

**Solid-State Fermentation of Soybean Curd Residue
by *Ganoderma lucidum* and *Lentinus edodes* and its
Activity Evaluation**

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Activity Evaluation**

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Abstract

In recent years, there has been an unprecedented increase in interest in more efficient utilization of agro-industrial residues because it provides an alternative way to reduce production costs and solve many environmental hazards. Soybean curd residue ^[1], a by-product of tofu, soymilk or soy protein manufacturing, is discharged as an agro-industrial waste. 0.7 million tons of SCR is disposed in Japan annually, and the most of SCR was incineration, which has caused severe environmental pollution.

In fact, SCR is rich in nutrients, such as proteins, fat, starch and sugar, which could allow SCR to be potentially utilized as a high quality media for the microbial fermentation.

Ganoderma lucidum (*G. lucidum*) is one of the most famous traditional Chinese medicines. Modern pharmaceutical research shows that *G. lucidum* polysaccharide has several physiological and health effects, including strong antioxidant activities, immuno-modulating activities, and anti-tumor activities.

Lentinus edodes (*L. edodes*), commonly known as the shiitake mushroom, is the second most widely used traditional Chinese medicinal mushroom in the global market. And *L. edodes* phenolic compound has been found to be an excellent antioxidant and synergist that is not mutagenic.

However, there were few reports about the production of *G. lucidum* polysaccharide and *L. edodes* phenolic compound using agricultural waste.

In this study, the optimal fermentation conditions of *G. lucidum* polysaccharide

and *L. edodes* phenolic compound using soybean curd residue as a substrate were investigated. Furthermore, the antioxidant activities and immunomodulatory activities of *G. lucidum* polysaccharide and *L. edodes* phenolic compound were assessed.

1. The effects of fermentation conditions on the production of polysaccharides from *G. lucidum* using soybean curd residue as a substrate were investigated. Based on the optimum conditions of solid-state fermentation, the fermented time, the inoculum size and the C/N ratio were optimized by response surface methodology. The optimal fermentation conditions for *G. lucidum* polysaccharide were determined to be the following: 14.53% of the inoculum size, 10.49 of the C/N ratio and 21.18 days incubation. The maximum polysaccharide yield of 48.14 ± 1.47 mg/g was obtained in the verification experiment.

2. The production of total polyphenol from *Lentinus edodes* using soybean curd residue were investigated. Based on the results of single-factor experiments, the inoculum size, the moisture content and the fermented time were optimized using central composite design in response surface methodology. As results, the optimal fermentation conditions of the total polyphenol production were determined as following: 12.13% of the inoculum size, 76.96% of the moisture content and 24 days incubation. Compared with unfermented soybean curd residue, the total polyphenol yield increased from 3.12 ± 0.02 to 22.93 ± 0.41 milligram gallic acid equivalent per gram, polysaccharide, proteins and various amino acid of fermented SCR were increased significantly.

3. *G. lucidum* polysaccharide was extracted from fermented soybean curd residue

by ultrasonics assisted extraction. The optimal extraction conditions were 30 min, 80 °C, 80 watt of the power with 10 of the water to solid ratio and *G. lucidum* polysaccharide of 115.47 ± 2.95 mg/g was obtained. Furthermore, the antioxidant and immunomodulatory activities of *G. lucidum* polysaccharide were investigated. The results showed that *G. lucidum* polysaccharide exhibited strong scavenging activities against DPPH radical, hydroxyl radical, hydrogen oxide, ABTS radical cation decolorization and reducing power, moderate ferrous chelating effect, and weak SOD-like activity. For immunomodulatory activities, *G. lucidum* polysaccharide was demonstrated to strongly stimulate the proliferation of the macrophage, the production of the nitric oxide, phagocytosis and protective effect on the macrophages from Doxorubicin (DOX) damage in a dose-dependent manner. *G. lucidum* polysaccharide seemed to play an important role in the exploration of natural antioxidants in food industry and pharmaceuticals.

4. *L. edodes* phenolic compounds (LEPC) were extracted from fermented soybean curd residue by ultrasonics assisted extraction. The optimal extraction conditions were 10 min, 40% ethanol concentration, 100 watt of the power with 30 of the ratio of water to solid and *L. edodes* phenolic compounds of 44.16 ± 2.35 mg GAE/g was obtained. *L. edodes* phenolic compounds (LEPC-I) were further purified by macroporous adsorption resins column SP825 (donated as LEPC-II). Antioxidant activities of LEPC-I and LEPC-II were investigated. The results showed that LEPC exhibited strong scavenging activities against DPPH radical, hydroxyl radical, hydrogen oxide, ABTS radical cation decolorization and reducing power, as well as

weak ferrous chelating effect and weak SOD-like activity. Furthermore antioxidant activities of LEPC-II were higher than those of LEPC-I. Therefore, LEPC-I and LEPC-II should be explored as the natural antioxidants in food industry and pharmaceuticals.

Briefly, reusing SCR for solid-state fermentation by *G. lucidum* and *L. edodes* was not only a simple and practicable method but also could reduce the effects on the environment. The fermented SCR was rich in nutritious substances and low in cost, resulting in excellent economic efficiency.

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Chapter 1 Introduction

1.1 Soybean curd residue

Up to now, various agro-industrial by-products have been used as inexpensive growth substrates for economical production of different mycelial species ^[1]. Producing tofu and soy milk generated considerable wastes soybean curd residue ^[2]. 0.7 million tons of SCR is disposed in Japan annually, mostly, it is incinerated and landfilled which has caused severe environmental pollution ^[3]. In fact, SCR is rich in carbohydrate, proteins and many other nutrients, suggesting that it is a potential source of low cost medium for the growth of mycelia. Many researchers have investigated the possibility of bioconversion of the residues by submerged and solid-state cultivation (SSC) ^[4]. The effective utilization of such agricultural waste not only solves environmental problems, but also promotes the economic value of the agricultural products.

1.1.1 Nutrition and functional properties of soybean curd residue

Soybean curd residue is low in fat, high in fiber, and also contains magnesium, iron, phosphorus and isoflavones. It contains 76% to 80% the moisture content, 20% to 40% solids and 3.5% to 4.0% proteins. On a dry weight basis soybean curd residue contains 36%-38% proteins, 9.3%-10.9% fat, 3.8%-5.3% carbohydrates and 52.8%-58.1% dietary fiber ^[5].

1.1.2 Protein and amino acid

Protein is the most important nutrient which is needed by the human body, accounting for 45% of total body dry matter and approximately 70% of the total muscle. The major components of body's cells, tissues, organs are all made up of protein and the body's metabolism, immune disease, fluid balance, genetic information transfer are also closely relating with proteins, so human protein is usually known as "the first nutrients" or "prime of life". However, it is reported that the protein in soybean curd residue is the better quality than that from other soy products ^[6]. Soy protein contains a large amount of essential amino acids and the components are extremely complete, therefore it is usually considered as one kind of complete and high-quality plant protein, and the nutritional value may equate to animal protein. The composition of amino acid in soy protein is similar to milk protein. On the other hand, it is also reported that the ratio of essential amino acids to total amino acids was similar to tofu and soy milk ^[7]. However the threonine and valine contents of soybean curd residue are high than those found in soymilk and tofu ^[8, 9].

1.1.3 Soybean dietary fiber

Soybean curd residue enriches in cell-wall polysaccharides. Characterization of this byproduct, including the protein, oil, dietary, and mineral composition, along with un-specified monosaccharides, and oligosaccharides can be found in the literature ^[1]. Soybean dietary fiber is the generic terms of macromolecular carbohydrates which are

not able to be digested by human digestive enzymes, including cellulose, pectin, xylan, mannose, etc. Soybean dietary fiber does not contain protein, vitamins, fats and other nutrients anymore after microbial degradation, but it still shows high physiological activity functions on the human body. Polysaccharide is one kind of the soluble dietary fiber of soybean which can significantly increase the growth and the ingestion rate index of body's macrophages and it also can stimulate antibody production and enhance the body's immune function.

1.2 *G. lucidum* and *G. lucidum* Polysaccharide

Ganoderma lucidum (*G. lucidum*), a medicinal fungus called “Lingzhi” in China and “Reishi” in Japan, is one of the most famous traditional Chinese medicines. *G. lucidum* has been used in dietary supplements and preventive medicines in the Far East for more than 2000 years and becomes a popular dietary supplement ingredient in western countries, with an annual global market value of over \$1.5 billion for *G. lucidum* extracts ^[10]. *G. lucidum* is a very important economic crop in certain regions of China and is used to prepare commercial bioactive extracts including the water-soluble health *G. lucidum* polysaccharide preparation (GLPL) ^[11]. Modern pharmaceutical researches showed that GLPL has some physiological and health effects, including strong antioxidant activities ^[12], immuno-modulating activities ^[13], and anti-tumor activities ^[14], etc. At present, *G. lucidum* was incubated by submerged culture using simple potato dextrose agar and there were few reports to culture *G. lucidum* and yield polysaccharide using agricultural waste with solid-state fermentation.

1.3 *L. edodes* and *L. edodes* phenolic compounds

Lentinus edodes (*L. edodes*), commonly known as the shiitake mushroom, is the second most widely used traditional Chinese medicinal mushroom in the global market. Also *L. edodes* phenolic compound (LEPC) has been found to be an excellent antioxidant and synergist that is not mutagenic ^[15]. In addition to phenolic compounds is obtainable from the biomass, mycelia also excrete polyphenol into the culture medium that differs somewhat in structure, composition and physiological activity. Therefore, mycelial cultivation has received great interest as an efficient method for industrial production of polyphenolic compounds.

1.4 Response surface methodology

One-factor-at-a-time-technique was the complex and uncertain associated the large-scale fungi fermentation usually and came from the lack of knowledge about the sophisticated interactions among various factors. Nowadays, response surface methodology (RSM) has been increasingly used for various phases of an optimization process in fermentation ^[1]. It is a powerful technique for testing multiple process variables because fewer experimental trials are needed compared to the study of one variable at a time. Also, interactions between variables can be identified and quantified by such a technique. In the last few years, RSM has been applied to optimize and evaluate interactive effects of independent factors in numerous chemical and biochemical processes such as fermentation of starch to lactic acid ^[5, 16].

1.5 Ultrasonic assisted extraction

Hot-water extraction has been widely used to extract the valuable compounds

from the plant materials, especially the extraction of polysaccharides ^[17]. However, the requirement for high temperatures and extended extraction times has disadvantages ^[18]. Development of an economical and efficient extraction technique for mushroom polysaccharides is of an urgent necessity. Various recently developed novel techniques for the extraction of bioactive substances from plants, including supercritical fluid extraction ^[19], microwave-assisted extraction ^[20] and ultrasonic assisted extraction ^[21]. Compared with the first two methods, ultrasonic-assisted extraction has the advantage of accelerating the extraction process, causing less damage to the structural and molecular properties of plant materials ^[22], and can be done at low temperatures ^[23]. Additionally, the ultrasonic device is cheaper and its operation is much easier ^[24, 25]. The former is suitable to sample preparation for analytical purpose and the latter is efficient in large-scale extraction ^[26]. For these reasons ultrasonic methods for assisting the extraction of polysaccharides from plant material are widely used today ^[27, 28]

1.6 Antioxidant activities

Cellular oxidative damage is a well-established general mechanism for cell and tissue injury and primarily caused by reactive oxygen species (ROS). These ROS can bind with most normal cellular components, and they react with unsaturated bonds of membrane lipids, denature proteins, and attack nucleic acids ^[29]. A disturbance of the balance between formation of active oxygen metabolites and the rate at which they are scavenged by enzymic and nonenzymic antioxidants is referred to as oxidative stress ^[30]. It has been suggested that oxidative stress plays an important role in some

physiological conditions and in many diseases, including diabetes mellitus (DM), myocardial infarction and carcinogenesis. Cells and biological fluids have an array of protective antioxidant mechanisms such as glucose-6-phosphate dehydrogenase, superoxide dismutase (SOD), catalase (CAT), glutathione-S-transferase (GST) and reduced glutathione, for both preventing the production of free radicals and repairing oxidative damage ^[31].

In the past decades, some natural polysaccharides and phenolic compounds have been demonstrated to play an important role as free radical scavengers in the prevention of oxidative damage in living organism and can be explored as novel potential antioxidants ^[32-34]. Moreover, previous studies indicated that antioxidant activity of polysaccharides and phenolic compounds might come from the ability to improve the activity of antioxidant enzymes, scavenge free radicals and inhibit lipid peroxidation ^[12]. It is generally admitted that the free radicals cause lipid peroxidation, decrease permeation, cause damage of membrane proteins and contribute to cellular inactivation ^[35].

Antioxidant activities of polysaccharide and phenolic compounds can be affected by many factors including their chemical components, molecular mass, structure, and conformation, even the drying methods, especially for the components that have been extracted or isolated from the raw material.

1.7 Immunomodulatory activities

Recent studies demonstrated that *G. lucidum* showed some pharmacological roles in promoting the immune system and antitumor activities ^[36, 37]. The main

bioactivity of the persimmon is beneficial for its rich polysaccharide content ^[38]. However, little reports about its immunomodulatory effects have been found. Much *in vivo* and *in vitro* evidence demonstrated that polysaccharides display immunomodulating function by stimulating both cellular and humoral immune-response ^[39].

The proliferation of the macrophages is an indicator of immune-enhancement. It has also been established that splenocytes synthesize and release factors, including nitric oxide (NO), interleukin-2 (IL-2) and tumor necrosis factor- α (TNF- α), which help to mediate a variety of cellular events in inflammation, activation of the immune response, and tissue regeneration. NO is reported to play an important role in the modulation of T helper cell differentiation and polarization ^[40]. Low concentrations of NO from activated splenocytes are beneficial as cells, along with other reactive nitrogen intermediates, they are responsible for cytostatic and cytotoxic activity against infectious organisms and tumor cells. In addition, NO plays a regulatory role in the function of natural killer cells and the expression of cytokines such as IFN- γ and transforming growth factor- β . However, overproduction of NO has been found to be associated with various diseases such as septic shock, autoimmune diseases, and chronic inflammation by increasing vascular permeability and the extravasations of fluid and proteins at the inflammatory site. Excess NO can react with DNA, causing mutations and eventually carcinogenicity. Furthermore, NO can react with superoxide to form peroxynitrite (ONOO⁻), a more potent oxidant and cytotoxic agent ^[41]. IL-2 can increase immunoglobulin synthesis and J-chain transcription in B cells, potently

augment the cytolytic activity of nature killer cells ^[42], and induce the cytolytic activity of lymphokine-activated killer (LAK) cells ^[43]. TNF- α is an important mediator involved in the onset and regulation of inflammatory and immune responses ^[44].

Phagocytosis, one of the apparent functions for macrophages, represents an early and crucial event in triggering host defenses against invading pathogens ^[45]. In addition to Fc γ R and CR3-mediated opsonic phagocytosis, macrophages can use another type of phagocytosis, the nonopsonic phagocytosis, to efficiently eradicate pathogens ^[46]. Moreover, the allogeneic cell nonopsonic phagocytosis of macrophages has played a great role in the rejection response of allografts ^[47]. Now, certain methods have been used in detecting the opsonic phagocytosis of macrophages in allogeneic immune response ^[48]. However, nonopsonic phagocytosis effects of macrophages have not been studied in allogeneic immune response due to the shortage of efficient phagocytosis analysis methods. Flow cytometry (FCM) offers rapid multi-parameter measurements of single cells in suspension. FCM techniques have been used for a while to evaluate phagocyte cellular defects and opsonic phagocytosis during diseases. Recently, multi-parameter assays by FCM have been developed to quantitatively reveal the antigen-specificity of opsonic phagocytic responses. The combined method of FCM and two photon laser scanning microscope (TPM) to evaluate the phagocytosis ability of macrophages is an elegant procedure, because it offers multi-parameter measurement of single cells ^[48-50].

1.8 Target and structure of the thesis

Soybean curd residue is an agro-industrial by-product, mostly, it was disposed. In fact, SCR generally is rich in nutrients for the solid-state fermentation of microorganisms. *Ganoderma lucidum* and *Lentinus edodes* are the most famous traditional Chinese medicines mushrooms. *G. lucidum* polysaccharide has several physiological and health effects, including strong antioxidant activities [51, 52], immuno-modulating activities [13], and anti-tumor activities, and *L. edodes* phenolic compound has been found to be an excellent antioxidant. Therefore, the purpose of this study was to investigate the optimum fermentation conditions of *G. lucidum* polysaccharide and *L. edodes* phenolic compounds by *Ganoderma lucidum* and *Lentinus edodes* using SCR as a growth medium, to extract these compounds using ultrasonic assisted extraction, and to evaluate the immunomodulatory activities and the antioxidant activities of these compounds.

Chapter 1 Introduction

To introduce the nutrients of SCR, the strains, the immunomodulatory activities and the antioxidant activities. And to explain the target of this thesis.

Chapter 2 Production of the polysaccharide and the total polyphenol from the fermented soybean curd residue by *Ganoderma lucidum* and *Lentinus edodes*

In this chapter, it investigated the single-factor fermentation conditions and the optimal fermentation conditions using response surface technology of *G. lucidum* polysaccharide and *L. edodes* phenolic compounds

Chapter 3 Extraction of *G. lucidum* polysaccharide (GLPL) and *L. edodes* polyphenol

(LEPC) from fermented soybean curd residue by ultrasonic assisted extraction

The purpose of this chapter was to investigate the optimal extraction conditions of *G. lucidum* polysaccharide and *L. edodes* phenolic compounds from the fermented SCR by the orthogonal experiments.

Chapter 4 Isolation and antioxidant activities of phenolic compounds from the fermented soybean curd residue by *Lentinus edodes*

The purpose of this chapter was to purify *L. edodes* phenolic compounds using macroporous adsorption resins column SP825 and to assess the antioxidant activities of phenolic compounds. The data showed that purified *L. edodes* phenolic compounds possessed a stronger antioxidant activities.

Chapter 5 Antioxidant activities of the crude polysaccharide from the fermented soybean curd residue by *Ganoderma lucidum*

The purpose of this chapter was to investigate the antioxidant activities of the crude *G. lucidum* polysaccharide and to assess the antioxidant activities of the polysaccharide. The data showed that *G. lucidum* polysaccharide possessed the strong antioxidant activities.

Chapter 6 Immunomodulatory activities of the crude polysaccharide from the fermented soybean curd residue by *Ganoderma lucidum*

The purpose of this chapter was to investigate the immunomodulatory activities of the crude *G. lucidum* polysaccharide. The data showed that the polysaccharide exhibited the immunomodulatory activities on the macrophage RAW 264.7 cells.

Chapter 7 Conclusions and future researches

In this chapter, the previous researches were concisely concluded, and future researches were prospected.

Chapter 2 Production of the crude polysaccharide and the total polyphenol from the fermented soybean curd residue by *Ganoderma lucidum* and *Lentinus edodes*

2.1 Introduction

A large amount of soybean curd residue arises from the food industry, especially the preparation of tofu (an Asian food which is produced from soybean). The residues are treated at great expense to prevent pollution because they have little commercial value. However, the residue is a good nutritional source for microorganisms. Some useful materials, such as riboflavin, lipase and okaramin, have been produced using soybean residue and cultures of microalgae^[53], and a lactic acid fermentation^[54] has been reported.

Solid-state fermentation (SSF) is a clean technology with great potential for application on production or extraction of biologically active compounds from natural sources. The agro-industrial residues reuse in this area is of particular interest due to their availability, low cost, and characteristics that allow obtaining different bioactive phenolic compounds, besides being an environment friendly alternative for their disposal. This area has great potential to expand in a near future due to the increased consumer desire to improve health through food.

Ganoderma lucidum is a fungus usually used in traditional Chinese medicine. Its fruiting body is called “Lingzhi” and contains a variety of chemical substances. The polysaccharide of *G. lucidum* is the major source of its biological activity and

therapeutic use. Recent studies on *G. lucidum* polysaccharide have showed many interesting biological activities, including antitumor and anti-inflammatory effects and cytotoxicity to hepatoma cells.

Lentinus edodes has been long-valued for its culinary and medicinal properties in small quantities by Native American and ethnic populations, and widely used for centuries by Asian cultures. Behind the common button and oyster mushrooms, *L. edodes* is the third most widely produced mushroom in the world. *L. edodes* is revered in Asian medicine for its health-promoting effects, including antiviral, antifungal, antioxidant, and antitumor effects boosts the immune system, lowers cholesterol, works as an anticoagulant and is helpful in cancer treatment ^[55]. Additionally, *L. edodes* phenolic compounds (LEPC) have been found to be an excellent antioxidant and synergist that is not mutagenic ^[15].

2.2 Materials and methods

2.2.1 Chemicals and reagents

D-glucose, sucrose, peptone, KH_2PO_4 , MgSO_4 , NaCl , Na_2CO_3 , NaOH and HCl , potato extract, yeast extract, agar, ethanol, trichloroacetic acid, Protein Quantification Kit-Rapid were obtained from Wako Pure Chemical, Osaka, Japan.

Folin–Ciocalteu reagent, sulfuric acid, phenol, gallic acid were purchased Sigma, Saint Louis, MO, USA.

2.2.2 The Microorganism and the culture conditions

The A50 strains of *G. lucidum* and 360 of *L. edodes* were purchased from agriculture and forestry strains Kaishas, Japan

The strains were maintained on potato dextrose agar (PDA) at 4°C. To maintain the strains activity, a mycelium square of size 5 mm × 5 mm was transferred to a fresh PDA agar every 3 months. The activation medium consisted of the following components: 2% glucose, 2% peptone, 0.4% potato extract, 0.3% KH₂PO₄, 0.15% MgSO₄, 2% agar. The initial pH was not adjusted (5.0-5.5). The mycelial agar petri dish was incubated at 25°C for 7 days. The 15 mL of liquid culture was performed in a 50 mL flask containing one unit of mycelial agar, which was 5 mm × 5 mm square that was obtained using a self-designed cutter. The flask of the liquid culture medium was composed of the following components: 2% sucrose, 2% yeast extract, 0.4% potato extract, 0.1% NaCl, 0.3% KH₂PO₄, 0.15% MgSO₄. The initial pH was from 5.0 to 5.5 and was incubated on a rotary shaker at 100 rpm and 25°C for 6 days. The seed for the solid culture was from the liquid culture. Solid-state culture experiment was performed in a 200 mL flask with wet SCR in different culture conditions and incubated at 25°C. All of the media were autoclaved at 121°C for 30 min.

2.2.3 The determination of polysaccharide

The fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The crushed powder (500 mg) was extracted with boiling water for two hours. The water-soluble polysaccharide was precipitated by adding eight volumes of 99.5% ethanol and stored at 4 °C overnight. The precipitated polysaccharide was collected by centrifuging at 7000 rpm for 30 min. Then the precipitate was dissolved in 10 mL of distilled water. The total polysaccharide was determined by the phenol-sulfuric acid method with some modifications^[56]. The color reaction was initiated by mixing 1 mL

of the polysaccharide solution with 0.5 mL of a 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubated in a boiling water bath for 15 min. After cooling to room temperature, the optical density ^[57] of the mixture was determined at 490 nm and the polysaccharide content was calculated with D-glucose as the standard. The results were expressed as milligram of glucose equivalent per gram of the fermented SCR.

2.2.4 The determination of phenolic compounds

Phenolic compounds were determined using the Folin-Ciocalteu method with some modifications ^[58]. 200 mg of fermented SCR was mixed with 7.5 mL (80% V/V) ethanol and placed on a rotary shaker at 100 rpm at 25 °C for 24 hours. Subsequently, the supernatant was collected by centrifugation at 8000 rpm for 20 min. The supernatant (0.125 mL) was mixed with distilled water (0.375 mL), the 0.5 mL of Folin–Ciocalteu reagent respectively. Three minutes later, 0.5 mL of Na₂CO₃ (20%) was added, and the mixture was brought to a final volume of 5 mL with distilled water. After being kept in the dark for 90 min, the O.D. of the mixture was read at 725 nm. The quantification was determined based on a standard curve of gallic acid (25–250 µg/mL) (Sigma). The results were expressed as milligram of gallic acid equivalent (GAE) per gram of the fermented SCR.

2.2.5 The determination of free amino acid

The fermented SCR was extracted with 80% ethanol in an 80 °C water bath for 20 min. The supernatant was recovered and the previous steps twice were repeated. After washing the solid matter with 80% ethanol, centrifuged and filtered all the

recovered supernatant. Next the supernatant was dried in bake oven at 40 °C and dissolved again with distilled water incubated at 4 °C for 12 h. Mixed the solution was with trichloroacetic acid at the ratio of 4:1 and incubated at 4 °C for 10 min followed by a centrifugation to remove the protein sediment. The pH value of the mixture was adjusted to 2-3 using NaOH and HCl (1 M), then the sample was filtered through a 0.45 µm diameter filter and determined by an auto amino acid analyzer (JLC-500/V2, Jeol Ltd., Tokyo, Japan) in accordance with the instructions of the manufacturer.

2.2.6 The determination of protein

The protein content of the fermented SCR was determined using the Protein Quantification Kit-Rapid (Wako Pure Chemical, Osaka). Briefly, 200 mg of fermented SCR was mixed with 4 mL of phosphate buffer (pH 7.6) and homogenized with a pulp refiner for 2 min. After 10 min, 6 µL of supernatant and 300 µL of Coomassie Brilliant Blue (CBB) were added into a 96-well plate separately, then the O.D. of the mixture was read at 595 nm and the protein content was calculated using a Bovine serum albumin (BSA) solution as the standard. The results were expressed as milligram of protein per gram of the fermented SCR.

2.2.7 The Experimental design and mathematical model

2.2.7.1 One-factor-at-a-time

In each experiment, one factor was changed, while holding all of the other factors constant. Different inoculum sizes (%), moisture content (%), pH value, C/N ratios and fermented time (days) were investigated by the single-factor experiments.

2.2.7.2 The optimization using response surface methodology for *G. lucidum* polysaccharide (GLPL)

The effects of process parameters in the optimum conditions for the production of GLPL were studied basing on a central composite rotatable design with three factors (the fermented time, the inoculum sizes and the C/N ratios) run by the software Stat-Ease Design-Expert 8.0.5 (Stat-Ease Corporation, USA). The ranges of independent variables were 10 to 30 days of the fermented time, 10% to 15 % of the inoculum sizes, and 5 to 15 of the C/N ratios (Table 1). Each treatment with a center point (i.e., 20 days of the fermented time, 12.5% of the inoculum size, and 10 of the C/N ratio) was replicated 3 times as previously described ^[59, 60]. This type of design was used to minimize the number of trials needed to obtain statistically valid results. Table 1 showed the coded and actual values of the process parameters. Subsequently, the experiments were conducted based on the design matrix shown in Table 2. Upon completion of all the experimental runs, the responses (the polysaccharide content) were obtained then fitted in a quadratic model using regression analysis.

2.2.7.3 The optimization using response surface methodology for *L. edodes* phenolic compounds (LEPC)

In this study, response surface methodology was used to determine the relationship of radial extension rate to the fermentation conditions (the fermented time, the moisture content and the inoculum size). The experiment (Table 3) was based on the central composite in cube design and consisted of a 3×2 the central composite design (CCD) (the fermented time, the moisture content and the inoculum size, each

at 2 levels). The ranges of independent variables were 10 to 30 days of the fermented time, 10% to 15 % of the inoculum size, and 60% to 80% of the moisture content. Each treatment with a center point (i.e., 20 days of the fermented time, 12.5% of the inoculum size, and 70% of the moisture content) was replicated 5 times as previously described. This type of design was used to minimize the number of trials needed to obtain statistically valid results.

A sequential procedure of collecting data, estimating polynomials, and checking the adequacy of the model was used. The method of least squares was used to estimate the parameters in the approximating polynomials. For the statistical analysis, Stat-Ease Design-Expert 8.0.5 was used to establish the experimental design and to test complex polynomials to model the data.

2.2.7.4. Mathematical modeling

After conducting the experiments, the coefficients of the polynomial model were calculated using the following equation :

$$y = b_0 + \sum_{i=1}^k b_i x_i + \sum_{i=1}^k b_{ii} x_i^2 + \sum_{i < j}^k b_{ij} x_i x_j \quad (1)$$

where y was the estimated response, and b_0 , b_i , b_{ii} and b_{ij} were the equation parameter estimates (constant: b_0 ; parameter estimates for linear terms: b_i ; for quadratic terms: b_{ii} ; for interaction term: b_{ij} . x_i and x_j were the levels of the factors and k was the number of factors). The model terms were selected or rejected based on the probability (P) value with a 95% confidence level. The results were completely analyzed using the analysis of variance (ANOVA) by Design Expert software. Three dimensional plots and their respective contour plots were obtained based on the

effects of the levels of the two factors. From these three-dimensional plots, the simultaneous interaction of the two factors on the responses was studied. The optimal region was also identified based on the main parameters in the overlay plot.

2.2.6 Statistical analysis

The experimental results were means \pm standard deviation (SD) of triple determinations. The data were analyzed by one-way analysis of variance (ANOVA). Tests of the significant differences were determined by Student's t-test analysis at $P = 0.05$ or independent sample t-test ($P = 0.05$).

2.3 Results

2.3.1 Screening of the single-factor for *G. lucidum* polysaccharide

Although the single-variable method was tedious and overlooked the interaction between different factors, this method was helpful for the selection of levels, making the results more reasonable and credible.

The inoculum size was an important factor for the growth rate of the mycelium and it needed to be suitable, when the inoculum size was too small, the fermentation starting time was long. In contrast, a greater inoculum size caused the nutrition to be consumed more quickly, and the fermentation could be interrupted. Figure 2-1. A indicated that 12.5% (as a ratio of the mass) was the optimum inoculum size for the production of polysaccharide, with the maximum yield of 20.44 mg/g. Greater inoculum size did not show any positive effect on the polysaccharide production.

The moisture content played an important role in solid-state fermentation. Although the fermentation with a rang from no moisture to very high initial moisture

levels has been reported ^[61], it has been observed that high moisture content led to the aggregation of the substrate particles, poor aeration and possible anaerobic conditions ^[62], whereas very low moisture content restricted the fungal growth ^[63]. The optimal moisture content depended on the nature of the microorganism and the substrate being used ^[64]. For the production of polysaccharide, 65% of the initial moisture was the optimum and the maximum polysaccharide yield was 24.45 mg/g by *G. lucidum*. Thereafter, the polysaccharide yield was reduced with increasing moisture content (Figure 2-1. B).

To estimate the effect of the initial pH values on polysaccharide production, the pH values were varied between 5.0 ± 0.2 and 7.0 ± 0.2 with 0.5 M phosphate buffer. The result was shown in Figure 2-1. C. The study suggested that 5.5 of an initial pH was the optimum pH with 29.12 mg/g of the maximum polysaccharide production. Further increasing initial pH value led to decrease in the polysaccharide yield. This result was similar to that of Ming Yao, who reported that 5.5 as the initial medium pH yielded the most GLPL ^[65].

The C/N ratio was found to be a crucial factor for the growth rate of the mycelium and for the content and the medical function of GLPL ^[66]. Therefore, it was important to adjust the C/N ratio for the production of GLPL. The total carbon and total nitrogen were 45.54% and 4.22% respectively in SCR, thus the C/N ratio was 10.8. C/N ratios of media from 5 to 40 were packed in a 200 mL flask to estimate the production of GLPL. Sucrose and yeast extract were used to adjust the C/N ratios. Figure 2-1.D showed that 10 of a C/N ratio was the optimum and that the maximum

polysaccharide yield was 44.96 mg/g by *G. lucidum*. Thereafter, the polysaccharide yield was reduced with increasing C/N ratios.

A time course of GLPL production in the solid-state fermentation was presented in Figure 2-1.E. The result clearly showed that the yield of the polysaccharide was significantly affected by the incubation time. The polysaccharide quickly increased up to 43.96 mg/g on 20 days. After that, a gradual decrease trend of polysaccharide yield was observed.

2.3.2 Screening of the single-factor for *L. edodes* phenolic compounds

The inoculum size decided the growth of the mycelium and the accumulation of LEPC, when the inoculum size was small, the fermentation starting time was long. In contrast, great inoculum size caused the nutrition to be consumed more quickly, the fermentation could be interrupted and bioactive compounds would be decreased. Therefore, suitable inoculum size was necessary to the fermentation. Figure 2-2. A indicated that 15% of the inoculum size was the optimum inoculum size for the production of LEPC, with 20.36 mg GAE/g of the maximum production.

The moisture content has an important role in SSC and although fermentation with a rang no moisture to very high initial moisture levels has been reported ^[61], it has been observed that high moisture content led to the aggregation of the substrate particles, poor aeration, and possible anaerobic conditions, whereas very low moisture content restricts the fungal growth ^[63]. The optimal moisture content depends on the nature of the microorganism and the substrate being used. For the production of LEPC, 75% of the initial moisture was the optimum and the maximum LEPC yield was 20.36

mg GAE/g from *L. edodes* (Figure 2-2.B).

L. edodes was cultivated in the basal medium with different values of initial pH value (5.0–7.0) and varied between 5.0 ± 0.2 and 7.0 ± 0.2 with 0.5 M phosphate buffer. Figure 2-2.C showed the influence of initial pH on LEPC production and the optimal initial pH was 5.5. The pH value of the medium is very important but it is often a neglected environmental factor. Shu and Lung claimed that the different morphology of the fungi mycelia under a different initial pH value was the critical factor in biomass accumulation and metabolite formation. The medium pH may affect cell membrane function, cell morphology and structure, the uptake of various nutrients, and product biosynthesis ^[67].

The C/N ratio is found to be a crucial factor for the growth rate of the mycelium and also for the content and the medical function of LEPC. Therefore, it is important to adjust the C/N ratio for the production of LEPC. The total carbon and total nitrogen were 45.54% and 4.22% respectively in SCR, thus C/N ratio was 10.8. C/N ratios of the media from 5 to 40 were packed in a 200 mL flask to investigate the production of LEPC (Figure 2-2.D). Sucrose and yeast extract were used to adjust the C/N ratio. For the production of LEPC, the maximum C/N ratio was 10, and increasing C/N ratio caused decline of LEPC. Therefore, the yield of LEPC was tapered off after C/N ratio of 10.

A time course of LEPC production in SSC was presented in Figure 2-2.E. The result clearly showed that yield of LEPC was significantly affected by the incubation time. It quickly increased up to 20.36 mg GAE/g on 25 days. A further decrease in

LEPC during the later stages of growth could be due to polymerization of the released phenolics caused by stress induced on the fungus. Our results were similar with the findings by Dhiraj A, who demonstrated that the total phenolics (17 mg/1 g pomace) was obtained after 20 days fermentation by *L. edodes* [68]. As well as, Patrick showed that *L. edodes* bioprocessing was very effective in mobilizing phenolic antioxidants from soybean powder. In ethanol extracts, the maximum LEPC (20.36 ± 0.94 mg GAE/g dry weight) was obtained for 25 days incubation [69]

2.3.3 The parameters of the solid-state fermentation of GLPL using response surface methodology

Based on the above results, a further study was conducted by response surface methodology. Three variables (the fermented time, the inoculum size and the C/N ratios) were used to determine the optimum levels of these parameters and their interactions based on 65% of the moisture content and 5.5 of the pH value. There was a considerable variation in the yield of polysaccharide depending upon the fermentation conditions, as shown in Table 2-2. The replication at the center point conditions resulted in higher yield of polysaccharide than at other levels. The predicted response Y for the production of polysaccharide was obtained as follows:

$$y = -88.75 + 2.63x_1 + 8.71x_2 + 7.93x_3 + 0.04x_1x_2 + 0.01x_1x_3 - 0.04x_2x_3 - 0.08x_1^2 - 0.29x_2^2 - 0.36x_3^2 \quad (2)$$

The statistical significance of Eq. (2); was confirmed by an F-test and the analysis of variance (ANOVA) for the response surface quadratic model was summarized in Table 2-4. The ANOVA of the quadratic regression model demonstrated that the model was significant, with an F-test of a very low probability

value ($P > F$) < 0.0001 . The goodness of the model was indicated by the determination coefficient (R^2) and the multiple correlation coefficient (R). The value of R^2 (0.9854) for Eq. (2) suggested that 98.54% of the sample variation for GLPL was attributed to the independent variables, and only approximately 1.46% of the total variation could not be explained by the model^[70]. The “Pred R-Squared” of 0.8536 was in reasonable agreement with the “Adj R-Squared” of 0.9666. The adequate precision was used to measure the ratio of signal to noise, which was generally desired to be greater than 4. In the present study, the value of this ratio (22.252) suggested that the polynomial quadratic model was an adequate signal, which could be used to navigate the design space (Table 2-4).

The P-values were used as a tool to check the significance of each coefficient, the smaller the value of P, the more significant was the corresponding coefficient^[71]. As can be seen from Table 2-4, three linear coefficients (X_1 – X_3) and two quadratic coefficients were significant. The insignificant coefficients were not omitted from Eq. (2), since it was a hierarchical model^[72].

The 3D-surface plot and 2D-projection were able to visually show the response over a region of interesting factor levels, the relationship between the response and experimental levels of each variable, and the type of interactions between the test variables to deduce the optimum conditions (Figure 2-3 (A–F)).

The 3D-surface plot and 2D-projection (Figure 2-3 (A, B)) depicted the effects of the fermented time and the inoculum size on the yield of polysaccharide, whereas the C/N ratio was fixed at its optimal concentration. The 3D-plot showed evidence

that the production of GLPL significantly increased upon increasing fermented time to approximately 20 days, but decreased sharply beyond this duration, reaching a maximum yield at approximately 22 days. The effect of the inoculum size on the yield of GLPL was also sensitive within the tested range, reaching a maximum yield at about 14. The same trends were indicated in Figure 2-3 (C-F).

By solving the inverse matrix (from Eq. (2)), the optimum values of the test variables were determined to be a 21.74 days of the fermented time, 13.68 of the inoculum size and 10.6 of the C/N ratio. In this situation, the maximum predicted production of polysaccharide was 48.98 mg/g.

The residual plots for the model and the experimental data set showed no patterns or trends (Figure 2-4). Therefore, it was concluded that the model was able to accurately predict the optimal fermentation conditions for the production of GLPL using SCR as a growth substrate for the solid-state fermentation. Cultivated with the optimized conditions, GLPL (48.14 ± 1.47 mg/g) was accumulated from fermented SCR.

2.3.4 The parameter of the solid-state cultivation of LEPC using response surface methodology

Based on the above results, further study was conducted by response surface methodology. Three variable factors (the fermented time, the moisture content and the inoculum size) were used to determine the optimum levels of these parameters and their interactions based on a 10 of the C/N ratio and 5.5 of the pH value. The design matrix of the variables in coded units was given in Table 2-3. The replication at the

center point conditions resulted in a higher total polyphenol yield than at other levels. The predicted response Y for the production of the total polyphenol was obtained as follows:

$$Y = -172.42 + 0.14x_1 + 8.98x_2 + 3.69x_3 - 0.014x_1x_2 + 0.028x_1x_3 - 0.01x_2x_3 - 0.045x_1^2 - 0.338x_2^2 - 0.028x_3^2 \quad (3)$$

where Y = experimental value of the total polyphenol (mg GAE/g), and x_i = independent variable i ($i = 1$ for the fermented time, 2 for the inoculum size, 3 for the moisture content). The fermentation conditions that the total polyphenol were assumed to be 20 days of the fermented time, 12.5% of the inoculum size and 70% of the moisture content, were used as a center point for RSM model building. To find the maximum production of the total polyphenol, increasingly complex equations from linear to quadratic were sequentially tested to model the data obtained from the trials in Table 2-5. When the data were analyzed using the various models, the P-value of regression was significant at the 0.1% α -level, whereas lack of fit was not significant at the 5% α -level only for the quadratic model.

An ANOVA using equation (3) was initially performed to investigate the possible interaction between variables (Table 2-5). The ANOVA of the quadratic regression model demonstrated that the model was significant, with an F-test of a very low probability value ($P > F$) < 0.0001 . The merit of the model was indicated by the determination coefficient (R^2) and the multiple correlation coefficient (R). The value of R^2 (0.9767) for Eq. (3) suggested that 97.67% of the sample variation was attributed to the independent variables, and only approximately 2.33% of the total variation could not be explained by the model. In addition, value of lack of fit F value

and lack of fit p-value were found to be 3.48 and 0.1298, respectively, which implied that the lack of fit was insignificant relative to the pure error due to noise. Insignificant lack of fit made the model fit. The results suggested that the proposed experimental design was suitable for the simulation of the production of the total polyphenol from *L. edodes* within the range of variables employed.

Table 2-5 showed that the fermented time \times the inoculum size, and the inoculum size \times the moisture content were not interdependent, respectively. Further statistical inspection demonstrated that the fermented time and the moisture content affected the production of *L. edodes* total polyphenol significantly at the 1% α -level. Therefore, the optimal quadratic model was used to describe the response surface of the total polyphenol yield was

$$Y = -160.22 - 0.03x_1 + 8.01x_2 + 3.56x_3 + 0.028x_1x_2 - 0.045x_1^2 - 0.338x_2^2 - 0.028x_3^2 \quad (4)$$

where Y = the experimental value of the total polyphenol content (mg GAE/g), and x_i = independent variable i ($i = 1$ for the fermented time, 2 for the inoculum size, 3 for the moisture content). The P-value of regression was significant at 0.1% α -level, whereas lack of fit was not significant at the 5% α -level only for the quadratic model (equation (4)). The determination coefficient (R^2) was 0.987 (Table 2-6). In addition, value of lack of fit F value and lack of fit p-value were found to be 2.85 and 0.166, respectively. Therefore, this equation was used to determine the conditions that would maximize the production of LEPC by setting the partial derivatives of the equation to zero with respect to the independent variables.

The 3D-plot and 2D-projection were able to visually show the response over a

region of interesting factor levels, the relationship between the response and experimental levels of each variable, and the type of interactions between the test variables to deduce the optimum conditions (Figure 2-5 (A–F)). The 3D-plot and 2D-projection (Figure 2-5 A and B) depicted the effects of the fermented time and the inoculum size on the production of LEPC, whereas the moisture content was fixed at its optimal concentration. The 3D-plot showed the evidence that LEPC significantly increased upon increasing fermented time, but decreased sharply beyond this duration, reaching a maximum yield at approximately 24 day. The effect of the inoculum size on the yield of LEPC was also sensitive within the tested range, reaching a maximum inoculum size at about 12%. The same trend is indicated in Figure 2-5 (C-F). By solving the inverse matrix (from Eq. (4)), the optimum values of the test variables in noncoding units were 12.13% of the inoculum size and 76.96% of the moisture content for 24 days of the incubation (Figure 2-5 (G, H)). The predicted maximum yield of LEPC was 23.38 mg GAE/g under the optimum condition.

The residual plots for the model and the experimental data set showed no patterns or trends (Figure 2-6). Therefore, it was concluded that the model was able to accurately predict optimal growth conditions for the production of LEPC using SCR as a growth substrate in SSC. Cultivated with the optimized conditions, the total polyphenol of fermented SCR (22.93 ± 0.41 mg GAE/g) was accumulated.

2.3.5 The improvements of nutrients fermented SCR by *G. lucidum*

Table 2-7 showed the changes of nutrients between the unfermented SCR and the fermented SCR. Cultivated with under the optimized conditions, the polysaccharide

produced on the fermented SCR (48.14 ± 1.47 mg/g) accumulated and as much as six-fold compared with that produced on the unfermented SCR. GLPL was reported to exhibit many biological activities, including antitumor, immunomodulatory, anti-viral, anti-hepatitis, antioxidant, anti-hypertension and anti-diabetic. In 2009, the in vivo antioxidant activity of *G. lucidum* polysaccharide was assessed by Jia and others using streptozotocin-induced diabetic rats. The results indicated that *G. lucidum* polysaccharide could significantly and dose-dependently increase nonenzymic/enzymic antioxidants and reduce lipid peroxidation ^[73].

For the total phenolic, the fermented SCR was 13.39 ± 0.14 mg GAE/g and fourfold contrasted with the unfermented SCR. The phenolic compounds of mushrooms have attracted more attention recently in the biochemical and medical fields because of their antioxidant activities. A very recent study demonstrated the presence of phenolic acids in both *L. deliciosus* and *C. cibarius*, which might account for their modest antioxidant effects ^[74]. Interestingly, in that experiment, no flavonoid derivatives were detected in these mushrooms.

The protein of the fermented SCR was 207.11 ± 14.38 mg/g (Table 2-7). The protein content of one kilogram of the fermented SCR is equal to the protein content of two kilogram lean meat, three kilograms of the egg or twelve kilogram the milk content, in addition this protein surpasses the above products and shows higher biological activity. After the fermentation, the protein of SCR was hydrolyzed to produce a peptide that had good solubility, the low viscosity ^[75]. Thus, this peptide was assimilated easily by in vivo digestion and was suitable for children and old

people.

In contrast to the unfermented SCR, the total amino acid content of the fermented SCR was $225.13 \pm 18.41 \mu\text{mol/g}$ representing an increase of more than twentyfold. Arginine, glycine, alanine, aspartic acid, phenylalanine and gaba amino acid were increased remarkably, and serine ($10.83 \pm 1.06 \mu\text{mol/g}$) and threonine content ($9.53 \pm 0.82 \mu\text{mol/g}$) of the fermented SCR were increased fiftyfold and forty fivefold respectively, compared with the unfermented SCR. Serine is a non-essential amino acid and contributes to the production of immunity globulin and immune bodies. Threonine is an essential amino acid for human, furthermore it is an important food enrichment that has the effects of reducing fatigue and accelerating growth. The gaba amino acid, it was increased more than eightfold. This amino acid can improve brain activity, decrease blood pressure, prevent arteriosclerosis and promote sleep. Moreover, isoleucine, lysine, leucine, taurine, histidine and neovaricaine were emerged, and glutamic acid ($20.17 \pm 1.57 \mu\text{mol/g}$) and valine ($10.23 \pm 1.44 \mu\text{mol/g}$) were more than $10 \mu\text{mol/g}$. It should be noted that lysine is an important essential amino acid for people and is beneficial for the physical growth and intelligence of children.

As the quantity and varieties of active substances in SCR were clearly evaluated after the fermentation, the fermented SCR could be a potential nutritious functional food.

2.3.6 The improvements of nutrients fermented SCR by *L. edodes*

Table 2-8 showed the changes of nutrients on the unfermented SCR and the

fermented SCR. Cultivated under the optimized conditions, total polyphenol produced on the fermented SCR (22.93 ± 0.41 mg GAE/g) accumulated and as much as sevenfold compared with that produced on the unfermented SCR. The ability of *L. edodes* to release ethanol and water soluble phenolic antioxidants from cranberry pomace via its high β -glucosidase activity, which can be of value to design food and nutraceutical products has been investigated [68]. For the polysaccharides of *L. edodes*, the fermented SCR was 50.55 ± 2.41 mg/g and fivefold contrasted with the unfermented SCR. The polysaccharides have attracted more attention recently in the biochemical and medical fields because of their immunomodulatory, antitumor effects and antibacterial effects [76-79]. The protein of the fermented SCR was 221.33 ± 16.07 mg/g (Table 2-8).

The amino acid, serine, threonine, glycine, alanine, and gaba amino acid were increased remarkably, especially, aspartic acid (56.99 ± 5.81 μ mol/g) and phenylalanine (41.10 ± 0.59 μ mol/g) of the fermented SCR were increased 185-fold and 130-fold respectively, compared with the unfermented SCR. The aspartic acid has the widespread use in the medicine, food and chemical industry. In the medicine, it uses for treating heart disease, the liver complaint and hypertension sickness, it also has the function of prevention weary. The gaba amino acid, it was increased more than sevenfold. Gaba amino acid can improve brain activity, decrease blood pressure, prevent arteriosclerosis and promote sleep. Moreover, glutamine acid, lysine, taurine and neovaricaine were emerged, and isoleucine (64.29 ± 1.78 μ mol/g), leucine (63.40 ± 4.03 μ mol/g) and valine (120.06 ± 6.41 μ mol/g) were more than 60 μ mol/g. It

should be noted that lysine is an important essential amino acid for people and is beneficial for the physical growth and intelligence of children. In contrast with the unfermented SCR, the total amino acid of the fermented SCR was 985.16 ± 46.27 $\mu\text{mol/g}$ and representing an increase of almost one hundred-fold.

As the quantity and varieties of active substances in SCR were clearly evaluated after the fermentation, the fermented SCR could be a potential nutritious functional food.

2.4 Discussion

With the popularity of bean products, the generation of their by-product, SCR, has increased massively. The annual production of SCR reaches as high as 20 million tons in China, and it is 0.7~0.8 million tons in Japan. Because the moisture ratio of fresh SCR is excessively high, that is, more than 80%, this product is acidified easily and releases a heavy stench when exposed to the air without treatments. Therefore, this material could cause severe environmental pollution. In Japan, most SCR is incinerated or landfilled directly, and the disposal cost of SCR is 10,000~20,000 yen per ton. Although there have been several reports about making SCR into a health food and feed, the processing techniques were too complex, and the cost was too high to be widely used^[80, 81]. The production of ecological feed by an edible mushroom is a new, simple and economical method to reuse SCR raw materials in solid-state fermentation.

Solid-state fermentation has gained new attention in recent years, mainly due to its advantages over submerged fermentation; these advantages include low capital

investment, solid waste management, reduced energy requirements, and improved product recovery ^[82]. Solid-state fermentation is a process whereby an insoluble substrate is fermented with sufficient moisture but without free water. The substrates generally used are low-cost agricultural residues, such as wheat straw, wheat bran, and soya hulls, which further reduce the production costs ^[83]. Thus, solid-state fermentation is a suitable way to produce ecological feed reusing SCR. Moreover, this material could be promoted for a wide range of functions and used widely. After fermentation, not only the flavor and mouth feel but also the quantity and varieties of active substances were clearly elevated in SCR.

In brief, reusing SCR for solid-state fermentation by *G. lucidum* and *L. edodes* was not only a simple and practicable method but also could reduce the effects on the environment. The fermented SCR was rich in nutritious substances and low in cost, resulting in excellent economic efficiency.

2.5 Summary

The effects of the fermentation conditions on the production of polysaccharides from *Ganoderma lucidum* (*G. lucidum*) using soybean curd residue as a substrate were investigated. Based on the optimum conditions of solid-state fermentation, the fermented time, the inoculum size and the C/N ratio were optimized by response surface methodology. The optimal fermentation conditions for *G. lucidum* polysaccharide were determined to be the following: 14.53% of the inoculum size, 10.49 of the C/N ratio and 21.18 days incubation. The maximum polysaccharide yield of 48.14 ± 1.47 mg/g was obtained in the verification experiment. Compared with the

unfermented soybean curd residue, the total phenolic compounds increased fourfold, and various amino acids were increased significantly in the fermented soybean curd residue.

The effects of the fermentation conditions on the production of the total polyphenol from *Lentinus edodes* using soybean curd residue were investigated in solid-state cultures. Based on the results of single-factor experiments, the inoculum size, the moisture content and the fermented time were optimized using central composite design in response surface methodology. As results, the optimal fermentation condition of the total polyphenol production was determined as following: 12.13% of the inoculum size, 76.96% of the moisture content for 24 days incubation. Compared with the unfermented SCR, the total polyphenol yield increased from 3.12 ± 0.02 to 22.93 ± 0.41 milligram gallic acid equivalent per gram, polysaccharides, proteins and various amino acid of the fermented SCR were increased significantly.

Table 2-1. Independent variable values of the process and their corresponding levels

Independent variable	Unit	Coded variables levels		
		-1	0	1
Fermented time	days	10	20	30
Inoculum size	%	10	12.5	15
C/N ratio	—	5	10	15

Table 2-2. The central composite experimental design matrix, and the responses and the predicted values for the polysaccharide content

Run	Independent variable			<i>G. lucidum</i> polysaccharide (mg/g)		
	Fermented time (days)	Inoculum size (%)	C/N ratio	Experimental (Y_0)	Predicted (Y_i)	$Y_0 - Y_i$
1	10	12.5	10	38.21 ± 2.25	38.32 ± 1.28	-0.11
2	10	10	15	24.79 ± 0.59	25.67 ± 0.48	-0.88
3	20	12.5	5	37.66 ± 1.14	38.26 ± 1.43	-0.60
4	20	12.5	10	49.14 ± 1.88	48.71 ± 0.81	0.43
5	10	15	5	28.59 ± 1.11	29.52 ± 1.69	-0.93
6	10	10	5	22.67 ± 1.24	21.42 ± 0.55	1.25
7	20	12.5	10	48.52 ± 1.02	48.71 ± 1.47	-0.19
8	20	12.5	15	40.07 ± 3.16	40.65 ± 2.23	-0.58
9	20	10	10	41.42 ± 0.57	42.12 ± 1.68	-0.70
10	30	15	5	35.35 ± 0.90	34.41 ± 2.12	0.94
11	10	15	15	32.32 ± 1.04	31.63 ± 0.24	0.69
12	30	12.5	10	42.23 ± 3.22	42.31 ± 0.95	-0.08
13	30	15	15	37.78 ± 1.77	38.38 ± 0.57	-0.60
14	20	15	10	50.79 ± 2.11	50.28 ± 0.09	0.51
15	30	10	15	29.74 ± 0.77	28.76 ± 0.66	0.98
16	20	12.5	10	44.85 ± 2.59	47.75 ± 0.33	-2.90
17	30	10	5	21.41 ± 1.34	22.52 ± 1.51	-1.11

With 65% of the moisture content and 5.5 of the pH value, the experiment was repeated four times and the response represents average values.

Table 2-3. CCD arrangement, the responses and the predicted values for the total polyphenol content

Run	Independent variable			Total polyphenol (Y) (mg GAE/g)		
	Fermented	Inoculum	Moisture	Experimental	Predicted	$Y_0 - Y_i$
	time (days)	size (%)	content (%)	(Y_0)	(Y_i)	
1	10	10	70	14.565	14.933	-0.368
2	30	10	70	17.797	18.353	-0.556
3	10	15	70	13.997	13.403	0.594
4	30	15	70	15.856	15.449	0.407
5	10	12.5	60	13.633	13.633	0.000
6	30	12.5	60	10.946	10.796	0.150
7	10	12.5	80	13.189	13.377	-0.188
8	20	12.5	80	22.189	22.038	0.151
9	20	10	60	15.896	15.721	0.175
10	20	15	60	13.217	13.755	-0.538
11	20	10	80	21.785	21.035	0.750
12	20	15	80	18.104	18.567	-0.463
13	20	12.5	70	21.878	22.138	-0.260
14	20	12.5	70	21.755	22.138	-0.383
15	20	12.5	70	21.936	22.138	-0.202
16	20	12.5	70	22.845	22.138	0.707
17	20	12.5	70	22.199	22.138	0.061

With 5.5 of the pH value and 10 of C/N ratio, the experiment was repeated four times and the response represents average values.

Table 2-4. Analysis of variance (ANOVA) for the regression equation of the polysaccharide content

Term	degree of	sum of	F value	Prob >F	significant
Model	9	1316.35	52.43	< 0.0001	**
X ₁	1	39.77	14.26	0.009	**
X ₂	1	200.65	71.93	< 0.0001	**
X ₃	1	36.19	12.97	0.0087	**
X ₁ X ₂	1	9.08	3.26	0.1141	
X ₁ X ₃	1	3.02	1.08	0.3327	
X ₂ X ₃	1	2.31	0.083	0.3932	
X ₁ ²	1	159.62	57.22	0.0001	**
X ₂ ²	1	9.02	3.24	0.1151	
X ₃ ²	1	220.59	79.08	< 0.0001	**
Residual	7	19.53		0.865	
Lack of fit	5	8.77	0.33		
Pure Error	2	10.76			
Cor Total	16	1335.88			
R ²		0.9854			
Adj R ²		0.9666			
Pred R ²		0.8536			
Adequate		22.522			

With 65% of the moisture content and 5.5 of the pH value, the experiment was repeated four times and the response represents average values.

p* < 0.05, ** p < 0.01

Table 2-5. Analysis of variance (ANOVA) for the regression equation of the total polyphenol content

Term	degree of freedom	sum of squares	F value	Prob >F	significant
Model	9	265.574	75.429	< 0.0001	**
X ₁	1	10.503	26.848	0.0013	**
X ₂	1	9.832	25.132	0.0015	**
X ₃	1	47.450	121.293	< 0.0001	**
X ₁ X ₂	1	0.472	1.206	0.3085	
X ₁ X ₃	1	17.045	43.570	0.0003	**
X ₂ X ₃	1	0.251	0.642	0.4495	
X ₁ ²	1	67.252	171.909	< 0.0001	**
X ₂ ²	1	16.832	43.025	0.0003	**
X ₃ ²	1	29.531	75.486	< 0.0001	**
Residual	7	2.738			
Lack of fit	3	1.980	3.480	0.1298	
Pure Error	4	0.758			
Cor Total	16	268.312			
R ²		0.9898			
Adj R ²		0.9767			
Pred R ²		0.8352			
Adequate Precision		23.642			

With 5.5 of the pH value and 10 of C/N ratio, the experiment was repeated four times and the response represents average values. p* < 0.05, ** p < 0.01

Table 2-6. Analysis of variance (ANOVA) for the optimal regression equation

Term	degree of	sum of	F value	Prob >F	significant
Model	7	264.851	98.38	< 0.0001	**
X ₁	1	10.503	27.31	0.0005	**
X ₂	1	9.832	25.56	0.0007	**
X ₃	1	47.450	123.38	< 0.0001	**
X ₁ X ₃	1	17.045	44.32	< 0.0001	**
X ₁ ²	1	67.252	174.87	< 0.0001	**
X ₂ ²	1	16.832	43.77	< 0.0001	**
X ₃ ²	1	29.531	76.79	< 0.0001	**
Residual	9	3.461			
Lack of fit	5	2.703	2.85	0.166	
Pure Error	4	0.758			
Cor Total	16	268.312			
R ²		0.9871			
Adj R ²		0.9771			
Pred R ²		0.9486			
Adequate		26.659			

With 5.5 of pH value and 10 of the C/N ratio, the experiment was repeated four times. p* < 0.05, ** p < 0.01

Table 2-7. The nutrients improvements on the unfermented SCR and the fermented SCR by *G. lucidum* under the optimum culture conditions

Nutrients	Unfermented SCR	Fermented SCR
Total amino acid	10.93 ±0.27	225.13 ±18.41
Serine	0.26 ±0.01	10.83 ±1.06
Aspartic acid	0.31 ±0.01	14.82 ±0.68
Threonine	0.21 ±0.01	9.53 ±0.82
Glycine	0.62 ±0.07	9.13 ±0.41
Alanine	1.21 ±0.14	15.69 ±1.27
Phenylalanine	0.31 ±0.01	5.08 ±0.31
Gaba amino acid	0.57 ±0.01	4.90 ±0.27
Arginine	0.56 ±0.06	6.73 ±0.36
Valine	0	10.23 ±1.44
Neovaricaine	0	2.3 ±0.03
Leucine	0	4.42 ±0.43
Isoleucine	0	4.71 ±0.38
Taurine	0	6.18 ±0.35
Glutamic acid	0	20.17 ±1.57
Lysine	0	3.42 ±0.13
Tyrosine	0	5.68 ±0.42
Histidine	0	1.74 ±0.09
Methionine	0	1.5 ±0.06
Polysaccharide (mg/g)	8.01 ±0.54	48.14 ±1.47
Total phenolic (mg GAE/g)	3.12 ±0.02	13.39 ±0.14
Protein (mg/g)	219.56 ±14.17	207.11 ±14.38

With the optimized fermentation conditions, which were 5.5 of the pH value, 65% of the moisture content, 13.68% of the inoculum size, 10.68 of the C/N ratio and 21.74 days of the fermented time, soybean curd residue was fermented by *Ganoderma lucidum*. The experiment was repeated four times.

Table 2-8. The nutrients change on the unfermented SCR and the fermented SCR by *L. edodes*

	Nutrients	Unfermented SCR	Fermented SCR
	Total amino acid	10.93 ±0.27	985.16 ±46.27
	Serine	0.26 ±0.01	15.27 ±0.25
	Aspartic acid	0.31 ±0.01	56.99 ±5.81
	Threonine	0.21 ±0.01	9.68 ±0.92
	Glycine	0.62 ±0.07	10.04 ±0.20
	Alanine	1.21 ±0.14	11.56 ±1.40
	Phenylalanine	0.31 ±0.01	41.10 ±0.59
Amino acid	Gaba amino acid	0.57 ±0.01	4.26 ±0.08
	Arginine	0.56 ±0.06	13.84 ±0.35
	Valine	0	120.06 ±6.41
	Neovaricaine	0	46.52 ±0.83
	Leucine	0	63.40 ±4.03
	Isoleucine	0	64.29 ±1.78
	Taurine	0	33.44 ±1.07
	Glutamic acid	0	26.88 ±1.95
	Lysine	0	9.25 ±0.63
	Total polyphenol (mg GAE/g)	3.12 ±0.02	22.93 ±0.41
	Polysaccharide (mg/g)	8.00 ±0.54	50.55 ±2.41
	Protein (mg/g)	219.56 ±14.17	221.33 ±16.07

With the optimized fermentation conditions, which were 5.5 of the pH value, 10 of the C/N ratio, 12.13% of the inoculum size, 76.96% of the moisture content and 24 days of the fermented time, soybean curd residue was fermented by *Lentinus edodes*. The experiment was repeated four times.

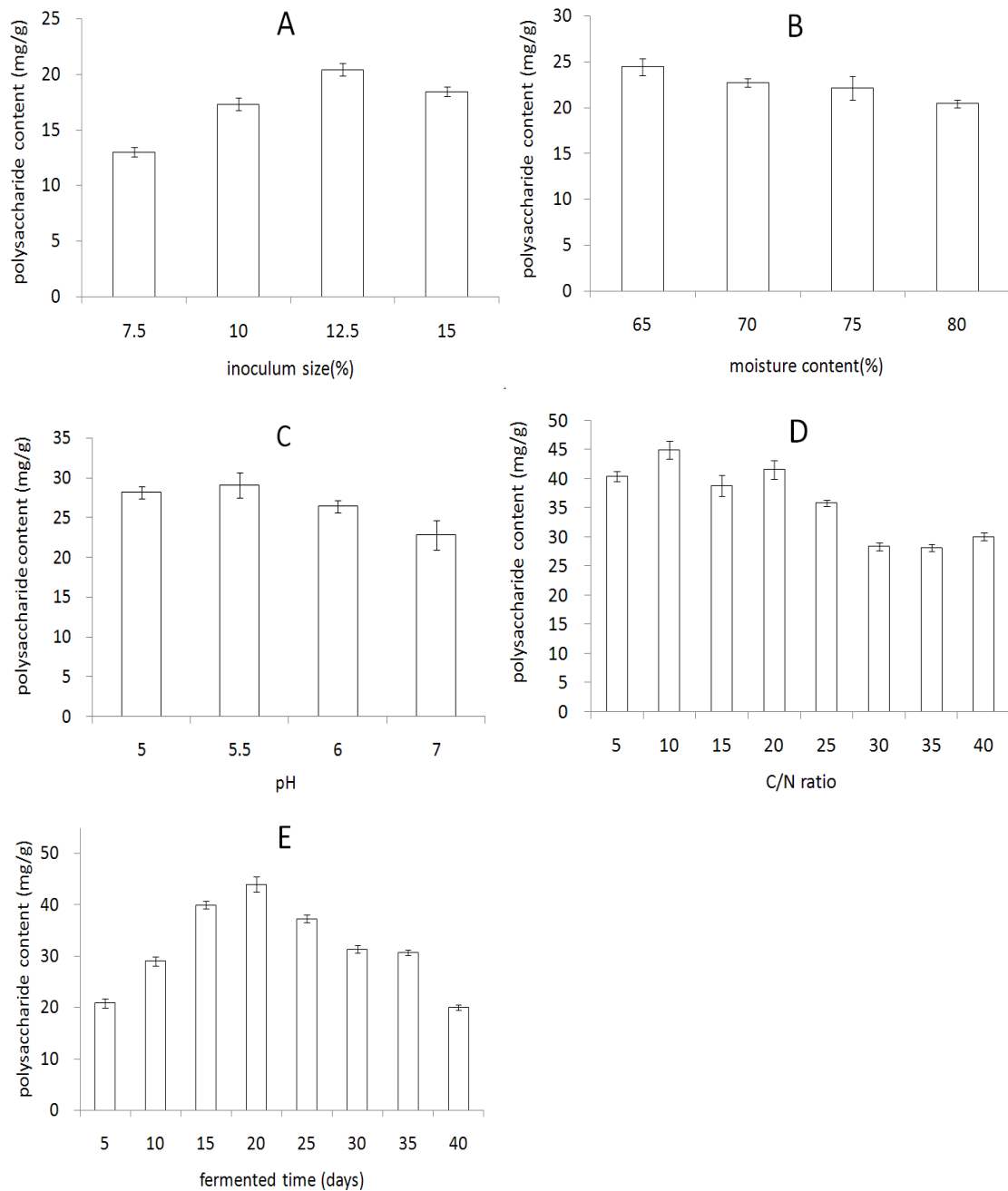


Figure 2-1. The production of polysaccharide in extracts of the fermented soybean curd residue following the solid-state bioprocessing with *G. lucidum* in different single-factor experiments.

The data were means of triplicate. The inoculum size (A); the moisture content (B); the pH value (C); the C/N ratio (D); the fermented time (E).

The bars designate standard deviations (a 95% confidence, t-test).

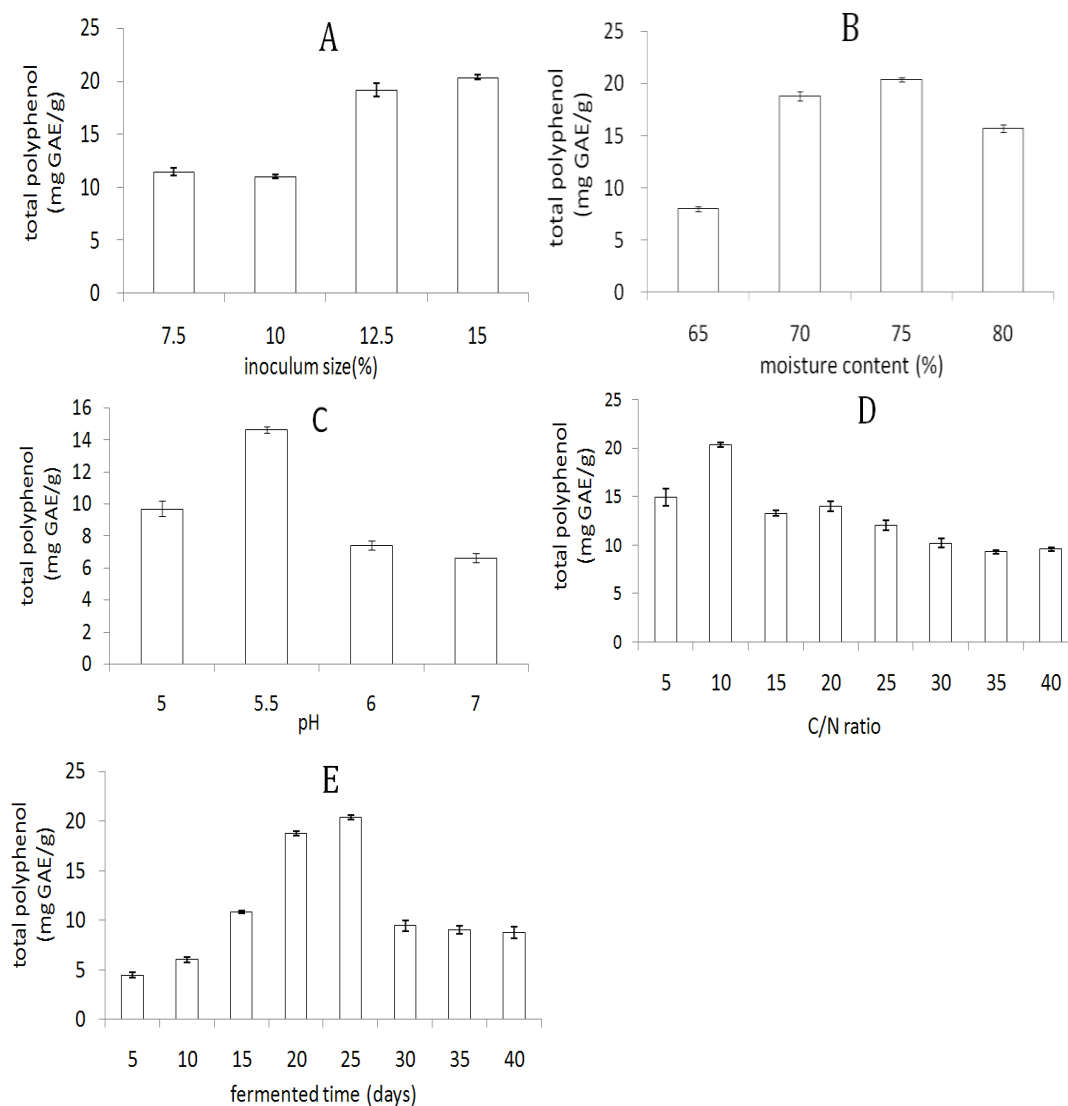


Figure 2-2. The production of total polyphenol in extracts of the fermented soybean curd residue following the solid-state bioprocessing with *L. edodes* in different single-factor experiments.

The data were means of triplicate. The inoculum size (A); the moisture content (B); the pH value (C); the C/N ratio (D); the fermented time (E).

The bars designate standard deviations (a 95% confidence, t-test).

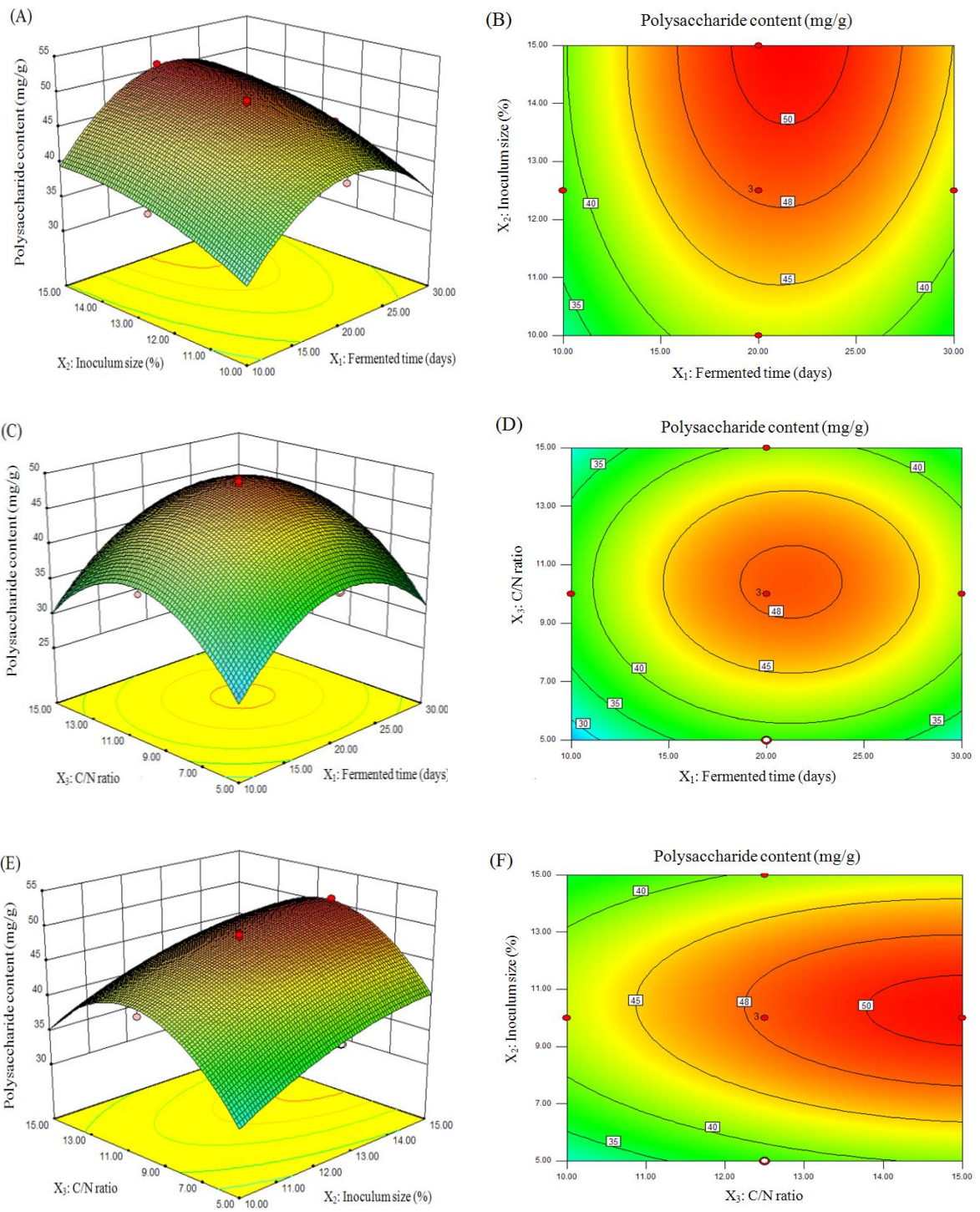


Figure 2-3. Response surface 3D-surface plot and 2D-projection showing the effects of the fermented time, the inoculum size and the C/N ratio on the yield of *G. lucidum* polysaccharide.

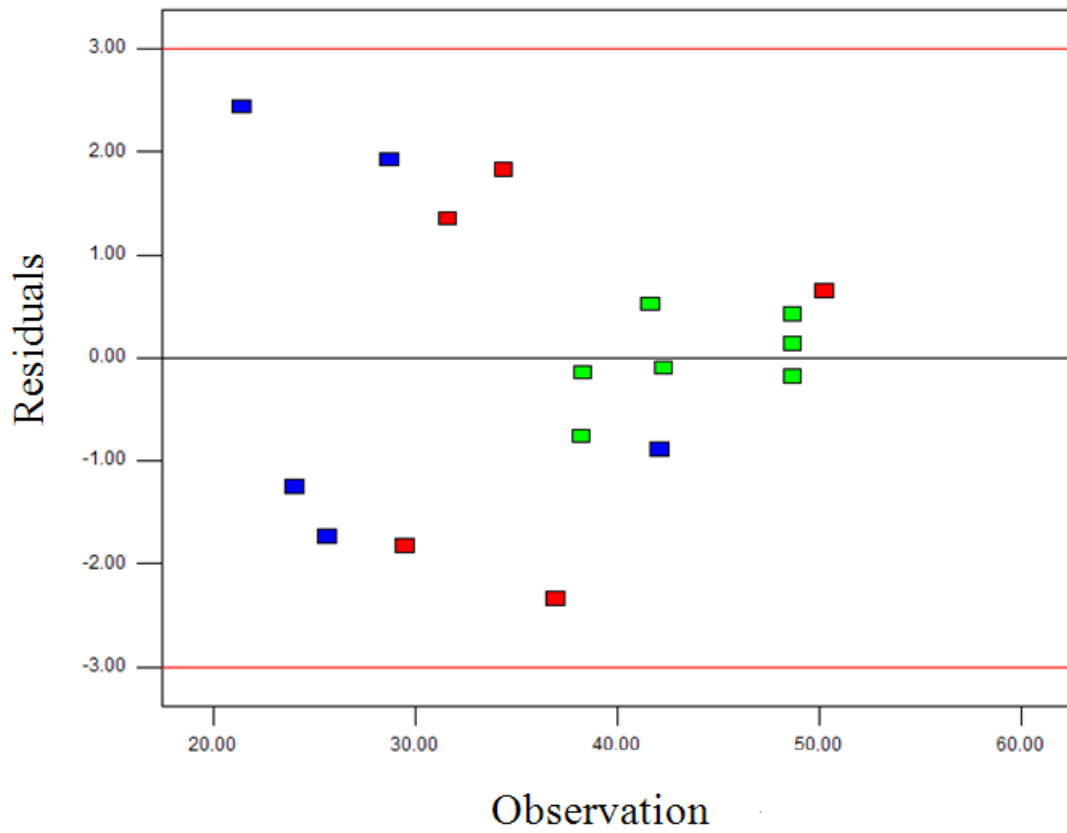


Figure 2-4. Residual plots of the quadratic model for the radial extension rate.

Each residual was calculated using the equation (2).

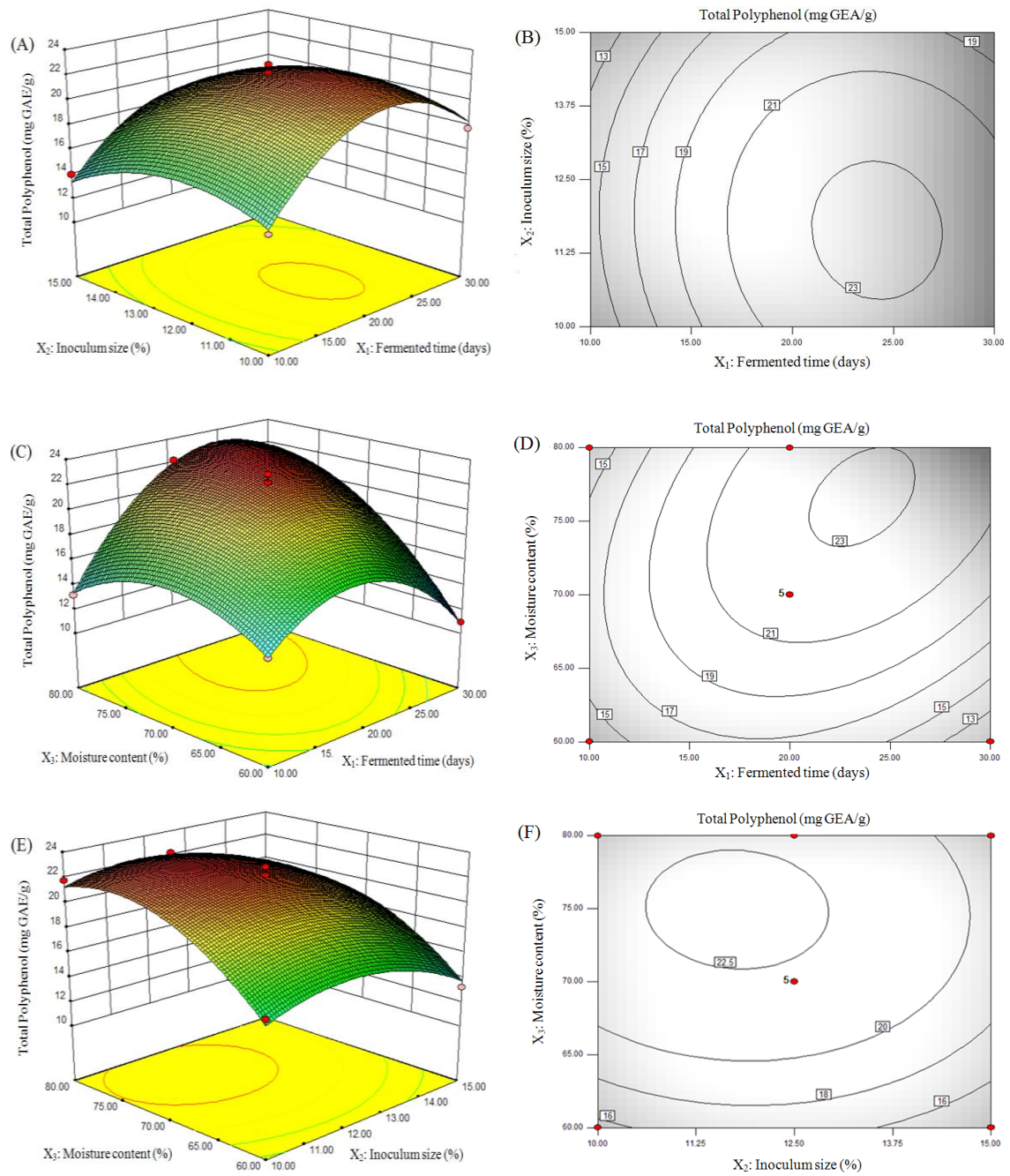


Figure 2-5. Response surface 3D-surface plot and 2D-projection showing the effects of the fermented time, the inoculum size and the moisture content on the yield of *L. edodes* total polyphenol.

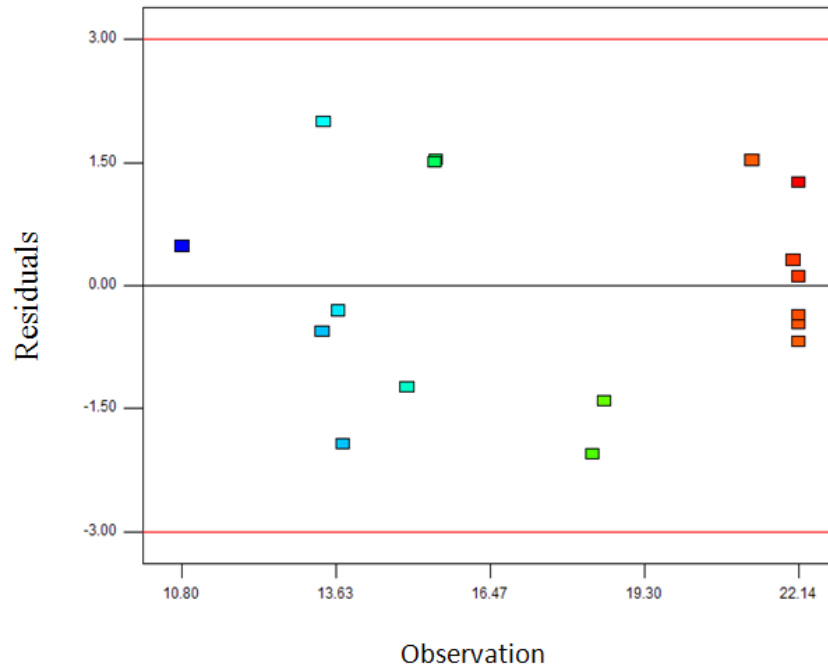


Figure 2-6. Residual plots of the quadratic model for radial extension rate.

Each residual was calculated using the equation (4).

Chapter 3 Extraction of *G. lucidum* polysaccharide (GLPL) and *L. edodes* polyphenol (LEPC) from the fermented soybean curd residue by ultrasonic assisted extraction

3.1 Introduction

Recently, various novel extraction techniques have been developed for the extraction of bioactive compounds such as ultrasonic assisted extraction, microwave assisted extraction and supercritical fluid extraction [25]. Among these, ultrasound-assisted extraction is one of the most inexpensive, simple and efficient techniques [27, 84, 85], which can increase the yield of extracted components, reduce extraction time and make higher processing throughput. It is very useful for the extraction of thermolabile and unstable compounds, presumably by avoiding degradation reactions [22]. Modern pharmaceutical researches show that GLPL and LEPC have some physiological and health effects, including strong antioxidant activities, immune-modulating activities, and anti-tumor activities, etc. However, due to the low purity and extraction efficiency, their practical value was limited. Therefore, in this study, the optimal extraction conditions of the polysaccharides and the total polyphenol were investigated from solid-state fermentation by *G. lucidum* and *L. edodes* using ultrasonic assisted extraction.

3.2 Materials and methods

3.2.1 Chemicals and reagents

Na₂CO₃, ethanol, D-glucose and concentrated sulfuric acid were obtained from

Wako Pure Chemical, Osaka, Japan. Folin–Ciocalteu reagent and gallic acid were obtained from Sigma, Saint Louis, MO, USA.

3.2.2 The extraction of GLPL from fermented SCR

By ultrasonic assisted extraction, GLPL was extracted from the fermented SCR, and it was determined by the phenol–sulphuric acid method using D-glucose as a standard ^[86]. Briefly, the fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The crushed powder (1 g) was extracted by ultrasonic assisted extraction with different conditions. Then the water-soluble polysaccharide was precipitated by adding eight volumes of 99.5% ethanol and stored at 4 °C overnight. The precipitated polysaccharide was collected by centrifuging at 7000 rpm for 30 min. Then the precipitate was dissolved in 10 mL of distilled water. The total polysaccharide was determined by the phenol-sulfuric acid method. The color reaction was initiated by mixing 1 mL of the polysaccharide solution with 0.5 mL of a 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubated in a boiling water bath for 15 min. The optical density ^[57] of the mixture was determined at 490 nm and the polysaccharide content was calculated with D-glucose as the standard. The results were expressed as milligram of glucose equivalent per gram of the fermented SCR.

3.2.3 The extraction of LEPC from the fermented SCR

By ultrasonic assisted extraction, LEPC were extracted from the fermented SCR, and LEPC were determined using the Folin-Ciocalteu method with some modifications ^[58]. Briefly, different concentrations of LEPC (0.125 mL) were mixed

with distilled water (0.375 mL), 0.5 mL of the Folin–Ciocalteu reagent respectively. Three minutes later, 0.5 mL of Na₂CO₃ (20%) was added, and the mixture was brought to a final volume of 5 mL with distilled water. After being kept in the dark for 90 min, the O.D. of the mixture was read at 725 nm. The quantification was determined based on a standard curve of gallic acid (25–250 µg/mL). The results were expressed as milligram of gallic acid equivalent (GAE) per gram of the fermented SCR.

3.2.4 Statistical analysis

The experimental results were means ± standard deviation (SD) of triple determinations. The data were analyzed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Student's t-test analysis at $P = 0.05$ or independent sample t-test ($P = 0.05$)

3.3 Results

3.3.1 The optimization of GLPL extraction

There were many factors affecting GLPL extraction by ultrasonic assisted extraction, such as extraction time, extraction temperature, the ultrasonic power and the ratio of liquid to solid (Table 3-1). According to the orthogonal experiment design L₉(3⁴), a total of four factors above, each with three different levels, was selected in the study. The results were showed in Table 3-2. This table showed the range of the product yield of GLPL varied from 37.35 mg/mL to 115.47 mg/mL and these data were taken as the original data to use in the range analysis and ANOVA. For each factor, a higher mean value (K_{ji}) indicates that the level has a larger effect on the

product yield. Therefore, the best level for each factor can be determined according to the highest mean value of the experimental condition (K_{ji}). In Table 3-2, the highest product yield of GLPL for each level was clearly distinguished, as extraction time was 30 min (73.46), extraction temperature was 80 °C (90.13), the ultrasonic power was 80 Watt (71.09) and the ratio of liquid to solid was 10 : 1 (71.59), since K_{ji} was the highest at these combinations ($A_3B_3C_2D_1$), which was number 9. The range value (R_j) indicates the significance of the factor's effect and a larger R_j means the factor has a bigger impact on the product yield. Therefore, compared with the range values of different factors (R_j), the factors' levels of significance were as follows: extraction temperature (42.00) > extraction time (17.83) > the ultrasonic power (12.99) > the ratio of liquid to solid (11.06). The range value was the largest, which meant a small change in extraction temperature could produce a significant change in the yield of GLPL. The product yield of GLPL changed slightly with changes in the ratio of liquid to solid because the range value of the ratio of liquid to solid was so small. The result of the orthogonal experiment also showed that all of the single-factor effects on the yield of GLPL were significant ($P < 0.01$) (Table 3-3). The mean yield of GLPL under the optimum extracted conditions was 115.47 ± 2.95 mg/g.

3.3.2 The optimization of LEPC extraction

There were many factors affecting LEPC extraction by ultrasonic assist extraction, such as extraction time, ethanol concentration, the ultrasonic power and the ratio of liquid to solid (Table 3-4). According to the orthogonal experiment design $L_9(3^4)$, a total of four factors above, each with three different levels, was selected in

the study. The results were showed in Table 3-5. This table showed that the range of the product yield of LEPC varied from 16.17 to 31.86 mg GAE/g and these data were taken as the original data to use in the range analysis and ANOVA. The mean values of K (K_{ji}) for different factors at different levels in the range analysis were shown in Table 3-5. For each factor, a higher mean value (K_{ji}) indicates that the level has a larger effect on the product yield. Therefore, the best level for each factor can be determined according to the highest mean value of the experimental condition (K_{ji}). In Table 3-5, the highest product yield of LEPC for each level was clearly distinguished, as extraction time was 10 min (25.44), ethanol concentration was 40% (27.24), the ultrasonic power was 100 Watt (26.57) and the ratio of liquid to solid was 30 : 1 (28.55), since K_{ji} was the highest at these combinations ($A_1B_1C_2D_3$). The range value (R_j) indicates the significance of the factor's effect and a larger R_j means the factor has a bigger impact on the product yield. Therefore, compared with the range values of different factors (R_j), the factors' levels of significance were as follows: the ratio of liquid to solid (11.07) > ethanol concentration (7.61) > the ultrasonic power (5.86) > extraction time (3.57). The range value was the largest, which meant a small change in the ratio of liquid to solid could produce a significant change in the product yield of LEPC. The product yield of LEPC changed slightly with changes in extraction time because the range value of extraction time was small. The result of the orthogonal experiment also showed that all of the single-factor effects on the yield of LEPC were significant ($P < 0.01$) (Table 3-6). According to the verification tests, the mean yield of LEPC under the optimum extraction conditions was 41.16 ± 2.35 mg GAE/g.

fermented SCR showed a high content of LEPC, compared with Ajila et al. who demonstrated that the polyphenol content of the extracts was found to be in the range of 5.78–16.12 mg GAE/g DW of samples ^[87].

3.4 Discussion

The traditional production of polysaccharides and phenolic compounds are extracted from *G. lucidum* fruiting bodies and *L. edodes* fruiting bodies, however, the time of the incubation is more than 60 days. And the content of extracted polysaccharides and phenolic compounds is below 100 mg/g. In this study, GLPL and LEPC were produced using SCR, it could greatly reduce the production time. Therefore, it is a promising new technology for GLPL production.

For the present, several conventional extraction techniques have been reported for the extraction of GLPL. Hot water technology is the main and most conventional extraction method for polysaccharides mentioned in recent studies. However, it is usually associated with longer extraction time and higher temperature but lower extraction efficiency. Development of an economical and efficient extraction technique for mushroom polysaccharides is of an urgent necessity. Various recently developed novel techniques for the extraction of bioactive substances from plants, including supercritical fluid extraction ^[19], microwave-assisted extraction ^[20] and ultrasonic-assisted extraction ^[88]. Compared with the first two methods, ultrasonic-assisted extraction has the advantage of accelerating the extraction process, causing less damage to the structural and molecular properties of plant materials ^[22], and can be done at low temperatures ^[23]. For these reasons ultrasonic methods for

assisting the extraction of polysaccharides from plant material are widely used today [27, 89]. Our results showed that yield of GLPL (11.55%) was increased significantly by ultrasonic-assisted extraction, and this extraction yield was higher than previous report, which was 10.29% from *G. lucidum* fruiting body [90].

Currently, several conventional extraction techniques have been reported for the extraction of phenolic compounds. Solvents extraction is the main and most conventional method for phenolic compounds mentioned in recent studies [91]. However, it is usually associated with longer extraction time but lower extraction efficiency. Recently, ultrasonic-assisted extraction has been widely employed in the extraction of target compounds from different materials owing to its facilitated mass transfer between immiscible phases through super agitation at low frequency [92]. It offers high reproducibility at shorter times, simplified manipulation, and lowered energy input, as well as solvent consumption [22]. By using conventional extraction under ultrasound irradiation (20–100 kHz), structural changes and degradation of target compounds can be avoided [93]. Thus, ultrasonic-assisted extraction may be an effective and advisable technique for the extraction of phenolic compounds. Our results showed that yield of LEPC (41.16 ± 2.35 mg GAE/g) was increased significantly by ultrasonic-assisted extraction.

3.5 Summary

The crude *G. lucidum* polysaccharide (GLPL) was extracted from fermented soybean curd residue by ultrasonics assisted extraction. The optimal extraction conditions were 30 min, 80 °C, 80 watt of the power with 10 of the water to solid

ratio and 115.47 ± 2.95 mg/g of GLPL yield was obtained

L. edodes phenolic compounds (LEPC) were extracted from fermented soybean curd residue by ultrasonics assisted. The optimal extracted conditions were 10 min, 40% ethanol concentration, 100 watt of the power with 30 of the ratio of water to solid and 44.16 ± 2.35 mg GAE/g of LEPC was obtained.

Table 3-1. Levels and factors affecting the yield of GLPL

Level	Factors			
	Factor A	Factor B	Factor C	Factor D
	Time (min)	Temperature (°C)	Power (W)	Liquid: Solid ^a
1	10	30	50	10 : 1
2	20	50	80	20 : 1
3	30	80	110	30 : 1

^a Liquid: Solid was Liquid: Solid (g)

Table 3-2. Experimental design and results of the orthogonal experiment L₉ (3⁴)

Run	Factor A	Factor B	Factor C	Factor D	Polysaccharide
No.	Time (min)	Temp (°C)	Power (W)	Liquid: Solid ^a	(mg/g)
1	10	30	50	10 : 1	44.64 ± 1.30 ^b
2	10	50	80	20 : 1	60.46 ± 1.15
3	10	80	110	30 : 1	85.95 ± 4.31
4	20	30	80	30 : 1	37.35 ± 1.40
5	20	50	110	10 : 1	54.65 ± 1.48
6	20	80	50	20 : 1	68.96 ± 2.40
7	30	30	110	20 : 1	48.49 ± 0.62
8	30	50	50	30 : 1	56.41 ± 2.96
9	30	80	80	10 : 1	115.47 ± 2.95
K ₁ ^c	63.68 ± 3.62	43.50 ± 2.35	56.67 ± 2.45	71.59 ± 2.89	
K ₂	53.65 ± 3.91	57.17 ± 3.44	71.09 ± 4.37	59.90 ± 1.57	
K ₃	73.46 ± 4.66	90.13 ± 5.09	63.03 ± 1.90	59.30 ± 2.84	
R ^d	17.83 ± 1.03	42.00 ± 2.72	12.99 ± 1.06	11.06 ± 1.03	
Optimal level	3	3	2	1	

^a Liquid: Solid was Liquid: Solid (g)

^b Values were mean of three determinations with standard deviation (±).

^c $K_i^A = \sum \text{polysaccharide yield at } A_i$. Values were mean of three determinations with standard deviation (±).

^d $R^A = \max\{K_i^A\} - \min\{K_i^A\}$. Values were mean of three determinations with standard deviation (±).

Table 3-3. Variance analysis of the orthogonal experiment

Variation source	d.f.	Sum of squares	F value	<i>P</i> (t-test)
Time (min)	2	1764.48	148.86	< 0.001
Temp (°C)	2	10342.13	872.48	< 0.001
Power (W)	2	940.32	79.33	< 0.001
Liquid ^[44] : Solid (g)	2	863.39	72.84	< 0.001
Error	2	94.83		
Sum	16	14009.04		

d.f. : degree of freedom

Table 3-4. Levels and factors affecting the yield of LEPC

Factors				
	Factor A	Factor B	Factor C	Factor D
Level	Time (min)	Ethanol concentration (%)	Power (W)	Liquid: Solid ^a
1	10	40	50	10 : 1
2	20	60	100	20 : 1
3	30	80	150	30 : 1

^a Liquid: Solid was Liquid: Solid (g)

Table 3-5. The experimental design and results of the orthogonal experiment $L_9 (3^4)$

Run	Factor A	Factor B	Factor C	Factor D	LEPC
No.	Time (min)	Concentration	Power (W)	Liquid: Solid ^a	(mg GAE/g)
1	10	40	50	10 : 1	18.25 ± 0.29 ^b
2	10	60	100	20 : 1	31.72 ± 0.56
3	10	80	150	30 : 1	26.33 ± 0.49
4	20	40	100	30 : 1	31.61 ± 0.93
5	20	60	150	10 : 1	17.82 ± 0.20
6	20	80	50	20 : 1	16.17 ± 0.10
7	30	40	150	20 : 1	31.86 ± 1.18
8	30	60	50	30 : 1	27.71 ± 1.13
9	30	80	100	10 : 1	16.38 ± 0.35
K_1^c	25.44 ± 1.53	27.24 ± 0.64	20.71 ± 0.88	17.48 ± 0.49	
K_2	21.87 ± 0.82	25.75 ± 1.03	26.57 ± 1.20	26.59 ± 1.48	
K_3	25.32 ± 1.09	19.63 ± 0.71	25.34 ± 1.35	28.55 ± 0.54	
R^d	3.57 ± 0.14	7.61 ± 0.37	5.86 ± 0.40	11.07 ± 0.56	
Optimal level	1	1	2	3	

^a Liquid: Solid was Liquid: Solid (g)

^b Values were mean of three determinations with standard deviation (\pm).

^c $K_i^A = \sum$ polysaccharide yield at A_i . Values were mean of three determinations with standard deviation (\pm).

^d $R^A = \max\{K_i^A\} - \min\{K_i^A\}$. Values were mean of three determinations with standard deviation (\pm).

Table 3-6. Variance analysis of the orthogonal experiment

Variation source	d.f.	Sum of squares	F values	<i>P</i> (t-test)
Time (min)	2	74.07	70.75	< 0.001
Ethanol concentration (%)	2	293.00	279.84	
Power (W)	2	171.80	164.09	< 0.001
Liquid ^[44] : Solid (g)	2	627.61	599.43	< 0.001
Error	2	8.38		
Sum	16	1175.15		

d.f. : degree of freedom

Chapter 4 Isolation and antioxidant activities of the phenolic compounds from the fermented soybean curd residue by *Lentinus edodes*

4.1 Introduction

In recent years, the studies on oxidative stress and its adverse effects on human health have become a subject of interest. Oxidative stress occurs when reactive oxygen species (ROS) overload the body's antioxidant defense or when the antioxidant defense system loses its capacity for response, which can lead to damage of cellular components ^[57]. Oxidation is an essential biological process for energy production in many living organisms. However, excessive ROS produced in vivo during some oxidative reactions, are strongly associate with the etiology and/or progression of a number of diseases, such as atherosclerosis, cancer and other degenerative diseases ^[58, 94-96]. In the recent years, increasing numbers of reports confirm that many fruits and vegetables may afford protection against some chronic diseases caused by oxidative stress. Because they contain a wide variety of free radical scavenging molecules, such as polyphenol, flavonoids, Vitamin C, carotenoids and tocopherols ^[97, 98], which can scavenge radicals by inhibiting initiation and breaking chain propagation or suppressing formation of free radicals by binding to the metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen. So these phytochemicals are supposed to play an important role in the prevention of these oxidation-induced diseases ^[99].

4.2 Materials and methods

4.2.1 Chemicals and reagents

Na_2CO_3 , phenol, sulphuric acid, ascorbic acid, hydrogen peroxide, chloride ferric, potassium ferricyanide, ferrous sulfate, potassium persulphate, trichloroacetic acid, sodium salicylate and ethylenediamine tetraacetic acid (EDTA) were obtained from Wako Pure Chemical, Osaka, Japan. SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Gallic acid, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All other chemical reagents were of analytical grade.

4.2.2 The extraction from the fermented SCR and the purification

L. edodes phenolic compounds were determined using the Folin-Ciocalteu method with some modifications^[58]. Briefly, different concentrations of LEPC (0.125 mL) was mixed with distilled water (0.375 mL), 0.5 mL of the Folin–Ciocalteu reagent (Sigma, Saint Louis, MO, USA) respectively. Three minutes later, 0.5 mL of Na_2CO_3 (20%) was added, and the mixture was brought to a final volume of 5 mL with distilled water. After being kept in the dark for 90 min, the optical density of the mixture was read at 725 nm. The quantification was determined based on a standard curve of gallic acid (25–250 $\mu\text{g}/\text{mL}$). The results were expressed as milligram of gallic acid equivalent (GAE) per gram of fermented SCR.

By ultrasonic assisted extraction, LEPC were extracted from fermented SCR with 10 min, 40% of ethanol concentration, 100 watt of the power of and 30 of the

ratio water to solid, denoted as LEPC-I fraction. Then the supernatant was collected by centrifugation at $8000\times g$ for 20 min and lyophilized. The LEPC-I was further purified by macroporous adsorption resins column SP825 (1.6×60 cm). The desorption conditions were as follows: eluted by 250 mL of water, followed by 250 mL of ethanol/water (40:60, v/v), 1 mL/min of flow rate. The ethanol–water eluate was collected and lyophilized under vacuum, denoted as LEPC-II fraction. LEPC-I and LEPC-II were stored at $4\text{ }^{\circ}\text{C}$ for determination of the content and antioxidant activities.

4.2.3 Measurement of chemical contents

The carbohydrate content was determined by the phenol–sulphuric acid method using D-glucose as a standard.

The protein content was quantified by the Protein Quantification Kit-Rapid (Wako Pure Chemical, Osaka) with bovine serum albumin as a standard.

4.2.4 Assay for antioxidant activities of LEPC-I and LEPC-II

4.2.4.1 HO• scavenging activity

HO• scavenging activity was measured according to a literature procedure with a few modifications^[100]. HO• were generated from FeSO_4 and H_2O_2 , and detected by their ability to hydroxylate salicylate. The reaction mixture (2.5 mL) contained 0.5 mL of FeSO_4 (1.5 mM), 0.35 mL of H_2O_2 (6 mM), 0.15 mL of sodium salicylate (20 mM), and 1 mL of different concentrations of LEPC-I and LEPC-II. Ascorbic acid was used as the positive control. After incubation for 1 h at $37\text{ }^{\circ}\text{C}$, the absorbance of the hydroxylated salicylate complex was measured at 562 nm. The percentage

scavenging effect was calculated as

$$\% \text{HO}\cdot \text{ scavenged} = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100\% \quad (1)$$

where A_1 was the absorbance of the sample or ascorbic acid, and A_0 was the absorbance of the solvent control, whereas A_2 was the absorbance of the reagent blank without sodium salicylate.

4.2.4.2 Ferrous metal ions chelating activity

Ferrous metal ions chelating activities of LEPC-I and LEPC-II were measured according to a literature procedure with a few modifications ^[101]. Sample or ethylenediaminetetraacetic acid (EDTA) solution (1 mL) were mixed with 50 μL of ferrous chloride (2 mM) and 0.2 mL of ferrozine (5 mM), shaken well, allowed to stay still for 10 min at room temperature, and the absorbance of the mixture was determined at 562 nm. EDTA was included as the positive control. The ion-chelating activity was calculated as

$$\text{chelating rate} = \left[1 - \frac{A_1 - A_2}{A_0}\right] \times 100\% \quad (2)$$

where A_0 was the absorbance of the control (without sample) and A_1 was the absorbance in the presence of the sample, A_2 was the absorbance without ferrozine.

4.2.4.3 Assay of H_2O_2 scavenging

The ability of samples to quench H_2O_2 was determined spectrophotometrically ^[102]. Samples were dissolved in 1 mL of 0.1 M pH 7.4 phosphate buffered saline (PBS) and mixed with 1 mL of 10 mM solution of H_2O_2 . Absorbance of H_2O_2 at 230 nm was determined 10 min later in a spectrophotometer. For each concentration, a separated

blank sample was used for background subtraction. The inhibition of H₂O₂ production was calculated as follows:

$$\text{inhibition rate (\%)} = [1 - (A_{\text{sample}} - A_{\text{sample blank}})/A_{\text{control}}] \times 100 \quad (3)$$

where A_{control} was the absorbance of the control group in the hydroxyl radicals generation system, A_{sample} was the absorbance of the test group and $A_{\text{sample blank}}$ was the absorbance of the samples only. EC₅₀ value (mg extract/mL) was the effective concentration at which the absorbance was 0.5 for reducing power and was obtained by interpolation from linear regression analysis.

4.2.4.4 DPPH free radical-scavenging

DPPH radical-scavenging activities of LEPC-I and LEPC-II were measured according to the method described by Blois (2002) with some modifications [103]. Aliquots (0.5 mL) of various concentrations (0.156–10.00 mg/mL) of LEPC-I and LEPC-II were mixed with 2 mL (25 µg/mL) of a MeOH solution of DPPH. Then the mixture was shaken vigorously and allowed to stand in the dark for 30 min. The absorbance was measured at 517 nm against a blank. Decrease of the DPPH solution absorbance indicated an increase of the DPPH radical-scavenging activity. Ascorbic acid was used as the positive control. DPPH free radical-scavenging activity was calculated according to the following equation:

$$\text{DPPH radical-scavenging activity (\%)} = [A_0 - A_1/A_0] \times 100 \quad (4)$$

where A_0 was the absorbance without samples and A_1 was the absorbance in the presence of the samples.

EC₅₀ value (mg extract/mL) was the effective concentration at which DPPH

radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

4.2.4.5 The determination of SOD-like activity

The levels of SOD-like activity in LEPC-I and LEPC-II were measured using the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular Technologies, Inc. Briefly, in a 96-well plate, 20 μL of sample solution was added to each sample and blank 2 well, and 20 μL of double distilled water was added to each blank 1 and blank 3 well. Then 200 μL of WST working solution was added to each well. After mixing, 20 μL of dilution buffer was added to each blank 2 and blank 3 well, and 20 μL of enzyme working solution was added to each sample and blank 1 well. The plate was incubated at 37 $^{\circ}\text{C}$ for 20 min and the O.D. was determined at 450 nm using a microplate reader (BIO-RAD Model 550, USA). The SOD-like activity was calculated by the following equation:

$$\text{SOD activity (inhibition rate \%)} = \frac{[(A_{\text{blank 1}} - A_{\text{blank 3}}) - (A_{\text{sample}} - A_{\text{blank 2}})]}{(A_{\text{blank 1}} - A_{\text{blank 3}})} \times 100 \quad (5)$$

Where $A_{\text{blank 1}}$, $A_{\text{blank 2}}$, $A_{\text{blank 3}}$, A_{sample} were the absorbance of blank 1, blank 2, blank and sample.

4.2.4.6 Reducing power

The reducing power of LEPC-I and LEPC-II were measured according to the method of Yen and Chen (1995) with slight modifications^[104]. An aliquot of each sample (1 mL), with different concentrations, was mixed with 1 mL of phosphate buffer (200 mM, pH 6.6) followed by 1 mL of 1% potassium ferricyanide

[K₃Fe(CN)₆]. The mixture was incubated for 20 min in a water bath at 50 °C. After incubation, 1 mL of 1% trichloroacetic acid (TCA) was added, followed by centrifugation at 6000×g for 10 min. The supernatant (2 mL) was mixed with 2 mL of distilled water and 0.4 mL of 0.1% ferric chloride (FeCl₃), then the absorbance was measured at 700 nm against a blank in the spectrophotometer. A higher spectrophotometrical absorbance means a higher reducing power activity. Ascorbic acid was used as the positive control.

4.2.4.7 ABTS radical-scavenging activity

ABTS was dissolved in distilled water at a final concentration of 7 mM and mixed with a potassium persulphate solution at a final concentration of 2.45 mM. The reaction mixture was left to settle at room temperature for 12–16 h in the dark before use [105]. For each experiment, freshly prepared ABTS⁺ solution was diluted with 0.01 M phosphate buffer saline (PBS, pH 7.4) to adjust its absorbance to within 0.70 ± 0.02 at 734 nm wavelength.

Then 0.15 mL of various concentrations of the sample (0.156–10.00 mg/mL) was mixed with 2.85 mL of ABTS⁺ solution. Finally, the absorbances were measured at 734 nm after incubation at room temperature for 10 min. The scavenging activity of ABTS free radical was calculated by using the following equation:

$$\text{ABTS scavenging activity (\%)} = \{(C-D) - (A-B)/(C-D)\} \times 100 \quad (6)$$

where, A = absorbance of ABTS solution + sample/standard, B = absorbance of potassium persulphate + sample/standard, C = absorbance of ABTS solution + distilled water/methanol and D = potassium persulphate + distilled water/methanol.

4.2.5 Statistical analysis

Experimental results were means \pm standard deviation (SD) of triple determinations. The data were analyzed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Student's t-test analysis at $P = 0.05$ or independent sample t-test ($P = 0.05$).

4.3 Results

4.3.1. Scavenging activity of hydroxyl radical

Hydroxyl radical (HO•) can easily cross cell membranes, and can readily react with biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death. Thus, removing HO• is important for the protection of living systems. As shown in Figure 4-1., the extracts from the fermented SCR exhibited dose-dependence (0.156–10 mg/mL) of hydroxyl radical scavenging activity. The scavenging effect of LEPC-II was higher than that of LEPC-I, and the maximum inhibition of LEPC-I and LEPC-II were 81.03% and 99.18% respectively, at 10 mg/mL. However, ascorbic acid could exhibit 99.28% hydroxyl radical scavenging activity at 1.25 mg/mL.

4.3.2. Ferrous metal ions chelating activity

Metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals and initiate the radical-mediated oxidative chain reactions in biological or food systems. Ion-chelating agents also may inhibit Fenton reaction and hydroperoxide decomposition. In the present study, LEPC-I and LEPC-II were compared to EDTA

for their Fe^{2+} -chelating capacity. The chelating actions of LEPC-I on ferrous ions increased with those concentrations (Figure 4-2.). The chelating abilities of LEPC-II were higher than those of LEPC-I. At 10 mg/mL, LEPC-I and LEPC-II chelated 22.08% and 33.65% ferrous ions respectively. EDTA (the positive control) showed a high chelating ability of 92.40% at 2.5 mg/mL.

4.3.3. H_2O_2 scavenging activity

H_2O_2 is an oxidative agent and is involved in the formation of other ROS molecules such as hydroxyl radical when it reacts with Fe^{2+} or the superoxide anion radical. Although H_2O_2 is not very reactive, it is one of the major inducers for cellular aging and may attack many cellular energy-producing systems because of its high penetrability of cell membranes. H_2O_2 scavenging by LEPC-I was in the range of 47.34–97.45% compared to 46.59–90.84% of LEPC-II at 0.156–5 mg/mL (Figure 4-3). However, ascorbic acid (the positive control) presented a stronger scavenging effect (99.69%) at a lower concentration (2.5 mg/mL).

4.3.4. DPPH radical scavenging activity

ROS produced in vivo including superoxide radical, hydrogen peroxide and hypochlorous acid. Hydrogen peroxide and superoxide can interact in the presence of certain transition metal ions to yield a highly-reactive oxidising species, the hydroxyl radical ^[106]. The antioxidants react with the stable free radical DPPH (deep violet colour) and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration. As shown in Figure 4-4, DPPH scavenging activity of LEPC-I was less effective than that of LEPC-II. It was generally observed that DPPH radical-scavenging effect increased as

the concentration of LEPC-I increased, to a certain extent, and then leveled off, even with further increases in the concentration. For example, LEPC-II at a concentration of 0.156–2.5 mg/mL exhibited 35.9–96.80% scavenging activity of DPPH radical, whilst its scavenging activity was 98.26% at the concentration of 5 mg/mL, no significant increase in the DPPH radical scavenging effect was observed with further increases in dosage. Ascorbic acid (the positive control) presented a stronger scavenging effect (97.25%) at a lower concentration (0.625 mg/mL).

4.3.5. SOD-like activity

All living bodies have a complex antioxidant defence system that includes various antioxidant enzymes, such as superoxide dismutase and catalase. A rapid and facile method for the assay of SOD-like activity, based on the ability to inhibit the auto-oxidation of pyrogallol, is widely used to predict antioxidant capability. In the present study, we found that SOD-like activity increased with the concentrations of LEPC-I, and treated with the concentration of 10 mg/mL, SOD-like activity of LEPC-I and LEPC-II were 36.50% and 67.51%, respectively (Figure 4-5).

4.3.6. Reducing power

The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. Antioxidant compounds cause the reduction of ferric (Fe^{3+}) form to the ferrous (Fe^{2+}) form because of their reductive capabilities. Prussian blue-colored complex is formed by adding FeCl_3 to the ferrous (Fe^{2+}) form. Therefore, reduction can be determined by measuring the formation of Perl's Prussian blue at 700 nm. All samples showed some degree of electron donation

capacity in a concentration-dependent manner (0.156–10 mg/ml), but the capacities were inferior to those of ascorbic acid and LEPC-I exhibited weaker reducing power than LEPC-II (Figure 4-6). The reducing power of LEPC-II was the highest, with 1.659 at 10 mg/mL, and LEPC-I showed highest reducing power with 0.966 at 10 mg/mL. The reducing power of ascorbic acid was 1.668 at the concentration of 5 mg/mL.

4.3.7. ABTS radical-scavenging activity

ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants and it employs a specific absorbance at a wavelength remote from the visible region and requires a short reaction time, can be used in both organic and aqueous solvent systems. The extent of scavenging of the $ABTS^{\cdot+}$ was plotted as a function of antioxidant concentration, as shown in Figure 4-7, LEPC-I scavenged $ABTS^{\cdot+}$ radicals dose-dependently and had more than 50% inhibitory activity at a low concentration of 1.25 mg/mL. At 10 mg/mL, ABTS radical-scavenging activity of LEPC-I was the highest, with 95.85%, whereas LEPC-II showed higher ABTS radical-scavenging activity, with 99.75% at 10 mg/mL. However, ascorbic acid could exhibit 100% inhibitory activity at low concentration, with 100% at 1.25 mg/mL.

4.3.8. EC_{50} value in antioxidant properties

The antioxidant properties are inversely correlated with their EC_{50} values. The antioxidant properties assayed herein were summarized in Table 4-1 and the results were normalized and expressed as inhibiting effects of 50% (EC_{50}) value (mg/mL) for

comparison. LEPC-I and LEPC-II revealed very different antioxidant properties (significant difference between EC₅₀ values, P < 0.05). The effective concentrations corresponding to EC₅₀ values of LEPC-II were significantly lower than those of LEPC-I on the hydroxyl radical scavenging, DPPH radicals scavenging, reducing power and ABTS radicals scavenging. The EC₅₀ value of LEPC-I was higher than that of LEPC-II on the hydrogen peroxide scavenging. However, the EC₅₀ values of ascorbic acid were lowest and showed strongest antioxidant activity, compared with LEPC-I and LEPC-II.

4.4 Discussion

Phenolic compounds, sometimes called phenolics, were reported to 8000 in various vegetables, fruits, cereal crops, etc. ^[107]. Phenolic compounds naturally occurring consisted of several groups such as simple phenols, phenolic acids, flavonoids, tannins, etc. Some phenolic compounds in plants were known as secondary metabolites, which were related to the protection of plants against ultraviolet radiation, pathogens, and herbivores ^[108]. Furthermore, phenolic compounds have also various biological activities like antioxidative, antimutagenic activities, antibacterial, atherosclerosis, coronary heart disease, or anticancer effects. Using in vitro or in vivo human and animal models, phenolics have been studied for minimising or eliminating an initiation or progress of various human diseases ^[42, 109]

Table 4-1 illustrated the major chemical content of the extracts from fermented SCR. It showed same chemical compositions of the LEPC-I and LEPC-II were existed. However the content of chemical compositions was significant difference.

The carbohydrate and protein content of LEPC-I were much higher than those of LEPC-II, and phenolic compounds content of LEPC-II was almost two times compared with that of LEPC-I. These results showed a part of carbohydrate and protein in LEPC-I were removed by macroporous adsorption resins and purer LEPC-I (denoted as LEPC-II) was obtained. These results were partly supported by Lianzhu Lin et al., who suggested after treatment with two macroporous resins columns (HP-20 and HP-20), phenolics were from 16.66% to 67.87% by HP-20 and to 58.81% by HP-20), and antioxidants were three times more than original ^[110]. Macroporous adsorption resins are often used to separate and purify phenolic compounds due to its less yielding cost, simpler operation, higher efficiency, more friendly to environmental protection and easier regeneration ^[111]. The adsorbents have no functional groups. The adsorption is due to their large surface areas and van der Waals forces, which forms weak bonds with molecules. Since the adsorption is reversible, it allows the recovery of products and the regeneration of resins by washing with common organic solvents such as higher concentration of alcohol and acetone ^[112, 113].

Phenolic compounds are one type of the most widely distributed plant secondary products. The ability of these compounds to act as antioxidants have been well established ^[114]. Mushrooms contain a variety of secondary metabolites, including various phenolic compounds, which have been shown to act as excellent antioxidants ^[58]. We produced *L. edodes* phenolic compounds using SCR as main culture. Seven antioxidant assays were used to evaluate the antioxidative effects of *L. edodes*

phenolic compounds in all directions in this study. Our results showed all of antioxidative effects increased with the concentrations of LEPC-I and LEPC-II.

LEPC-I and LEPC-II showed moderate chelating effect on ferrous ions. At 10 mg/mL, LEPC-I and LEPC-II chelated 22.08% and 33.65% of ferrous ions respectively (Figure 4-2). Tsai et al. (2007) reported that ethanolic extracts of *A. blazei* had chelating abilities of 50% at 10 mg/mL ^[115]. These authors suggest that moderate to high ferrous-ions chelating abilities showed beneficial to health by mushrooms. Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxy and alkoxy radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation.

LEPC-I and LEPC-II exhibited strong DPPH radical scavenging activities as well as EC₅₀ values of LEPC-I and LEPC-II were 1.77 and 0.66 mg/mL respectively (Figure 4-4). DPPH radical scavenging activities of LEPC were superior to those of phenolic compounds extracted from *L. edodes*, with EC₅₀ of 3.5 mg/mL ^[116]. The antioxidant property of LEPC was due to the supply of hydrogen, which combined with radicals and it formed a stable radical to terminate the radical chain reaction by acting as a chain break antioxidant. The other possibility was that LEPC could combine with the radical ions that were necessary for the radical chain reaction, then the reaction was terminated. The exact mechanism of action, however, is still unknown.

SOD-like activities of LEPC-I and LEPC-II were 36.50% and 67.51%

respectively, at 10 mg/mL (Figure 4-5). SOD is the abbreviation of Super Oxide Dimutase which is an important antioxidant enzyme in vivo and is widely distributed in various biological body, such as animals, plants, microorganisms. SOD has a special physiological activity and primary material to scavenging free radical as well as SOD is direct indicator for aging and death in vivo. SOD-like activities of extracts of buckwheat sprouts were almost equivalent to that of rutin, isoorientin and orientin. In addition, sulforaphane, present in broccoli sprouts, has been found to lead to marked SOD-like activity ^[117]. Our results suggested that with its SOD-like activity, LEPC from fermented SCR could be a beneficial component that provides comparable biological effect such as those of rutin and sulforaphane.

The reducing power of LEPC-I and LEPC-II increased with their concentrations, and the highest reducing power of LEPC-I and LEPC-II were 0.966 and 1.659 at 10 mg/mL, respectively (Figure 4-6). In previous studies, a reducing power around 0.7 was found for an ethanolic extract of the mushroom at the concentration of 10 mg/mL ^[115]. With regard to reducing powers, LEPC were obtained from fermented SCR in the present study, were superior to those of edible and medicinal mushroom in general significantly ^[118]. On the other hand, exceptionally high reducing power has been described for some medicinal mushrooms. For example, 4 mg/mL methanolic extracts of *Ganoderma tsugae* and *G. lucidum*, present reducing powers of 2.38 and 2.28, respectively ^[58].

ABTS radical-scavenging of LEPC-I and LEPC-II were more than 50% inhibitory activity at a low concentration of 2.5 mg/mL (Figure 4-7). This method,

used for the screening of antioxidant activity, is applicable to both lipophilic and hydrophilic antioxidants ^[119]. Ke-Xue et al. found that phenolic compounds extracted from defatted wheat germ and the EC₅₀ value of ABTS radical-scavenging was 5.6 mg/mL, which was higher than EC₅₀ values of LEPC-II (1.36 mg/mL) and LEPC-I (3.31 mg/mL) ^[91].

The antioxidant properties are inversely correlated with their EC₅₀ values and values lower than 10 mg/mL are indicative of the effective antioxidant activity. In the previous reports on plant extracts, it was found that phenolic compounds were the main antioxidant components and its total content was directly proportional to the antioxidant activity. And in this paper, a similar trend was observed. LEPC-II showed stronger antioxidant activities than LEPC-I, except H₂O₂ scavenging activity, as table 4-1 showed LEPC-II (59.73%) contained more phenolic compounds than LEPC-I (33.58%).

Antioxidant activities of phenolic compounds were correlated to their chemical structures. Structure-activity relationship of some phenolic compounds (e.g. flavonoids, phenolic acids, tannins) has been studied ^[120]. In general, free radical scavenging and antioxidant activity of phenolics (e.g. flavonoids, phenolic acids) mainly depend on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules, and are also affected by other factors, such as glycosylation of aglycones, other H-donating groups (-NH, -SH), etc. For example, flavonol aglycones such as quercetin, myricetin, and kaempferol, containing multiple hydroxyl groups, had higher antioxidant activity than their glycosides such as

rutin, myricitrin, astragalin. The glycosylation of flavonoids reduced their activity. There were many reports on antioxidant components, generally focusing on flavonoids and phenolic acids ^[112, 113], but relatively fewer on other phenolic antioxidant components. Therefore, further chemical identification of LEPC-I and LEPC-II in the tested fermented SCR will be required to reveal the possible structure-activity relationship of various types of phenolics.

4.5 Summary

On the basis of the above results, it could be concluded that *L. edodes* phenolic compounds (LEPC-I) was purified by macroporous adsorption resins column SP825 (denoted as LEPC-II). Antioxidant activities of LEPC-I and LEPC-II were investigated. The results showed that LEPC-I and LEPC-II exhibited strong scavenging activities against DPPH radical, hydroxyl radical, hydrogen oxide, ABTS radical cation decolorization and reducing power, as well as weak ferrous chelating effect and weak SOD-like activity. Furthermore antioxidant activities of LEPC-II were higher than those of LEPC-I.

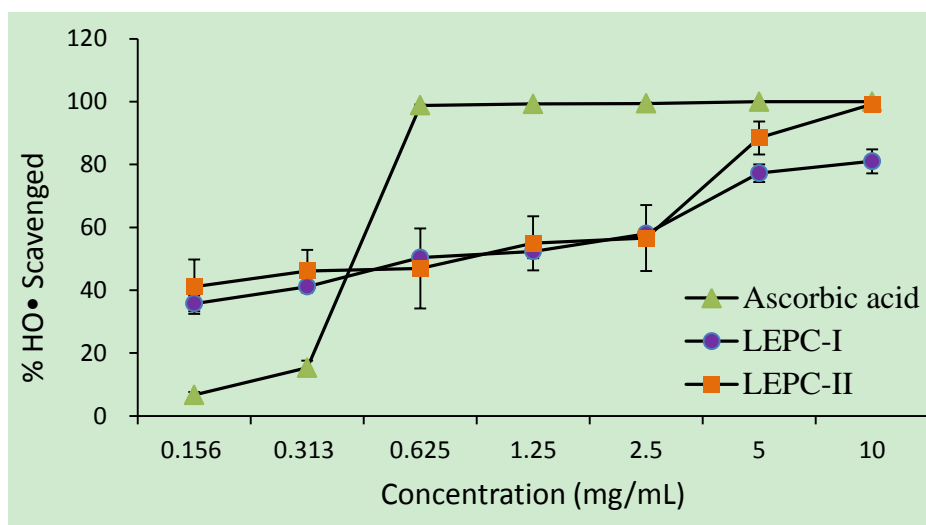


Figure 4-1. Hydroxyl radical scavenging activities of LEPC

All treatments were conducted in triplicate, and mean values were reported.

Ascorbic acid was positive control.

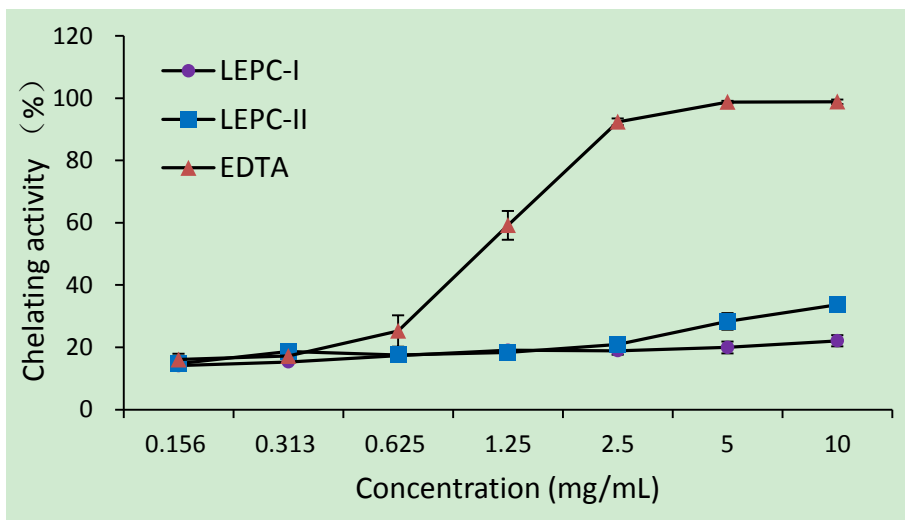


Figure 4-2. Chelating activity of LEPC

All treatments were conducted in triplicate.

The vertical bars represent the standard deviation of each data point.

EDTA was the positive control.

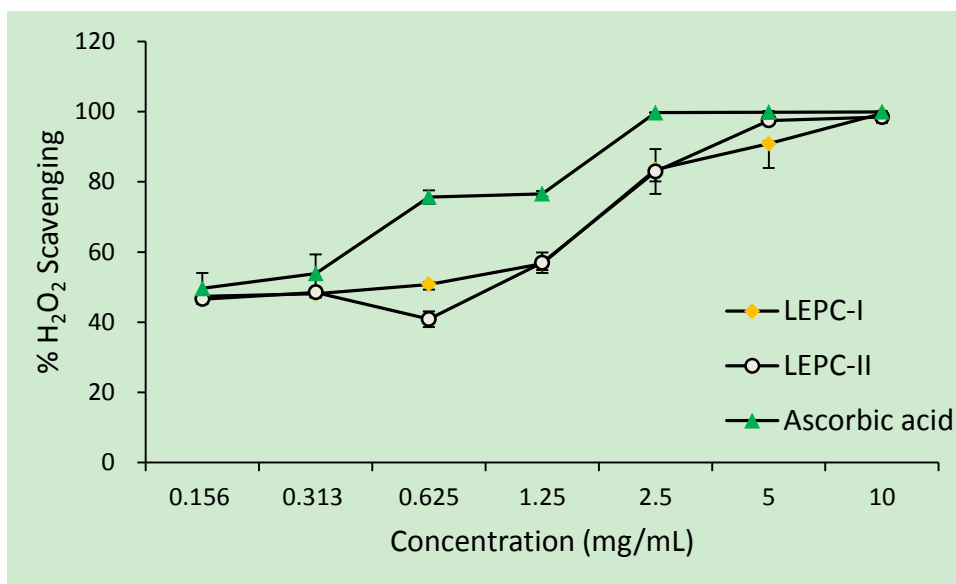


Figure 4-3. Hydrogen oxide scavenging activities of LEPC

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

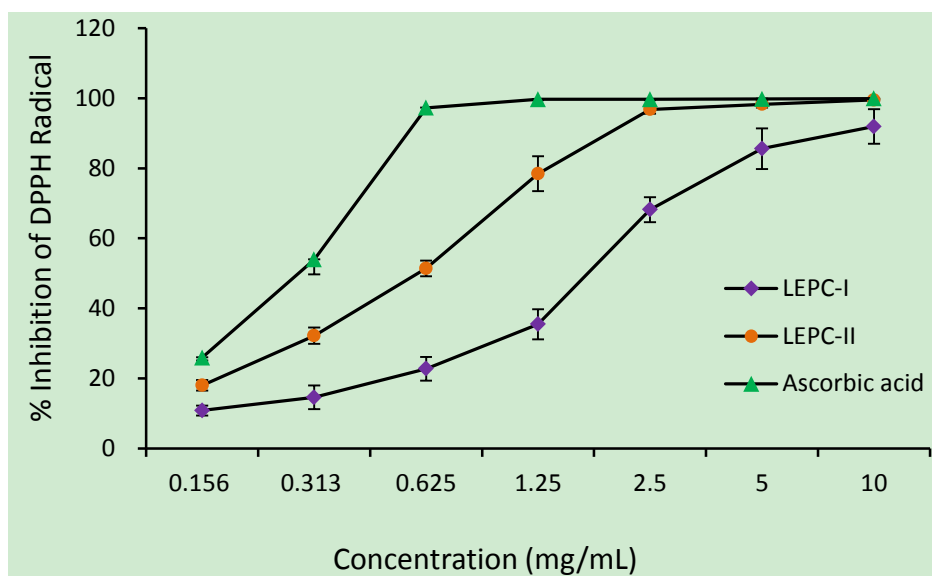


Figure 4-4. DPPH radical inhibition capacity of LEPC

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

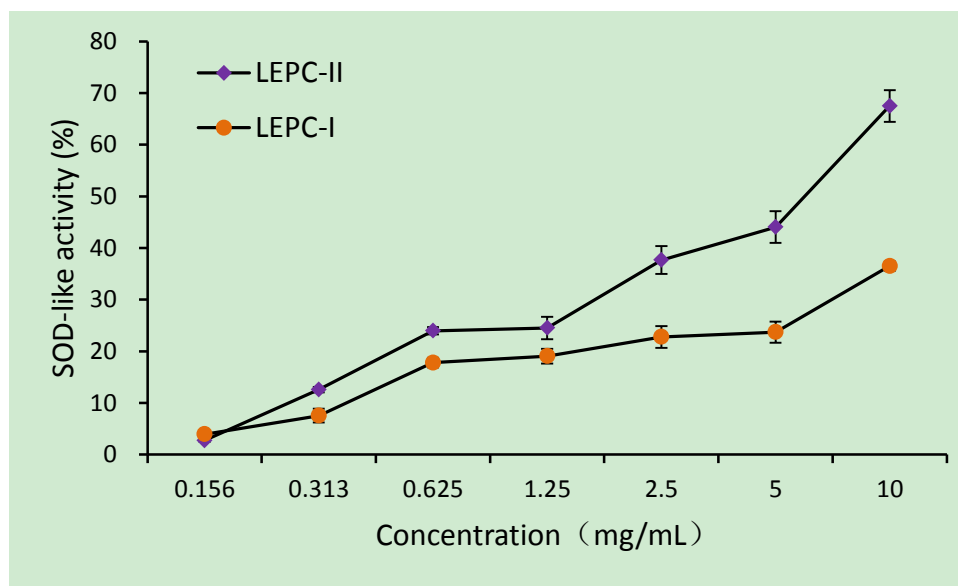


Figure 4-5. SOD-like activity of LEPC

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

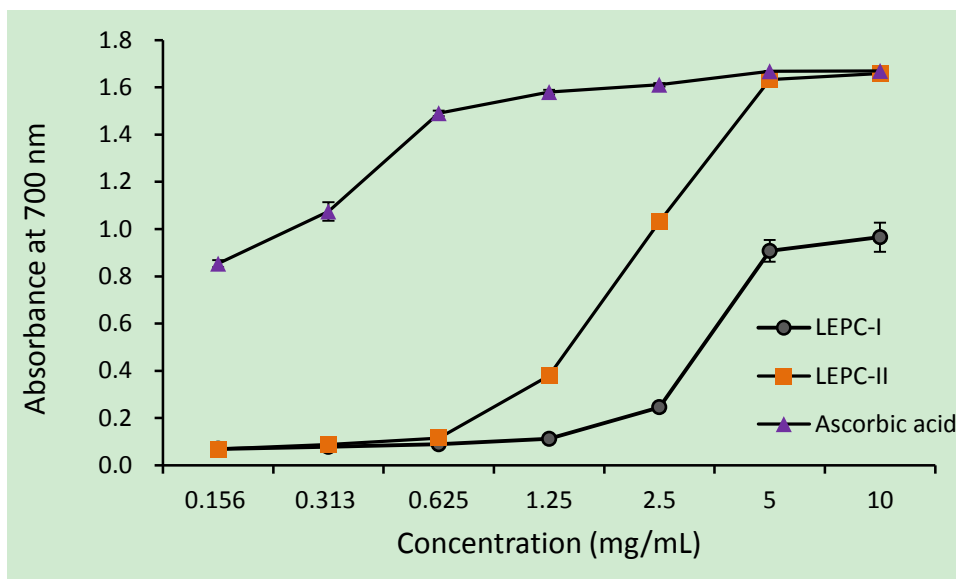


Figure 4-6. Reducing power of LEPC

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

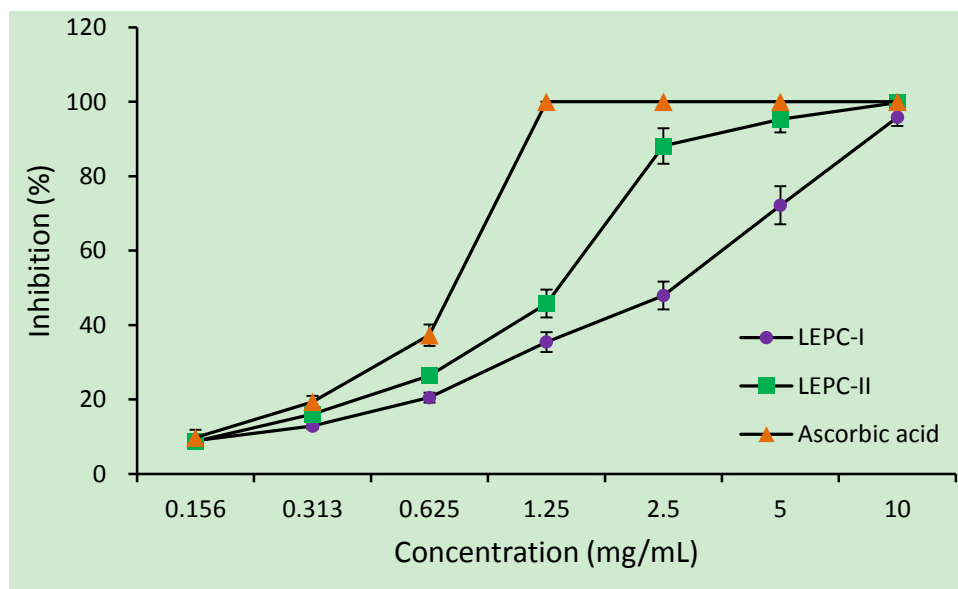


Figure 4-7. Inhibition (%) of LEPC on the stable ABTS +
 All treatments were conducted in triplicate, and mean values were reported.
 The vertical bars represented the standard deviation of each data point.
 Ascorbic acid was the positive control.

Table 4-1. Major chemical content of the extracts from the fermented SCR

	LEPC-I	LEPC-II
Phenolic compounds content (%)	33.58 \pm 2.83	59.73 \pm 2.18
Carbohydrate content (%)	24.33 \pm 2.13	16.36 \pm 1.03
Protein content (%)	25.03 \pm 1.10	10.34 \pm 0.39

Chapter 5 Antioxidant activities of the crude polysaccharide from the fermented soybean curd residue by *Ganoderma lucidum*

5.1 Introduction

An earlier study has shown that treatment with antioxidant reduces diabetic complications ^[121]. Efforts to discover antioxidants as useful drug candidates to combat diabetic complications are going on relentlessly. *Ganoderma lucidum* is a fungus usually used in traditional Chinese medicine. Recent studies on *G. lucidum* have shown many interesting biological activities, including anti-tumour and anti-inflammatory effects and cytotoxicity to hepatoma cells. The polysaccharides of *G. lucidum* are the major source of its biological activity and therapeutic uses. Polysaccharide extracts from many species of fungi exhibit immunostimulating and/or anti-tumor activities.

Scientific investigations show that polysaccharide of *G. lucidum* promises to be a new type of carcinostatic agent, which might be useful in immunotherapy ^[122]. Recently, it has been reported that *G. lucidum* polysaccharide has the ability to scavenge the free radicals ^[123, 124].

The importance of the antioxidant constituents of *G. lucidum* polysaccharide in the maintenance of health is also raising interest among scientists, food manufacturers and consumers as the trend for the future is toward functional food with specific health effects.

5.2 Materials and methods

5.2.1 Chemicals and reagents

D-glucose, phenol, sulphuric acid, ascorbic acid, hydrogen peroxide, chloride ferric, potassium ferricyanide, ferrous sulfate, potassium persulphate, trichloroacetic acid, sodium salicylate and ethylenediamine tetraacetic acid (EDTA) were obtained from Wako Pure Chemical, Osaka, Japan. SOD Assay Kit-WST was purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan). Gallic acid, 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picryl-hydrazyl radical (DPPH) were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA). All other chemical reagents were of analytical grade.

5.2.2 Extraction from fermented SCR

The fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The crushed powder (1 g) was extracted by ultrasonic assisted extraction with 30 min, 80 °C, 80 watt of the power with 10 of the water to solid ratio. The water-soluble polysaccharide was subsequently precipitated by adding eight volumes of 99.5% ethanol and stored at 4 °C overnight. The precipitated polysaccharide was collected by centrifuging at 8000 rpm for 30 min and lyophilized. Then the powder was dissolved in 10 mL of distilled water. Total polysaccharide was determined by the phenol-sulfuric acid method. The color reaction was initiated by mixing 1 mL of GLPL solution with 0.5 mL of a 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubated in a boiling water bath for 15 min. The optical density of the mixture was determined at 490 nm and the GLPL

content was calculated with D-glucose as the standard. The results were expressed as milligram of glucose equivalent per gram of fermented SCR.

5.2.3 Assay for antioxidant activities of GLPL

5.2.3.1 HO• scavenging activity

HO• scavenging activity was measured according to a literature procedure with a few modifications^[100], as mentioned in 2.4.1 of Chapter 4.

5.2.3.2 Ferrous metal ions chelating activity

Ferrous metal ions chelating activities of GLPL were measured according to a literature procedure with a few modifications^[101], as mentioned in 2.4.2 of Chapter 4.

5.2.3.3 Assay of H₂O₂ scavenging

The ability of samples to quench H₂O₂ was determined spectrophotometrically^[102], as mentioned in 2.4.3 of Chapter 4.

5.2.3.4 DPPH free radical-scavenging

DPPH radical-scavenging activities of GLPL were measured according to the method described by Blois (2002) with some modifications^[103], as mentioned in 2.4.4 of Chapter 4.

EC₅₀ value (mg extract/mL) was the effective concentration at which DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis.

5.2.3.5 The determination of SOD-like activity

The levels of SOD-like activity in GLPL were measured using the SOD Assay Kit-WST according to the technical manual provided by Dojindo Molecular

Technologies, Inc., as mentioned in 2.4.5 of Chapter 4.

5.2.3.6 Reducing power

The reducing power of GLPL were measured according to the method of Yen and Chen (1995) with slight modifications ^[104], as mentioned in 2.4.6 of Chapter 4.

5.2.3.7 ABTS radical-scavenging activity

ABTS was dissolved in distilled water at a final concentration of 7 mM and mixed with a potassium persulphate solution at a final concentration of 2.45 mM. The reaction mixture was left to settle at room temperature for 12–16 h in the dark before use ^[105], as mentioned in 2.4.7 of Chapter 4.

5.2.4 Statistical analysis

Experimental results were means \pm standard deviation (SD) of triple determinations. The data were analysed by one-way analysis of variance (ANOVA). Tests of significant differences were determined by Student's t-test analysis at $P = 0.05$ or independent sample t-test ($P = 0.05$).

5.3 Results

5.3.1. Scavenging activity of hydroxyl radical by GLPL

Hydroxyl radical (HO•) can easily cross cell membranes, and can readily react with biomolecules including carbohydrates, proteins, lipids, and DNA in cells, and cause tissue damage or cell death. Thus, removing HO• is important for the protection of living systems. As shown in Figure 5-1, GLPL could scavenge HO•, and the maximum inhibition of GLPL was 98.83% at 5 mg/mL. However, ascorbic acid exhibited 99.28 % of hydroxyl radical scavenging activity at 1.25 mg/mL.

5.3.2. Ferrous metal ions chelating activity of GLPL

Metal chelating capacity is important since it reduces the concentration of transition metals that may act as catalysts to generate the first few radicals and initiate the radical-mediated oxidative chain reactions in biological or food systems. Ion-chelating agents also may inhibit Fenton reaction and hydroperoxide decomposition. In the present study, GLPL was compared to EDTA for their Fe^{2+} -chelating capacity. The chelating actions of GLPL on ferrous ions increased with its concentrations (Figure 5-2). At 10 mg/mL, GLPL chelated $51.27 \pm 1.48\%$ of ferrous ions. EDTA (the positive control) showed the higher chelating ability of 92.4% at 2.5 mg/mL.

5.3.3. H_2O_2 scavenging activity of GLPL

H_2O_2 is an oxidative agent and is involved in the formation of other ROS molecules such as hydroxyl radical when it reacts with Fe^{2+} or the superoxide anion radical. Although H_2O_2 is not very reactive, it is one of the major inducers for cellular aging and may attack many cellular energy-producing systems because of its high penetrability of cell membranes. H_2O_2 scavenging by GLPL was in the range of 42–97% compared to 49–99% of ascorbic acid at 0.156–2.5 mg/mL (Figure 5-3). The EC_{50} values for H_2O_2 scavenging activities of GLPL and ascorbic acid were 0.483 and 0.122 mg/mL, respectively.

5.3.4. DPPH radical scavenging activity

ROS produced in vivo including superoxide radical, hydrogen peroxide and hypochlorous acid. Hydrogen peroxide and superoxide can interact in the presence of

certain transition metal ions to yield a highly-reactive oxidising species, the hydroxyl radical ^[106]. The antioxidants react with the stable free radical DPPH (deep violet colour) and convert it to 1,1-diphenyl-2-picryl hydrazine with decoloration. The scavenging effects of GLPL increased with its concentrations, 92% at the concentration of 1.25 mg/mL, with an EC₅₀ value of 0.229 mg/mL (Figure 5-4). Ascorbic acid (positive control) presented a scavenging effect of 97% at the concentration of 0.625 mg/mL with an EC₅₀ value of 0.186 mg/mL. The radical scavenging capacity of GLPL was considerably superior to those found for several other edible mushroom methanolic extracts ^[125].

5.3.5. SOD-like activity of GLPL

All living bodies have a complex antioxidant defence system that includes various antioxidant enzymes, such as superoxide dismutase and catalase. A rapid and facile method for the assay of SOD-like activity, based on the ability to inhibit the auto-oxidation of pyrogallol, is widely used to predict antioxidant capability. In the present study, we found that SOD-like activity increased with the concentrations of GLPL, and treated with GLPL at the concentration of 10 mg/mL, SOD-like activity was $63.56 \pm 1.18\%$ (Figure 5-5).

5.3.6. Reducing power of GLPL

The reducing power assay measures the electron-donating ability of antioxidants using the potassium ferricyanide reduction method. Figure 5-6, showed the reducing power of GLPL and ascorbic acid. The reducing power of ascorbic acid was 1.611 at the concentration of 2.5 mg/mL. GLPL exhibited weak reducing power and the

maximum reducing power by GLPL was 1.438 at the concentration of 10 mg/mL under the experiment conditions. With regard to reducing powers, GLPL obtained in the present study, was appreciable and comparable to those of edible and medicinal mushroom in general. On the other hand, exceptionally high reducing power has been described for some medicinal mushrooms. For example, 4 mg/mL of methanolic extracts of *Ganoderma tsugae*, presented reducing powers of 2.38^[126].

5.3.7 ABTS radical-scavenging activity of GLPL

ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants and it employs a specific absorbance at a wavelength remote from the visible region and requires a short reaction time, can be used in both organic and aqueous solvent systems^[127]. The extent of scavenging of the ABTS⁺ was plotted as a function of antioxidant concentration, as shown in Figure 5-7, GLPL scavenged ABTS⁺ radicals dose-dependently and had more than 50% inhibitory activity at a low concentration of 1.25 mg/mL, with EC₅₀ of 1.21 mg/mL. However, ascorbic acid could exhibit 100% inhibitory activity at this concentration with EC₅₀ of 0.69 mg/mL.

5.4 Discussion

G. lucidum was reported to have effective antioxidant functions including inhibition of lipid peroxidation, foam cell formation, and necrosis of macrophages^[126]. We produced GLPL using SCR as main culture. Seven antioxidant assays were used to evaluate the antioxidative effects of GLPL in all directions in this study. Our results showed all of antioxidative effects increased with the concentrations of GLPL.

Treated with 5 mg/mL GLPL, HO• scavenging was 98.83%, H₂O₂ scavenging was 98.37%, reducing power was 1.2 and chelating effect on ferrous ions of 48.10% respectively (Figure 5-1, 5-2, 5-3, 5-6). Wei Liu et al showed that purified GLPL of 8 mg/mL exhibited weak reducing power (0.303), 50% of H₂O₂ scavenging, 58% of chelating effect on ferrous ions and 78.3% of HO• scavenging ^[128]. Thus, GLPL exhibited greater capacities in HO• scavenging, H₂O₂ scavenging and reducing power than previous reports.

GLPL showed strong DPPH radical scavenging activity, with an EC₅₀ value of 0.229 mg/mL (Figure 5-4), compared with the results of polysaccharides yield of *Inonotus obliquus*, with EC₅₀ of 2.5mg/mL ^[129]. The antioxidant property of GLPL was due to the supply of hydrogen by GLPL, which combined with radicals and it formed a stable radical to terminate the radical chain reaction by acting as a chain break antioxidant. The other possibility was that GLPL could combine with the radical ions that were necessary for the radical chain reaction, then the reaction was terminated. The exact mechanism of action, however, is still unknown.

SOD-like activity was 63.56 ± 1.18% at 10 mg/mL GLPL (Figure 5-5). SOD is the abbreviation of super oxide dimutase which is an important antioxidant enzyme in vivo and is widely distributed in various biological body, such as animals, plants, microorganisms. SOD has a special physiological activity and primary material to scavenging free radical as well as SOD is direct indicator for aging and death in vivo. SOD-like activities of extracts of buckwheat sprouts were almost equivalent to that of rutin, isoorientin and orientin. In addition, sulforaphane, present in broccoli sprouts,

has been found to lead to marked SOD-like activity^[117]. Our results suggested that with its SOD-like activity, GLPL from fermented SCR could be a beneficial component that provides comparable biological effect such as those of rutin and sulforphane.

ABTS radical-scavenging of GLPL was more than 50% inhibitory activity at a low concentration of 1.25 mg/mL (Figure 5-7). This method, used for the screening of antioxidant activity, is applicable to both lipophilic and hydrophilic antioxidants^[119]. Compared with the results of polysaccharides of *P. asiatica L.* seed, with EC₅₀ of 0.18mg/mL, the activity of GLPL had weak scavenging power on hydroxyl ABTS radicals^[127].

The reason of high antioxidative effects of GLPL was that ultrasonic-assisted extraction might disrupt the cell wall and liberate antioxidant compounds from insoluble portion of mushroom. Another reason for improved antioxidant activities of GLPL could be due to formation of novel compounds having antioxidant activity during fermentation using SCR as the main culture.

5.5 Summary

G. lucidum polysaccharide (GLPL) was extracted using ultrasonic assisted extraction from fermented soybean curd residue. The in vitro antioxidant activities study indicated that GLPL exhibited the great capacities in hydroxyl radical scavenging, DPPH radical scavenging, ABTS radical cation decolorization, hydrogen oxide scavenging and reducing power, additionally moderate chelating effect on ferrous ions.

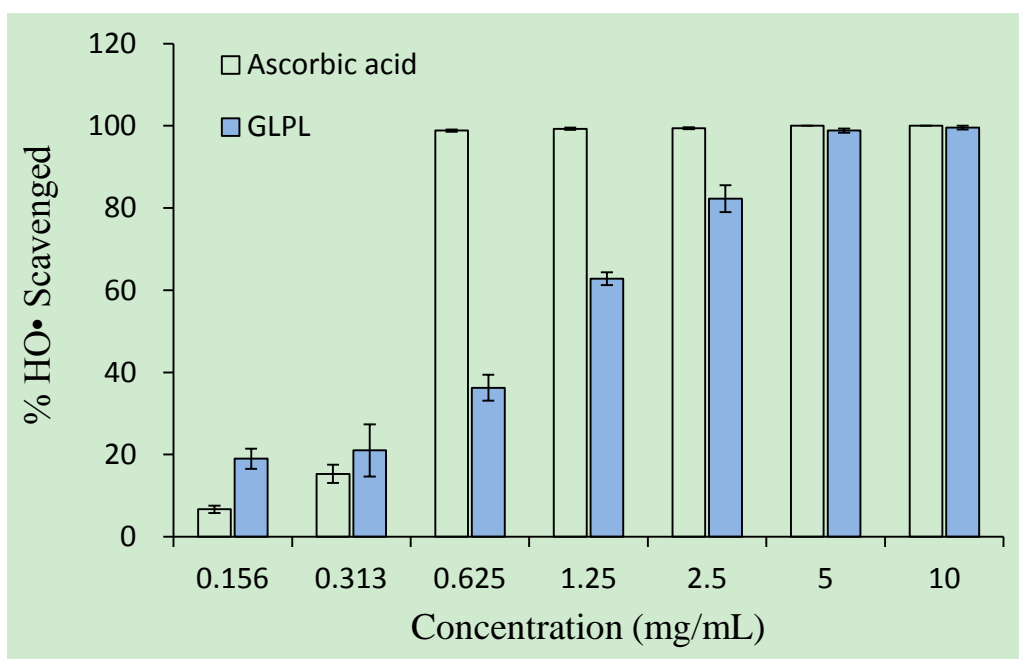


Figure 5-1. Hydroxyl radical scavenging activities of GLPL

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

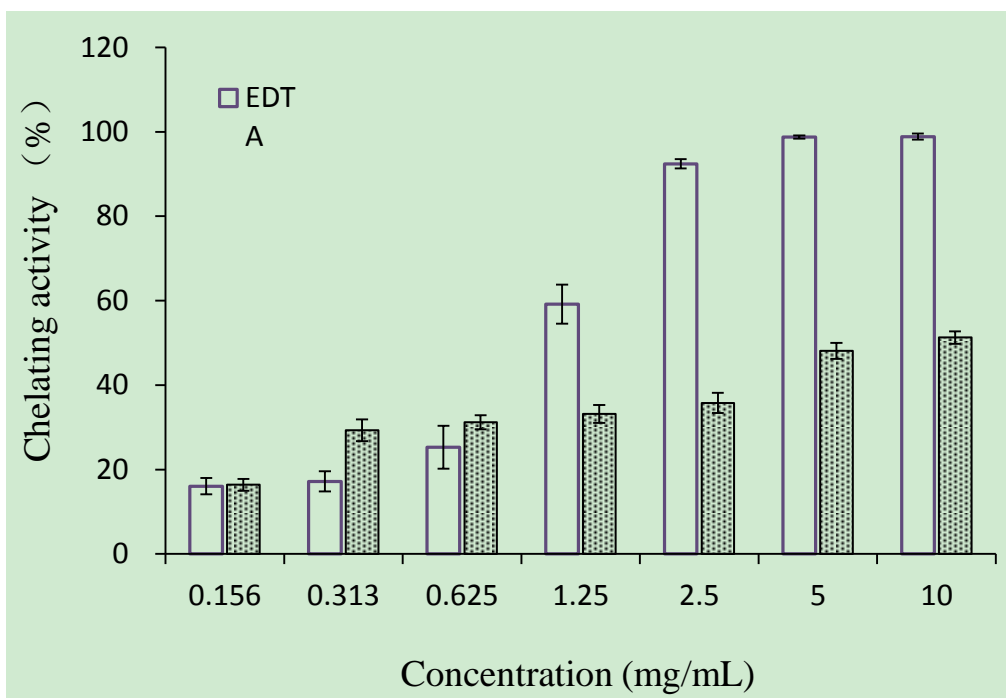


Figure 5-2. Chelating activity of GLPL

All treatments were conducted in triplicate.

The vertical bars represent the standard deviation of each data point.

EDTA was positive control.

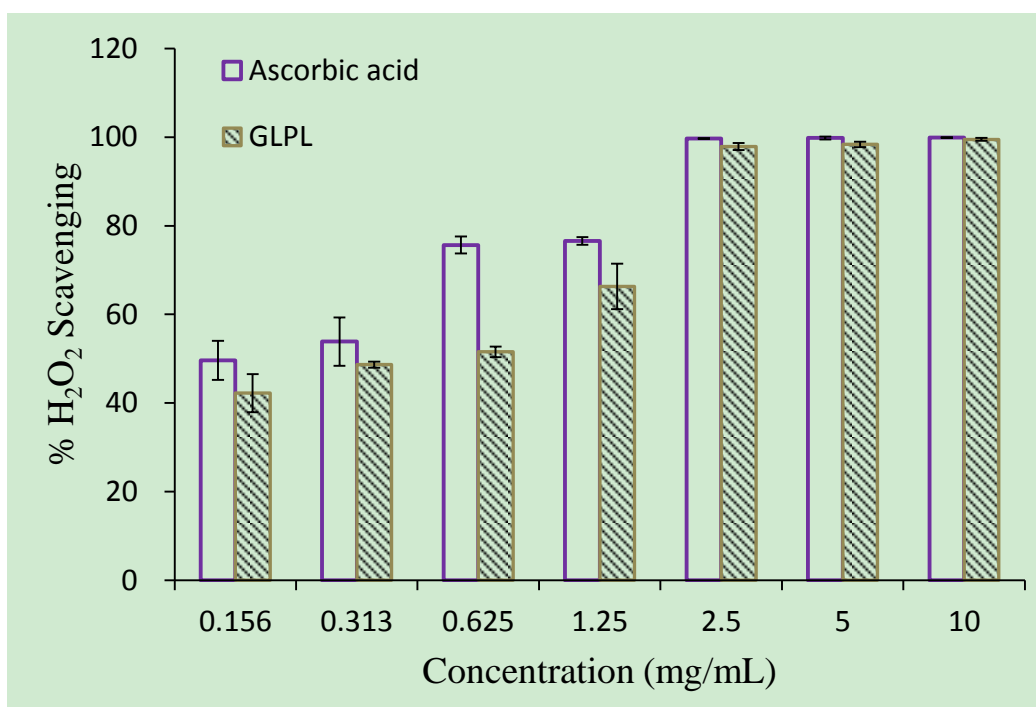


Figure 5-3. Hydrogen oxide scavenging activities of GLPL

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

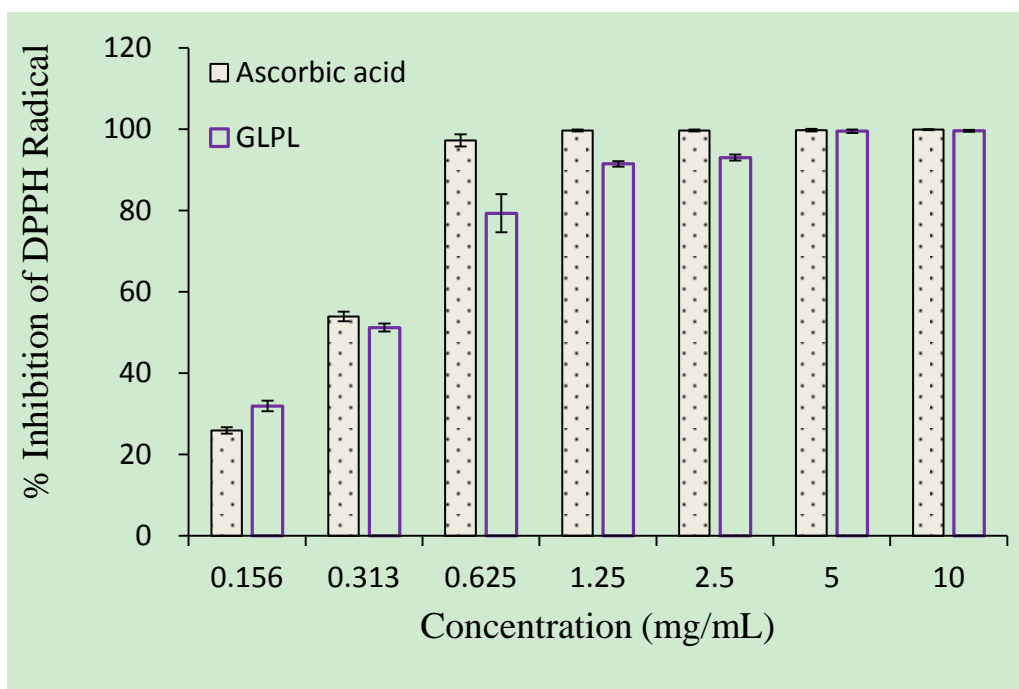


Figure 5-4. DPPH radical inhibition capacity of GLPL

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

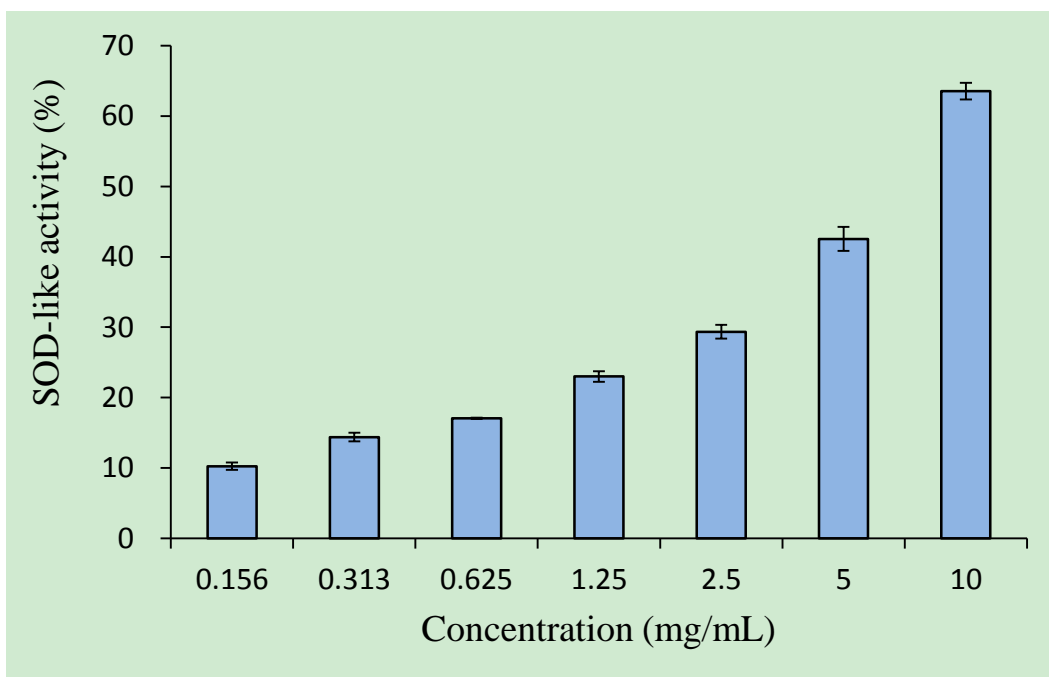


Figure 5-5. SOD-like activity of GLPL

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

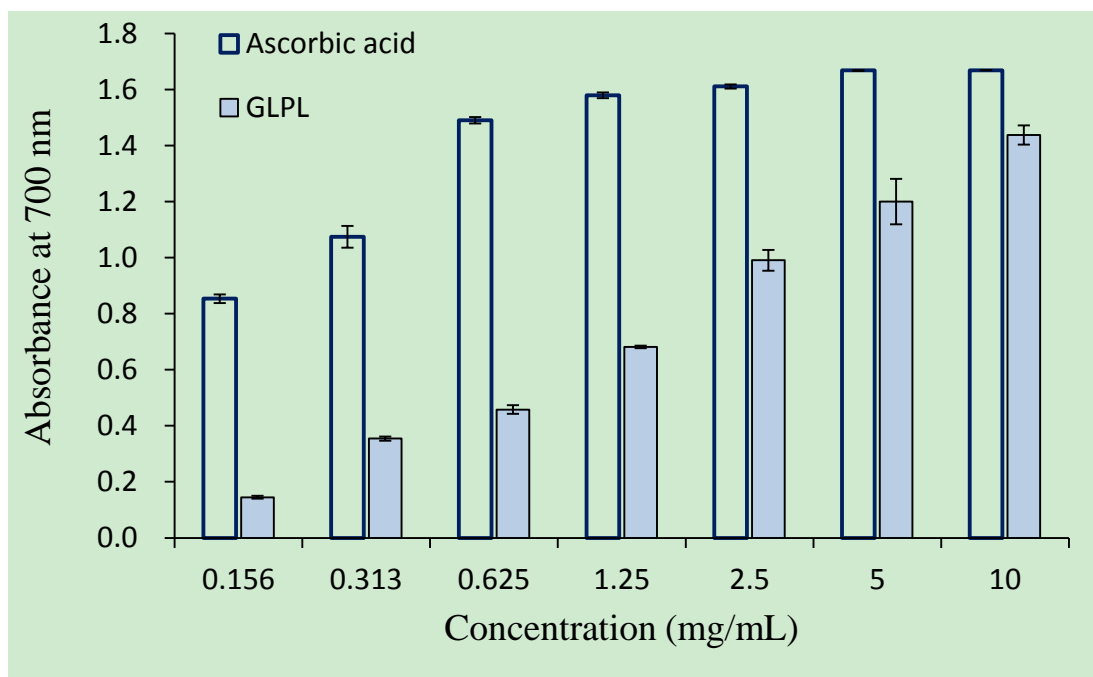


Figure 5-6. Reducing power of GLPL

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

Ascorbic acid was the positive control.

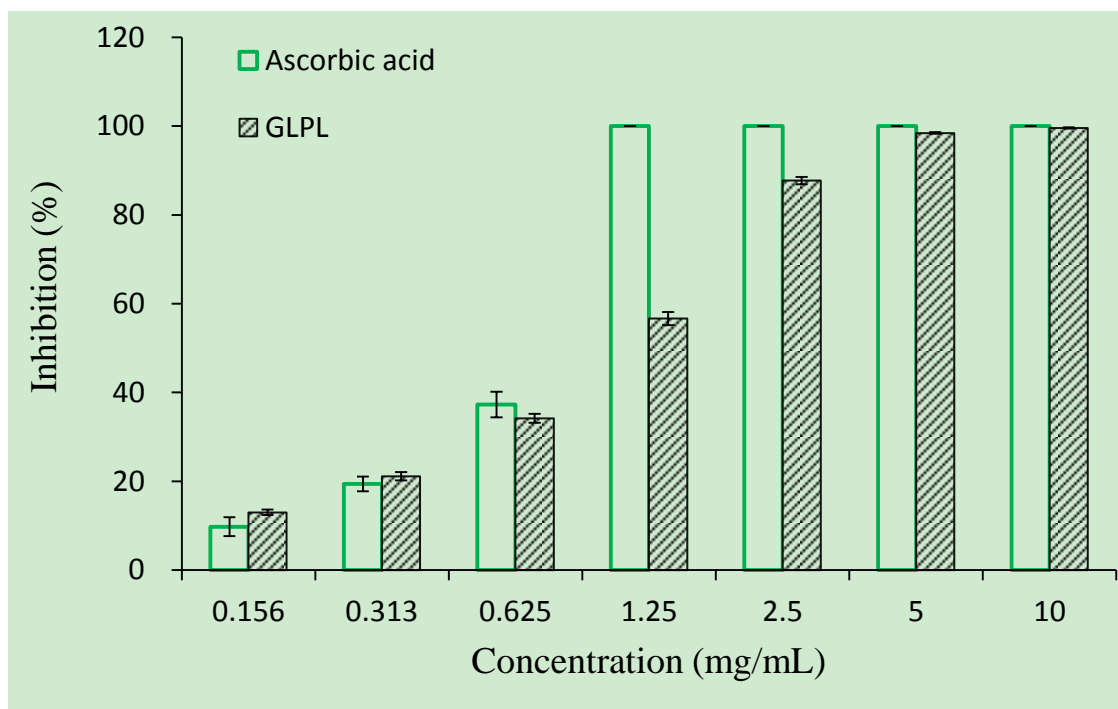


Figure 5-7. Inhibition (%) of GLPL on the stable ABTS +
 All treatments were conducted in triplicate, and mean values were reported.
 The vertical bars represented the standard deviation of each data point.
 Ascorbic acid was the positive control.

Chapter 6 Immunomodulatory activities of the crude polysaccharide from the fermented soybean curd residue by *Ganoderma lucidum*

6.1 Introduction

Macrophages play a significant role in the host defense mechanism. When activated, they activate phagocytic activity, produce and release reactive oxygen species (ROS) and nitric oxide (NO) in response to stimulation with various agents and can inhibit the growth of a wide variety of tumor cells and micro-organisms^[130]. Moreover, the immunomodulatory activity not only involves effects on macrophages activation but also on cell proliferation and differentiation. Papers report that polysaccharide from mushroom can enhance and activate macrophages immune responses, leading to immunomodulation, anti-tumor activity, wound-healing and other therapeutic effects^[131, 132].

Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂) and hydroxyl radical (HO•), are continuously generated by light, ultraviolet, ionizing radiation, some chemical reactions, metabolic processes and aging^[133]. In recent years, lots of studies have shown that ROS is responsible for various diseases such as cancer, Alzheimer's diseases, Parkinson's diseases, epilepsy, inflammation, retrolental fibroplasias, atherosclerosis, lung injury, ischemia-reperfusion injury and other disorders^[134]. Although almost all organisms possess antioxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to entirely prevent the damage. Against this background, antioxidants that have an important role in the prevention of these diseases must be obtained. Because lots of synthetic antioxidants such as butylated hydroxyanisole and butylated hydroxytoluene used in foods are suspected to have cytotoxicity^[135], more and more

attention has been paid to natural non-toxic antioxidants. In recent years, an increasing pile of evidences highlights that some polysaccharides isolated from plants, herbs and fungi had antioxidant activities but low cytotoxicity^[136].

The purpose of this study was to extract GLPL by ultrasonic assisted extraction from the fermented SCR, which was fermented to use SCR as the main substance by *G. lucidum*, and to investigate its immunomodulatory activities of on macrophage RAW 264.7 cells.

6.2 Materials and methods

6.2.1 Chemicals and reagents

Minimum Essential Medium Eagle (MEM) medium, fetal bovine serum (FBS), were purchased from Sigma Aldrich, Inc. (Saint Louis, MO, USA), Lipopolysaccharide (LPS) from *E. coli* 055 was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), SOD Assay Kit-WST and Cell Counting Kit-8 (CCK-8) were purchased from Dojindo Molecular Technologies, Inc. (Kumamoto, Japan), Doxorubicin (DOX) was purchased from TopoGEN, Inc. (Florida, USA).

6.2.2 Cell lines

The murine macrophage cell line, RAW 264.7 was obtained from the Riken Cell Bank (Tsukuba, Japan) and maintained in MEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL of streptomycin at 37 °C in a humidified 5% CO₂ atmosphere (ESPEC CO₂ Incubator). The cells were cultured for 2–3 days to reach the logarithmic phase and then used for experiments.

6.2.3 Preparation of *G. lucidum* polysaccharide (GLPL)

The fermented SCR was dried in a convection oven at 50 °C and ground to a powder. The crushed powder (1 g) was extracted by ultrasonic assisted extraction with 30 min, 80 °C, 80 watt of the power with 10 of the water to solid ratio. The water-soluble polysaccharide was subsequently precipitated by adding eight volumes of 99.5% ethanol and stored at 4 °C overnight. The precipitated polysaccharide was collected by centrifuging at 8000 rpm for 30 min and lyophilized. Then the powder was dissolved in 10 mL of distilled water. The total polysaccharide was determined by the phenol-sulfuric acid method. The color reaction was initiated by mixing 1 mL of the GLPL solution with 0.5 mL of a 5% phenol solution and 2.5 mL of concentrated sulfuric acid, and the reaction mixture was incubated in a boiling water bath for 15 min. The optical density of the mixture was determined at 490 nm and the GLPL content was calculated with D-glucose as the standard. The results were expressed as milligram of glucose equivalent per gram of the fermented SCR.

6.2.4 Immunomodulation activities of GLPL

6.2.4.1 Bioactivity assay

The effect of GLPL on the proliferation of RAW 264.7 cells was estimated using the Cell Counting Kit-8 (CCK-8). RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4 cells/mL at 37 °C in a 5% CO₂ atmosphere for 24 h. Next the cells were incubated with GLPL at 37 °C for 24 h. After this incubation, 10 µL of CCK-8 solution was added and incubated at 37 °C for 4 h. The cell viability was determined by the O.D. at the wavelength of 450 nm with a microplate reader. The data were

expressed as percentages of the control.

6.2.4.2 Measurement of the production of the nitric oxide

The nitrite accumulation was measured using Griess reagent and used as an indicator of nitric oxide (NO) production in the medium ^[137, 138]. The macrophage cells (1×10^5 cells/mL) were dispensed into a 96-well plate for 24 h. Next the cells were stimulated with LPS (1 $\mu\text{g/mL}$) and various concentrations of GLPL for 24 h. After this incubation, 50 μL of the culture supernatants were mixed with 50 μL of Griess reagent in a 96-well plate and incubated at 25 $^\circ\text{C}$ for 10 min. The absorbance at 570 nm was measured on a microplate reader. The nitrite concentrations in the culture supernatants were measured to assess the NO production in the RAW 264.7 cells. NaNO_2 was used as standard to calculate the nitrite concentrations.

6.2.4.3 Phagocytosis assay

The phagocytic ability of the macrophages was measured by neutral red uptake ^[139]. The macrophages RAW 264.7 cells (5×10^4 cells/mL) were cultured in the presence of various concentrations of GLPL and LPS (1 $\mu\text{g/mL}$) in a 96-well plate in a total volume of 100 μL per well for up to 48 h at 37 $^\circ\text{C}$. 100 μL /well of 0.075% neutral red was added and incubated for another 1 h. The media were discarded and the macrophages were washed twice with PBS (pH: 7.2-7.4). Then, 100 μL /well of the cell lysing solution [50% ethanol: 50% acetic acid (v/v) = 1:1] was added and incubated for 2 h. The absorbance at 570 nm was measured using a microplate reader.

6.2.4.4 Protective activity

The RAW 264.7 cells were cultured in a 96-well plate at a density of 5×10^4

cells/mL for 24 h at 37 °C in a 5% CO₂ atmosphere. Then cells were incubated with DOX (5 µM) in the presence or absence of various concentrations of GLPL for 24 h. After drug exposure, 10 µL of the CCK-8 solution was added and incubated at 37 °C for 4 h. The cell numbers were quantitated by reading the absorbance at 450 nm. The data were expressed as percentages of the control.

6.2.5 Statistical analysis

Statistical significant differences between groups were determined using Student's t- test analysis. P values < 0.05 were considered significant.

6.3 Results

6.3.1. The effect of GLPL on the proliferation of the macrophages

The stimulatory effect of GLPL extracted from the fermented SCR on the proliferation of the macrophages was tested. The results showed the exposure of GLPL activated the proliferation of the macrophages (Table 6-1). At the concentration of 20 µg/mL, the stimulatory effect reached maximum, was 171.43%. Actually the higher concentrations (40 µg/mL) decreased the proliferation rate of the macrophages and this may be related with the immunologic paralysis caused by the high dosage. It was suggested that GLPL possessed stimulatory effect on the proliferation of the macrophage RAW 264.7 cells with low cytotoxicity. Xie et al. (2006) previously demonstrated that GLPL have the inhibition of the cancer cell proliferation ^[140]. However, the mechanism(s) responsible for the inhibitory effects of GLPL on the cancer cells has not been fully elucidated. In this study, the immunity activity of GLPL was examined at the cellular level, which helped explain the mechanism of

inhibitory effects of GLPL on cancer cells. Some results have also found the similar effect of others mushroom polysaccharide in animal models. Likewise, intravenous injection of lentinan increased the absolute number of monocytes in peripheral blood, as well as number of granulocyte–macrophage progenitor cells in spleen and bone marrow ^[131].

6.3.2. The effect of GLPL on the production of the nitric oxide

It is reported that LPS showed strong immunomodulating activities. It stimulates the macrophages to produce pro-inflammatory cytokines and secondary mediators, such as NO which is a gaseous molecule synthesized from L-arginine by nitric oxide synthase (NOS). It is a highly reactive free radical and it can form a number of oxidation products such as NO₂, NO₂⁻, N₂O₃ and S-nitrosothiols. NO participates in the physiology and pathophysiology of many systems ^[141]. It is an important mediator of the non-specific host defense against invading microbes and tumors. Thus NO can be used as a quantitative index of the macrophages activation. The results of stimulatory effect of GLPL on the NO production were showed incubation with GLPL stimulated RAW 264.7 cells NO production in a dose-dependent manner. Treatment with GLPL at the concentration of 40 µg/mL significantly stimulated the NO production (21.16 µM) in comparison with the control (6.32 µM) (p < 0.01), and was higher than 20.52 µM of 1 µg/mL LPS (the positive control) produced (Table 6-1).

6.3.3. The effect of GLPL on the pinocytic activity of the macrophages

Because the macrophages play an important role in host defense that phagocytize the pathogens ^[142]. Thus phagocytosis is an important indicator of the macrophage

effector activity and it represents the final and most indispensable step of the immunological defense system. The phagocytic activity of the macrophages was monitored by measuring the amount of neutral red internalized in the macrophages. The results showed that GLPL significantly and dose-dependently increased the phagocytosis of the RAW 264.7 cells in comparison with the control (Table 6-1) ($p < 0.01$). Moreover, the O.D. value of treatment with GLPL at 1.25 $\mu\text{g}/\text{mL}$ was higher than that of the positive control, which was treated with LPS at 1 $\mu\text{g}/\text{mL}$. The results demonstrated that administration of GLPL may result in the initiation of immune reaction against foreign materials such as pathogen and tumors. Indeed, a variety of plant polysaccharides have been reported to exhibit beneficial pharmacological effects via their ability to modulate the macrophages function. One of the most distinguished features of the macrophages activation would be an increase in pinocytic activity. Yi et al. (2008) also reported Glycyrrhiza uralensis fish polysaccharide could activate peritoneal macrophages and enhanced pinocytic activity^[143].

6.3.4. Protective effect of GLPL on DOX-induced macrophages viability

Doxorubicin (DOX) is a drug used in cancer chemotherapy. It is an anthracycline antibiotic, closely related to the natural product daunomycin, and like all anthracyclines it works by intercalating DNA. Treatment with DOX resulted in a decrease of the macrophages survival rate, which was 58.45% (Figure 6-1). But in the presence of GLPL, the macrophages viability was significantly increased and in a dose-dependent manner. For example, incubation with 40 $\mu\text{g}/\text{mL}$ of GLPL, the cell survival rate (97.36%) was significantly higher than the negative control (exposure of

5 μM DOX). This result was similar with Hu et al., who showed that the cell survival rate was 96%, treatment with 5 μM DOX and 0.5 mg/mL of the extract of Chaga ^[144].

6.4 Discussion

Activated macrophages not only participate in both specific and non-specific immune reactions, but also are the “bridge cells” of these two kinds of immune reactions. When activated, they produce large amount of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) which both inhibit the growth of a wide variety of tumor cells and microorganisms. In this study, we detected the phagocytosis, which is important in uptake of antigen, and measured the NO production, which is crucial in killing microbes, parasites and tumor cells. After phagocytic uptake, macrophages turn their role into antigen-presenting cells with expression of higher levels of costimulatory molecules, such as CD80 and CD86, and then mediate an interaction between T cells and macrophages ^[145]. In the present study, phagocytosis assay of neutral red showed that GLPL significantly and dose dependently increased the phagocytosis of RAW264.7 cells. These results demonstrated that administration of GLPL may result in the initiation of immune reaction against foreign materials such as pathogen and tumors. As well as, it has been stated that NO has dual effects ^[146]. Low concentration of NO has been shown to have protective effects while high concentration to be cytotoxic. Our results suggested that GLPL treatment increased the nitrite concentration in the supernatants in a dose dependent manner. Comparing to some other polysaccharides, GLPL increased the nitrite concentration to a proper level which will not be cytotoxic ^[147].

6.5 Summary

G. lucidum polysaccharide demonstrated the immunomodulatory activities, including the effect on the proliferation of the cells, the production of the nitric oxide, phagocytosis and protection effect on the macrophages from Doxorubicin (DOX) damage in a dose-dependent manner.

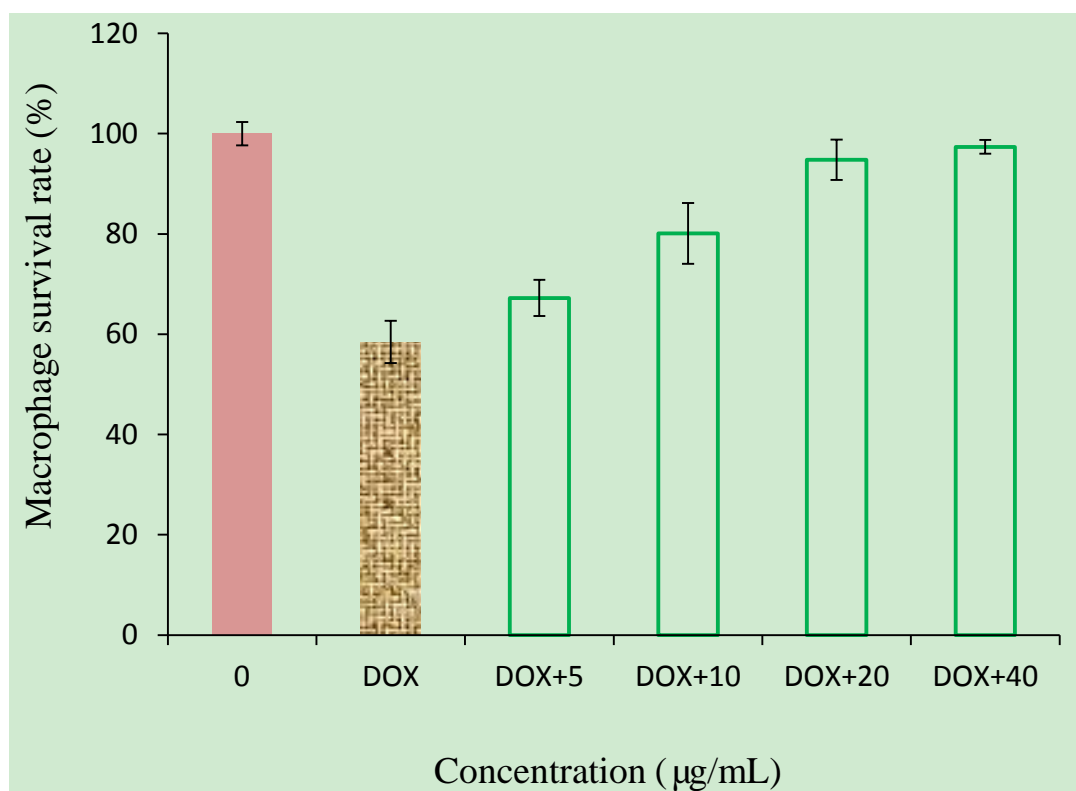


Figure 6-1. Protective effect of GLPL on DOX-induced macrophage viability

All treatments were conducted in triplicate, and mean values were reported.

The vertical bars represented the standard deviation of each data point.

LPS was the positive control.

Table 6- 1. Immunomodulation activities of GLPL

Concentrations of GLPL ($\mu\text{g/mL}$)	the proliferation of the macrophages (%)	the production of the nitric oxide (μM)	Phagocytosis (O.D.540 nm)
0	100.01 \pm 0.60	6.32 \pm 0.18	0.093 \pm 0.006
1.25	120.18 \pm 2.57	8.38 \pm 0.41	0.127 \pm 0.004
2.5	123.79 \pm 5.89	16.92 \pm 0.49	0.131 \pm 0.004
5	144.31 \pm 4.73	17.02 \pm 0.32	0.135 \pm 0.005
10	155.86 \pm 6.82	18.61 \pm 0.64	0.141 \pm 0.006
20	171.43 \pm 8.96	21.05 \pm 0.73	0.146 \pm 0.014
40	158.02 \pm 13.12	21.16 \pm 1.65	0.155 \pm 0.003
LPS ^a (1 $\mu\text{g/mL}$)	----	20.52 \pm 0.18	0.122 \pm 0.011

^a LPS was the positive control.

Chapter 7 Conclusions and Future Researches

We produced the polysaccharide and phenolic compounds from fermented soybean curd residue by *G. lucidum* and *L. edodes*, and assessed the antioxidant activities and immunomodulatory activities of these compounds.

The experimental objectives were realized and the main results and conclusions were showed as follows:

7.1 Conclusions

1. The effects of fermentation conditions on the production of polysaccharide from *Ganoderma lucidum* (*G. lucidum*) using soybean curd residue as a substrate were investigated. Based on the optimum conditions of solid-state fermentation, the fermented time, the inoculum size and the C/N ratio were optimized by response surface methodology. The optimal fermentation conditions for *G. lucidum* polysaccharide were determined to be the following: 14.53% of the inoculum size, 10.49 of the C/N ratio and 21.18 days incubation. The maximum polysaccharide yield of 48.14 ± 1.47 mg/g was obtained in the verification experiment.

2. The production of total polyphenol from *Lentinus edodes* (*L. edodes*) using soybean curd residue was investigated. Based on the results of single-factor experiments, the inoculum size, the moisture content and the fermented time were optimized using central composite design in response surface methodology. As results, the optimal fermentation conditions of the total polyphenol production were determined as following: 12.13% of the inoculum size, 76.96% of the moisture

content and 24 days incubation. Compared with the unfermented SCR, the total polyphenol yield increased from 3.12 ± 0.02 to 22.93 ± 0.41 milligram gallic acid equivalent per gram, polysaccharides, protein and various amino acid of fermented SCR were increased significantly.

3. *G. lucidum* polysaccharide (GLPL) was extracted from fermented soybean curd residue by ultrasonics assisted extraction. The optimal extraction conditions were 30 min, 80 °C, 80 watt of the power with 10 of the water to solid ratio and GLPL of 115.47 ± 2.95 mg/g was obtained. Furthermore, the antioxidant and immunomodulatory activities of GLPL were investigated. The results showed that GLPL exhibited strong scavenging activities against DPPH radical, hydroxyl radical, hydrogen oxide, ABTS radical cation decolorization and reducing power, moderate ferrous chelating effect, and weak SOD-like activity. For immunomodulatory activities, GLPL was demonstrated to strongly stimulate macrophage proliferation, nitric oxide production, phagocytosis and protection effect on macrophages from Doxorubicin (DOX) damage in a dose-dependent manner. GLPL seemed to play an important role in the exploration of natural antioxidants in food industry and pharmaceuticals.

4. *L. edodes* phenolic compounds were extracted from fermented soybean curd residue by ultrasonics assisted (donated as LEPC-I). The optimal extraction conditions were 10 min, 40% ethanol concentration, 100 watt of the power with 30 of the ratio of water to solid and LEPC of 44.16 ± 2.35 mg GAE/g was obtained. LEPC-I were further purified by macroporous adsorption resins (donated as LEPC-II).

Antioxidant activities of LEPC-I and LEPC-II were investigated. Results showed that LEPC-I and LEPC-II exhibited strong scavenging activities against DPPH radical, hydroxyl radical, hydrogen oxide, ABTS radical cation decolorization and reducing power, as well as weak ferrous chelating effect and weak SOD-like activity. Furthermore antioxidant activities of LEPC-II were higher than that of LEPC-I. Therefore, LEPC-I and LEPC-II should be explored as the natural antioxidants in food industry and pharmaceuticals.

7.2 Further researches

1. Further works should be in progress on the isolation, purification, characterization of *G. lucidum* polysaccharide and *L. edodes* phenolic compounds from fermented SCR, and fermented SCR may possess a potential research and development value in the field of functional foods.

2. Although fermented SCR contained low contents of *G. lucidum* phenolic compounds and *L. edodes* polysaccharide, there were some reports about these compounds. Therefore, in the future, *G. lucidum* phenolic compounds and *L. edodes* polysaccharide should be determined.

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Appendix

The main content of this thesis have been published and will be submitted to journals for publication as the following:

1. **Min Shi**, Zhenya Zhang, Yingnan Yang, Li Yiting, Yuepeng Wang. (2012). Optimum Condition of Ecologic Feed Fermentation by *Pleurotus Ostreatus* Using Soybean Curd Residue as Raw Materials. *International Journal of Biology*. 34, 2-12.
2. Yuepeng Wang, Yingnan Yang, **Min Shi**, Li Yiting, Ying Zhang, Zhenya Zhang. (2012). Effect of Cucumber Extract on Soybean Lipoxygenase Activity and the Functional Properties of Soybean Protein Concentrate. *International Journal of Biology*. 4 (1), 111-119.
3. **Min Shi**, Yingnan Yang, Qinghong Wang, Ying Zhang, Yuepeng Wang, Zhenya Zhang. (2012). Optimum Condition of Fermentation by *Lentinus edodes* Using Soybean Curd Residue as Raw Materials. *Journal of Food Science and Technology*. DOI:10.1111/j.1365-2621.2012.02961.x.
4. **Min Shi**, Yingnan Yang, Di Guan, Ying Zhang, Zhenya Zhang. (2012). Bioactivity of the Crude Polysaccharides from Fermented Soybean Curd Residue by *Flammulina Velutipes*. *Carbohydrate Polymers*. DOI:10.1016/j.carbpol.2012.04.047.
5. **Min Shi**, Yingnan Yang, Di Guan, Yuepeng Wang, Zhenya Zhang. (2012). Evaluation of Solid-State Fermentation by *Ganoderma lucidum* Using Soybean Curd Residue. *Food and Bioprocess Technology* DOI:10.1007/s11947-012-0857-y.
6. **Min Shi**, Yingnan Yang, Zhenya Zhang. Isolation, antioxidant activities and immunomodulatory activities of polysaccharide from fermented soybean curd residue

by *Ganoderma lucidum*. **Submitted to Food Chemistry and under review.**

7. **Min Shi**, Yingnan Yang, Yuepeng Wang, Zhenya Zhang. Isolation, purification and antioxidant activities of phenolic compounds from fermented soybean curd residue by *Lentinus edodes*. **The manuscript was finished and supervisor is checking.**