaccharide. The points were adjacent to an original line, and the graph allowed the normal distribution of errors with a mean zero. Figure 2b shows the comparison between the actual and predicted values from the polysaccharide's total sugar concentration after ethanol precipitation. The predicted values were estimated by design using Eq. (1). This plot exhibited great agreement, which interacted with the actual and predicted results. The adjusted R^2 (0.9941) of total sugar concentration was close to 1.0 and consented to the results in the graph [30]. Moreover, Fig. 2c reveals the relationship between the externally studentized residuals and predicted results to set the standard deviations for normal probability. The results in the plot showed preliminary errors and the plot specified normality for these values. Lastly, the residuals and experimental run numbers have assessed the model to good fit using an internally studentized arrangement of the total sugar concentration model, as presented in Fig. 2d.

In addition, the effect of the mushroom content and ethanol concentration on the total sugar concentration of the polysaccharide was evaluated by analysis of variance (ANOVA), as concluded in Table 3. *P*-value and *F*-value as statistical terms indicate the significance of the model and each factor (*P*-value < 0.05) at a 95.0% confidence level [31]. The model *F*-value of 602.91 indicated that the model was significant. There was only a 0.01% chance which an *F*-value, this large could occur because of noise. ANOVA model demonstrated the greatest *F*-value of 595.41 at model A (the mushroom content), while model B (ethanol concentration) has the least effect on the response with an *F*-value of 5.47. *P*-values < 0.05 implied model terms are significant. In this case, A, B, AB, A^2, and B^2 were significant model terms. *P*-value > 0.10 signified that the model terms were not significant. The lack of fit *F*-value of 3.48 indicated that the lack of fit was nonsignificant relative to the pure error. There was a 5.81% chance that a lack of fit *F*-value, this large could happen because of noise. The insignificance of lack of fit is good, leading the model to fit. The predicted R^2 of 0.9912 was reasonable with the adjusted R^2 of 0.9941, namely the difference < 0.2. Adequate precision analyzed the signal-to-noise ratio. A ratio > 4 was desirable. The ratio of 73.9591 signified an adequate signal. ANOVA model of total sugar concentration could be applied to direct the design space.

Moreover, the contour plot and response surface plot depicted the interactions of two independent variables and the coincident effect on the total sugar concentration of the polysaccharide (Fig. 3). The interaction of AB was negative significantly at *F*-value of 217.91 and *P*-value < 0.0001 (Table 3, Eq. (1)). When analyzing the graph shape of

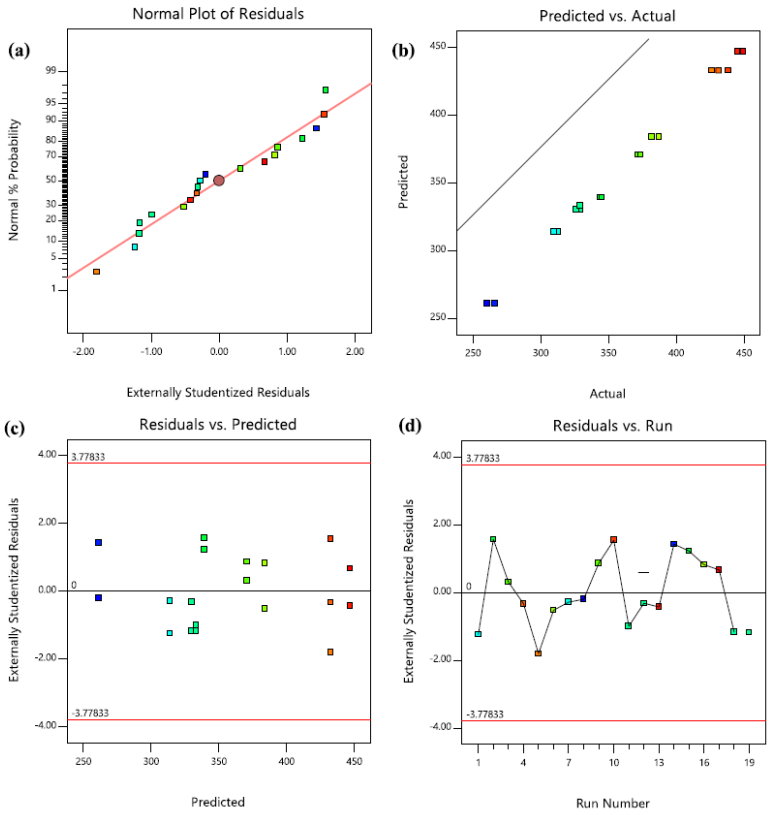


Fig. 2 Total sugar concentration analytical plots of normal plot of residuals (**a**); predicted versus actual plot (**b**); residuals versus predicted plot (**c**); and residuals versus the experimental run from the polysaccharide (**d**)

Table 3 ANOVA model analysis for optimization of total sugar concentration of the polysaccharide

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	60,194.08	5	12,038.82	602.91	< 0.0001
Split gill mushroom content (A)	11,889.03	1	11,889.03	595.41	< 0.0001
Ethanol concentration (B)	109.15	1	109.15	5.47	0.0360
AB	4351.13	1	4351.13	217.91	< 0.0001
A^2	1316.24	1	1316.24	65.92	< 0.0001
B^2	40,736.09	1	40,736.09	2040.10	< 0.0001
Residual	259.58	13	19.97		
Lack of fit	132.67	3	44.22	3.48	0.0581
Pure error	126.91	10	12.69		
Cor total	60,453.67	18			
Std. dev	4.47		R^2	0.9957	
Mean	360.91		Adjusted R^2	0.9941	
C.V. %	1.24		Predicted R^2	0.9912	
			Adeq precision	73.9591	

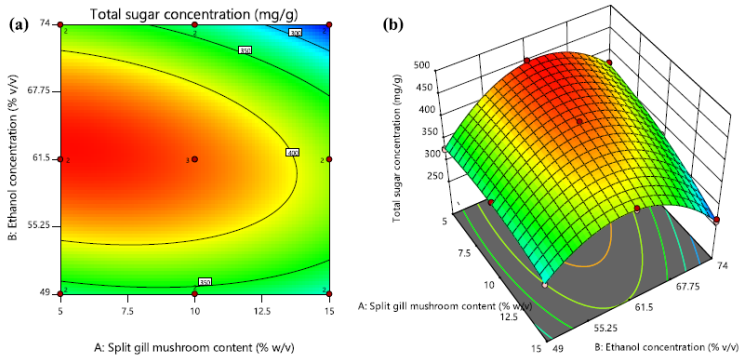


Fig. 3 Contour plot (**a**) and 3D plot (**b**) obtained from total sugar concentration of the polysaccharide as a function of the mushroom content and precipitation ethanol concentration

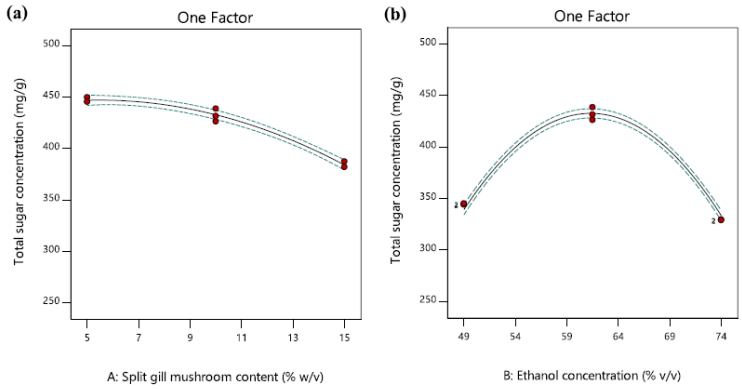


Fig. 4 Total sugar concentration of the polysaccharide in each factor: the mushroom content (**a**) and ethanol concentration (**b**)

separation on one factor of total sugar concentration models from Fig. 4, Fig. 4a was the negative non-linear relationship, i.e., total sugar concentration decreased when the mushroom content increased, while Fig. 4b was the negative quadratic relationship, i.e., total sugar concentration raised with increasing ethanol concentration during 49.0 to 61.5% (v/v), and total sugar concentration fell with more than 61.5% (v/v) ethanol.

Furthermore, β-glucan, a polysaccharide of mushroom, is extracted using hot water to solubilize it into solution, as well as an acidic, alkaline, organic solvent or enzyme-assisted extraction, and so on [27, 32], because these treatments could degrade greatly mushroom's cell wall and liberate water-soluble intracellular compositions to external of the cells [8]. However, in this case, hydrogen bonds that are intermolecular forces for maintaining the triple-helical and single-helical conformer of β-glucan are broken. Consequently, the conformation of β-glucan expressed changes from triple helix to single helix and random coil [6].

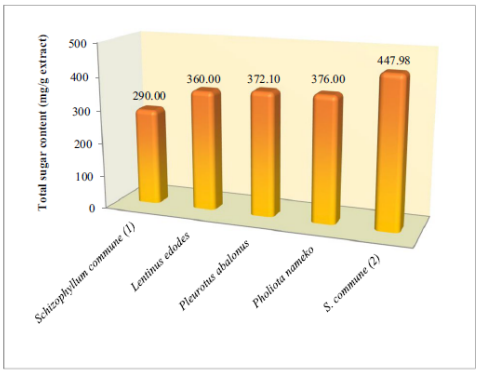


Fig. 5 Total sugar concentration of the polysaccharide extract from the different mushrooms. *(1) from Klaus et al. [8]; (2) from this study

Besides, the total sugar concentration of the schizophyllan by hot water extraction with ethanolic precipitation from *S. commune*, compared to that from other mushroom species, is illustrated in Fig. 5. It was noticed that the optimized total sugar concentration of this schizophyllan was superior to the polysaccharide extract of this mushroom using hot water extraction combined with ethanol precipitation around 1.5 folds [8]. Likewise, its total sugar concentration was slightly greater than polysaccharide-protein complexes of *Lentinus edodes* by ultrasonic extraction [33], the polysaccharide-peptide complex of *Pleurotus abalonus* using hot water extraction combined with ethanol treatment [34], and the extract of *Pholiota nameko* by enzyme-assisted extraction with Alcalase [35].

3.2 Reducing sugar concentration of schizophyllan extracts

The results of lowering sugar concentration are shown in Table 4 to analyze the influence of split gill mushroom content and ethanol concentration on polysaccharide reducing sugar concentration. The coded equation (Eq. (2)) demonstrated the association between the reducing sugar concentration and the coefficients of the factors obtained from calculating the predicted data. In addition, Eq. (2) presents the influence of each significant parameter and the interaction of the variables on reducing sugar concentrations. In this experimental design, the optimized extraction conditions were predicted to be the mushroom content 15.00% (w/v) and precipitation ethanol concentration 74.00% (v/v) for the minimized reducing sugar concentration of 64.99 mg/g extract.

Table 4 Experimental design results in reducing sugar concentration of the polysaccharide

Std	Run	Factor 1 A: Split gill mushroom content (% w/v)	Factor 2 B: Ethanol concentration (% v/v)	Reducing sugar concentration (mg/g)	
				Actual	Predicted
4	1	15	49.0	91.75	92.09
14	2	10	49.0	95.75	95.21
5	3	5	74.0	76.25	76.72
19	4	10	61.5	80.75	81.83
18	5	10	61.5	82.75	81.83
12	6	15	61.5	77.25	77.80
3	7	15	49.0	92.75	92.09
7	8	15	74.0	65.25	64.99
6	9	5	74.0	76.50	76.72
17	10	10	61.5	81.25	81.83
15	11	10	74.0	70.75	69.92
1	12	5	49.0	100.25	100.20
10	13	5	61.5	88.50	87.71
8	14	15	74.0	64.75	64.99
13	15	10	49.0	94.75	95.21
11	16	15	61.5	78.00	77.80
9	17	5	61.5	88.00	87.71
2	18	5	49.0	99.75	100.20
16	19	10	74.0	69.75	69.92

$$\text{Reducing sugar concentration (mg/g extract)} = 81.83 - 4.96A - 12.65B - 0.91AB + 0.93A^2 + 0.74B^2 \quad (2)$$

Besides, Fig. 6a depicts the normal plot of residual's distribution from the polysaccharide. These points were close to a primary line. The chart approved the normal distribution of errors with a mean zero. The comparison of the actual and the predicted data from reducing sugar concentration of the polysaccharide is shown in Fig. 6b. The predicted data were analyzed by design using Eq. (2). The graph expressed a great arrangement cooperating between the actual and predicted data. The adjusted R^2 (0.9964) of reducing sugar concentration was adjacent to 1.0 and concurred with the values in the plot [30]. Figure 6c also presents the association between the predicted values and externally studentized residuals to organize the standard deviations for normal probability. The values in the chart illustrated initial errors. The plot indicated normality for the results. Finally, the experimental run numbers and residuals have estimated the model to good fit using an internally studentized configuration of the reducing sugar concentration model, as revealed in Fig. 6d.

Moreover, the influence of the mushroom content and ethanol concentration on reducing sugar concentration of the polysaccharide was analyzed by analysis of variance (ANOVA), as summarized in Table 5. The model was significant at a F-value of 1008.48 with a 0.01% chance that this large could happen because of noise. ANOVA model illustrated the highest F-value of 4344.18 at model B, ethanol concentration, while model A, the mushroom content, there is the fewest influence on the response with an F-value of 667.86. In this case, A, B, AB, A^2, and B^2 were significant model terms (P-value < 0.05). The lack of fit was not significant relative to the pure error at F-value of 1.06 with a 40.77% chance that F-value, this large could occur because of noise. As a result, the model was to fit. The predicted R^2 of 0.9950 was reasonable with the adjusted R^2 of 0.9964; namely, the difference was lower than 0.2. Adequate precision measured the signal-to-noise ratio. A ratio of more than 4 was pleasing. The ratio of 94.2676 indicated an adequate signal. This ANOVA model could be utilized to navigate the design space.

In addition, the contour plot and 3D plot portrayed the interactions of two parameters and the concomitant effect on reducing sugar concentration of the polysaccharide (Fig. 7).

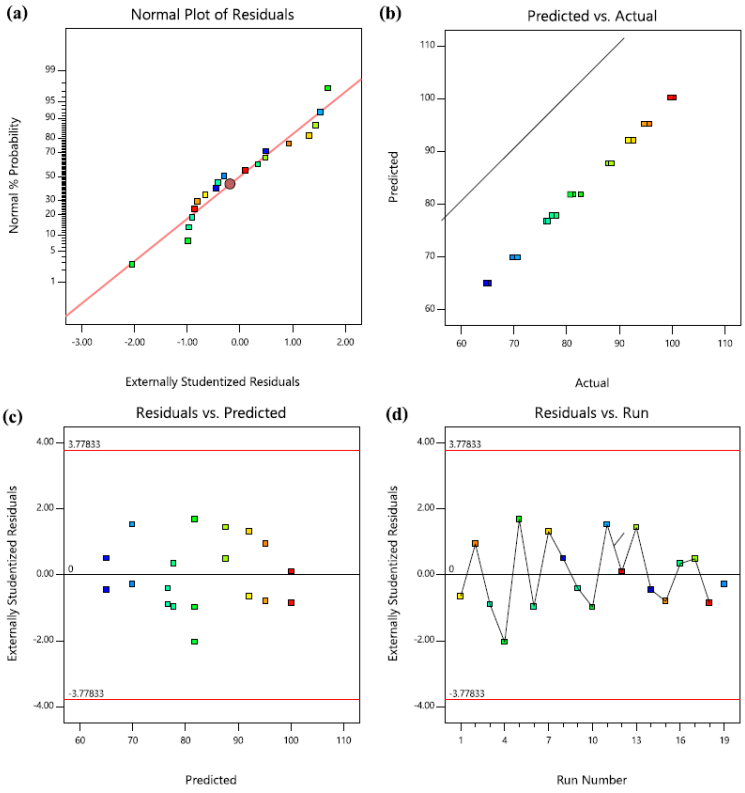


Fig. 6 Reducing sugar concentration analytical plots of normal plot of residuals (**a**); predicted versus actual plot (**b**); residuals versus predicted plot (**c**); and residuals versus the experimental run from the polysaccharide (**d**)

Table 5 ANOVA model analysis for optimization of reducing sugar concentration of the polysaccharide

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	2227.43	5	445.49	1008.48	<0.0001
Split gill mushroom content (A)	295.02	1	295.02	667.86	<0.0001
Ethanol concentration (B)	1919.01	1	1919.01	4344.18	<0.0001
AB	6.57	1	6.57	14.87	0.0020
A^2	3.78	1	3.78	8.57	0.0118
B^2	2.41	1	2.41	5.46	0.0361
Residual	5.74	13	0.4417		
Lack of fit	1.39	3	0.4628	1.06	0.4077
Pure error	4.35	10	0.4354		
Cor total	2233.17	18			
Std. dev	0.6646		R^2	0.9974	
Mean	82.88		Adjusted R^2	0.9964	
C.V. %	0.8019		Predicted R^2	0.9950	
			Adeq precision	94.2676	

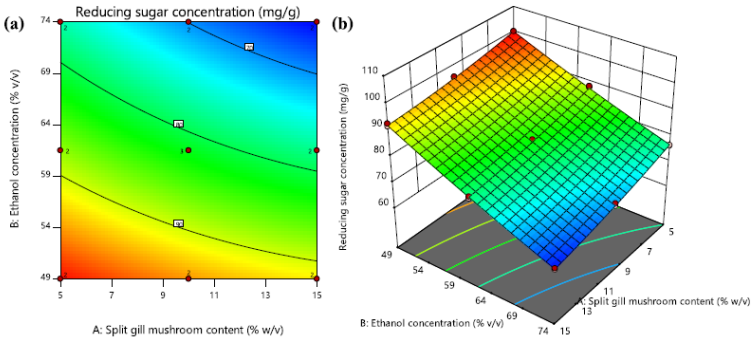


Fig. 7 Contour plot (**a**) and 3D plot (**b**) obtained from reducing sugar concentration of the polysaccharide as a function of the mushroom content and precipitation ethanol concentration

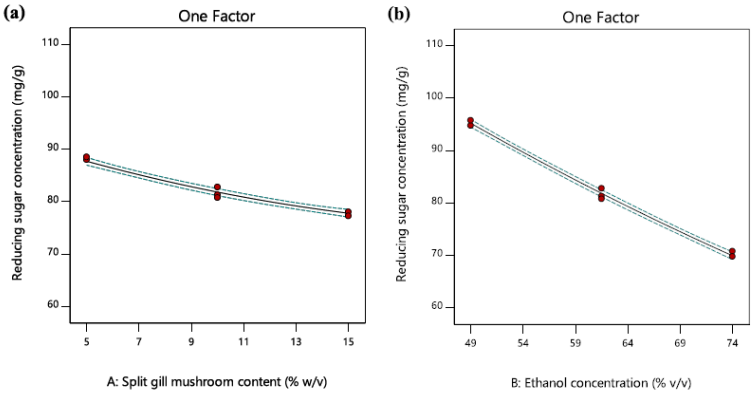


Fig. 8 Reducing sugar concentration of the polysaccharide in each factor: the mushroom content (**a**) and ethanol concentration (**b**)

The AB interaction was significantly negative at F-value of 14.87 and P-value of 0.0020 (Table 5, Eq. (2)). In the case of investigating the chart shape of separation on one factor of reducing sugar concentration models from Fig. 8, Fig. 8a is the negative non-linear relationship, namely reducing sugar concentration augmented when the mushroom content declined. As Fig. 8b was the negative linear relationship, namely reducing sugar concentration dropped considerably with raising of ethanol concentration.

3.3 Degree of polymerization of schizophyllan extracts

The reaction on the DP values is shown in Table 6 to study the influence of mushroom content and ethanol concentration on polysaccharide polymerization (DP). The coded equation (Eq. (3)) revealed the relationship between the DP values and the coefficients of the variables from the calculation of the trial data. In addition, Eq. (3) shows the effect of each significant factor and the interaction of the parameters on the DP values. In this experiment, the optimal extraction conditions were predicted to be the mushroom content 8.05% (w/v) and precipitation ethanol concentration 65.10% (v/v) for the maximal DP value of 5.40.

$$\text{Degree of polymerization (DP)} = 5.29 - 0.14A + 0.56B - 0.23AB - 0.27A^2 - 1.13B^2 \quad (3)$$

Moreover, the normal plot of residual's distribution is shown in Fig. 9a. The points were adjacent to a preliminary line. The plot allowed errors' be the normal distribution with a mean zero. Figure 9b portrays the predicted and actual results from the DP value of the polysaccharide. The predicted results were estimated by design using Eq. (3). The chart performed good agreement that cooperated between the predicted and the actual results. The adjusted R^2 (0.9881) of DP value was close to 1.0 and accepted the data in the graph [30]. The relationship between the externally studentized residuals and predicted data arranged the standard deviations for normal probability, as revealed in Fig. 9c. The data in the graph showed former errors. The chart signified normality for the data. Eventually, Fig. 9d presents the experimental run numbers versus residuals. These data have

Table 6 Experimental design results in degree of polymerization (DP) of the polysaccharide

Std	Run	Factor 1 A: Split gill mushroom content (% w/v)	Factor 2 B: Ethanol concentration (% v/v)	Degree of polymerization (DP)	
				Actual	Predicted
4	1	15	49.0	3.38	3.42
14	2	10	49.0	3.60	3.60
5	3	5	74.0	4.88	4.82
19	4	10	61.5	5.34	5.29
18	5	10	61.5	5.15	5.29
12	6	15	61.5	4.94	4.88
3	7	15	49.0	3.37	3.42
7	8	15	74.0	3.99	4.08
6	9	5	74.0	4.89	4.82
17	10	10	61.5	5.40	5.29
15	11	10	74.0	4.66	4.72
1	12	5	49.0	3.28	3.24
10	13	5	61.5	5.03	5.16
8	14	15	74.0	4.11	4.08
13	15	10	49.0	3.63	3.60
11	16	15	61.5	4.96	4.88
9	17	5	61.5	5.11	5.16
2	18	5	49.0	3.27	3.24
16	19	10	74.0	4.72	4.72

analyzed the model to good fit using an internally studentized arrangement of the DP value model.

Furthermore, Table 7 summarizes the evaluation of these parameters by analysis of variance (ANOVA) that affected the DP value of the polysaccharide. Furthermore, -glucan is important in reducing free radical-induced oxidative damage. ANOVA model showed the superior F-value of 528.69 at model B: ethanol concentration, while model A: the mushroom content, there is the inferior effect on the result with an F-value of 34.04. In this case, A, B, AB, A^2, and B^2 were significant model terms at P-value lower than 0.05. The lack of fit was not significant relative to the pure error at F-value of 3.37 with an 6.27% chance that F-value, this large could happen due to noise, resulting in the model to fit. The predicted R^2 of 0.9828 was the rational agreement with the adjusted R^2 of 0.9881, i.e., the difference < 0.2. Adequate precision determined the signal-to-noise ratio. A ratio higher than 4 was desirable. The ratio of 43.2390 implied an adequate signal. This ANOVA model could be applied to guide the design space.

Besides, Fig. 10 reveals the contour plot and response surface plot of DP value of the polysaccharide extract as a function of the mushroom content and ethanol concentration, including the interactions of two variables and the concurrent influence on the DP value. The interaction of AB was negative significantly at F-value of 61.11 and P-value < 0.0001 (Table 7, Eq. (3)). In the case of evaluating the graph shape of separation on one factor of DP value models, Fig. 11a is the negative quadratic relationship, i.e., DP value went up slightly with increasing of the mushroom content during 5 to 10% (w/v), and DP value fell insignificantly with greater than 10% (w/v) the mushroom. This case meaned that the mushroom content affected slightly on DP value of the polysaccharide. Nevertheless, although Fig. 11b is the negative quadratic relationship, DP value climbed up exceedingly with raising ethanol concentration between 49.0 and 61.5% (v/v), and DP value declined with higher than 61.5% (v/v) ethanol. These graphs could be summarized that the high mushroom content did not have an effect on efficient extraction of the polysaccharide with greater DP value (Fig. 11). Hence, further, scale-up, the polysaccharide consisting of higher DP value will be extracted with the low mushroom content due to reduction of the production cost.

In addition, the conformational complexity of β-glucan, the mushroom polysaccharide, is essential in the immune system's function [36]. The triple-helical β-glucan, containing a higher DP value with more branching structural complexity, has a greater potency for immune-enhancing and anticarcinogenic properties [32] than linear or lower branched β-glucan consisting of low DP value obviously [7].

4 Conclusion

Schizophyllum commune is an abundant potential schizophyllan substance with various pharmaceutical, biological properties, namely antioxidative, anti-inflammatory, immunomodulatory, and anticancer capacity. From this optimization of the ethanol precipitation of the schizophyllan, it could be summarized that the optimized condition of this schizophyllan extraction was the ranges of the mushroom content 5–8% (w/v) and ethanol concentration 62–65% (v/v) with the greatest DP value (around 5), maximal total sugar, and optimal reducing sugar concentration. Therefore, this polysaccharide consisting of higher DP value and branching structure was declared more potential for immune-enhancing and anticarcinogenic activities. Additionally, the low-cost commercial extraction of the polysaccharide with superior DP value will be achieved with less mushroom content and cost-effective ethanol concentration. Consequently, this functional schizophyllan has more potential to produce nutraceutical products or medicines with high bioactive compounds to promote health. However, the biological properties of the polysaccharide should be studied more in further in vitro and/or in vivo experiments.

Acknowledgements The authors are sincerely grateful to the Center of Excellence in Agricultural Innovation for Graduate Entrepreneur and the Program in Biotechnology, Faculty of Science, Maejo University,

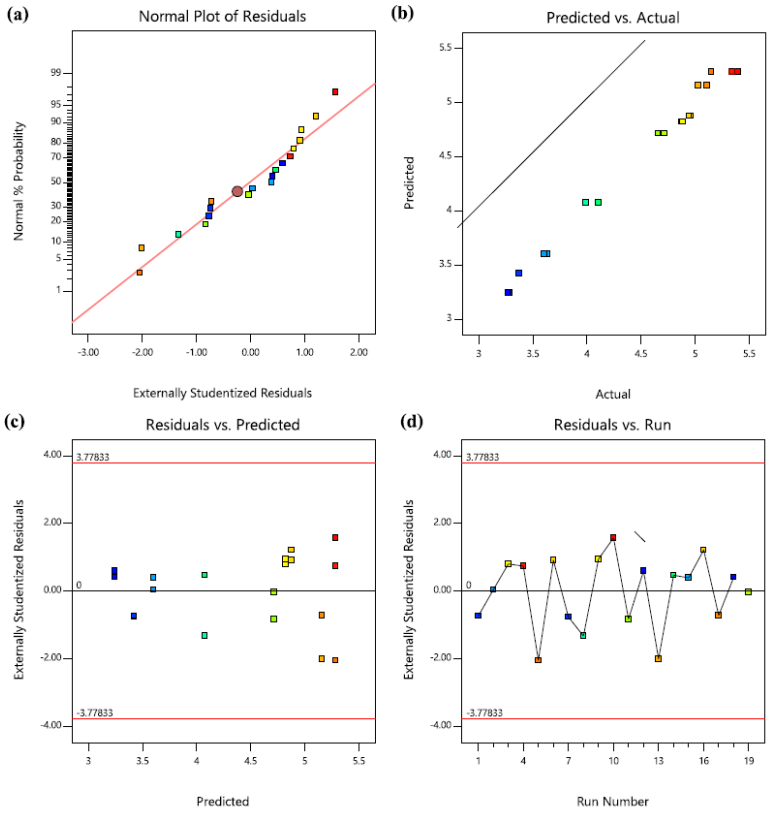


Fig. 9 Degree of polymerization (DP) analytical plots of normal plot of residuals (**a**); predicted versus actual plot (**b**); residuals versus predicted plot (**c**); and residuals versus the experimental run from the polysaccharide (**d**)

Table 7 ANOVA model analysis for optimization of degree of polymerization (DP) of the polysaccharide

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-value
Model	10.61	5	2.12	299.67	< 0.0001
Split gill mushroom content (A)	0.2410	1	0.2410	34.04	< 0.0001
Ethanol concentration (B)	3.74	1	3.74	528.69	< 0.0001
AB	0.4326	1	0.4326	61.11	< 0.0001
A^2	0.3138	1	0.3138	44.33	< 0.0001
B^2	5.57	1	5.57	786.68	< 0.0001
Residual	0.0920	13	0.0071		
Lack of fit	0.0463	3	0.0154	3.37	0.0627
Pure error	0.0457	10	0.0046		
Cor total	10.70	18			
Std. dev	0.0841		R^2	0.9914	
Mean	4.41		Adjusted R^2	0.9881	
C.V. %	1.91		Predicted R^2	0.9828	
			Adeq precision	43.2390	

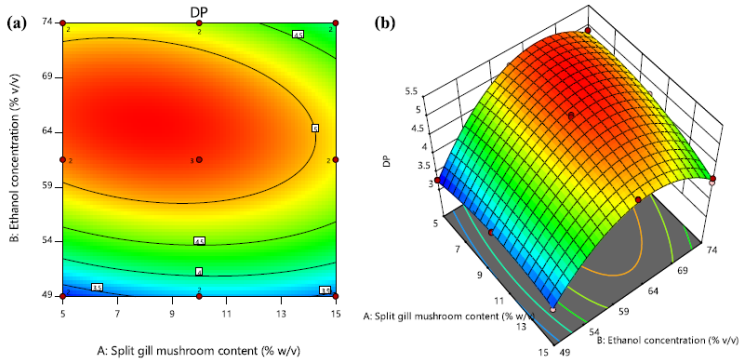


Fig. 10 Contour plot (**a**) and 3D plot (**b**) obtained from degree of polymerization (DP) of the polysaccharide as a function of the mushroom content and precipitation ethanol concentration

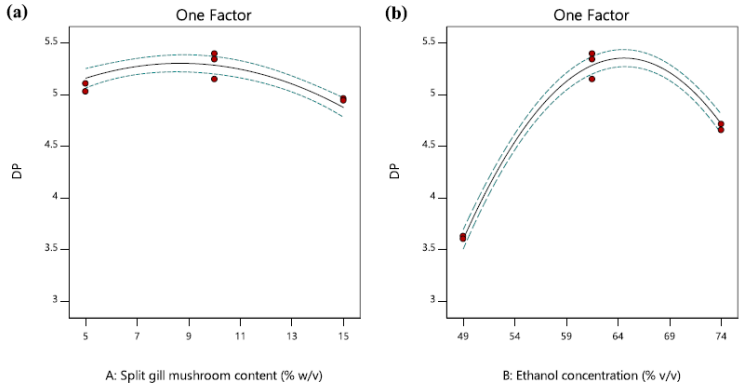


Fig. 11 Degree of polymerization (DP) of the polysaccharide in each factor: the mushroom content (**a**) and ethanol concentration (**b**)

Chiang Mai, Thailand, for providing the necessary facilities to accomplish the experimental study.

Author contribution Nuttapong Saetang: laboratory analysis, investigation, methodology, and writing (original draft preparation); Rameshprabu Ramaraj: methodology, resources, visualization, and writing (reviewing and editing); Yuwalee Unpaprom: data analysis, supervision, conceptualization, visualization, methodology, and writing (reviewing and editing).

Data availability The authors confirm that the data supporting the findings of this study are available within the article.

Declarations

Ethics approval Not applicable.

Conflict of interest The authors declare no competing interests.

References

1. Commission E (2010) Functional foods. Publications Office of the European Union Vol. 7
2. Yim HS, Chye FY, Rao V, Low JY, Matanjun P, How SE, Ho CW (2013) Optimization of extraction time and temperature on antioxidant activity of *Schizophyllum commune* aqueous extract using response surface methodology. J Food Sci Technol 50:275–283
3. Maity P, Sen IK, Maji PK, Paloi S, Devi KS, Acharya K, Maiti TK, Islam SS (2015) Structural, immunological, and antioxidant studies of beta-glucan from edible mushroom *Entoloma lividoalbum*. Carbohydr Polym 123:350–358
4. Yelithao K, Surayot U, Lee C, Palanisamy S, Prabhu NM, Lee J, You S (2019) Studies on structural properties and immune-enhancing activities of glycomannans from *Schizophyllum commune*. Carbohydr Polym 218:37–45
5. Hobbs C (2005) The chemistry, nutritional value, immunopharmacology, and safety of the traditional food of medicinal split-gill fugus *Schizophyllum commune* Fr.:Fr. (Schizophyllaceae). A literature review. Int J Med Mushrooms 7:127–139

6. Leung MY, Liu C, Koon JC, Fung KP (2006) Polysaccharide biological response modifiers. Immunol Lett 105:101–114
7. Lee S, Ki CS (2020) Inflammatory responses of macrophage-like RAW264. 7 cells in a 3D hydrogel matrix to ultrasonicated schizophyllan. Carbohydr Polym 229:115555
8. Klaus A, Kozarski M, Niksic M, Jakovljevic D, Todorovic N, Van Griensven LJLD (2011) Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. LWT - Food Sci Technol 44:2005–2011
9. Basso V, Schiavenin C, Mendonca S, de Siqueira FG, Salvador M, Camassola M (2020) Chemical features and antioxidant profile by *Schizophyllum commune* produced on different agroindustrial wastes and byproducts of biodiesel production. Food Chem 329:127089
10. Gonzalez A, Nobre C, Simoes LS, Cruz M, Loredo A, Rodriguez-Jasso RM, Contreras J, Texeira J, Belmares R (2021) Evaluation of functional and nutritional potential of a protein concentrate from *Pleurotus ostreatus* mushroom. Food Chem 346:128884
11. Kim K, Choi B, Lee I, Lee H, Kwon S, Oh K, Kim AY (2011) Bioproduction of mushroom mycelium of *Agaricus bisporus* by commercial submerged fermentation for the production of meat analogue. J Sci Food Agric 91:1561–1568
12. Saetang N, Ramaraj R, Pumisutapon P, Unpaprom Y (2022) Influence of hot water treatment on alternative mycoprotein from *Schizophyllum commune*. Proceeding of the 3rd International Conference on Renewable Energy, Sustainable Environmental & Agri-Technological and Innovation (i-RESEAT 2021), Maejo University, Chiang Mai, p 1–9
13. Wongaem A, Reamtong O, Srimongkol P, Sangtanoo P, Saisavoey T, Karnchanatat A (2021) Antioxidant properties of peptides obtained from the split gill mushroom (*Schizophyllum commune*). J Food Sci Technol 58:680–691
14. Akanbi MH, Post E, van Putten SM, de Vries L, Smisterova J, Meter-Arkema AH, Wosten HA, Rink R, Scholtmeijer K (2013) The antitumor activity of hydrophobin SC3, a fungal protein. Appl Microbiol Biotechnol 97:4385–4392
15. Han CH, Zhang GQ, Wang HX, Ng TB (2010) Schizolysin, a hemolysin from the split gill mushroom *Schizophyllum commune*. FEMS Microbiol Lett 309:115–121
16. Zhao S, Gao Q, Rong C, Wang S, Zhao Z, Liu Y, Xu J (2020) Immunomodulatory effects of edible and medicinal mushrooms and their bioactive immunoregulatory products. J Fungi 6:269
17. Chandrawanshi NK, Tandia DK, Jadhav S (2017) Nutraceutical properties evaluation of *Schizophyllum commune*. Indian J Sci Res 13:57–62
18. Du B, Yang Y, Bian Z, Xu B (2017) Characterization and anti-inflammatory potential of an exopolysaccharide from submerged mycelial culture of *Schizophyllum commune*. Front Pharmacol 8:252
19. Smirnou D, Knotek P, Nesporova K, Smejkalova D, Pavlik V, Franke L, Velebny V (2017) Ultrasound-assisted production of highly-purified β-glucan schizophyllan and characterization of its immune properties. Process Biochem 58:313–319
20. Zhong K, Liu L, Tong L, Zhong X, Wang Q, Zhou S (2013) Rheological properties and antitumor activity of schizophyllan produced with solid-state fermentation. Int J Biol Macromol 62:13–17
21. Zhong K, Tong L, Liu L, Zhou X, Liu X, Zhang Q, Zhou S (2015) Immunoregulatory and antitumor activity of schizophyllan under ultrasonic treatment. Int J Biol Macromol 80:302–308
22. Chaikliang C, Wichienchot S, Youravoug W, Graidist P (2015) Evaluation on prebiotic properties of β-glucan and oligo-β-glucan from mushrooms by human fecal microbiota in fecal batch culture. Funct Foods Health Dis 5:395–405
23. Van Loo J (2006) Inulin-type fructans as prebiotics. In: Gibson GR, Rastall RA (eds) Prebiotics: development and application. John Wiley & Sons, Hoboken, p 59
24. Singdevsachan SK, Auroshree P, Mishra J, Baliyarsingh B, Tayung K, Thatoi H (2016) Mushroom polysaccharides as potential prebiotics with their antitumor and immunomodulating properties: a review. Bioact Carbohydr Diet Fibre 7:1–14
25. Saetang N, Rattanapot T, Manmai N, Amornlerdpison D, Ramaraj R, Unpaprom Y (2022) Effect of hot water extraction process on schizophyllan from split gill mushroom. Biomass Convers Biorefin. https://doi.org/10.1007/s13399-021-02286-z
26. Ruthes AC, Smiderle FR, Iacomini M (2015) D-glucans from edible mushrooms: a review on the extraction, purification and chemical characterization approaches. Carbohydr Polym 117:753–761
27. Zhu F, Du B, Xu B (2016) A critical review on production and industrial applications of beta-glucans. Food Hydrocoll 52:275–288
28. Dubois M, Gilles KA, Hamilton JK, Pt R, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356
29. Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428
30. Manmai N, Unpaprom Y, Ponnusamy VK, Ramaraj R (2020) Bioethanol production from the comparison between optimization of sorghum stalk and sugarcane leaf for sugar production by chemical pretreatment and enzymatic degradation. Fuel 278:118262
31. Biniaz P, Farsi M, Rahimpour M (2016) Demulsification of water in oil emulsion using ionic liquids: statistical modeling and optimization. Fuel 184:325–333
32. Khan AA, Gani A, Khanday FA, Masoodi FA (2018) Biological and pharmaceutical activities of mushroom β-glucan discussed as a potential functional food ingredient. Bioact Carbohydr Diet Fibre 16:1–13
33. Cheung Y-C, Siu K-C, Liu Y-S, Wu J-Y (2012) Molecular properties and antioxidant activities of polysaccharide–protein complexes from selected mushrooms by ultrasound-assisted extraction. Process Biochem 47:892–895
34. Li N, Li L, Fang JC, Wong JH, Ng TB, Jiang Y, Wang CR, Zhang NY, Wen TY, Qu LY, Lv PY, Zhao R, Shi B, Wang YP, Wang XY, Liu F (2012) Isolation and identification of a novel polysaccharide-peptide complex with antioxidant, anti-proliferative and hypoglycaemic activities from the abalone mushroom. Biosci Rep 32:221–228
35. Rodrigues D, Freitas AC, Sousa S, Amorim M, Vasconcelos MW, da Costa JP, Silva AM, Rocha-Santos TA, Duarte AC, Gomes AM (2017) Chemical and structural characterization of *Pholiota nameko* extracts with biological properties. Food Chem 216:176–185
36. Chan GC, Chan WK, Sze DM (2009) The effects of β-glucan on human immune and cancer cells. J Hematol Oncol 2:25

APPENDIX E

PETTY PATENT

แบบ สป/สผ/อสป/001-ก
หน้า 1 ของจำนวน 3 หน้า

คำขอรับสิทธิบัตร/อนุสิทธิบัตร

☐ การประดิษฐ์
☐ การออกแบบผลิตภัณฑ์
☑ อนุสิทธิบัตร

ข้าพเจ้าผู้ลงลายมือชื่อในคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ขอรับสิทธิบัตร/อนุสิทธิบัตร ตามพระราชบัญญัติสิทธิบัตร พ.ศ. 2522 แก้ไขเพิ่มเติมโดยพระราชบัญญัติสิทธิบัตร (ฉบับที่ 2) พ.ศ. 2535 และ พระราชบัญญัติสิทธิบัตร (ฉบับที่ 3) พ.ศ. 2542

สำหรับเจ้าหน้าที่	
วันที่รับคำขอ	เลขที่คำขอ
วันที่ยื่นคำขอ - 4 พ.ย. 2563	2003002998
สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	
ใช้กับแบบผลิตภัณฑ์ ประเภทผลิตภัณฑ์	
วันประกาศโฆษณา	เลขที่ประกาศโฆษณา
วันออกสิทธิบัตร/อนุสิทธิบัตร	เลขที่สิทธิบัตร/อนุสิทธิบัตร
ลายมือชื่อเจ้าหน้าที่	

1. ชื่อที่แสดงถึงการประดิษฐ์/การออกแบบผลิตภัณฑ์
กระบวนการผลิตชุปเห็ดเสริมสมุนไพร

2. คำขอรับสิทธิบัตรการออกแบบผลิตภัณฑ์นี้เป็นคำขอสำหรับแบบผลิตภัณฑ์อย่างเดียวกันและเป็นคำขอลำดับที่ .....
ในจำนวน ..... คำขอ ที่ยื่นในคราวเดียวกัน

3. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☐ บุคคลธรรมดา ☑ นิติบุคคล ☑ หน่วยงานรัฐ ☐ มูลนิธิ ☐ อื่นๆ .....
ชื่อ มหาวิทยาลัยแม่โจ้
ที่อยู่ 63 หมู่ 4
ตำบล/แขวง หนองหาร อำเภอ/เขต สันทราย จังหวัด เชียงใหม่ รหัสไปรษณีย์ 50290 ประเทศ ไทย
อีเมล
☐ เลขประจำตัวประชาชน ☐ เลขทะเบียนนิติบุคคล ☑ เลขประจำตัวผู้เสียภาษีอากร 0 9 9 4 0 0 0 4 2 2 9 0 3 ☑ เพิ่มเติม(ดังแนบ)
ในกรณีที่กรมฯ สื่อสารกับท่าน ท่านสะดวกใช้ทาง ☐ อีเมลผู้ขอ ☑ อีเมลตัวแทน

3.1 สัญชาติ	ไทย
3.2 โทรศัพท์	0 5387 5635
3.3 โทรสาร	0 5387 5637

4. สิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☑ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

5. ตัวแทน (ถ้ามี) 5.1 ตัวแทนเลขที่ 1323
ชื่อ นายภัคย์ ภัลยาณมิตร 5.2 โทรศัพท์ 0 5387 5635
ที่อยู่ อุทยานวิทยาศาสตร์เทคโนโลยีเกษตรและอาหาร มหาวิทยาลัยแม่โจ้ 63 หมู่ 4 5.3 โทรสาร 0 5387 5635
ตำบล/แขวง หนองหาร อำเภอ/เขต สันทราย จังหวัด เชียงใหม่ รหัสไปรษณีย์ 50290 ประเทศ ไทย
อีเมล kalayanamitra@gmail.com
เลขประจำตัวประชาชน 3 5 3 9 9 0 0 2 6 4 2 6 6 ☐ เพิ่มเติม (ดังแนบ)

6. ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ ☐ ชื่อและที่อยู่เดียวกับผู้ขอ
ชื่อ ผู้ช่วยศาสตราจารย์ ดร.ยุวดี อันพาพรม
ที่อยู่ 179/10 หมู่ที่ 4
ตำบล/แขวง แคนเหนือ อำเภอ/เขต บ้านไผ่ จังหวัด ขอนแก่น รหัสไปรษณีย์ 40110 ประเทศ ไทย
อีเมล
เลขประจำตัวประชาชน 3 4 0 9 7 0 0 2 1 8 0 7 5 ☑ เพิ่มเติม (ดังแนบ)

7. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิม
ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้ถือว่าได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ ในวันเดียวกับคำขอรับสิทธิบัตร
เลขที่ ..... วันยื่น ..... เพราะคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้แยกจากหรือเกี่ยวข้องกับคำขอเดิมเพราะ
☐ ผู้ประดิษฐ์/ผู้ออกแบบ ☐ ผู้รับโอน ☐ ผู้ขอรับสิทธิโดยเหตุอื่น

<u>หมายเหตุ</u> ในกรณีที่ไม่อาจระบุรายละเอียดได้ครบถ้วน ให้จัดทำเป็นเอกสารแนบท้ายแบบพิมพ์นี้โดยระบุหมายเลขกำกับข้อและหัวข้อที่แสดงรายละเอียด เพิ่มเติมดังกล่าวด้วย

สำหรับเจ้าหน้าที่

จำแนกประเภทสิทธิบัตร/อนุสิทธิบัตร

☐ กลุ่มวิศวกรรม
สิทธิบัตรการประดิษฐ์ (วิศวกรรม)
สิทธิบัตรการประดิษฐ์ (ไฟฟ้า)
สิทธิบัตรการประดิษฐ์ (ฟิสิกส์)

☐ กลุ่มเคมี
สิทธิบัตรการประดิษฐ์ (เคมีเทคนิค)
สิทธิบัตรการประดิษฐ์ (ปิโตรเคมี)
สิทธิบัตรการประดิษฐ์ (เทคโนโลยีชีวภาพ)
สิทธิบัตรการประดิษฐ์ (เภสัชภัณฑ์)

สิทธิบัตรการออกแบบ
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 1)
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 2)
☐ สิทธิบัตรการออกแบบ (ออกแบบผลิตภัณฑ์ 3)

อนุสิทธิบัตร
☐ อนุสิทธิบัตร (วิศวกรรม)
☐ อนุสิทธิบัตร (เคมี)

8. การยื่นคำขอนอกราชอาณาจักร ☐ PCT ☐ เพิ่มเติม (ดังแนบ)

วันยื่นคำขอ	เลขที่คำขอ	ประเทศ	สัญลักษณ์จำแนกการประดิษฐ์ระหว่างประเทศ	สถานะคำขอ
8.1				
8.2				
8.3				

8.4 ☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอสิทธิให้ถือว่าได้ยื่นคำขอนี้ในวันที่ได้ยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรในต่างประเทศเป็นครั้งแรกโดย
☐ ได้ยื่นเอกสารหลักฐานพร้อมคำขอนี้ ☐ ขอยื่นเอกสารหลักฐานหลังจากวันยื่นคำขอนี้

9. การแสดงการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรได้แสดงการประดิษฐ์ที่หน่วยงานของรัฐเป็นผู้จัด
วันแสดง ..... วันเปิดงานแสดง ..... ผู้จัด .....

10. การประดิษฐ์เกี่ยวกับจุลชีพ

10.1 เลขทะเบียนฝากเก็บ	10.2 วันที่ฝากเก็บ	10.3 สถาบันฝากเก็บ/ประเทศ
.....	.....	.....
.....	.....	.....

11. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอยื่นเอกสารภาษาต่างประเทศก่อนในวันยื่นคำขอนี้ และจะจัดยื่นคำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ที่จัดทำเป็นภาษาไทยภายใน 90 วัน นับจากวันยื่นคำขอนี้ โดยขอยื่นเป็นภาษา
☐ อังกฤษ ☐ ฝรั่งเศส ☐ เยอรมัน ☐ ญี่ปุ่น ☐ อื่นๆ .....

12. ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ขอให้อธิบดีประกาศโฆษณาคำขอรับสิทธิบัตร หรือรับจดทะเบียน และประกาศโฆษณาอนุสิทธิบัตรนี้ หลังจากวันที่ .....
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตรขอให้ใช้รูปเขียนหมายเลข ..... ในการประกาศโฆษณา

13. คำขอรับสิทธิบัตร/อนุสิทธิบัตรนี้ประกอบด้วย

ก. แบบพิมพ์คำขอ		3	หน้า
ข. รายละเอียดการประดิษฐ์ หรือคำพรรณนาแบบผลิตภัณฑ์		9	หน้า
ค. ข้อถือสิทธิ		2	หน้า
ง. รูปเขียน	..... รูป		หน้า
จ. ภาพแสดงแบบผลิตภัณฑ์			
☐ รูปเขียน	..... รูป	.....	หน้า
☐ ภาพถ่าย	..... รูป	.....	หน้า
ฉ. บทสรุปการประดิษฐ์		1	หน้า

14. เอกสารประกอบคำขอ
☑ เอกสารแสดงสิทธิในการขอรับสิทธิบัตร/อนุสิทธิบัตร
☐ หนังสือรับรองการแสดงการประดิษฐ์/การออกแบบผลิตภัณฑ์
☑ หนังสือมอบอำนาจ
☐ เอกสารรายละเอียดเกี่ยวกับจุลชีพ
☐ เอกสารการขอนับวันยื่นคำขอในต่างประเทศเป็นวันยื่นคำขอในประเทศไทย
☐ เอกสารขอเปลี่ยนแปลงประเภทของสิทธิ
☑ เอกสารอื่นๆ

15. ข้าพเจ้าขอรับรองว่า
☑ การประดิษฐ์นี้ไม่เคยยื่นขอรับสิทธิบัตร/อนุสิทธิบัตรมาก่อน
☐ การประดิษฐ์นี้ได้พัฒนาปรับปรุงมาจาก .....

16. ลายมือชื่อ
☐ ผู้ขอรับสิทธิบัตร/อนุสิทธิบัตร ☑ ตัวแทน

(นายกัลย์ กัลยาณมิตร)
ตัวแทน

หมายเหตุ บุคคลใดยื่นขอรับสิทธิบัตรการประดิษฐ์หรือการออกแบบผลิตภัณฑ์ หรืออนุสิทธิบัตร โดยการแสดงข้อความอันเป็นเท็จแก่พนักงานเจ้าหน้าที่ เพื่อให้ได้ไปซึ่งสิทธิบัตรหรืออนุสิทธิบัตร ต้องระวางโทษจำคุกไม่เกินหกเดือน หรือปรับไม่เกินห้าพันบาท หรือทั้งจำทั้งปรับ

ใบต่อแนบท้าย สป/สผ/อสป/001ก

ผู้ประดิษฐ์/ผู้ออกแบบผลิตภัณฑ์ (เพิ่มเติม)
1. ผู้ช่วยศาสตราจารย์ ดร. ดวงพร อมรเลิศพิศาล
ที่อยู่เลขที่ 96 หมู่ที่ 15 ถนนคันคลองชลประทาน ตำบลสุเทพ อำเภอเมือง จังหวัดเชียงใหม่ รหัสไปรษณีย์ 50200
เลขประจำตัวประชาชน 3659900057529

2. นายนัฐพงษ์ แซ่ตั้ง
ที่อยู่เลขที่ 151 หมู่ 7 ตำบลไชยสถาน อำเภอสารภี จังหวัดเชียงใหม่ 50140
เลขประจำตัวประชาชน 1509900744130

Form PI/PD/PP/301

The Kingdom of Thailand
Ministry of Commerce
Department of Intellectual Property
Patent Office

Certification of Petty Patent Application

This is to certify that annexed hereto is a true copy of the following application as filed with this office

Application Number : 2003002998

Filing Date : November 4, 2020

Applicant : Maejo University

Issued on July 5, 2022

(Mr.Suwatchai BOON-AREE)
Director
Patent Office

Bangkok

APPENDIX F

CERTIFICATE OF PRESENTATION AND AWARDS

CERTIFICATE

i-RESEAT

This is to certify that

Nuttapong Saetang, Rameshprabu Ramaraj, Ruenkaew Praphruet and Yuwalee Unpaprom

for the **"best poster award"** with a paper entitled

Characterization of split gill mushroom, chemical feature, and prebiotic property of schizophyllan extract stimulating probiotic bacteria

at the 4th International Conference on Renewable Energy, Sustainable Environmental and Agri-Technological Innovation (i-RESEAT) from December 1st to 2nd, 2022, Warsaw University of Technology, Warsaw, Poland.

Assoc. Prof. Dr. Weerapon Thongma

Chair of the 4th i-RESEAT

National Taiwan University

No. 1, Section 4, Roosevelt Road, Taipei City 10617, Taiwan

CERTIFICATE OF AWARD

This is to certify that

Nuttapong Saetang

as **THE SPEAKER**

on the sub-theme ***Smart Agriculture***

2021 UNIVERSITY CONSORTIUM GRADUATE FORUM

"Agricultural Adaptation Strategies for Coping with Climate Change"

co-organized by

College of Bioresources and Agriculture, National Taiwan University (HOST)

and

Southeast Asian Regional Center for Graduate Study and Research in Agriculture

December 2nd and 3rd, 2021

Dean Huu-Sheng LUR

College of Bioresources and Agriculture
National Taiwan University

Director Glenn B. GREGORIO

Southeast Asian Regional Center for Graduate Study and Research in Agriculture

This certificate was issued electronically. No signature required. To verify its authenticity email ntuciaeae@ntu.edu.tw

CERTIFICATE
OF ACHIEVEMENT

GOLD AWARD 2022

International Innovation Competition
(AAACU-MJU-2022)

GOLD AWARD

The award is hereby awarded to

N.Saetang, Y.Unpaprom, R.Ramaraj, D.Amornlerdpison, P.Junluthin

for the creative invention of Split gill mushroom essence supplemented with herbs

---

Innovation Competition is part of the AAACU 23rd Biennial Conference with Maejo University
July 5-6, 2022, Furama Hotel, Chiang Mai, Thailand (online & on-site)

DR. SATORU TSUCHIKAWA
President
Asian Association of Agricultural Colleges and Universities

DR. WEERAPON THONGMA
President
Maejo University

CERTIFICATE

OF RECOGNITION

This certificate is presented to

NUTTAPONG SAETANG, YUWAIEE UNPAPROM*, RAMESHPRABU RAMARAJ, DOUNGPORN AMORNLERDPISON, PATTRANAN JUNLUTHIN

For the 1st prize winner of food and byproduct application in title
"Beta-G Plus Splitgill mushroom essence supplemented with herbs"

in the International Seminar and Innovation Competition
of Technology and Innovation for High Value Creation
on Agricultural Products under New Southbound Policy Collaboration
Given this 9th of May 2023 at Vanung University, Taiwan

Professor Dr.Thomas Chuang
President of Vanung University

SAFE NETWORK
Asia Pacific Network for Sustainable Agriculture Food and Energy

HOME FOR CONNECTING PEOPLE.

2023

SAFE-Network & Maejo University Product Innovation Competition

Beta-G Plus

Splitgill mushroom essence supplemented with herbs

Nuttapong Saetang, Asst. Prof. Dr. Yuwalee Unpaprom, Assoc. Prof. Dr. Rameshprabu Ramaraj, Assoc. Prof. Dr. Doungporn Amornlerdpison, Pattranan Junluthin
Maejo University, Thailand

Chiang Mai , Thailand
May 28 - 29, 2023

ASS.PROF.DR. YUWALEE UNPAPROM
Maejo University. Thailand

ASSOC.PROF. NGUYEN HUY BICH, PH.D
NongLam University, Hochiminh City, Vietnam

ASSOC.PROF.DR. RAMESHPRABU RAMARAJ
Maejo University. Thailand

ASSOC.PROF.DR. SITI NORASMAH
Universiti Teknologi MARA (UiTM), Malaysia

APPENDIX G

CERTIFICATE OF HUMAN RESEARCH ETHICS AND BIOSAFETY

มหาวิทยาลัยแม่โจ้

มอบประกาศนียบัตรนี้เพื่อแสดงว่า

นายนัฐพงษ์ แซ่ตั้ง

ได้เข้ารับการอบรม

โครงการอบรมจริยธรรมการวิจัยในคน

สำหรับการวิจัยทางสังคมศาสตร์ รุ่นที่ ๒ ประจำปี ๒๕๖๓

วันพุธที่ ๒๙ กรกฎาคม ๒๕๖๓

ณ ห้องประชุมข้าวหอมมะลิ อาคารเฉลิมพระเกียรติสมเด็จพระเทพรัตนราชสุดา

มหาวิทยาลัยแม่โจ้

ประกาศนียบัตรมีอายุ ๒ ปี ให้ไว้ ณ วันที่ ๒๙ กรกฎาคม ๒๕๖๓

(ผู้ช่วยศาสตราจารย์ ดร.ลิวา ผาดไธสง)
ประธานคณะกรรมการจริยธรรมวิจัยในคน
มหาวิทยาลัยเชียงใหม่

(รองศาสตราจารย์ ดร.วีระพล กองมา)
อธิการบดีมหาวิทยาลัยแม่โจ้

MJU MAEJO UNIVERSITY

มหาวิทยาลัยแม่โจ้ จังหวัดเชียงใหม่
เลขที่ ๗๓ หมู่ ๔ ต.หนองหาร อ.สันทราย จ.เชียงใหม่ ๕๐๒๙๐
โทรศัพท์ · ๐๕๓-๘๗๓๐๐๐ แฟกซ์ · ๐๕๓-๘๗๓๐๑๕

CMU SH&E
Center for Safety, Occupational Health and Environment, Chiang Mai University

มหาวิทยาลัยเชียงใหม่

ประกาศนียบัตรฉบับนี้ให้ไว้เพื่อแสดงว่า

นายนัฐพงษ์ แซ่ตั้ง

ได้สำเร็จการฝึกอบรมหลักสูตร

"ความปลอดภัยทางชีวภาพ (Biosafety) และการรักษาความปลอดภัยทางชีวภาพ (Biosecurity)"

รุ่นที่ 6

- หลักสูตรนี้ได้รับการรับรองจากกรมวิทยาศาสตร์การแพทย์เรียบร้อยแล้ว -

ให้ไว้ ณ วันที่ 20 ธันวาคม พ.ศ. 2563

หมดอายุ วันที่ 19 ธันวาคม พ.ศ. 2566

(ศาสตราจารย์คลินิก นายแพทย์นิเวศน์ นันทจิต)

อธิการบดีมหาวิทยาลัยเชียงใหม่

APPENDIX H

CERTIFICATE OF IELTS

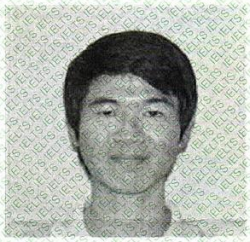
IELTS™

Test Report Form ACADEMIC

NOTE *Admission to undergraduate and post graduate courses should be based on the ACADEMIC Reading and Writing Modules. GENERAL TRAINING Reading and Writing Modules are **not** designed to test the full range of language skills required for academic purposes. It is recommended that the candidate's language ability as indicated in this Test Report Form be re-assessed **after two years** from the date of the test.*

Centre Number	TH011	Date	14/SEP/2019	Candidate Number	009185

Candidate Details



Family Name	SAETANG
First Name	NUTTAPONG
Candidate ID	1509900744130

Date of Birth	17/06/1989	Sex (M/F)	M	Scheme Code	Private Candidate

Country or Region of Origin	
Country of Nationality	THAILAND
First Language	THAI

Test Results

Listening	Reading	Writing	Speaking	Overall Band Score	CEFR Level
5.0	6.0	5.0	5.0	5.5	B2

Administrator Comments

Centre stamp

Validation stamp

Administrator's Signature

Date 26/09/2019

Test Report Form Number 19TH009185SAEN011A

BRITISH COUNCIL idp Cambridge Assessment English

The validity of this IELTS Test Report Form can be verified online by recognising organisations at http://ielts.ucles.org.uk

REFERENCES

Akanbi, M. H., Post, E., van Putten, S. M., de Vries, L., Smisterova, J., Meter-Arkema, A. H., Wosten, H. A., Rink, R. & Scholtmeijer, K. 2013. The antitumor activity of hydrophobin SC3, a fungal protein. **Applied Microbiology and Biotechnology**, 97(10), 4385-4392.

AOAC. 2012. **Official Methods of analysis of AOAC International (19th ed)**. Gaithersburg, MD: AOAC.

AOAC. 2019. **Official Methods of analysis of AOAC International (21st ed)**. Gaithersburg, MD: AOAC.

Arbaayah, H. H. & Umi Kalsom, Y. 2013. Antioxidant properties in the oyster mushrooms (*Pleurotus* spp.) and split gill mushroom (*Schizophyllum commune*) ethanolic extracts. **Mycosphere**, 4(4), 661-673.

Asghari, A., Fazilati, M., Latifi, A. M., Salavati, H. & Choopani, A. 2016. A review on antioxidant properties of *Spirulina*. **Journal of Applied Biotechnology Reports**, 3(1), 345-351.

Basso, V., Schiavenin, C., Mendonca, S., de Siqueira, F. G., Salvador, M. & Camassola, M. 2020. Chemical features and antioxidant profile by *Schizophyllum commune* produced on different agroindustrial wastes and byproducts of biodiesel production. **Food Chemistry**, 329(1), 127089.

Boulekbache-Makhlouf, L., Medouni, L., Medouni-Adrar, S., Arkoub, L. & Madani, K. 2013. Effect of solvents extraction on phenolic content and antioxidant activity of the byproduct of eggplant. **Industrial Crops and Products**, 49(1), 668-674.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Analytical Biochemistry**, 72(1-2), 248-254.

Chaari, F., Belghith-Fendri, L., Zaouri-Ellouzi, S., Driss, D., Blibech, M., Kallel, F., Bouaziz, F., Mehdi, Y., Ellouz-Chaabouni, S. & Ghorbel, R. 2016. Antibacterial and antioxidant properties of mixed linkage beta-oligosaccharides from extracted β-glucan hydrolysed by *Penicillium occitanis* EG_L lichenase. **Natural Product**

Research, 30(12), 1353-1359.

Chaikliang, C., Wichienchot, S., Youravoug, W. & Graidist, P. 2015. Evaluation on prebiotic properties of β-glucan and oligo-β-glucan from mushrooms by human fecal microbiota in fecal batch culture. **Functional Foods in Health and Disease**, 5(11), 395-405.

Chan, G. C., Chan, W. K. & Sze, D. M. 2009. The effects of β-glucan on human immune and cancer cells. **Journal of Hematology & Oncology**, 2(1), 25.

Chandrawanshi, N. K., Tandia, D. K. & Jadhav, S. 2017. Nutraceutical properties evaluation of *Schizophyllum commune*. **Indian Journal of Scientific Research**, 13(2), 57-62.

Cheung, Y.-C., Siu, K.-C., Liu, Y.-S. & Wu, J.-Y. 2012. Molecular properties and antioxidant activities of polysaccharide–protein complexes from selected mushrooms by ultrasound-assisted extraction. **Process Biochemistry**, 47(5), 892-895.

Choi, Y., Nguyen, H. T. K., Lee, T. S., Kim, J. K. & Choi, J. 2020. Genetic diversity and dye-decolorizing spectrum of *Schizophyllum commune* population. **Journal of Microbiology and Biotechnology**, 30(10), 1525-1535.

Chumkhunthod, P., Rodtong, S., Lambert, S. J., Fordham-Skelton, A. P., Rizkallah, P. J., Wilkinson, M. C. & Reynolds, C. D. 2006. Purification and characterization of an *N*-acetyl-D-galactosamine-specific lectin from the edible mushroom *Schizophyllum commune*. **Biochimica et Biophysica Acta**, 1760(3), 326-332.

Commission, E. 2010. **Functional foods**. Publications Office of the European Union.

Concier, S. B. 2019. **Beta-glucan (*2) a moisture-rich essence taken from mushrooms**. [Online]. Available https://www.spabeaute-concier.com/spa_treatment/component/beta-glucan.html (10 March 2021).

Du, B., Yang, Y., Bian, Z. & Xu, B. 2017. Characterization and anti-inflammatory potential of an exopolysaccharide from submerged mycelial culture of *Schizophyllum commune*. **Frontiers in Pharmacology**, 8(1), 252.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. t. & Smith, F. 1956. Colorimetric method for determination of sugars and related substances. **Analytical**

Chemistry, 28(3), 350-356.

Erjavec, J., Kos, J., Ravnikar, M., Dreo, T. & Sabotič, J. 2012. Proteins of higher fungi - from forest to application. **Trends in Biotechnology**, 30(5), 259-273.

Fu, Y., Cheng, L., Meng, Y., Li, S., Zhao, X., Du, Y. & Yin, H. 2015. *Cellulosimicrobium cellulans* strain E4-5 enzymatic hydrolysis of curdlan for production of (1 ⟶ 3)-linked β-D-glucan oligosaccharides. **Carbohydrate Polymers**, 134(1), 740-744.

Garfin, D. E. (1990). One-dimensional gel electrophoresis. In **Methods in enzymology** (Vol. 182, pp. 425-441): Elsevier.

Gbolagade, J., Ajayi, A., Oku, I. & Wankasi, D. 2006. Nutritive value of common wild edible mushrooms from southern Nigeria. **Global Journal of Biotechnology and Biochemistry**, 1(1), 16-21.

Giese, E., Dekker, R. F., Barbosa, A. d. M. & Silva, R. d. 2011. Production of β-(1,3)-glucanases by *Trichoderma harzianum* Rifai: optimization and application to produce gluco-oligosaccharides from paramylon and pustulan. **Fermentation Technology**, 1(1), 1-5.

González, A., Nobre, C., Simoes, L. S., Cruz, M., Loredo, A., Rodriguez-Jasso, R. M., Contreras, J., Texeira, J. & Belmares, R. 2021. Evaluation of functional and nutritional potential of a protein concentrate from *Pleurotus ostreatus* mushroom. **Food Chemistry**, 346(1), 128884.

Guan, N., Yu, J., Liu, W. & Chen, R. 2018. A High-yielding enzymatic process for production of bioactive β-1,3-glucan oligosaccharides with defined size. **Journal of Glycobiology**, 07(01), 1-4.

Gufe, C., Ngenyoung, A., Rattanarojpong, T. & Khunrae, P. 2022. Investigation into the effects of CbXyn10C and Xyn11A on xylooligosaccharide profiles produced from sugarcane bagasse and rice straw and their impact on probiotic growth. **Bioresource Technology**, 344(1), 126319.

Halsted, C. H. 2003. Dietary supplements and functional foods: 2 sides of a coin? **The American Journal of Clinical Nutrition**, 77(4), 1001S-1007S.

Han, C. H., Zhang, G. Q., Wang, H. X. & Ng, T. B. 2010. Schizolysin, a hemolysin from the split gill mushroom *Schizophyllum commune*. **FEMS Microbiology Letters**,

309(2), 115-121.

Hobbs, C. 2005. The chemistry, nutritional value, immunopharmacology, and safety of the traditional food of medicinal split-gill fugus *Schizophyllum commune* Fr.:Fr. (Schizophyllaceae). A literature review. **International Journal of Medicinal Mushrooms**, 7(1-2), 127-139.

Ivanova, T., Titova, L. & Megalinska, G. 2015. Compositional study of *Schizophyllum commune* Fr.: Fr. grown on the new substrate breadcrumb. **Наукові доповіді НУБіП України**, 8(1), 57.

Jantan, I., Haque, M. A., Ilangkovan, M. & Arshad, L. 2019. An insight into the modulatory effects and mechanisms of action of *Phyllanthus* species and their bioactive metabolites on the immune system. **Frontiers in Pharmacology**, 10(1), 878.

Jin, Z., Hou, Q. & Niu, T. 2020. Effect of cultivating *Pleurotus ostreatus* on substrates supplemented with herb residues on yield characteristics, substrates degradation, and fruiting bodies' properties. **Journal of the Science of Food and Agriculture**, 100(13), 4901-4910.

Jung, E.-J., Bae, M.-S., Jo, E.-K., Jo, Y.-H. & Lee, S.-C. 2011. Antioxidant activity of different parts of eggplant. **Journal of Medicinal Plants Research**, 5(18), 4610-4615.

Kabuyi, M. K., Kapepula, P. M., Kabengele, J. K., Moni, B., Makengo, G., Mungitshi, P. M., Mukuta, A. T., Mutunda, C. M., Mpulusu, S. D., Ngbolua, J. P. K.-T.-N., Kabamba, N. N. & Fundu, T. M. 2017. Selenium content and antioxidant potential of some edible wild mushrooms from Bandundu area, DR Congo. **Natural Resources**, 8(2), 103-113.

Khan, A. A., Gani, A., Khanday, F. A. & Masoodi, F. A. 2018. Biological and pharmaceutical activities of mushroom β-glucan discussed as a potential functional food ingredient. **Bioactive Carbohydrates and Dietary Fibre**, 16(1), 1-13.

Kim, K., Choi, B., Lee, I., Lee, H., Kwon, S., Oh, K. & Kim, A. Y. 2011. Bioproduction of mushroom mycelium of *Agaricus bisporus* by commercial submerged

fermentation for the production of meat analogue. **Journal of the Science of Food and Agriculture**, 91(9), 1561-1568.

Kim, K. H., Kim, Y. W., Kim, H. B., Lee, B. J. & Lee, D. S. 2006. Anti-apoptotic activity of laminarin polysaccharides and their enzymatically hydrolyzed oligosaccharides from *Laminaria japonica*. **Biotechnology Letters**, 28(6), 439-446.

Klaus, A., Kozarski, M., Niksic, M., Jakovljevic, D., Todorovic, N. & Van Griensven, L. J. L. D. 2011. Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. **LWT - Food Science and Technology**, 44(10), 2005-2011.

Kumar, M., Tomar, M., Potkule, J., Verma, R., Punia, S., Mahapatra, A., Belwal, T., Dahuja, A., Joshi, S., Berwal, M. K., Satankar, V., Bhoite, A. G., Amarowicz, R., Kaur, C. & Kennedy, J. F. 2021. Advances in the plant protein extraction: Mechanism and recommendations. **Food Hydrocolloids**, 115(1), 106595.

Lam, K.-L. & Chi-Keung, C. P. 2013. Non-digestible long chain beta-glucans as novel prebiotics. **Bioactive Carbohydrates and Dietary Fibre**, 2(1), 45-64.

Lee, S. & Ki, C. S. 2020. Inflammatory responses of macrophage-like RAW264. 7 cells in a 3D hydrogel matrix to ultrasonicated schizophyllan. **Carbohydrate Polymers**, 229(1), 115555.

Leung, M. Y., Liu, C., Koon, J. C. & Fung, K. P. 2006. Polysaccharide biological response modifiers. **Immunology Letters**, 105(2), 101-114.

Li, N., Li, L., Fang, J. C., Wong, J. H., Ng, T. B., Jiang, Y., Wang, C. R., Zhang, N. Y., Wen, T. Y., Qu, L. Y., Lv, P. Y., Zhao, R., Shi, B., Wang, Y. P., Wang, X. Y. & Liu, F. 2012. Isolation and identification of a novel polysaccharide-peptide complex with antioxidant, anti-proliferative and hypoglycaemic activities from the abalone mushroom. **Bioscience Reports**, 32(3), 221-228.

Li, Q., Zhang, H.-H., Claver, I. P., Zhu, K.-X., Peng, W. & Zhou, H.-M. 2011. Effect of different cooking methods on the flavour constituents of mushroom (*Agaricus bisporus* (*Lange*) *Sing*) soup. **International Journal of Food Science and Technology**, 46(5), 1100-1108.

Li, Y., Zheng, Y., Zhang, Y., Xu, J. & Gao, G. 2018. Antioxidant activity of coconut (*Cocos nucifera* L.) protein fractions. **Molecules**, 23(3), 707.

Liu, Z., Shi, Y., Liu, H., Jia, Q., Liu, Q. & Tu, J. 2021. Purification and identification of pine nut (*Pinus yunnanensis* Franch.) protein hydrolysate and its antioxidant activity *in vitro* and *in vivo*. **Chemistry & Biodiversity**, 18(1), e2000710.

Maeng, J.-H., Muhammad Shahbaz, H., Ameer, K., Jo, Y. & Kwon, J.-H. 2017. Optimization of microwave-assisted extraction of bioactive compounds from *Coriolus versicolor* mushroom using response surface methodology. **Journal of Food Process Engineering**, 40(2).

Maity, P., Sen, I. K., Maji, P. K., Paloi, S., Devi, K. S., Acharya, K., Maiti, T. K. & Islam, S. S. 2015. Structural, immunological, and antioxidant studies of beta-glucan from edible mushroom *Entoloma lividoalbum*. **Carbohydrate Polymers**, 123(1), 350-358.

Megazyme, L. (2021). **Mushroom and yeast beta-glucan assay procedure (K-YBGL 02/21)** (pp. 1-15). Bray,Co. Wicklow, Ireland: Bray Business Park.

Menezes, R. C., Kai, M., Krause, K., Matthaus, C., Svatos, A., Popp, J. & Kothe, E. 2015. Monitoring metabolites from *Schizophyllum commune* interacting with H*ypholoma fasciculare* combining LESA-HR mass spectrometry and Raman microscopy. **Analytical and Bioanalytical Chemistry**, 407(8), 2273-2282.

Mensor, L. L., Menezes, F. S., Leitao, G. G., Reis, A. S., dos Santos, T. C., Coube, C. S. & Leitao, S. G. 2001. Screening of Brazilian plant extracts for antioxidant activity by the use of DPPH free radical method. **Phytotherapy Research**, 15(2), 127-130.

Miller, G. L. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. **Analytical Chemistry**, 31(3), 426-428.

Munir, N., Jahangeer, M., Bouyahya, A., El Omari, N., Ghchime, R., Balahbib, A., Aboulaghras, S., Mahmood, Z., Akram, M., Ali Shah, S. M., Mikolaychik, I. N., Derkho, M., Rebezov, M., Venkidasamy, B., Thiruvengadam, M. & Shariati, M. A. 2021. Heavy metal contamination of natural foods is a serious health issue: a review. **Sustainability**, 14(1), 1-20.

Nam, W., Kim, S. P., Nam, S. H. & Friedman, M. 2017. Structure-antioxidative and anti-inflammatory activity relationships of purpurin and related anthraquinones in chemical and cell assays. **Molecules**, 22(2), 265.

Nam, W., Nam, S. H., Kim, S. P., Levin, C. & Friedman, M. 2019. Anti-adipogenic and anti-obesity activities of purpurin in 3T3-L1 preadipocyte cells and in mice fed a high-fat diet. **BMC Complementary and Alternative Medicine**, 19(1), 364.

Nitschke, J., Modick, H., Busch, E., von Rekowski, R. W., Altenbach, H. J. & Molleken, H. 2011. A new colorimetric method to quantify beta-1,3-1,6-glucans in comparison with total beta-1,3-glucans in edible mushrooms. **Food Chemistry**, 127(2), 791-796.

Nowak, R., Nowacka-Jechalke, N., Juda, M. & Malm, A. 2018. The preliminary study of prebiotic potential of Polish wild mushroom polysaccharides: the stimulation effect on *Lactobacillus* strains growth. **European Journal of Nutrition**, 57(4), 1511-1521.

Okwulehie, I. C., Nwosu, C. & Okoroafor, C. J. 2007. Pharmaceutical and nutritional prospects of two wild macro-fungi found in Nigeria. **Research Journal of Applied Sciences**, 2(6), 715-120.

Oli, A. N., Edeh, P. A., Al-Mosawi, R. M., Mbachu, N. A., Al-Dahmoshi, H. O. M., Al-Khafaji, N. S. K., Ekuma, U. O., Okezie, U. M. & Saki, M. 2020. Evaluation of the phytoconstituents of *Auricularia auricula-judae* mushroom and antimicrobial activity of its protein extract. **European Journal of Integrative Medicine**, 38(1), 101176.

Otaka, K. 2006. Functional oligosaccharide and its new aspect as immune modulation. **生物高分子**, 6(1), 3-9.

Pasakawee, K., Laokuldilok, T., Srichairatanakool, S. & Utama-ang, N. 2018. Relationship among starch digestibility, antioxidant, and physicochemical properties of several rice varieties using principal component analysis. **Current Applied Science and Technology**, 18(3), 133-144.

Paudel, M. R., Chand, M. B., Pant, B. & Pant, B. 2018. Antioxidant and cytotoxic activities of *Dendrobium moniliforme* extracts and the detection of related compounds by GC-MS. **BMC Complementary and Alternative Medicine**, 18(1), 1-9.

Poojary, M. M., Orlien, V., Passamonti, P. & Olsen, K. 2017. Enzyme-assisted extraction enhancing the umami taste amino acids recovery from several cultivated

mushrooms. **Food Chemistry**, 234(1), 236-244.

Ramalingam, L., Menikdiwela, K., LeMieux, M., Dufour, J. M., Kaur, G., Kalupahana, N. & Moustaid-Moussa, N. 2017. The renin angiotensin system, oxidative stress and mitochondrial function in obesity and insulin resistance. **Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease**, 1863(5), 1106-1114.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. **Free Radical Biology and Medicine**, 26(9-10), 1231-1237.

Rodrigues, D., Freitas, A. C., Sousa, S., Amorim, M., Vasconcelos, M. W., da Costa, J. P., Silva, A. M., Rocha-Santos, T. A., Duarte, A. C. & Gomes, A. M. 2017. Chemical and structural characterization of *Pholiota nameko* extracts with biological properties. **Food Chemistry**, 216(1), 176-185.

Ruthes, A. C., Smiderle, F. R. & Iacomini, M. 2015. D-glucans from edible mushrooms: a review on the extraction, purification and chemical characterization approaches. **Carbohydrate Polymers**, 117(1), 753-761.

Saetang, N., Amornlerdpison, D., Rattanapot, T., Ramaraj, R. & Unpaprom, Y. 2022a. Processing of split gill mushroom as a biogenic material for functional food purpose. **Biocatalysis and Agricultural Biotechnology**, 41(1), 1-6.

Saetang, N. & Khanongnuch, C. 2014. Production of β-mannanase by a lipase defective mutant *Bacillus subtilis* M7 using copra meal as substrate. p. ST83-ST88. In **International Graduate Research Conference (*i*GRC 2014)**.

Saetang, N., Rattanapot, T., Manmai, N., Amornlerdpison, D., Ramaraj, R. & Unpaprom, Y. 2022b. Effect of hot water extraction process on schizophyllan from split gill mushroom. **Biomass Conversion and Biorefinery**, 1–10.

Sari, Y. W., Mulder, W. J., Sanders, J. P. & Bruins, M. E. 2015. Towards plant protein refinery: Review on protein extraction using alkali and potential enzymatic assistance. **Biotechnology Journal**, 10(8), 1138-1157.

Sawangwan, T., Wansanit, W., Pattani, L. & Noysang, C. 2018. Study of prebiotic properties from edible mushroom extraction. **Agriculture and Natural Resources**, 52(6), 519-524.

Shi, Y., Liu, J., Yan, Q., You, X., Yang, S. & Jiang, Z. 2018. *In vitro* digestibility and prebiotic potential of curdlan (1 ⟶ 3)-β-D-glucan oligosaccharides in *Lactobacillus* species. **Carbohydrate Polymers**, 188(1), 17-26.

Singdevsachan, S. K., Auroshree, P., Mishra, J., Baliyarsingh, B., Tayung, K. & Thatoi, H. 2016. Mushroom polysaccharides as potential prebiotics with their antitumor and immunomodulating properties: A review. **Bioactive Carbohydrates and Dietary Fibre**, 7(1), 1-14.

Singh, S., Raj, C., Singh, H. K., Avasthe, R. K., Said, P., Balusamy, A., Sharma, S. K., Lepcha, S. C. & Kerketta, V. 2021. Characterization and development of cultivation technology of wild split gill *Schizophyllum commune* mushroom in India. **Scientia Horticulturae**, 289(1), 110399.

Smirnou, D., Knotek, P., Nesporova, K., Smejkalova, D., Pavlik, V., Franke, L. & Velebny, V. 2017. Ultrasound-assisted production of highly-purified β-glucan schizophyllan and characterization of its immune properties. **Process Biochemistry**, 58(1), 313-319.

Stankovic, M. S. 2011. Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. extracts. **Kragujevac Journal of Science**, 33(1), 63-72.

Stephan, A., Ahlborn, J., Zajul, M. & Zorn, H. 2018. Edible mushroom mycelia of *Pleurotus sapidus* as novel protein sources in a vegan boiled sausage analog system: functionality and sensory tests in comparison to commercial proteins and meat sausages. **European Food Research and Technology**, 244(5), 913-924.

Sun, Y., Lv, F., Tian, J., Ye, X. Q., Chen, J. & Sun, P. 2019. Domestic cooking methods affect nutrient, phytochemicals, and flavor content in mushroom soup. **Food Science & Nutrition**, 7(6), 1969-1975.

Tejedor-Calvo, E., Morales, D., Marco, P., Sanchez, S., Garcia-Barreda, S., Smiderle, F. R., Iacomini, M., Villalva, M., Santoyo, S. & Soler-Rivas, C. 2020. Screening of bioactive compounds in truffles and evaluation of pressurized liquid extractions (PLE) to obtain fractions with biological activities. **Food Research International**,

132(1), 109054.

Thongsiri, C., Nagai-Yoshioka, Y., Yamasaki, R., Adachi, Y., Usui, M., Nakashima, K., Nishihara, T. & Ariyoshi, W. 2021. *Schizophyllum commune* beta-glucan: Effect on interleukin-10 expression induced by lipopolysaccharide from periodontopathic bacteria. **Carbohydrate Polymers**, 253(1), 117285.

Van Loo, J. (2006). Inulin-type fructans as prebiotics. In G. R. Gibson, and Rastall R.A. (Ed.), **Prebiotics: development and application** (pp. 59). Hoboken, New Jersey: John Wiley & Sons.

Wasunan, P., Maneewong, C., Daengprok, W. & Thirabunyanon, M. 2022. Bioactive earthworm peptides produced by novel protease-producing *Bacillus velezensis* PM 35 and its bioactivities on liver cancer cell death via apoptosis, antioxidant activity, protection against oxidative stress, and immune cell activation. **Frontiers in Microbiology**, 13(1), 892945.

Wongaem, A., Reamtong, O., Srimongkol, P., Sangtanoo, P., Saisavoey, T. & Karnchanatat, A. 2021. Antioxidant properties of peptides obtained from the split gill mushroom (*Schizophyllum commune*). **Journal of Food Science and Technology**, 58(2), 680-691.

Xu, X., Yan, H., Chen, J. & Zhang, X. 2011. Bioactive proteins from mushrooms. **Biotechnology Advances**, 29(6), 667-674.

Yelithao, K., Surayot, U., Lee, C., Palanisamy, S., Prabhu, N. M., Lee, J. & You, S. 2019. Studies on structural properties and immune-enhancing activities of glycomannans from *Schizophyllum commune*. **Carbohydrate Polymers**, 218(1), 37-45.

Yim, H. S., Chye, F. Y., Rao, V., Low, J. Y., Matanjun, P., How, S. E. & Ho, C. W. 2013. Optimization of extraction time and temperature on antioxidant activity of *Schizophyllum commune* aqueous extract using response surface methodology. **Journal of Food Science and Technology**, 50(2), 275-283.

Yin, C., Fan, X., Liu, C., Fan, Z., Shi, D., Yao, F., Cheng, W. & Gao, H. 2019. The antioxidant properties, tyrosinase and **α**-glucosidase inhibitory activities of phenolic compounds in different extracts from the golden oyster mushroom,

Pleurotus citrinopileatus (Agaricomycetes). **International Journal of Medicinal Mushrooms**, 21(9), 865-874.

Zhao, J. & Cheung, P. C. 2013. Comparative proteome analysis of *Bifidobacterium longum* subsp. *infantis* grown on β-glucans from different sources and a model for their utilization. **Journal of Agricultural and Food Chemistry**, 61(18), 4360-4370.

Zhao, S., Gao, Q., Rong, C., Wang, S., Zhao, Z., Liu, Y. & Xu, J. 2020. Immunomodulatory effects of edible and medicinal mushrooms and their bioactive immunoregulatory products. **Journal of Fungi**, 6(1), 269.

Zhong, K., Liu, L., Tong, L., Zhong, X., Wang, Q. & Zhou, S. 2013. Rheological properties and antitumor activity of schizophyllan produced with solid-state fermentation. **International Journal of Biological Macromolecules**, 62(1), 13-17.

Zhong, K., Tong, L., Liu, L., Zhou, X., Liu, X., Zhang, Q. & Zhou, S. 2015. Immunoregulatory and antitumor activity of schizophyllan under ultrasonic treatment. **International Journal of Biological Macromolecules**, 80(1), 302-308.

Zhu, F., Du, B. & Xu, B. 2016. A critical review on production and industrial applications of beta-glucans. **Food Hydrocolloids**, 52(1), 275-288.

มหาวิทยาลัยแม่โจ้
MAEJO UNIVERSITY

CURRICULUM VITAE

NAME	Mr. Nuttapong Saetang
DATE OF BIRTH	17 June 1989
EDUCATION	2008 High school, The Prince Royal's College, Chiang Mai 2012 Bachelor of Science (Honors) Class 1 in Agro-Industrial Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 2012 - 2015 Master of Science in Biotechnology, Graduate School, Chiang Mai University, Chiang Mai
WORK EXPERIENCE	March - May 2011 Trainee, National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Pathum Thani 2016 - 2018 Academic team, section biology, Ondemand Education Co., Ltd., Bangkok 2019 - 2020 Research assistant, Center of Excellence in Agricultural Innovation for Graduate Entrepreneur and Program in Biotechnology, Faculty of Science, Maejo University, Chiang Mai