Effects of Fluorides and Nanobubble Water on the Immunomodulatory and Antioxidative Activities of Polysaccharides from *Cordyceps militaris*

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Abstract

Cordyceps militaris (*C. militaris*) has traditionally been used as medicine for hundreds of years in some Asia countries. Previous research has shown its significant pharmacological properties on a variety of diseases and functional food application. The polysaccharides of *C. militaris* possess various activities, such as immunomodulatory, anti-inflammatory, anti-tumor and antioxidant activities. Nanobubble water (NBW) can promote internal pressure, gas stability, high gas dissolution rate, resulting in efficient mass transfer with enhanced chemical reactions. Up to now, however, little information is available regarding the effect of NBW-based fermentation on mycelia of *C. militaris*. In order to enhance the production of *C. militaris* and application, the effects of chemical (fluoride) and physical (NBW) conditions were evaluated on bioactivities of crude polysaccharides from the mycelia of *C. militaris.*

In this study, the liquid fermentation system was first established. The strain was submerged in culture medium for fermentation, to which fluoride and NBW were supplemented at different concentrations. The crude polysaccharides were extracted with boiled water for 3 hours, and then precipitated 3 times. Extracts from the optimal conditions were selected for further purification with their antioxidant and immunomodulatory activities being evaluated. The main results can be concluded as follows.

(1) The supplementation of KF 1.0 mg/L during the *C. militaris* fermentation was beneficial for the mycelia accumulation, reaching the highest concentration of 3.4 mg/mL. And the crude polysaccharide extracts exhibited a higher DPPH scavenging activities and macrophage proliferation activity (123.04%). The DPPH inhibition rates were higher in all the test groups, especially for B2 (with 1.0 mg/L KF addition) group. After purification, three major fractions were obtained from the crude polysaccharides in B2 group. The CMPF-3 exhibited the strongest SOD activity (27%) and macrophage proliferation activity (122.01%) in all the extracts.

(2) The highest mycelium concentration (3.90 mg/mL) and crude polysaccharides extraction yield (12.76%) were obtained in 25%-NBW group. The antioxidant activities of mycelia were significantly promoted after NBW supplementation. The polysaccharides from 25%-NBW, 75%-NBW, and 50%-NBW groups exhibited the strongest DPPH and ABTS radical scavenging activities, and reducing power, respectively, achieving the highest radical scavenging rate (nearly 100% at 1.2 mg/mL), the lowest IC_{50} value (1.09 mg/mL) and the highest OD value (2.13 at 2.0 mg/mL).

(3) G2/M phase was noticed to significantly increase in the 25% NBW group, with the maximum percentage of 9.67%. 25%-NBW extracts showed a significant promotion effect on

macrophage cells, the viability reached highest 123.6 ± 6.9% at 6.25 μg/mL. When compared with the untreated cells, the highest cytokines expression observing in the 25%-NBW treated groups were IL-2 (180.4 pg/mL), IL-4 (86.7 pg/mL), and IL-4 (152.2 pg/mL), respectively. In addition, the fluorescence intensity in cells treated with 25%-NBW was significantly increased in a dose-dependent manner in comparison to the control group, demonstrating that 25%-NBW treatment may mediate the upregulation of intracellular reactive oxygen species (ROS) production.

In conclusion, fluoride and NBW were first found to significantly influence *C. militaris* biomass and its crude polysaccharides accumulation. The NBW supplementation showed a greater effect on the mycelia of *C. militaris* when compared with fluoride. The polysaccharides extract from 25%-NBW group exhibited stronger DPPH and ABTS radical scavenging activities and stronger reducing power. Besides, the higher macrophage proliferation activity was also detected in the 25%-NBW group, which is probably associated with cell viability, G2/M arrest, cytokines generation and ROS production. Results from this study provide a cheaper and simpler fermentation method. NBW-based fermentation, for a large quantity and more effective production of *C. militaris*. This study also contributes to a new concept for the development and application of *C. militaris* in the future.

Keywords: Nanobubble water; *Cordyceps militaris*; Fluoride; Polysaccharide; Immunomodulatory; Antioxidant

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Chapter 1 Introduction

1.1. *Cordyceps militairs*

Cordyceps is a wild parasitic fungus, usually parasitizes on the larvae of *Hepialidae insects*. The larvae of insects are used as a medium to grow and reproduce. When the external conditions are suitable, they will reproduce sexually, and the ascomycetes will reproduce germinating spores. It was used as a traditional Chinese medicine more than a thousand years ago. The value of wild *Cordyceps* was due to its scarcity and precious medicinal effects. In 2012, China declared wild *Cordyceps* as an endangered species. In recent decades, many countries in Asia have begun to study the artificial cultivation of *Cordyceps*.

Cordyceps militaris (*C. militaris*), also called “Yong Chong Cao” in Chinese, is a very precious herbal medicine (Shrestha et al., 2012). It has received considerable attention as an important biomaterial for pharmaceutical and functional food application owing to its various bioactive components (Ng and Wang, 2005; Song et al., 1998; Zeng et al., 2014). Among them, polysaccharides play a critical role in pharmacological functions of *C. militaris* such as immunomodulatory (Lee et al., 2014), antioxidant (Chen et al., 2013), anti-cancer (Mizuno et al., 1999), anti-inflammatory (Jo et al., 2010), hepatoprotective (Tian et al., 2020), antinociceptive and anti-aging activities (Zhang et al., 2019). In recent decades, lots of research works focused on the identification of bioactive components from its fruiting body. On the other hand, the polysaccharide extracted from mycelia has been recognized as a potential substitute with the advantages of short growth cycle, easy control and some better bioactivities (Liu et al., 2019). *C. militaris* is a *Cordyceps* species that was widely artificially cultivated. Most of the products are *Cordyceps* powder prepared from *Cordyceps* fruiting bodies. These products are produced from fruit bodies grown in rice medium. The studies have found that artificially cultivated mycelium of *Cordyceps militaris* has similar or better content of certain active ingredients than wild *Cordyceps*, such as polysaccharides, cordycepin, ergosterol and mannitol. That indicates that the cultivated mycelium of *C. militaris* could be an ideal substitute.

In recent decades, with the advancement of science and technology in Asia, some specific traditional medicines of Asia have been continuously discovered. Functional mushrooms (edible or medicinal) possess many biological activities as a natural product (Zhou et al., 2020). As one of the most important traditional Chinese medicines recorded in "Compendium of Materia Medica" is *Cordyceps* (an entomopathogenic fungus) (Figure 1-1). The wild fungal larvae of *C. militaris* are widely distributed in worldwide (Shrestha et al., 2012). As an

artificially cultivated fungus, it is treated as a special type of *C. militaris* was found to be very easy to cultivate in artificial liquid and solid media compared to *Cordyceps sinensis* which must be infested with insects. Also, its chemical composition is similar to that of *Cordyceps sinensis*, therefore it was considered as a similar substitute, especially in China (Huang et al., 2016). It has been used in traditional Chinese medicine as a treatment for many diseases, and recent studies have demonstrated its pharmacological properties, including tumors, aging, kidney deficiency, coronary disease, hyperlipidemia (Song et al., 1998; Ng and Wang, 2005). Its pharmacological properties will be continually developed in the future. However, the existing production capacity is still unable to supply the market demand on the other side. For this reason, some new fermentation techniques to increase *C. militaris* production and other substitute fungal strains were being used (Sung et al., 2006).

1.2. Polysaccharides

Polysaccharides are a group of polymeric sugar polymeric carbohydrates formed by multiple (more than 10) monosaccharide molecules. Polysaccharides are mostly found in living organisms and are not low in content, and they play a very important role in living organisms. Polysaccharides are known to us as the important components of cell walls of plants and animals, such as pectin and cellulose. Besides, polysaccharides possess specific biological activities in the cells, such as antioxidant activity (Chen et al., 2013), anti-inflammatory (Hou et al., 2020), antiviral (Zambare and Christopher, 2012), etc. However, all these properties do not occur individually, the physiological activities of polysaccharides are often complex and overlapping. *Cordyceps* polysaccharides are an important part of its pharmacological effects (Zhong et al., 2009), and the content and quality of *Cordyceps* polysaccharides have been used as criteria for determining *C. militaris* products.

1.2.1. The structure of polysaccharides

The most basic structural unit of polysaccharides is the monosaccharide, which is linked by glycosidic bonds. Their relative molecular masses can reach tens of millions. The biological activity of polysaccharides is influenced by the chemical structure and the straight-chain and branched configurations of the glycosidic bonds. In particular, the different conformations affect the water solubility of polysaccharides and have a great impact on the activity of polysaccharides in living organisms. In addition, polysaccharides in microorganisms such as fungi, bacteria and algae are often entangled with other macromolecules and co-create bioactive

effects (Yang and Zhang, 2009). Therefore, the structure of polysaccharides, and the form of polysaccharides in organisms is worthy of more in-depth study.

1.2. Chemical composition and structure characteristics of *Cordyceps militaris*

Polysaccharide is a mixture, and polysaccharides are complex and variable in nature. Polysaccharide from *C. militaris* also possess a complex chemical structure. Different species of polysaccharides are often difficult to separate because they have similar chemical properties, and the polysaccharides extracted from the same source are slightly different each time. Monosaccharide composition and glycosidic bonds directly influence the chemical structure of polysaccharides (Ji et al, 2019).

1.2.1. Monosaccharide composition

Carbohydrate that cannot be rehydrolyzed are called monosaccharides. The common monosaccharides found in nature are mainly including D-(-)-ribose, D-(-)-2-deoxyribose, D-(+)-xylose, L-(+)-arabinose, D-(+)-glucose, D-(+)-mannose, D-(+)-galactose and D-(-)-fructose. Ribose is present in yeast and cells in the form of glycosides and is a component of nucleic acids as well as some enzymes and vitamins, and some derivatives of galactose are widely distributed in the plant kingdom. Monosaccharides are the basic components of polysaccharides. The study of monosaccharides helps to understand the structure of polysaccharides and the process of their formation.

1.2.2. Chemical structures

There are many studies on structures of *C. militaris* polysaccharides, most of them are extracted by hot water. (1 → 4)-linked-galactose were found in the polysaccharide (CPS-3) which could generate glucose phosphate to enter the glucose metabolic pathway (Yu et al., 2004). The α-(1 → 4)-D-mannose was inferred existing in the *C. militaris* polysaccharides. The D-mannose is widely distributed in body fluids and tissues, especially in the nerves, skin, testes, retina, liver and intestine. It participated in immune regulation; increasing wound healing; anti-inflammatory effect; inhibiting tumor growth and metastasis.

1.3. Bioactivities of *Cordyceps militaris* polysaccharides

The natural activity of *C. militaris* polysaccharides is excellent and has been widely researched and extracted in recent years as a health product and functional food. *C. militaris*

polysaccharides have various bioactivities such as antioxidant activity and immunomodulatory activity (Liu et al., 2016). The effective antioxidant activity can prevent diabetes, aging, atherosclerosis and many other diseases. The analysis shown the polysaccharides from *C. militaris* could significantly affect the oxygen free radicals and concentration of antioxidant enzymes (Liu et al., 2016). In addition, when the free radical content in the cells is too high, oxidative stress is generated and a large amount of reactive oxygen species (ROS) can lead to a decrease in cell viability and even death. Some of researcher found that the antioxidant ability of polysaccharides from *C. militaris* even higher than the polysaccharides extracted from *Cordyceps sinensis in vitro* and *vivo* which could be substituted in the future (Wang et al., 2012).

The effect of polysaccharides on immune activity was mostly assessed by their effect on macrophages. There is reported the polysaccharide from *C. militaris* could enhancing the inflammatory cytokines in RAW 264.7 macrophage cells, and phagocytic uptake (Lee et al., 2015). Besides it could increase the phagocytosis and simulate the production of NF-κB (Chen et al., 2010).

The structure determines the properties of polysaccharides. It has been shown that the composition of the sugar units in the primary structure of polysaccharides and the connection of adjacent monosaccharide groups determine the activity of polysaccharides; while the type of branched chains in the higher structure, the degree of polymerization and their distribution on the main chain determine the strength of polysaccharide activity. The exact antioxidant mechanism of polysaccharides still needs to be studied. At present, the antioxidant mechanism of polysaccharides is considered to include the following four aspects: first, the hydrogen atoms on the polysaccharide structure can react with free radicals to generate water, and the reaction generates single electrons that can be further reduced; second, polysaccharides can capture free radicals generated in lipid reactions or chelate with metal ions, which are essential factors for the formation of free radicals; third, polysaccharides can enhance the antioxidant activity of certain antioxidant enzymes in the body, such as superoxide dismutase. Thirdly, polysaccharides can enhance the activity of certain antioxidant enzymes such as superoxide dismutase in the body, so as to better play the antioxidant ability; fourthly, polysaccharides can indirectly achieve the antioxidant effect by regulating the immunity of the body.

1.4. Fluoride development

Moderate intake of fluoride (F^-) is beneficial to one's health. Tea is a perfect natural fluoride provider. Studies have found that the oral health of adults over the age of 25 in the UK

is significantly better than in the US, which is related to the tea culture of the British. Therefore drinking 0.48mg of tea containing fluoride daily will prevent dental caries (Onishi et al., 1981). However, excessive intake of fluoride can lead to fluorosis. Excessive fluoride concentration is usually caused by industrial waste or excessive pesticides. Research on organic fluoride is currently applied only to chemical synthesis. Some synthetic drugs have been found to be more potent and have higher biological activity, such as anti-tumor drug: fluorouracil, Fludarabine phosphate; Anti-inflammatory drug: Diflucan; Hormonal drugs: Flurandrenolide and Fludrocortisone. The research on fluorine pesticides has also been greatly developed.

1.5. Nanobubbles water

Nanobubbles (NBs) are tiny bubbles with respective diameters less than 1000 nm. In contrast to microbubbles and coarse bubbles, NBs have longer retention times, superior stability, high mass transfer efficiency, larger surface area, excellent electrical conductivity (Alheshibri et al., 2016; Ulatowski et al., 2019) and higher zeta potential (ζ) value (Uchida et al., 2011). Nanobubbles were discovered in the last century, however, they have been developed and applied in recent decades, and their current applications are mainly focused on the agriculture, food, wastewater treatment, and medical industries. The surface rigidity of NBs is so strong that is not easily broken. According to the stokes' theorem, the bubble floating speed is proportional to the square of the bubble diameter for nano scale bubbles, and the rising speed of microbubbles is independent of the diameter due to the change in shape, so the smaller the diameter of nanobubbles has a longer residence time in water (Grunsky, 1983; Takahashi, 2005; Ushikubo et al., 2010; Ohgaki et al, 2010). The stability observed and reported by Zimmerman Agarwal et al. (2011). And Agarwal et al. (2011) also conjectured that there is relationship between hydrogen bonding structure and NBs stability.

1.6. Objective and synopsis of the thesis

The main objective of this study was for the development of, and the article tried both physical (nanobubble) and chemical (fluoride) ways to promote the enhancement of *C. militaris* products. And it is also the first time to add fluoride and nanobubble water during the process of cultivating the *C. militaris*, and the effect of the antioxidant activity and immunomodulatory activities of *C. militaris* was evaluated.

In order to accomplish the targets, the experiments were represented by four chapters.

Chapter 1 Introduction

The background was provided in the chapter one, the introduction was arranged with polysaccharide, fluoride, nanobubbles, bioactivities of *C. militaris* polysaccharides and target of this thesis.

Chapter 2 Effects of fluoride on biomass accumulation and bioactivities of polysaccharide extracts from *C. militaris*

In this chapter, the new fermentation system induced by fluoride have been built, and then the bioactive effects were evaluated. The optimal fluoride concentration was selected, the crude polysaccharide was purified and analyzed after that.

Chapter 3 Effects of air-nanobubble water supplementation on biomass accumulation of *Cordyceps militaris* and the antioxidant and immunomodulatory activities of extracted polysaccharides

In this chapter, 25%-NBW crude polysaccharide was selected and purified. The antioxidant was assessed by DPPH, ABTS radical scavenging assay. Besides, the immunomodulatory activities were estimated with macrophage cell viability, cell cycle, cytokines and ROS production.

Chapter 4 Conclusions and future study

This chapter provides a summary and evaluation of the expected results, including which conditions could promote higher yield and better biological activity of *C. militaris*. In addition, the future research direction is designed.

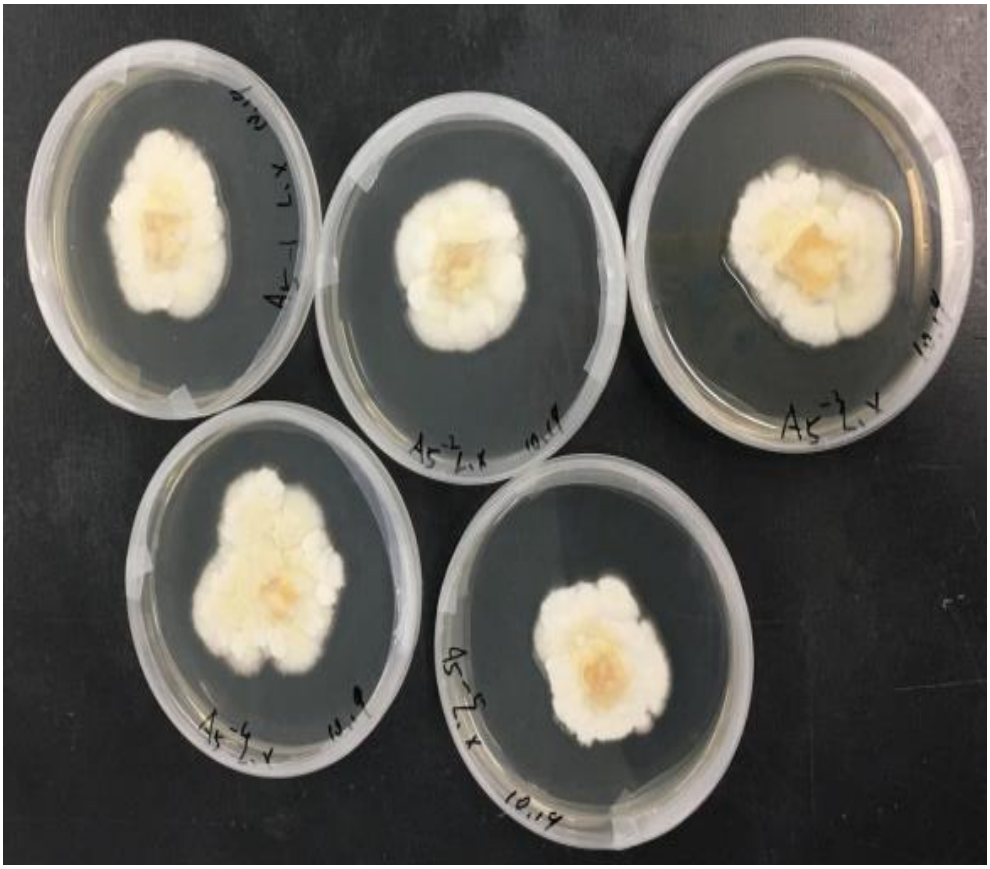


Figure 1-1 *C. militaris* from Wuhan Academy of Agricultural Sciences (Wuhan, China).

Chapter 2 Effects of fluoride on biomass accumulation and bioactivities of polysaccharides extracted from *C. militaris*

2.1. Introduction

Fluorine is widely distributed in nature, among which tea and fish are richer in fluorine among the foods naturally consumed by human beings. Fluorine is one of the essential trace elements in human body, and the normal adult contains 2.6g of fluorine, the right amount of fluoride can have a certain positive effect on the body's metabolism and play a role in preventing the occurrence of diseases, and fluorine has a significant inhibitory effect on tooth decay. The United States has since taken the lead in adding fluoride to tap water, then many other parts of the world quickly followed suit, Health Canada recommended that the optimal fluoride concentration in drinking water is 0.8 to 1.0 mg/L. In 2015, the recommended fluoride concentration in U.S.A. for drinking water was reduced to 0.7 mg/L. Fluoride is closely related to the metabolism of calcium and phosphorus, and small amounts of fluoride can accelerate the mineralization of bone and dental hard tissue components and increasing its stability. It can also promote the development of strong bones under a safe dose. British researchers have also found that daily consumption of moderate amounts of fluoridated tea has a positive effect on oral health (Onishi et al., 1981). Organic fluorides are widely used in the pharmaceutical industry. Due to the special bioactivity and bio adaptability of fluorinated organic compounds, the efficacy of fluorinated drugs is several times stronger than that of ordinary drugs, such as anti-tumor drug: fluorouracil, Fludarabine phosphate; Anti-inflammatory drug: Diflucan; Hormonal drugs: flurandrenolide, fludrocortisone. The fluoride content of raw natural foods is generally very low, such as fresh vegetables, fruits, eggs and milk, while many processed foods, especially beverages such as soda and fruit juices, generally have a higher fluoride content. Fluoride pesticides have a wide market in China, for example fosfomycin, fluralin, ethoxyfluorfen and other herbicides and insecticides. Besides, the performance of herbicides made of fluoropyridine derivatives such as Steadicam and Dinotefuran has both insecticidal and sterile functions.

There is no research to identify the effects of fluoride on the edible mushroom. Thus, this research was established to evaluate the effects of low concentration fluoride supplementation on the biomass accumulation and bioactivities during the *C. militaris* fermentation.

2.2. Materials and methods

2.2.1. Strain and mycelia fermentation

The strain used in this study was purchased from Wuhan Academy of Agricultural Sciences (Wuhan, China). The culture medium was containing Peptone 10 g/L, Sucrose 20 g/L, Yeast 1 g/L, magnesium sulphate 0.5g/L, Potassium hydrogen phosphate 1.0 g/L. The pH was adjusted to 6.0. 200 mL of the culture medium was placed in a 300 mL flask. Two different fluoride (NH_4F, KF) were added with three different concentrations of 10 mg/L, 1.0 mg/L, and 0.1 mg/L. Each flask was inoculated with four pieces of 1×1 cm2 seed strain after sterilization. The strain was fermented in shaker with 80 r/min at 22°C under dark space. The C. *militaris* was collected after fermentation for 15 days. C. *militaris* treated with NH_4F and KF at different concentrations was recorded as group A (A1, A2, A3 were the mycelia induced by 10 mg/L, 1.0 mg/L, 0.1 mg/L NH_4F, respectively) and group B (B1, B2 B3 were the mycelia induced by 10 mg/L, 1.0 mg/L, 0.1 mg/L KF, respectively).

2.2.2. Crude polysaccharide extraction

The mycelium was collected after fermentation. The mixture was centrifuged at 10000 g for 10 min to remove the culture medium. The residue was then collected and oven under 50°C. The dry powder weight was recorded. After that, the mycelium was extracted with ten times of boiling distilled water for 3 hours. And then concentrated with rotary evaporator. This step should be repeated at least for three times. The supernatant was concentrated and filtrated with 0.45μm membrane, and then added with 4 times of ethanol and mixed thoroughly for one night. After 3 times the precipitate was deproteinized with trichloroacetic acid following the Sevag method, the residue was filtrated. The supernatant was concentrated and lyophilized to obtain the *C. militaris* crude polysaccharide induced by fluoride (CMPF).

2.2.3. Hydroxyl radical scavenging activity assay

The hydroxyl radical scavenging activity of CMPF was measured following the method analyzed at 1989 (Nicholas and Quinton, 1989). Firstly, Preparing 200mL $FeSO_4$ solution, H_2O_2 and 50mL sodium salicylate with volumetric flask. The concentration of three reagent was 1.5 mM, 60 mM and 20 mM, respectively. Next to mix ferrous chloride (50 μL) with 100 μL sample solutions of different concentration increment by multiples (0.2-1.0 mg/mL), and then add 35 μL of hydrogen perhydro slowly. After that, each sample was supplemented with 15 μL of sodium salicylate. The mixture was shaken thoroughly for 60 minutes. The Trolox was used as the positive control. The absorbance of the reaction solutions was measured at 562 nm by using a spectrophotometer.

The percentage of scavenging capacity was calculated according to the following formula:

Hydroxyl radical scavenging ability = $[1-(A_1–A_2)/A_0] \times 100\%$

whereas, A_1 represented the absorbance of samples, A_2 represented the absorbance of the solution without sodium salicylate which could eliminate the effect of sample color, A_0 represented the absorbance of the control group.

2.2.4. Scavenging ability on DPPH radicals

The DPPH radical scavenging activity of crude polysaccharide was assayed according to the procedure as described by Brand-Williams et al. (1995) with some modifications. The sample solutions were prepared at first. Make each sample at different concentrations (0.2-1.0 mg/mL). The 25 μg/mL of DPPH was prepared with ethanol solution and stored at dark space. Taking 2 mL of sample at different concentrations (0.2-1.0 mg/mL) to mix with 2 mL of DPPH. Then the solution was reacted for 30minutes in dark space. Absorbance was recorded at 517 nm. Trolox was used as the positive control, and deionized water was used as the blank control. The DPPH radical scavenging ability was calculated by the equation below.

DPPH radical scavenging activity (%) = $[1 – (A_1- A_2) /A_0] \times 100\%$

Where, A_1 is the absorbance of the sample treated by DPPH solution (tested polysaccharides); A_2 is the absorbance of the sample added with DW; and A_0 is the control.

2.2.5. Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was identified by using a SOD assay kit-WST. Firstly, 20 μL of different concentration (0.00001 mg/mL, 0.0001 mg/mL, 0.001 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 1.0 mg/mL) of sample solution were prepared, and then 20 μL of ddH_2O was added to blank 1 and blank 3 well as the blank group. Then supplement with 200 μL of WST was mixed to each well, shaken for a while. After that, 20 μL of dilution buffer was added to each blank 2 and blank 3 well. At last, added 20 μL of enzyme working solution to each sample and blank 1 well, and the reaction mixtures were shaken vigorously. Incubated the plate at 37°C for 20 min. The absorbance was measured at 450 nm by microplate reader.

The SOD activity was calculated as following equation:

SOD activity (inhibition rate %) = $100\times[(A_{blank1}-A_{blank3}) - (A_{sample}-A_{blank2})] / (A_{blank1}-A_{blank3})$

2.2.6. Cell lines and culture

Mouse macrophage cell RAW264.7 was obtained from Riken Cell Bank (Tsukuba, Japan). Dulbecco's modified Eagle's medium (DMEM) with low sugar was used as culture medium. DMEM supplemented with thawing 10% (v/v) of fetal bovine serum (FBS) and 1% of penicillin and streptomycin antibiotics solution. The cell cultivation incubator was adjusted to 37°C and 5% of carbon dioxide

2.2.7. Cytotoxicity evaluation on RAW264.7 cells

The RAW264.7 cell viability after treated by the extracts was employed as the MTT assay following the method designed by Fang et al., 2015 with some modification. The macrophage cells were seeded into the 96-well plates at a density of 5000 cell for each well. The macrophage cells were treated as designed dosage of CMPF for forty-eight hours (6.25-100 μg/mL). The absorbance recorded at 490 nm for microplate reader (BIO-RAD, Tokyo, Japan).

2.2.8. Purification of crude polysaccharide

The polysaccharide from B2 group was prepared by using distilled water and separated by an ultrafiltration membrane with a molecular weight cut-off of 5 kDa. About 250 mg of CMPF-B2 was dissolved in 10 mL of ultrapure water and fractionated by DEAE fast flow column (2.6 cm × 30 cm) using distilled water and NaCl-gradient solution (0.1, 0.3 and 0.5 M, respectively) as eluent (1 mL/min). Then, three fractions were collected and lyophilized, named as CMPF-1, CMPF-2 and CMPF-3, respectively. The antioxidant activities of purified fractions were identified according to the procedure motioned above.

2.2.9. Statistical analysis

The experiment data were made independently by at least three times. The data are presented as mean ±SD. One-way analysis of variance (ANOVA) was applied to the data, representing a significance level $p < 0.05$.

2.3. Results and discussion

2.3.1. Mycelia kinetics curve

As shown in Fig. 2-1, it is clear that the initial 3 days is the lag phase. In this period, fungus adapt themselves to growth conditions and get ready to rapid multiply. From day 4 on, the cells began to grow fast, and the cell concentration increase logarithmically, so this period is called log phase. Since the quality and quantity of cells are best in this period, the cells in log phase

could be used as inoculum for further culture. The stationary phase started to appear from day 15 to day 28 which resulted from equal division and death rate. In this phase, the fungus produced the highest number of secondary metabolites. And then the fungus went into the decline phase after 28 days.

2.3.2. Effects of fluoride on biomass accumulation

According to Fig. 2-2, A1, A2, A3 groups were collected 2.41mg/mL, 2.63 mg/mL, 2.01 mg/mL of mycelia concentration, respectively. B1, B2, B3 groups were obtained 2.31 mg/mL, 3.4096 mg/mL, 1.74 mg/mL of mycelia concentration, respectively. The concentration of mycelia from six groups induced by different kinds of fluoride were all increased compared with normal control group which harvested 1.50 mg/mL. The results indicated that fluoride application could increase the biomass production, and among all the experiment groups, B2 (1.0 mg/L KF) was the optimum condition.

2.3.3. Effects of fluoride on polysaccharide accumulation

As showed in Fig. 2-3, the crude polysaccharide production was a little different with the results of biomass accumulation. The polysaccharide generation in A1, A2, and A3 group were 534 mg, 312 mg, 258 mg crude polysaccharides, respectively. B1, B2, B3 were obtained 300 mg, 392 mg, 346 mg crude polysaccharides, respectively. The A1 group (10 mg/L NH_4F) harvest the maximum polysaccharides. And the crude polysaccharide production in the fluoride treatment groups were all increased compared with the control group (176 mg). The results indicated that the fluoride application could also increase the polysaccharide production. The polysaccharide extraction ratio was 22%, 12%, 13%, 13%, 12%, 20% and 12% in A1, A2, A3, B1, B2, B3 and control groups, respectively (Fig. 2-4). So, the extraction ratio results showed the culture condition A2, A3, B1, B3 have no significant effect on polysaccharide accumulation. The culture condition A1 and B2 could promote the crude polysaccharide accumulation.

2.3.4. Antioxidant activities

(1) SOD inhibition assay

As shown in Fig. 2-5, the SOD activity of CMPF in all the groups were increased with the increasing of concentrations (10^{-5} to 10^{-1}mg/mL). With higher inhibition capacity, the polysaccharide in blank group reached the strongest inhibition ability $52 \pm 0.04\%$ at concentration of 10^{-1} mg/mL, which was significantly higher than the fluoride treatment groups,

which indicated that fluoride treatment might have a negative effect on SOD ability.

(2) DPPH free radical scavenging ability

As shown in Fig. 2-6, there are no significant differences of DPPH radical scavenging abilities among all groups. The highest inhibition rate of 98%, 93%, 95%, 100%, 96%, 90%, and 88% in A1, A2, A3, B1, B2, B3, and Blank groups were obtained when the concentration reached to 1.0 mg/mL, respectively.

(3) Hydroxyl radical scavenging ability

The hydroxyl radical scavenging ability of polysaccharides were identified with result showing in Fig. 2-7. Similar with the results obtained above, fluoride treatment did not play a positive effect on the hydroxyl radical scavenging ability of polysaccharides obtained. And all the polysaccharides in the fluoride-treated groups showed similar or lower inhibition capacities at concentrations range from 0.31 to 5.0 mg/mL.

2.3.5. Macrophages proliferation effect of polysaccharides induced by fluoride

The proliferation effect of polysaccharides on macrophage cell was analyzed by MTT assay. As shown in Fig. 2-8, compared with other groups, only B2 at higher concentration (100 μg/mL) exhibited significant macrophage cell proliferation ability, which could be selected for future research.

2.3.6. Isolation and separation of polysaccharides extracted from B2 group

In this work, with significant biomass accumulation, relative higher bioactive compound production, antioxidant and macrophages proliferation effect, polysaccharide in B2 group was selected for further separation. After separation, 3 fractions (CMPF-1, CMPF-2 and CMPF-3) were obtained as shown in Fig. 2-9. The CMPF-2 was the mainly content in the CMPF.

(1) Antioxidant activities of purified fractions

In this study, the antioxidant activities of purified extracts were identified. As shown in Fig. 2-10, the SOD activity of three fractions were increased and followed a dose dependent manner with the increasing of concentrations (10^{-5} to 10^{-1} mg/mL). The CMPF-3 reached highest inhibition ratio (27%) at concentration of 0.1 mg/mL, which were lower than the other fractions. The DPPH scavenging ability receded after the purification. Although three purified fractions exhibited similar trend on DPPH inhibition capacity, it was slight lower than CMPF-B2. The Hydroxyl radical scavenging results suggested that the CMPF-2 and CMPF-3 exhibited higher scavenging ability against hydroxyl radical than CMPF-1and CMPF-B2.

(2) Macrophages proliferation activities after purification

The proliferation activity of purified fractions was analyzed by MTT assay. As shown in Fig. 2-11, Among all the purified fraction, CMPF-3 at concentration of 100 μg/mL exhibited significant macrophage cell proliferation ability. Fraction CMPF-1 exhibited no significant effect at all the concentrations tested and fraction CMPF-2 showed toxicity effect on macrophage cell at higher concentrations.

2.4. Summary

In a conclusion, three major fractions were purified from crude polysaccharide extracts induced by fluoride. The CMPF-1, CMPF-2, and CMPF-3 differed in antioxidant and immunomodulatory. CMPF-3 exhibited a strong SOD activity compared with other two fractions. Further, it is necessary to determine molecular weight, monosaccharide composition, and ultrastructure. The properties of polysaccharides are closely related to their structural properties, and we hope these properties will be further discovered in future research.

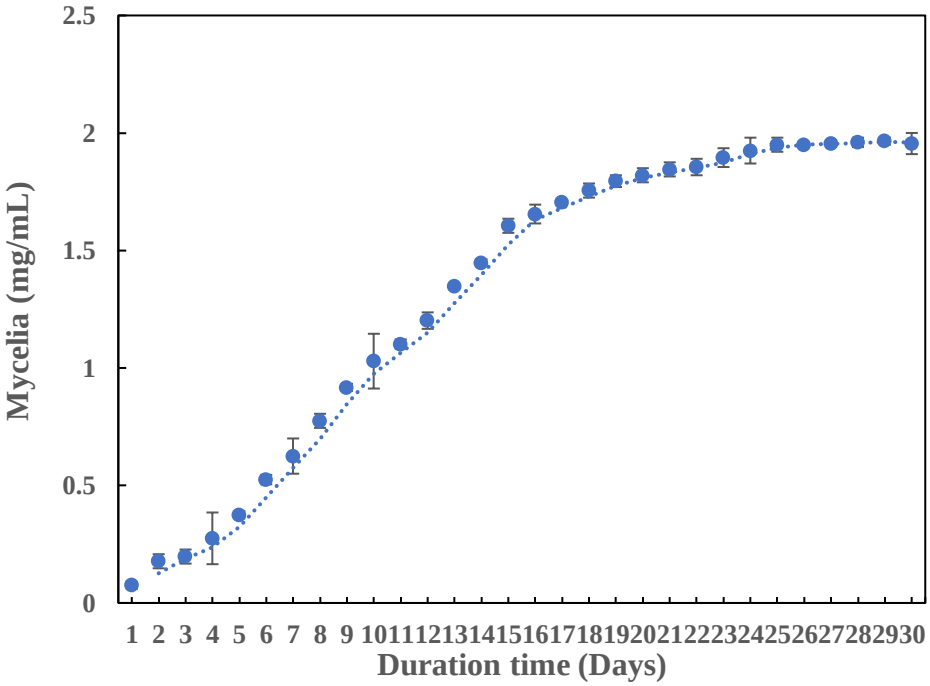


Figure 2-1 The kinetics of mycelia submerged culture. Data represent the means ± SD of three experiments.

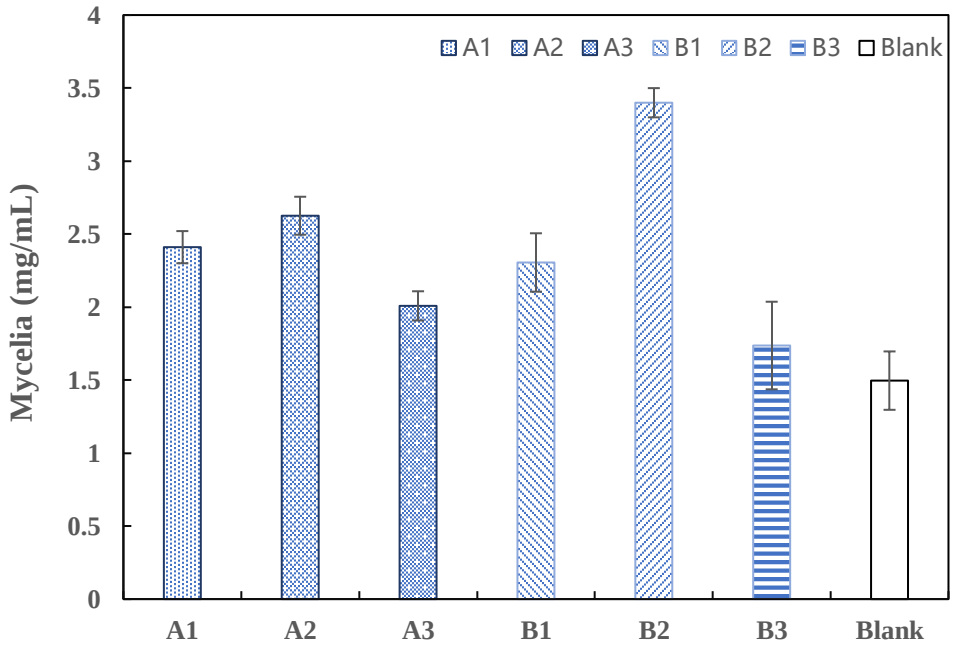


Figure 2-2 The mycelia concentration induced by NH_4F (A group) and KF (B group). A1, A2, A3 were the mycelia induced by 10 mg/L, 1.0 mg/L, 0.1 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 10 mg/L, 1.0 mg/L, 0.1 mg/L of KF, respectively. Blank was the normal control group without fluorides supplementation.

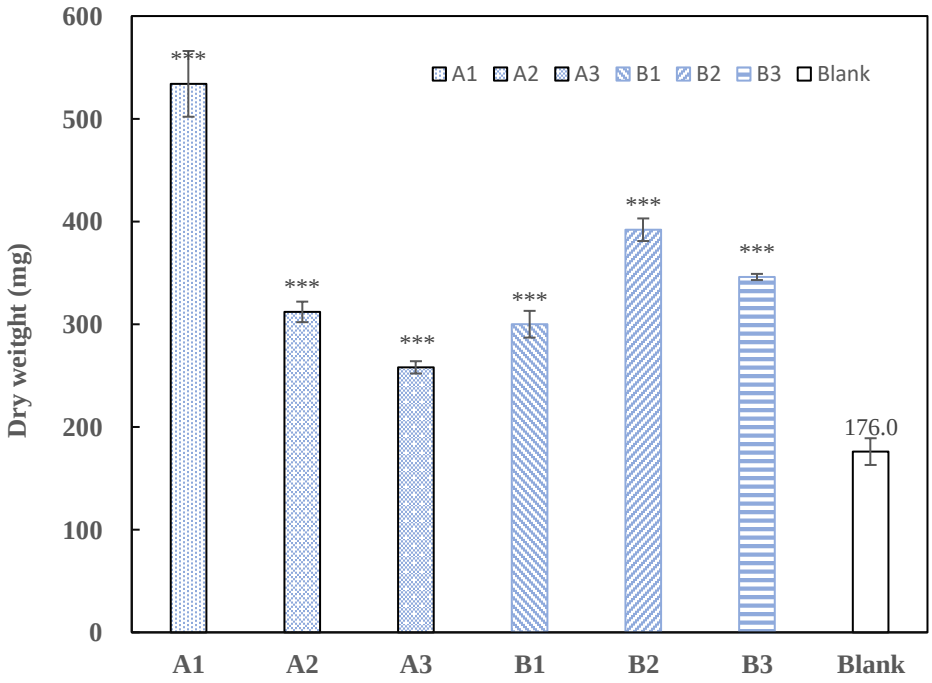


Figure 2-3 The dry weight of crude polysaccharide induced by NH_4F (A group) and KF (B group). Data represent the means ± SD of three experiments. A1, A2, A3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of KF, respectively. Blank was the normal control group without fluorides supplementation. Compared with the control group, ***$p < 0.001$.

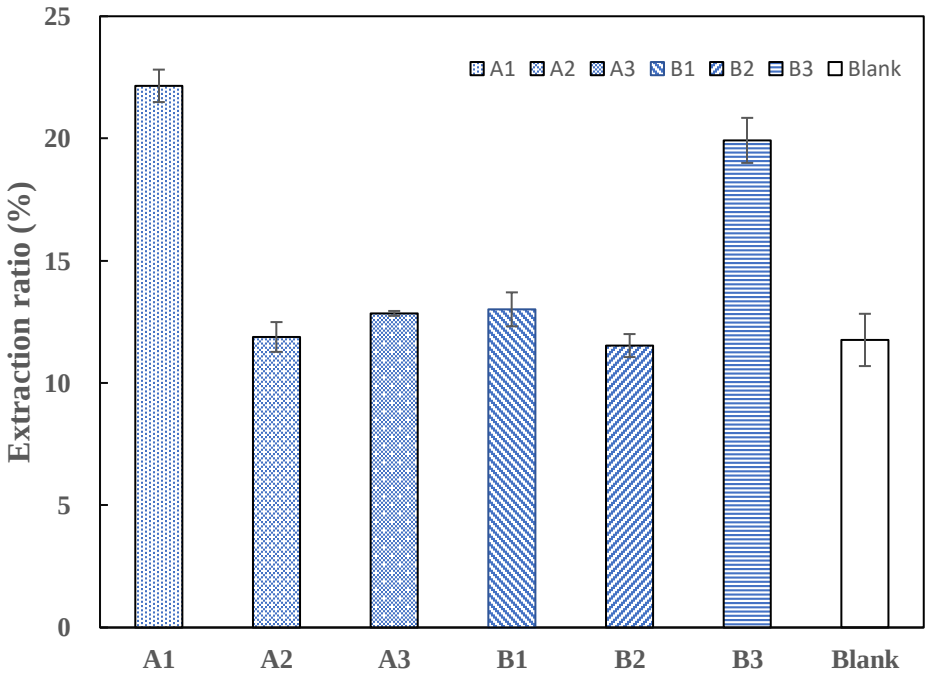


Figure 2-4 The extraction yield of mycelia induced by NH_4F (A group) and KF (B group). Data represent the means ± SD of three experiments. A1, A2, A3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of KF, respectively. Blank was the normal control group without fluorides supplementation.

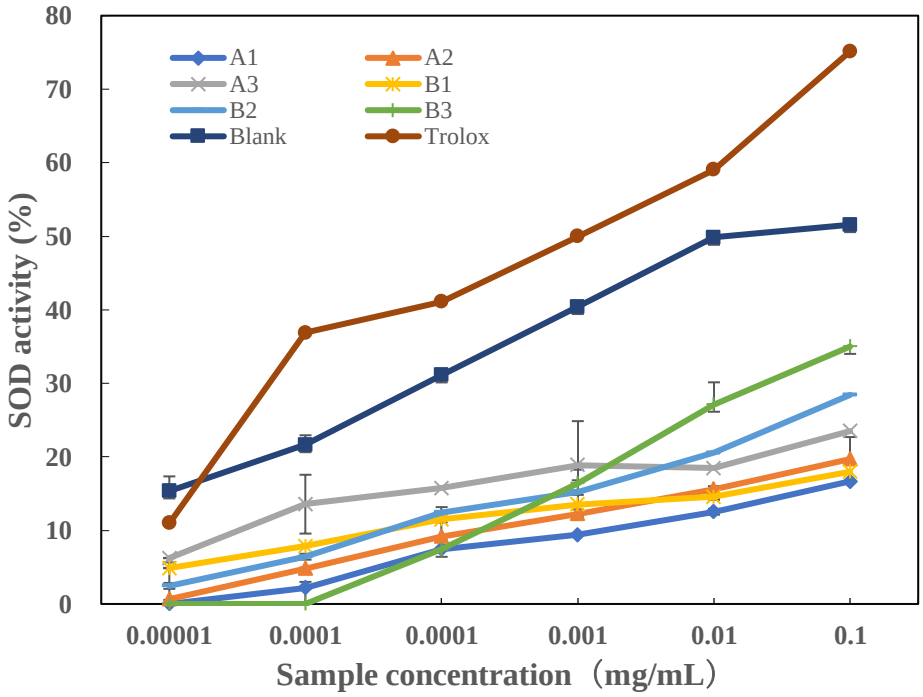


Figure 2-5 The SOD activities of crude polysaccharide induced by NH_4F (A group) and KF (B group). Data represent the means ± SD of three experiments. A1, A2, A3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of KF, respectively. Blank was the normal control group.

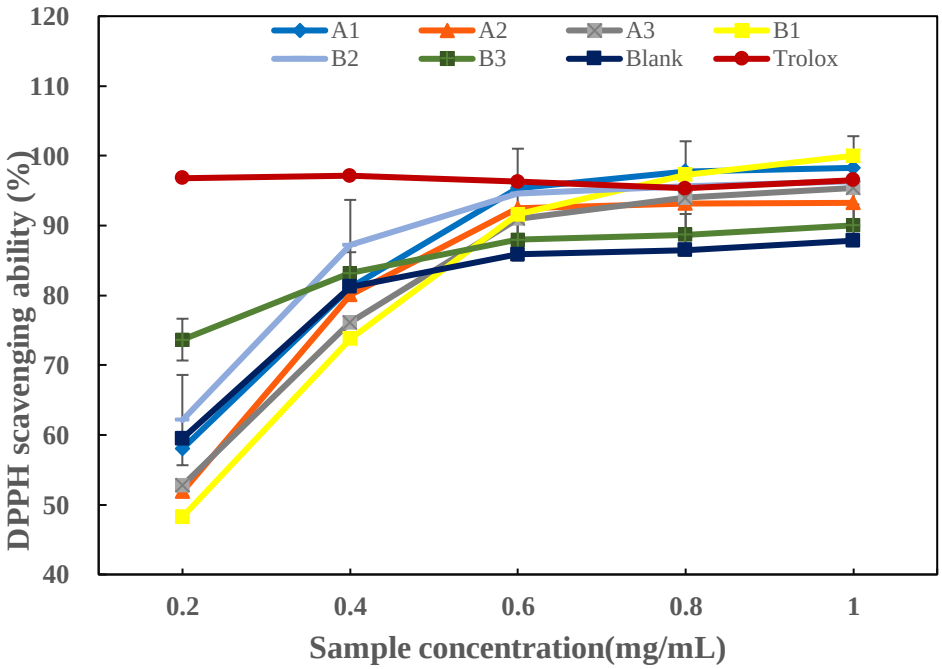


Figure 2-6 The DPPH inhibition rate of polysaccharides induced by NH_4F (A group) and KF (B group). Data represent the means ± SD of three experiments. A1, A2, A3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of KF, respectively. Blank was the normal control group.

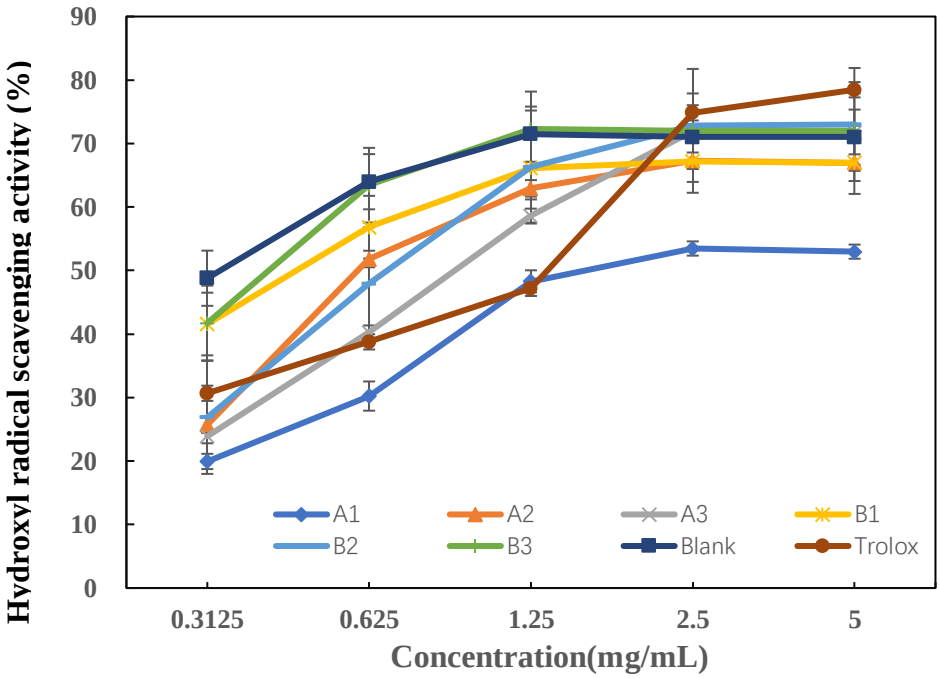


Figure 2-7 The Hydroxyl radical inhibition rate of polysaccharides induced by NH_4F (A group) and KF (B group). Data represent the means ± SD of three experiments. A1, A2, A3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of KF, respectively. Blank was the normal control group.

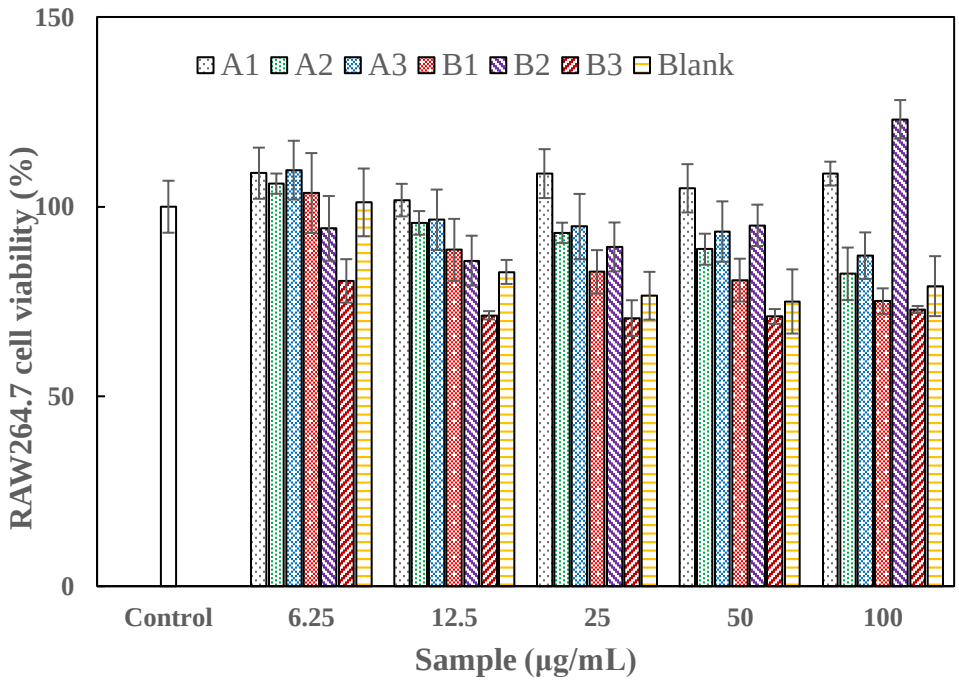


Figure 2-8 RAW 264.7 cell MTT assay. The cells were treated by crude polysaccharides induced by NH_4F (A group) and KF (B group). Data represent the means ± SD of three experiments. A1, A2, A3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of NH_4F, respectively. B1, B2 B3 were the mycelia induced by 0.1 mg/L, 1.0 mg/L, 10 mg/L of KF, respectively. Blank was the normal control group.

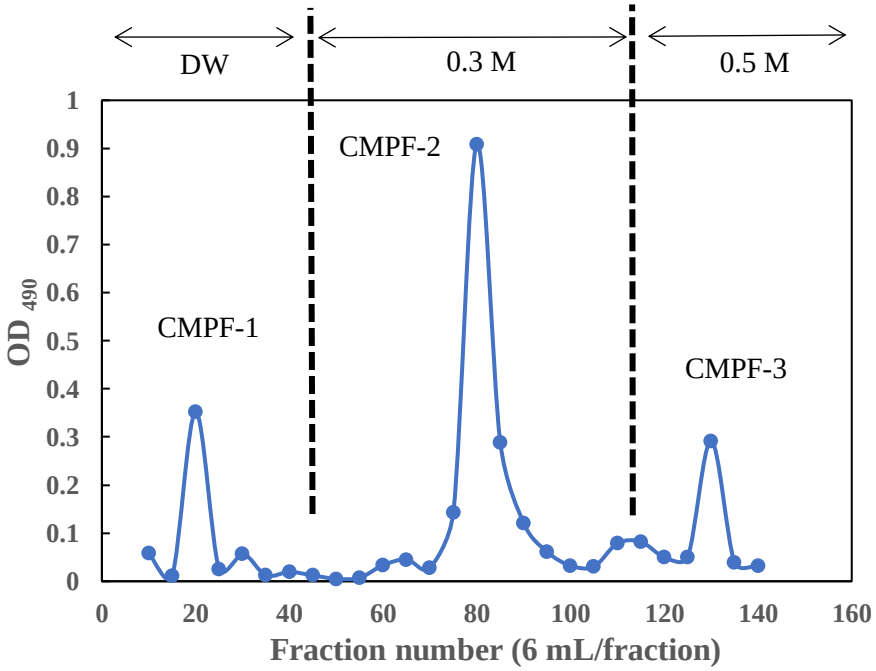
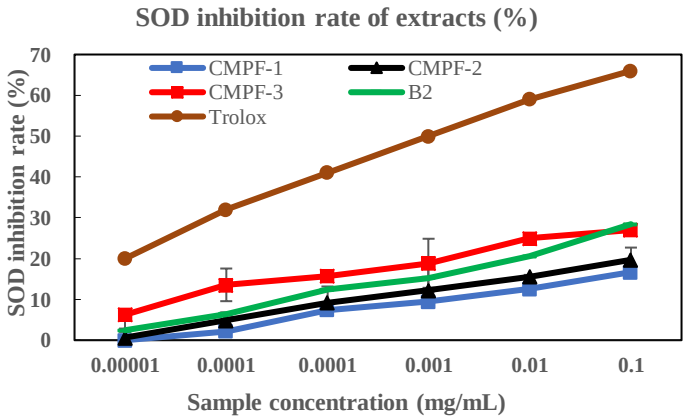
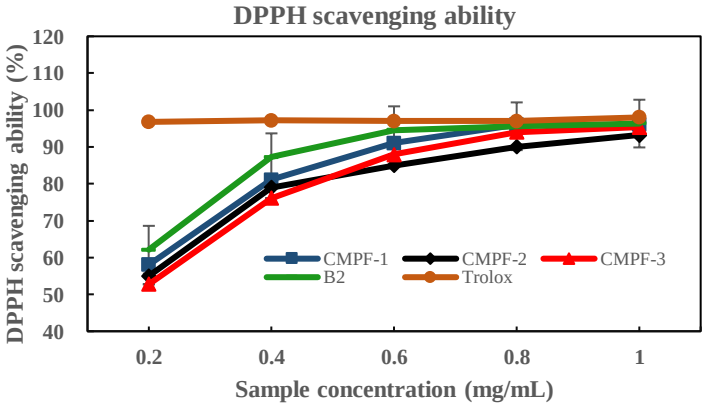


Figure 2-9 Isolation and purification of polysaccharides extracted from B2 group (mycelia induced by 1.0 mg/L KF).

(a)



(b)



(c)

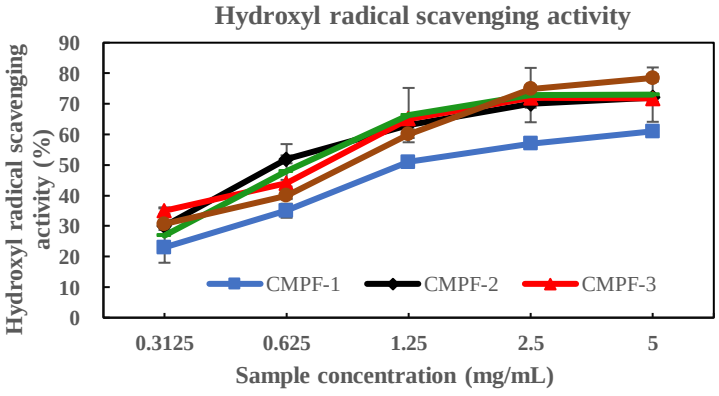


Figure 2-10 The SOD, DPPH and Hydroxyl radical inhibition rate of separated polysaccharide fractions showed in figure (a), (b) and (c), respectively. CMPF-1, CMPF-2 and CMPF-3 is the purified compounds from B2 group (induced by 1.0 mg/mL of KF).

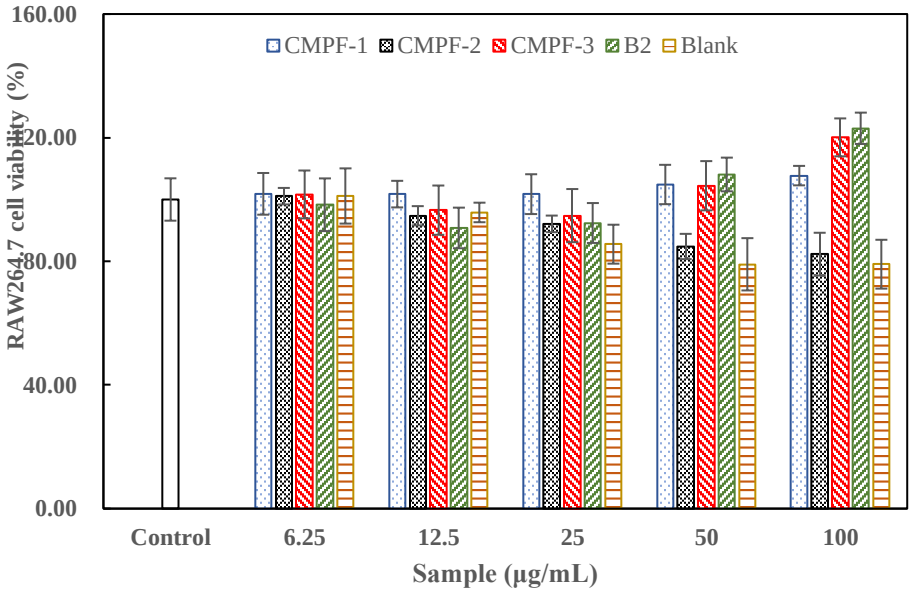


Figure 2-11 RAW 264.7 cell proliferation activity of purified polysaccharide fractions. CMPF-1, CMPF-2 and CMPF-3 is the purified compounds from B2 group (induced by 1.0 mg/mL of KF).

Chapter 3. Effects of air-nanobubble water supplementation on biomass accumulation of *Cordyceps militaris* and the antioxidant and immunomodulatory activities of extracted polysaccharides

3.1. Introduction

Nanobubble water (NBW) is a kind of water containing ultra-fine gas bubbles with diameter less than 1,000 nm. Due to the nano size of the bubbles ranging from 0 nm to 1,000 nm in aqueous solution, nanobubble (NB) has high surface areas and stagnation time in NBW (Meegoda et al., 2018). The characteristics of high internal pressure (Zhang and Seddon, 2016), good physical stability (Temesgen et al., 2017), high gas dissolution rate (Ushikubo et al., 2010) and zeta potential can result in an efficient mass transfer and chemical reactions at the gas-liquid interfaces (Bunkin et al., 2009; Sun et al., 2016). The application of NBs is more and more popular in recent decades. Reports show that application of NBW can increase seed germination rate, promote the growth of plants, mouse and shellfish, and inhibit tumor cell development individually or cooperatively with other substances (Lukianova-Hleb et al., 2014; Ebina et al., 2013; Wang et al., 2010). Most recently, N_2-NBW was claimed to have promotion effects on the growth of the probiotic *Lactobacillus acidophilus* 1028 at the lag and logarithmic phases (Guo et al., 2019). However, up to the present, little information is available on the effects of NBW on *C. militaris* growth. Moreover, no systematic evaluation can be found on the effects of NBW on the bioactive compounds accumulation in *C. militaris*.

The present study aims to explore the effects of NBW supplementation on the growth of *C. militaris*. This is also the first study about the evaluation of the effects of NBW on fungus.

3.2. Materials and methods

3.2.1. Preparation of NBW

In this study, Air-NBW was used, which was produced by a NBW generator (HACK UFB Co., Ltd., Yamanashi, Japan) according to a previous work (Guo et al., 2019). Firstly, 2 L of deionized water (DW) was injected into a transparent plastic beaker which was recycled through the NBW generator with the introduction of air. During the generation period, the gas intake speed and DW circulation rate were maintained at 0.05 L/min and 3 L/min, respectively. The generator was operated for about 20 min, and the produced NBW was labelled as Air-NBW. Then, the Air-NBW was mixed with DW at different volume ratios (25%, 50%, 75%, and

100%) to make the test water at different NB densities.

3.2.2. Identification of NBW properties

The bubble size and density were measured before NBW being added into the culture with the nanoparticle tracking analysis method by using NanoSight instrument (NanoSight-LM10, MALVERN, UK). The liquid suspension with particles was loaded into the laser module sample chamber and viewed in close proximity to the optical element. The number of captures was adjusted to 3, with the capture duration of 20 s, the screen gain of 8 and the camera level of 16. The figures were composed by the software of Nanosight. The NB size distribution and NB density were estimated by using Brownian motion and Einstein-Stokes equations (Sutherland, 1905).

$$Dt=kB\ T/3\pi\mu d$$

where Dt is the diffusion coefficient; KB is the Boltzmann constant; T is the absolute temperature; μ is the dynamic viscosity; and d is the diameter of the particle.

Zeta potential was measured by Zetasizer Nano ZSP (MALVERN, UK).

3.2.3. Strain and mycelia fermentation

The culture medium composed of 1% peptone (w/v), 2% sucrose (w/v), yeast 0.1%(w/v), 0.1% magnesium sulphate (w/v), and 0.2% potassium dihydrogen phosphate (w/v) was prepared at different volume ratios of Air-NBW as indicated above, with DW being used as the normal control group. The medium was autoclaved at 121°C for 15 min and incubated on a shaker at 90 rpm and 22°C in dark for 14 days after inoculation before the biomass and antioxidant activity being analysed.

The strain was then transferred into a 2 L culture flask for the kinetics analysis. 30 mL of mycelium liquid was taken out for analysis every day. The mycelium liquid was filtrated through a 0.45 μm filter paper and then the solid part was dried at 60°C for 10 h to obtain the biomass concentration. pH and dissolved oxygen (DO) values were recorded once per day for 20 days.

3.2.4. Extraction and isolation of crude polysaccharides

After culture, the mycelium was collected, and oven dried at 50°C to a constant weight with the dry weight being recorded. Then the dried mycelium was extracted with DW at 100°C for 3 h at a ratio of 1:10 (w/v). After 3 times of repetition, the supernatant was collected, and

filtered through a 0.45 μm filter paper. After concentrated by rotary evaporator at 50°C, the solution was then mixed with 4 times of ethanol overnight at 4°C. This step was repeated for 3 times to thoroughly remove the ethanol soluble part. Then the precipitate was dissolved in DW and dialyzed by using a dialysis bag with molecular weight cut off of 5kD for 48 hours. Finally, the fraction was lyophilized, and the crude polysaccharide was obtained. The total sugar content was measured by phenol sulfuric acid method (Dubois et al., 1956). And the total protein content and total phenolic content were quantified using Lowry's method and Folin-Ciocalteu method with bovine serum albumin (BSA) and gallic acid as the standard, respectively (Lowry et al., 1951).

3.2.5. Antioxidant activity assay

(1) DPPH radical scavenging assay

The DPPH radical scavenging activity of crude polysaccharide was assayed according to the procedure as described in Chapter 2 (2.2.4). The DPPH radical inhibition capacity of different concentrations (0.4, 0.8, 1.2, 1.6, or 2.0 mg/mL) of polysaccharides in NBW treatment groups were identified with Trolox served as positive control.

(2) ABTS radical scavenging assay

The ABTS radical scavenging activity of extracted polysaccharides was assessed by previous studies (Lee et al., 2015). Preparing 7mM ABTS and 140 mM $K_2S_2O_8$. Then mixing at least 20 mL of ABTS solution with 0.352 mL of $K_2S_2O_8$. Then the mixture should be adjusted after 12 hours in dark space. The ABTS solution was diluted with a phosphate buffer solution (PBS, pH 7.4) to adjust the absorbance around 0.70 ± 0.02 at 734 nm. Then 0.15 mL of tested sample at different concentration (from 0.4 to 2.0 mg/mL) was mixed with 2.85 mL of ABTS solution. The absorbance was measured at 734 nm after incubation at room temperature for 10 min. Trolox was used as the positive control. The ABTS free radical scavenging activity was calculated according to the following equation.

$$\text{ABTS scavenging activity (\%)} = [1- (A - B) / (C - D)] \times 100$$

where A is the absorbance of the sample mixed with ABTS solution; B is the absorbance of the sample mixed with PBS; C is the absorbance of ABTS solution mixed with DW; and D is PBS mixed with DW.

(3) Reducing power assay

The reducing power for ferricyanide of the extracted polysaccharides was determined according to the procedure described by Khaskheli et al. (2015). The different concentration of

each sample solution should be prepared at first. Then taking 1 mL of sample solution mixing with 1 mL of phosphate buffer (200 mM, pH 6.6) and 1 mL of 1% potassium hexacyanoferrate [$K_3Fe(CN)_6$]. The mixture was shaken well. Next, the mixture was incubated at 50°C for 20 min. Finally, added 1mL of 10% trichloroacetic acid. The solution transferred into centrifugal machine (1500 rpm/min, 10 min). The supernatant (2 mL) was collected and mixed with 2 mL of deionized water and 0.4 mL of 0.1% ferric chloride. The absorbance was measured at 700 nm, and trolox was used as the positive control.

(4) Ferrous ion chelating ability

The chelating ability of ferrous ion (Fe^{2+}) was following to Decker and Welch (1990) with some modification. The different concentrations of sample (0.4 - 2 mg/mL) were preparing at first. 7.5 μL of a 2 mM ferrous chloride solution ($FeCl_2$) was mixed with 277.5 μL of sample. The mixture was supplemented with 15 μL of a 5mM ferrozine solution. The absorbance was measured after 10 minutes at 560 nm. Trolox was used as the positive control.

The ability chelating ferrous ion Fe^{2+} was calculated as follows:

Ferrous iron chelating capacity (%) = $100\times(A_0-A_S)/A_0$

where, A_0 was blank absorbance, A_S was sample absorbance.

(5) Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was identified by using a SOD assay kit-WST as described in Chapter 2 (2.2.5).

(6) Hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of crude polysaccharide was identified according to Chapter 2 (2.2.3).

3.2.6. Scanning electron microscope (SEM) analysis

Scanning electron microscope (SEM) was used to represent surface morphologies of samples. SEM can be used to observe and analyze three-dimensional morphology and to analyze the composition of micro-areas while observing the morphology. The SEM (JSM6330F, Japan) used in this study by magnification at 200× and 1,000×.

3.2.7. FTIR spectroscopy

The functional groups showed a similar absorption frequency in infrared spectroscopy. So, the fourier-transform infrared spectroscopy (FTIR) are generally used to identify functional

groups. The functional groups are considered to be the internal structural units of polysaccharides which could help us to figure out the structure of polysaccharides in this study. The extracts power was grounded and mixed with KBr powder and measured by FTIR (FT/IR-300, JASCO, Japan) at a frequency range of 4,000-400 cm^{-1}.

3.2.8. Cell line and culture

RAW264.7 was the murine macrophage cell which purchased from Riken Cell Bank (Tsukuba, Japan). The culture medium was DMEM media supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL). The incubator was adjusted to 37°C with 5% carbon dioxide supplemented.

3.2.9. *In vitro* immunomodulatory activity

(1) RAW264.7 cell proliferation and morphological observations

The viability effects of 0%-NBW and 25%-NBW on RAW 264.7 cell were evaluated by using MTT assay as described in 2.2.7. The macrophage cells were treated with different concentrations of 0%-NBW and 25%-NBW (3.125-50 μg/mL) extracts for twenty-four hours. The absorption values were recorded at 490 nm by the counter (Model 550 microplate reader, BIO-RED, Tokyo, Japan). The morphology of cells in different groups were observed by using a microplate reader (Leica-DMi8, Germany).

(2) Cell cycle analysis

RAW 264.7 cells were seeded at a density of 5×10^5 cells/well in 6-well plates. After 24 h of seeding, cells were treated with different concentrations of 0%-NBW and 25%-NBW (3.125-50 μg/mL) for 24 h. The cells harvested and collected after adding trypsin. The cell pellets were cooling at -20°C supplement with 70% ethanol. Then it was taken out and centrifuged at 3000 rpm for 5 min. The supernatant was assimilated, and the residues were washed with cold 1 mL of PBS. The clean cells were dyed with 500 μL of Cell Cycle reagent (Beyotime) in dark space, then transfer to the cell cycle analysis using flow cytometer (Beckman) and Modfit Software after thirty minutes.

(3) Measurement of cytokines production

Macrophage cells were incubated in 48-well plates with different doses of 0%-NBW and 25%-NBW (3.125-50 μg/mL). The culture supernatant was collected after 24 hours, and the cytokines IL-2, IL-4, and IL-10 production were determined by the IL-2, IL-4, and IL-10 ELISA Assay Kits, respectively. The normal cells supplemented with same volume of sterile

water instead of 0%-NBW and 25%-NBW were regarded as control group.

(4) ROS secretion assay

Reactive oxygen species (ROS) production by RAW264.7 was analysed by Mito-Tracker Red CMXRos (Beyotime). The macrophage cell was prepared in 6-well plate (5×10^5 cells/well) 12 hours ago. The macrophage cells were treated 25%-NBW extracts in different concentration (6.25-25 μg/mL). After 24 hours' incubation, the culture medium was carefully removed, followed by the addition of Mito-Tracker Red CMXRos working solution (200 nM) and Hoechst 33342 (1×) in serum free medium. The strained cells were washed carefully with PBS after 15 min. The macrophage cells were transferred to the microplate reader (Leica-DMi8, Germany). The Fluorescence intensity was recorded and listed in the figure.

3.2.10. Statistical analysis

All the data were expressed as mean ± standard deviation (SD), and the results used in this work were from at least three independent experiments performed in triplicate. In addition, statistical analysis was conducted using one-way analysis of variance (ANOVA) with the Duncan's multiple-range test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ were considered as statistically significant.

3.3.Results and discussion

3.3.1. NBs distribution and concentration in Air-NBW

Different percentage of NBW solution contains different NBs concentration, in which the coalescence and self-organization processes might be affected by zeta potential, which is particularly important and related with their colloidal behavior (Bunkin et al., 2009; Gurung et al., 2016; Sun et al., 2016). Fig. 3-1 illustrates the distribution and concentration of nanoparticles in the NBW. The nanoparticle concentrations in 0%, 25%, 50%, 75% and 100%-NBW were $1.16\pm0.12 \times10^7$, $5.53\pm0.53 \times10^7$, $1.03\pm0.52 \times10^8$, $1.47\pm0.42 \times10^8$ and $1.90\pm0.82 \times10^8$ particles/mL, respectively. The size of NBs is quite important, which has a strong relationship with NB stability, spin-spin relaxation time, electric conductivity properties and zeta potential value (Ushikubo et al., 2010; Nirmalkar et al., 2018; Zhang et al., 2020). As shown in Fig. 3-1a, the particles were dispersed in the DW (0%-NBW), with size ranging from 0 to 600 nm in the 0%-NBW group. In contrast, the particle size was then centralized with the increase in NBW percentage or the concentration of NBs. The average nanoparticle size in 0%-, 25%-, 50%-, 75%-, or 100%-NBW was 440±84.85 nm, 160±24.87 nm, 170±19.1 nm,

151±22.73 nm, or 187±14.1 nm, respectively in this study.

3.3.2. Effect of autoclaving on stability of NBW

The stability of bubbles is controlled by pH value, temperature and pressure. However, these conditions will be different after the NBW being autoclaved. Therefore, it is also necessary to investigate the effect of autoclaving on NBs before its being used for fermentation. As shown in Fig. 3-2, after autoclaving, the nanoparticle numbers were decreased to 1.11±0.07 $\times 10^7$, (4.53±0.37 $\times 10^7$, 7.83±0.39 $\times 10^7$, 1.13±1.16$\times 10^8$, and 1.49±0.84$\times 10^8$ particles/mL in the 0%-, 25%-, 50%-, 75%- and 100%-NBW groups, respectively. The high stability of NBs may be attributable to the high pressure inner-gas and the electric charge interface of the bubbles (Ushikubo et al., 2010). The reduction of nanoparticles might be brought about by the inner gas pressure of NBs which would break up due to the autoclaving, leading to fewer vapor atoms inside NBs. Moreover, the interior gas pressure would not be high enough to support the force balance of a NB. However, the remaining bubbles may recover their balance after the autoclaving. As shown in Fig. 3-2, there is almost no significant effect of autoclaving on the average size of NBs in the present study, except the 0%-NBW group. As shown in Fig. 3-1, being different from the NBW solutions, the particle size in the normal control group (0%-NBW) ranged from 0 nm to 600 nm. Experimental data indicated that smaller particles with high zeta potential are much stable. Conversely, the larger the particle size, the more unstable it is (Meegoda et al., 2018). The large size particles in the 0%-NBW group will break up during autoclaving, and this phenomenon may result in a decrease in the average size of particles in the 0%-NBW group. Anyway, compared to the NBW groups, the changes in the particle size in the control group were much less due to its much lower particle concentration.

The dispersed mycelial filaments and pellets (densely interwoven mycelia) of fungi have an important impact on fungal fermentation (Papagianni, 2004). Zeta potential (ζ) is an important indicator for the colloidal dispersions: a higher zeta potential absolute value represents a stable colloidal system, while in the low zeta potential solution, attractive forces may lead to aggregation (Hanaor et al., 2012). As depicted in Fig. 3-3, the zeta potential followed a dose dependent manner after supplemented with a different ratio of air-NBW, about -3.07±1.88, -18.60±2.56, -22.90±2.12, -24.77±1.09, and -27.24±2.82 mV in the 0%-, 25%-, 50%-, 75%-, and 100%-NBW groups, respectively. And these potentials increased to -1.68±0.99, -14.44±2.32, -15.99±3.79, -18.83±1.42, and -22.13±2.50 mV after autoclaving, probably due to the decrease of NBs concentration.

The changes in dissolved oxygen (DO) and pH of NBW and culture medium before and after autoclaving was also recorded (Tables 3-1 and 3-2). As seen, the DO value was decreased after autoclaving in all the samples, and a similar trend was noticed in the culture medium prepared with NBW. The pH values in different NBW groups were similar, indicating that supplementation of air-NBW has limited effect on its pH value (Table 3-2).

Although the pH value of NBW increased by 1.3 - 1.5 after being autoclaved, only 0.02-0.13 of pH increase occurred in the culture medium after being autoclaved under the same operational conditions. This observation might be contributed by the buffering capacity of the buffer solution added into the culture medium

3.3.3. Effect of NBW on biomass growth and polysaccharides accumulation

Fig. 3-4a demonstrates the biomass accumulation in different NBW groups during the 20 days fermentation. The *C. militaris* in 0%-NBW group ended the lag phase after day 6 or 7; however, this lag phase was shortened to 4 days when supplemented with 25%-NBW. The 25%-NBW treatment accelerated the *C. militaris* growth, achieving the highest mycelia dry weight during the fermentation period compared with other test groups. The biomass concentration reached to 3.96 mg/mL after 20 days' fermentation, which is remarkably higher than the 0%-NBW group (1.30 mg/mL). The DO value maintained at 2-4 mg/L in the NBW groups, while the DO in 0%-NBW group was decreased to 0.31 mg/L after the 20 days' fermentation. No significant difference in pH value was detected during the entire experimental period (Fig. 3-4b). The difference of DO value in the control deionized water and NBW is attributed to the existing form of oxygen in water. Generally, the molecular oxygen exists in the gap of the water molecule, and the oxygen dissolved from air keeps a balance with the oxygen in water. During the NBW generation, the superfluousness oxygen is introduced into the nanobubble generator by a pump, which can establish a balance at high oxygen concentration. The superfluousness oxygen molecules may be assembled by electric force, which cannot be detected by the standard DO meter (Wang et al., 2020). After the small oxygen molecules are consumed, the extra gap may be replenished from the congregative oxygen. Thus, the NBW group can exhibit a stable DO value.

As stated, the growth of mycelial filaments exhibits a high-density medium that is similar to the non-Newtonian liquid (Papagianni, 2004), leading to the mass transfer and gas-liquid exchange problems. NBW, with a higher spin-spin relaxation time (T_2), could increase the mobility of water molecules (Ushikubo et al., 2010). On the other hand, the feature of high zeta

potential value of NBW could create repulsion force to avoid the coalescence and keep electrical stability (Wang et al., 2013). Therefore, NBW supplementation may have positive effects on biomass accumulation. In this study, NBW application exhibited some promotion effect on the growth of mycelium (Fig. 3-5), achieving biomass concentrations of 3.90±0.84, 1.89±0.54, 1.99±0.94, and 2.70±0.65 mg/mL in the 25%-, 50%-, 75%-, and 100%-NBW groups, respectively after 20 days' fermentation, in comparison to 1.25±0.36 mg/mL in the control group (0%-NBW).

Crude polysaccharides were extracted from the mycelium obtained in the different NBW groups as shown in Fig. 3-5. 25%-NBW supplementation produced the highest polysaccharides yield of 12.76%, followed by the control (6.35%), 50%-NBW (4.14%), 75%-NBW (4.00%), and 100%-NBW groups (2.74%). This observation shows that 25%-NBW treatment might be beneficial for polysaccharide accumulation, owing to the enhanced mass transfer, high mobility of water molecules, suitable NB size and concentration, and stable electrochemical environment. The in-depth mechanisms involved deserve further investigation. The total sugar, protein and polyphenol contents in each crude polysaccharide extracted were also quantified in the present study as shown in Table 3-3. In the present study, NBW application exhibited promotion effect on the growth of mycelium, especially the 25%-NBW group which accelerated the growth cycle of strain and passed the lag phase in the shortest time, resulting in a higher polysaccharide obtained. Compared with other test groups, 25%-NBW treatment might be more beneficial for polysaccharide accumulation, owing to the enhanced mass transfer, high mobility of water molecules, suitable NB size and concentration, and stable electrochemical environment. However, the accumulation of polysaccharides is also related to other factors. For instance, the size of mycelium pellets is negatively correlated with the production of polysaccharides. That is, the size of mycelium pellets is large while the yield of polysaccharides is low, which may be related to the contact area between mycelia and the culture medium. Besides, the reduction of polysaccharides accumulation in 50%, 75%, and 100%-NBW may also be associated with the increase of other metabolites. Therefore, the in-depth mechanisms involved deserve further investigation. With a longer spin-spin relaxation time (T_2), NBW could increase the mobility of water molecules, and at the same time, promote the transmission of nutrients. As shown in Fig. 3-4a, the 25%-NBW accelerated the growth cycle of strain and passed the lag phase in the shortest time, indicating that the oxygen content and the mass transfer in 25%-NBW group is more suitable for the strain growth. On the other hand, the relatively high zeta potential value

of NBW could avoid the coalescence and electrical stabilization. Therefore, probably the zeta potential value in 25%-NBW group is more applicable for the *C. militaris* fermentation. The mechanisms underlying still need further investigation.

3.3.4. Evaluation on antioxidant activities

The extra free radicals in human body will disturb the balance of oxidative stress, leading to some diseases like cancer, Parkinson's disease, aging and so on (Halliwell, 2007; Rains and Jain, 2011). Thus, discovery and identification of compounds with antioxidant properties have been paid more and more attention in biology and medicine fields. In the present study, the antioxidant activities of the extracted polysaccharide samples were evaluated as shown in Fig. 3-6.

The DPPH radical scavenging assay is a common method widely used to determine the free radical scavenging activity of natural antioxidants. Seen from Fig. 3-6a, the polysaccharides from NBW treatment groups exhibited significantly stronger scavenging ability against DPPH radicals when compared with the control group, and the inhibition ability increased obviously with the increase in concentration. When the concentration approached to 2 mg/mL, the radicals scavenging rates by the polysaccharides from 25%-, 50%-, 75% -and 100%- NBW groups were 100%, 88%, 92%, and 78%, respectively, which were significantly higher than the control group (35%). This result is comparable with or even higher than those from previous *C. militaris* studies (Jing et al., 2015; Chen et al., 2014; Zhan et al., 2006; Dang et al., 2018), in which the scavenging ability for DPPH radicals ranged between 50-70% at a concentration of 0.9-2.5 mg/mL. Results from this study indicate that NBW application can largely promote the growth of mycelium, from which the polysaccharide possesses much stronger DPPH radical scavenging ability.

The ABTS radical scavenging assay is extensively applied to measure the total antioxidant power of compounds extracted from various plants. The ABTS radical scavenging potentials of the tested samples are presented in Fig. 3-6b, reflecting a concentration-dependent manner, in which the NBW groups exhibited higher ABTS radical scavenging ability at all concentrations tested. All the maximum ABTS radical inhibition abilities of 25%, 50%, 75% and 100%-NBW groups were obtained at the concentration of 2 mg/mL, about 58%, 73%, 78%, and 66%, respectively.

Reducing power assay is always served as a significant indicator of potential antioxidant activity of samples. As shown in Fig. 3-6c, the reducing potentials of polysaccharides from

25%-, 50%-, 75%-, and 100%- NBW groups were significant, with their OD values increased to 0.95, 2.13, 2.00, and 1.29, respectively as the concentration was increased to 2 mg/mL, which were obviously stronger than the control group (0.32 at 2 mg/mL) and the result from a previous study (<0.1 at a polysaccharide fraction concentration of 2.0 mg/mL) (Zhan et al., 2006). These results indicated that polysaccharides from NBW groups exhibited stronger reducing power, possessing high potentials for being developed as value-added antioxidants. Many reports have proved that the structural properties of polysaccharides take a close influence on the biological activity of polysaccharides in organisms (Bellich et al., 2019). Some common antioxidant activity has been shown to be associated with specific molecules, for example the DPPH radical scavenging activity has associated with the content of arabinose and galactose (Wu et al., 2020). Furthermore, the reducing power are found to greatly correlate with galactose amounts (Wu et al., 2020). Related to this, the ABTS radical scavenging activity is significantly related with the content of uronic acid (Xiao et al., 2016). In the present study, polysaccharides from 25%-NBW, 75%-NBW, and 50%-NBW groups exhibited the strongest DPPH radical, ABTS radical scavenging activities, and reducing power, probably due to the different contents of monosaccharides in the extracted samples.

3.3.5. SEM analysis

The ppolysaccharides from different condition showed different surface structures under SEM analysis (Fig. 3-7). The result show that crude polysaccharide in 0%-NBW group was thin porous lamellar structure, 25%-NBW group was loose roughness structure, 50 %-NBW group was fine powdered with some pieces of thin porous lamellar structure, 75 %-NBW group was thin waviness lamellar structure, and 100 %-NBW group was fine powdered structure.

3.3.6. FT-IR spectra analysis

There were some absorptions observed in 0%-NBW and 25%-NBW groups spectroscopy. As shown in Fig. 3-8, polysaccharide of 0%-NBW and 25%-NBW exhibited characteristic in the 4,000-500 cm^{-1} range. The strong peaks around 3,392 and 3,410 cm^{-1} were corresponded with O-H in the sugar molecule. A weak peak ranging from 2,975 to 2,988 cm^{-1} was considered as the C-H bond (Hu et al., 2015). The broad bands approximately 1,638-1,647 cm^{-1} and 1,406-1,418 cm^{-1} originate from COO^{-}, indicating the uronic acids appearing in the extracts (Wu et al., 2012; Zhang et al., 2013). Moreover, the arabinofuranose units and galactopyranose might existed which is supported by the peaks at 1,078-1,079 cm^{-1} and 1,079cm^{-1}. Finally, the signals

around at 830-848 cm^{-1} indicating the extracts contain α-configurations structure (Zeng et al., 2020).

3.3.7. Effects of polysaccharides on the viability of RAW264.7 cells

Macrophages could phagocytose apoptotic cell debris and pathogens and activate lymphocytes or other immune cells. Macrophages are found in various parts of the body and have a very important role in the immune system. It is used as a model cell in the immunomodulatory activity study. The mouse macrophage cells: RAW 264.7 cells were used in the present study to analyse the effect of 0%-NBW and 25%-NBW extracts on immunomodulatory activity. According to Fig. 3-9, 25%-NBW extracts showed an significant positive effect on macrophage cells viability which reached a highest cell viability of 123.6 ± 6.9% at 6.25 μg/mL. Furthermore, the macrophage cell viability increased after supplemented with 25%-NBW extracts in every concentration, comparing with control group (0%-NBW).

3.3.8. Cell cycle analysis

Based on the above data, the effects of polysaccharides on macrophage cell cycle progression were identified. As shown in Fig. 3-10, the percentage of macrophage cells in G2/M phase increased from 3.67% in control to 9.67% in 6.25 μg/mL of 25%-NBW-treated cells, which was obvious higher than the population in other groups. The increase in cell population in G2 stage was accompanied by a decrease in number of cells in G0/G1 phase. This result represented a selective proliferative effect of 25%-NBW to macrophages, indicating that 25%-NBW promote the macrophage cell proliferation by increasing the cell cycle.

3.3.9. Cytokines generation in macrophages

Cytokines are a series of small molecular proteins or peptides. Cytokines plays an important role in the immune system, it could regulate cell growth, differentiation, immune response and other functions after binding receptor with. There is some important lymphokine such as IL-2, IL-4, and IL-10 act on intercellular interactions, immunomodulation and inflammation. As shown in Fig. 3-11, the expression of IL-2, IL-4, and IL-10 was significantly strong after treated with 25%-NBW extracts. The highest IL-2, IL-4, and IL-10 expression in 25%-NBW treated groups were 180.4, 86.7, and 152.2 pg/mL, respectively, which were significantly higher than the results in control and 0%-NBW groups.

3.3.10. Effect of 25%-NBW on ROS production in RAW264.7 cells

Under normal conditions, ROS could act as messenger of cellular signal transduction and the cell cycle. When the body immune system faced some environmental stress, ROS levels would increase dramatically which causing damage to cellular structures. ROS could trigger oxidative stress, leading to the destruction of intracellular biomolecules. The main source of ROS comes from the substrate end of the respiratory chain in the mitochondrial inner membrane. It is associated with aging, cancer, diabetes and so on. The ability to estimate ROS levels in culture media is important to help us understanding the mechanisms of disease processes. The production of ROS by 25%-NBW was summarized in Fig. 3-12. The results were observed clearly, the fluorescence intensity of 25%-NBW supplemented groups was significantly increased in a dose-dependent manner, compared with the dyer cells of control group under fluorescence microscopy (Fig. 3-12). This result demonstrated that 25%-NBW treatment mediated the upregulation of intracellular ROS production. This was also an evidence of the increased cytokines (IL-2, IL-4, and IL-10) level caused by 25%-NBW treatment.

3.3.11. Possible mechanism of NBW effect on mycelia from C. militaris

There is a serious problem of the artificial culture in the liquid fermentation. the growth of mycelial filaments exhibits a high-density medium that is similar with the non-Newtonian liquid. Which would lead to the mass transfer and gas-liquid exchange problems. And the filament mycelia would become a ball with the cohesion force, So the central part would lack of nutrient and died. The related cytokines of apoptosis would flow out. The whole system would be influenced. The NBW could provide a negative charge and increase the mobility of water molecules. And the high zeta potential could create repulsion force to avoid the coalescence and keep electrical stability. Therefore, NBW supplementation might have positive effects on biomass accumulation (Fig.3-13).

3.4.Summary

Results from this work suggest that NBW supplementation is beneficial for *C. militaris* fermentation, especially under 25%-NBW addition condition, achieving the highest biomass accumulation (3.90 mg/mL) and crude polysaccharides extraction yield (12.76%). All the polysaccharides from the NBW groups possess higher antioxidant abilities at the tested concentrations when compared with the control group. With higher polysaccharide production and relatively higher antioxidant activity, 25%-NBW was selected for further

immunomodulatory activity identification. Compared with 0%-NBW, 25%-NBW was found to exhibit stronger macrophage cell proliferation ability and cytokines generation. Therefore, NBW supplementation can be used as a potential approach in fermentation field, and its effects on other bioactivities of *C. militaris* should also be explored in the future in addition to the real mechanisms involved.

Table 3 - 1 Changes in dissolved oxygen in NBW and medium after autoclaving (mg/L).

Group	NBW	NBW after autoclaving	CM	CM after autoclaving
0%-NBW	8.52±0.08	7.12±0.23	8.21±0.52	7.80±0.48
25%-NBW	8.11±0.23	7.10±0.08	8.32±0.42	7.43±0.24
50%-NBW	8.42±0.60	7.14±0.27	8.13±0.25	7.17±0.24
75%-NBW	8.46±0.21	7.12±0.42	8.14±0.63	7.12±0.53
100%-NBW	8.19±1.11	7.11±0.94	8.52±0.25	7.14±0.14

Results are expressed as the mean ± SD (n = 3). CM, culture medium; NBW, nanobubble water. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote statistically significant difference between the dissolved oxygen data before and after autoclaving.

Table 3 - 2 Variations of pH value in NBW and culture medium after autoclaving.

Group	NBW	NBW after autoclaving	CM	CM after autoclaving
0%-NBW	5.39±0.02	6.67±0.02***	5.64±0.00	5.75±0.03**
25%-NBW	5.43±0.00	6.85±0.01***	5.66±0.00	5.72±0.01***
50%-NBW	5.38±0.60	6.88±0.04	5.63±0.00	5.76±0.02***
75%-NBW	5.40±0.00	6.99±0.62**	5.65±0.01	5.67±0.02
100%-NBW	5.44±1.11	6.95±0.94	5.62±0.00	5.67±0.03*

Results are expressed as the mean ± SD (n = 3). CM, culture medium; NBW, nanobubbles water. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote statistically significant difference between the pH values before and after autoclaving.

Table 3 -3 Chemical composition of crude polysaccharide.

Group	TSR (%)	TPNR (%)	TPC (mg GAE/g)
0%-NBW	41 ± 0.86	11.28 ± 0.03	30.61 ± 0.26
25%-NBW	52 ± 0.62	7.38 ± 0.02	30.61 ± 0.42
50%-NBW	60 ± 0.22	6.09 ± 0.02	33.62 ± 0.65
75%-NBW	55 ± 1.04	3.47 ± 0.00	22.06 ± 0.55
100%-NBW	42 ± 0.59	6.03 ± 0.02	31.19 ± 0.90

TSR, total sugar ratio; TPNR, total protein ratio; TPC, total phenolic content was calculated as gallic acid equivalent (GAE) mg/g of dry extract.

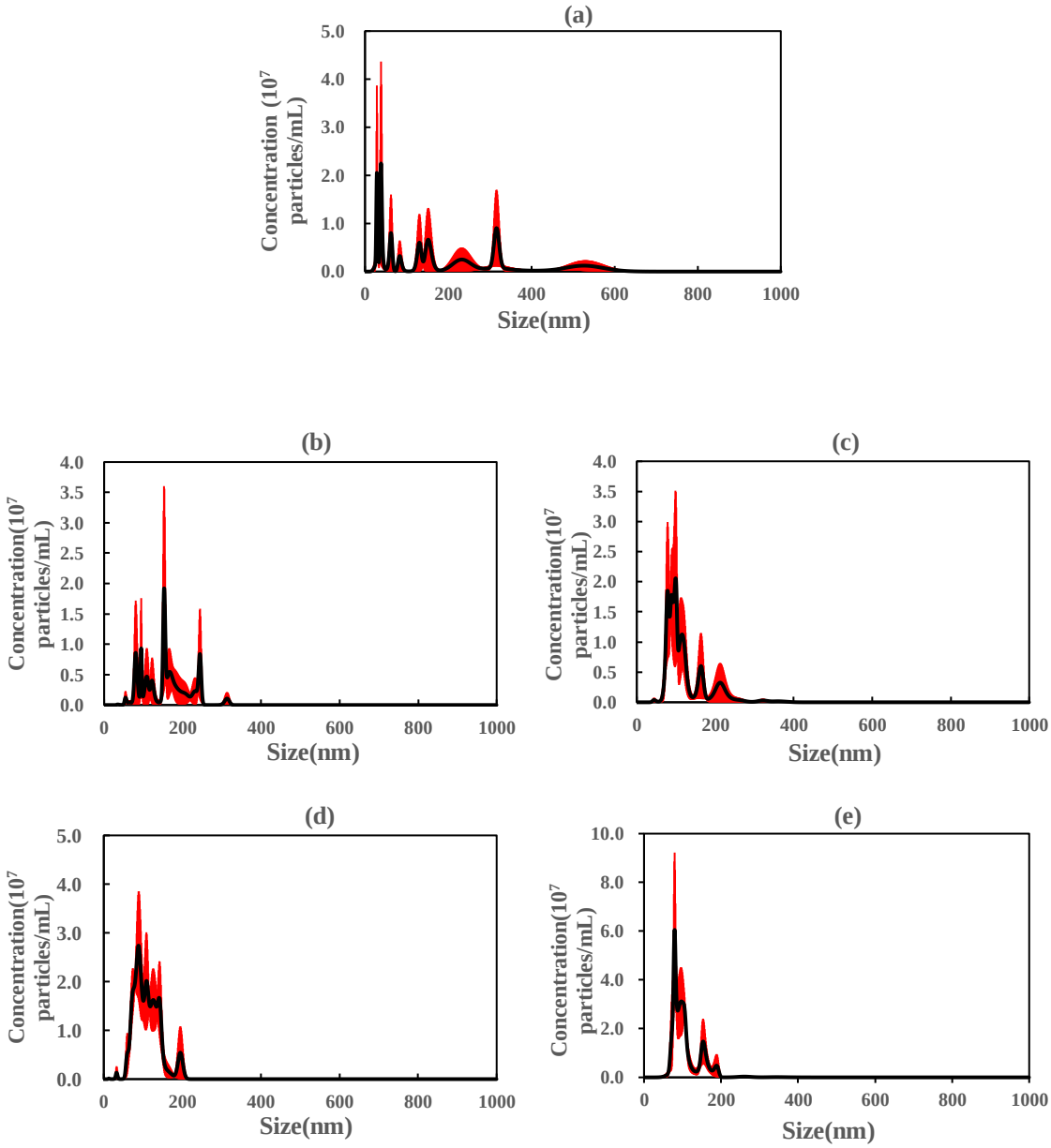


Figure 3-1 Size and concentration of NBs in Air-NBW observed by nano sight: (a) 0%-NBW, (b) 25%-NBW, (c) 50%-NBW, (d) 75%-NBW and (e) 100%-NBW. NBs, nanobubbles; NBW, nanobubble water.

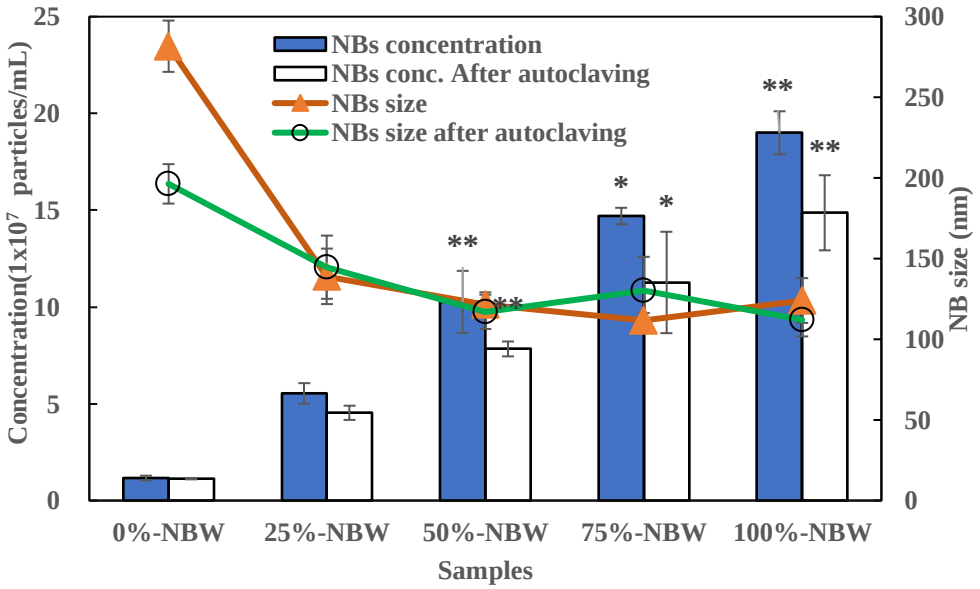


Figure 3-2 Effects of autoclaving on the concentration and size of NBs in different percentage NBW solutions. NBs, nanobubbles; NBW, nanobubbles water. Results are represented as mean ± SD, n = 5. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistically significant difference between the data before and after autoclaving.

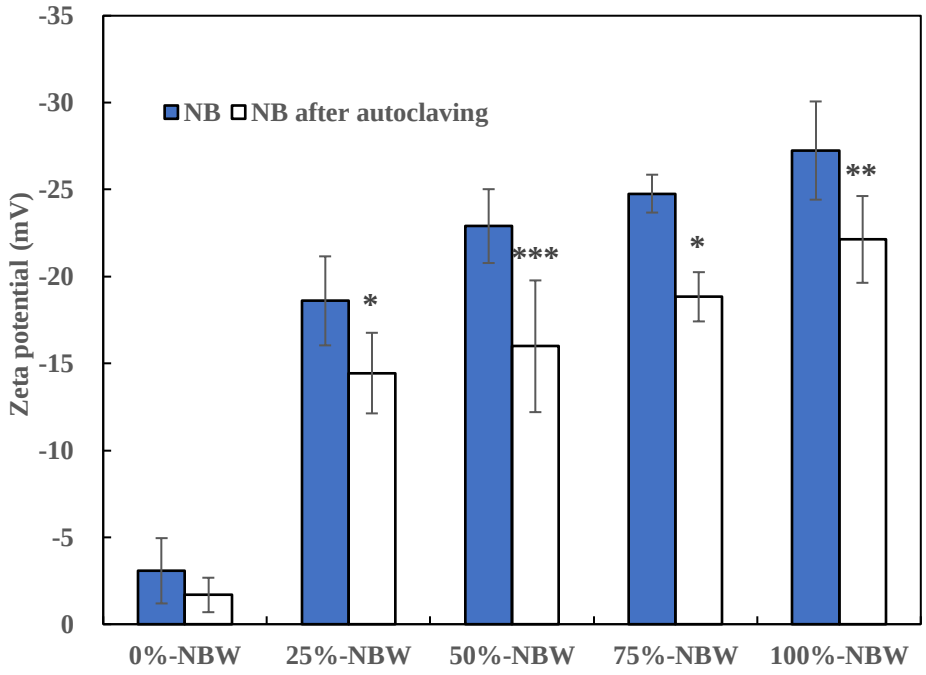
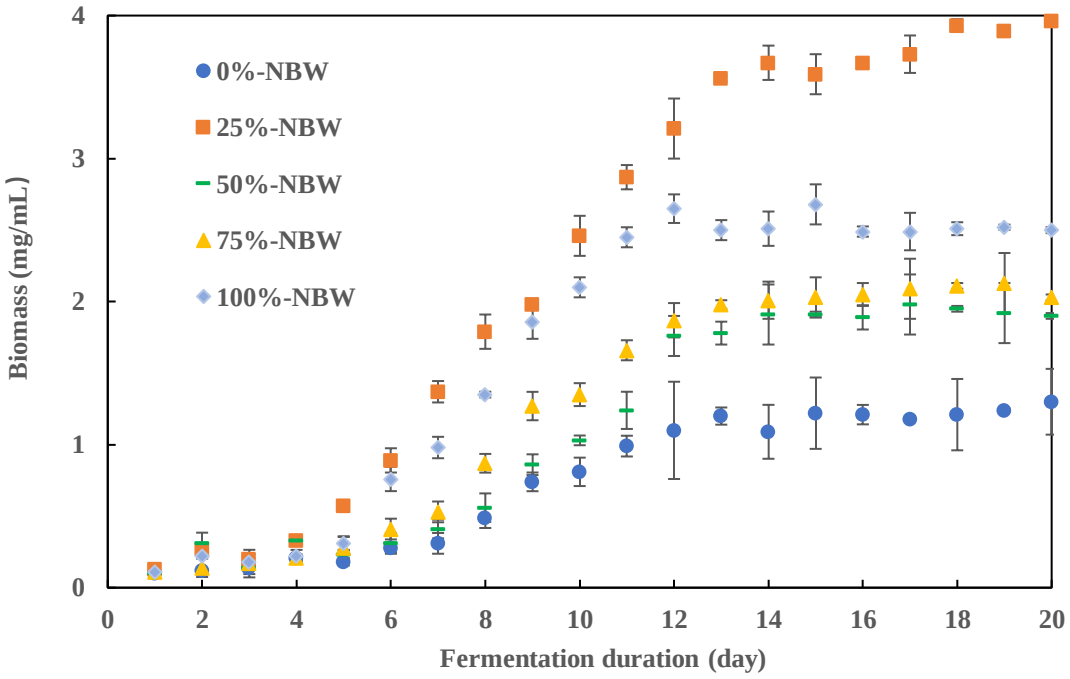


Figure 3-3 Effect of autoclaving on zeta potential of NBW. NBW, nanobubbles water. Results are represented as mean ± SD, n = 5. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ denote statistically significant difference between the data before and after autoclaving.

(a)



(b)

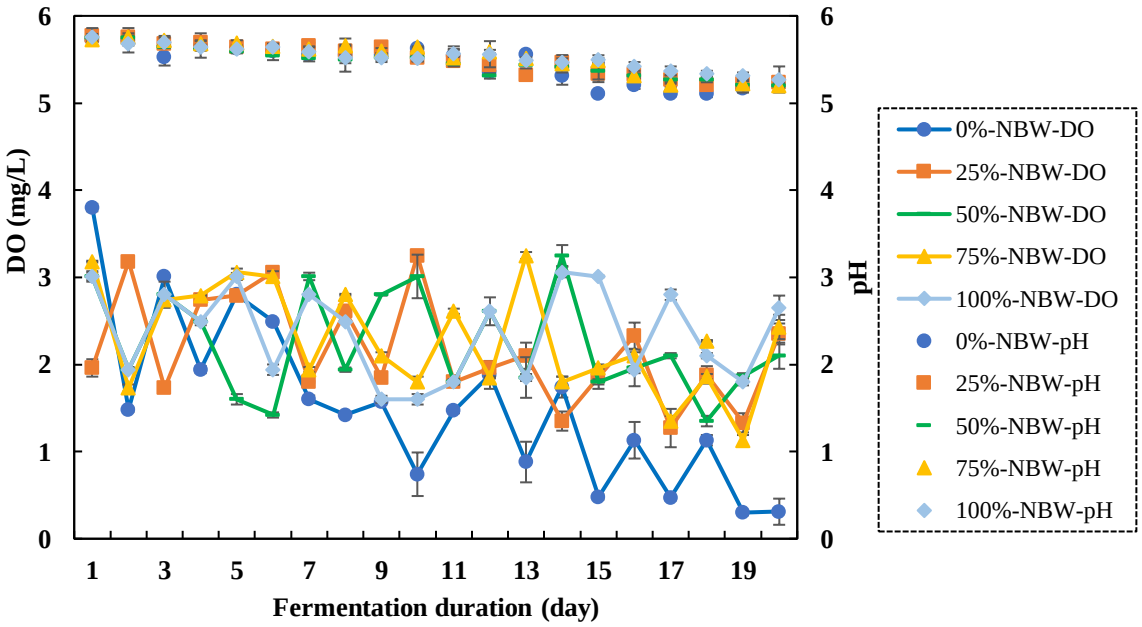


Figure 3-4 Changes of biomass accumulation (a), DO and pH value (b). NBW, nanobubble water; DO, dissolved oxygen.

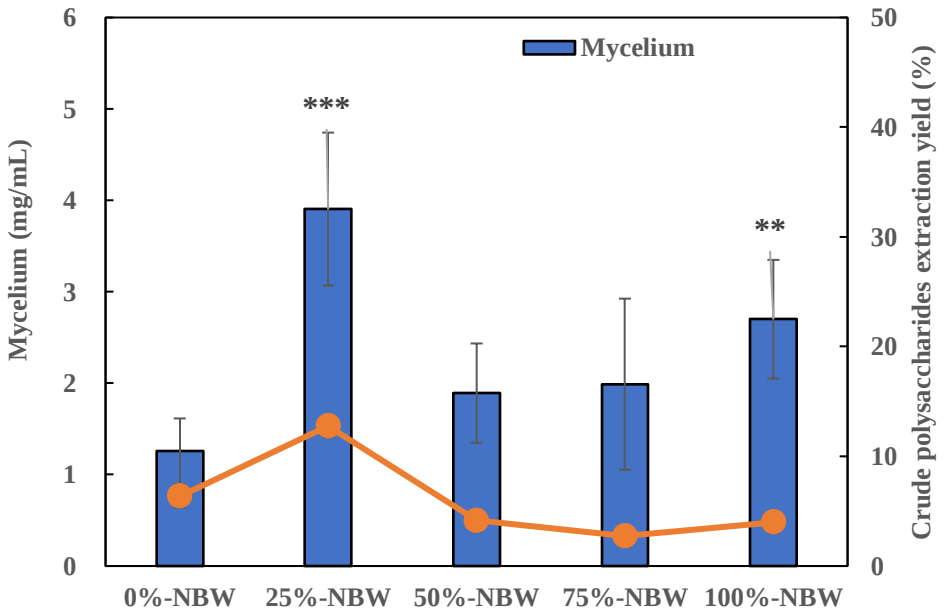


Figure 3-5 Mycelium concentrations and crude polysaccharide extraction yields from different NBW group cultures. NBW, nanobubble water. Results are represented as mean ± SD, n = 3. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ denote statistically significant difference between the NBW treated and control groups.

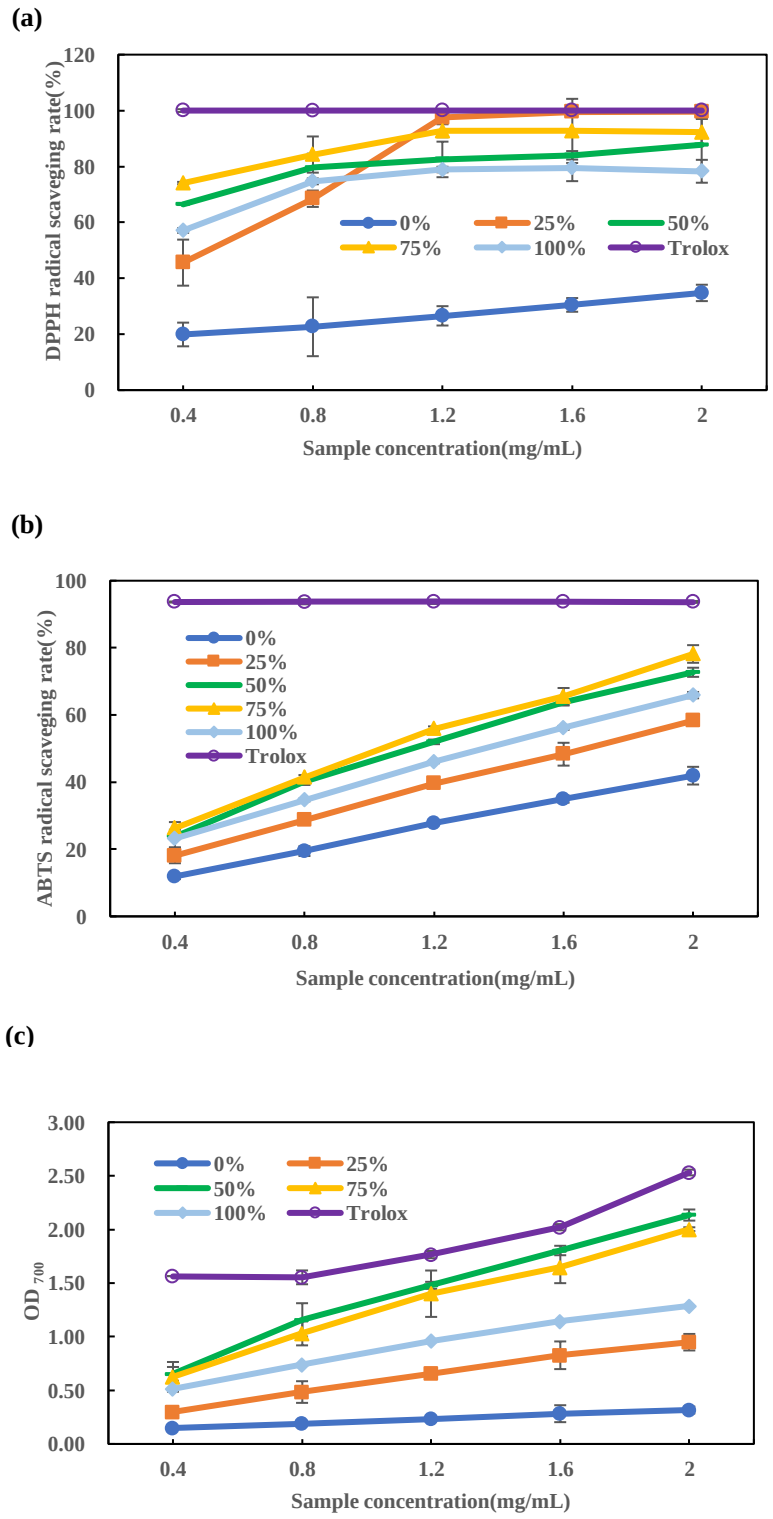


Figure 3-6 Antioxidant activities of polysaccharides extracted from NBW groups: (a) DPPH radical scavenging activity, (b) ABTS radical scavenging activity, and (c) reducing power. NBW, nanobubble water.

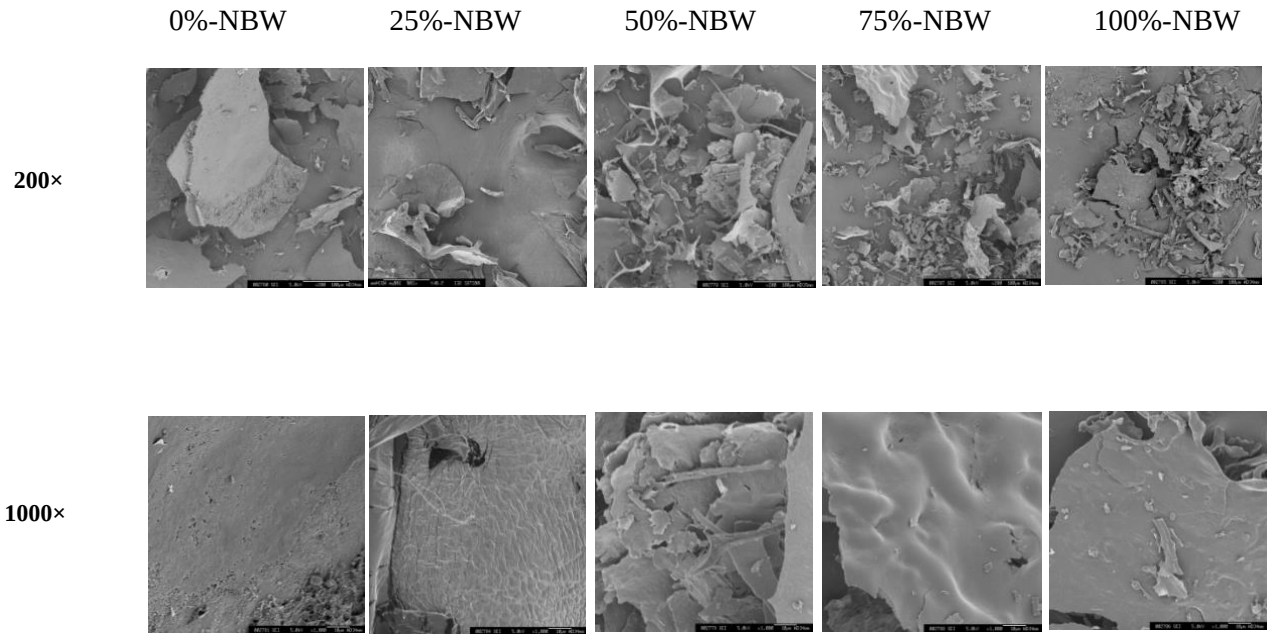


Figure 3-7 SEM analysis of polysaccharide fractions.

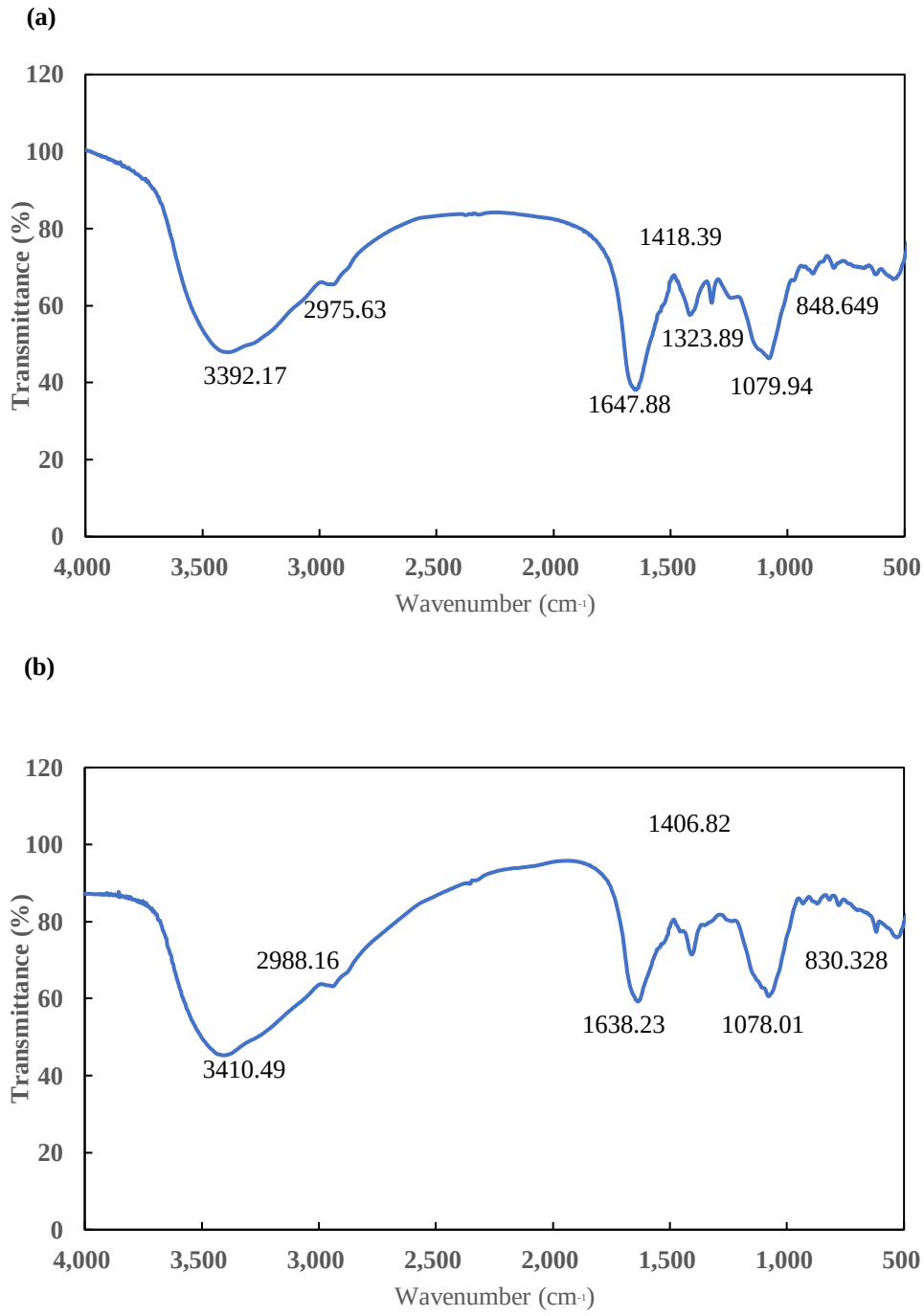


Figure 3-8 FTIR spectrum of polysaccharide 0%-NBW and 25%-NBW. (a) 0%-NBW group. (b) 25%-NBW group.

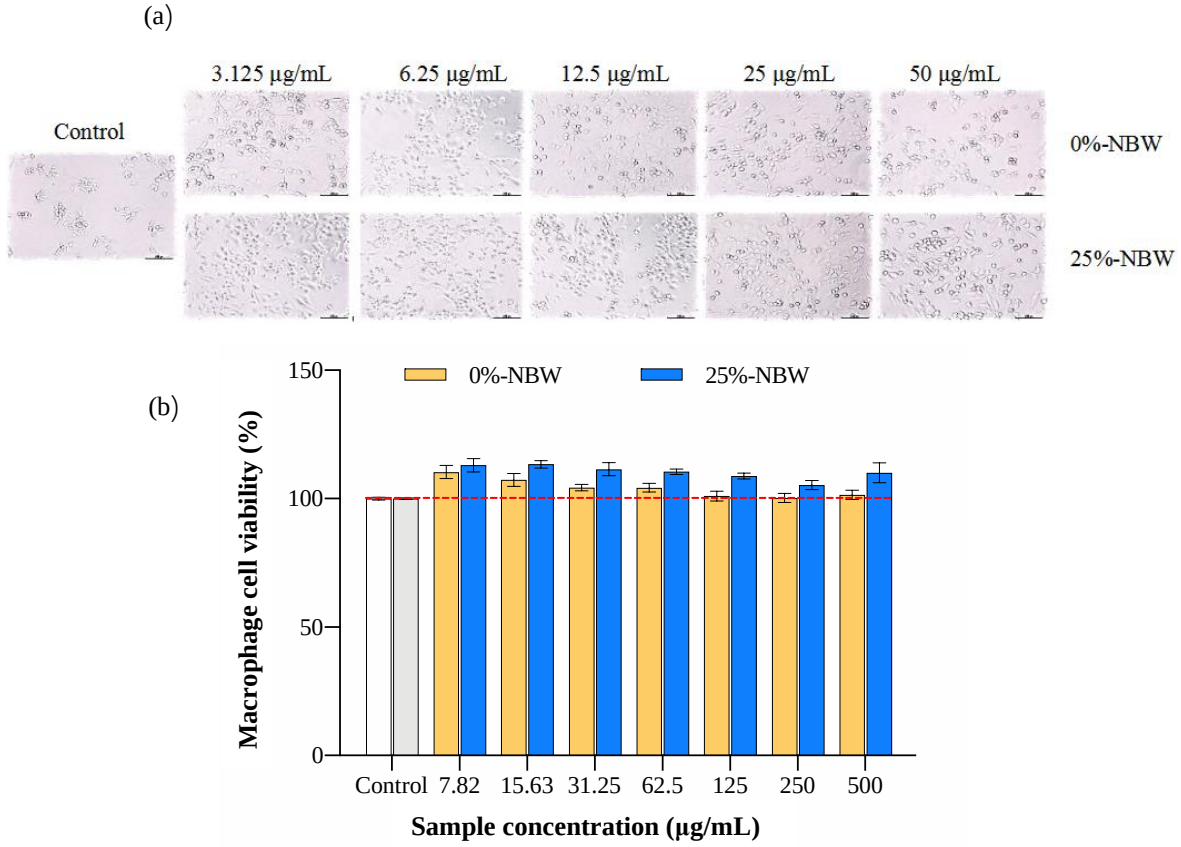


Figure 3-9 Effects of 25%-NBW on the viability of RAW264.7 macrophage cells. The cells were treated with various concentrations of 25%-NBW or 0%-NBW for 24 h. (a) Microscope photos of macrophage cells after cultivation in different concentration. (b) Macrophage cell viability%.

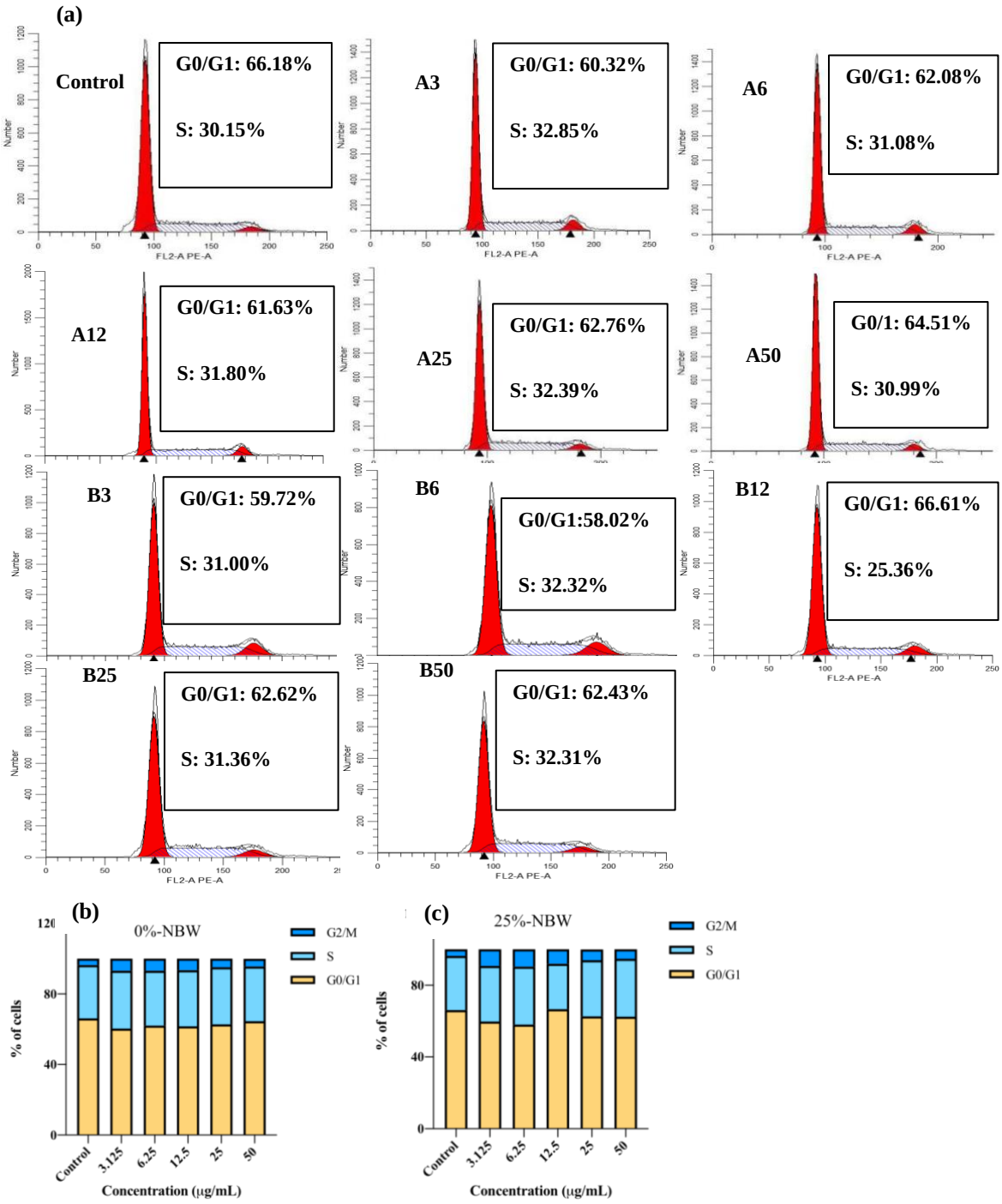


Figure 3-10 Effects of 25%-NBW on cell cycle in macrophage cells. The cells were treated with various concentrations of 25%-NBW or 0%-NBW for 24 h. (a) Percentage of cell amounts in each phase treated by different concentration. (b) Percentage of cells treated by 0%-NBW extracts. (c) Percentage of cells treated by 25%-NBW extracts.

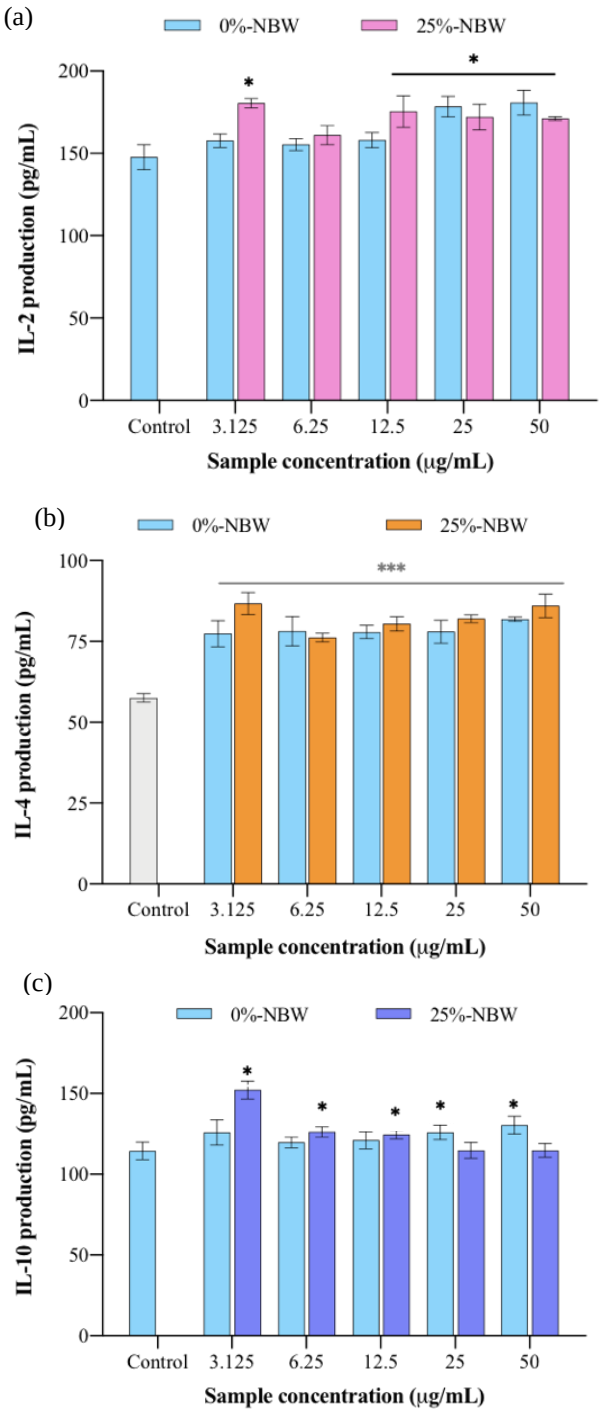


Figure 3-11 Effects of 25%-NBW on the Il-2, IL-4 and IL-10 generation in macrophage cells showed in figure (a), (b) and (c), respectively. The cells were treated with various concentrations of 25%-NBW or 0%-NBW for 24 h. Quantitation date from three independent experiments (mean ± SD, n=5). *$p < 0.05$ and ***$p < 0.001$.

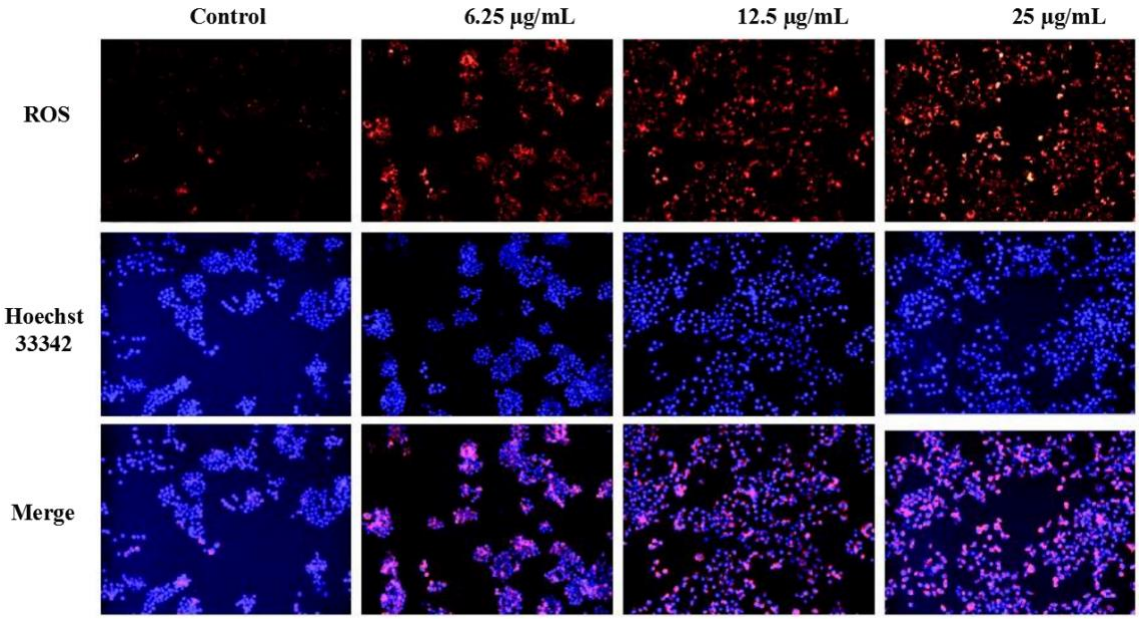
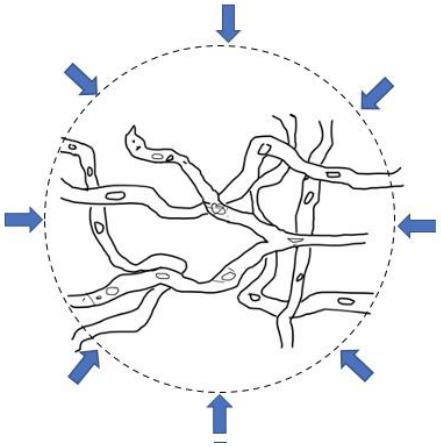
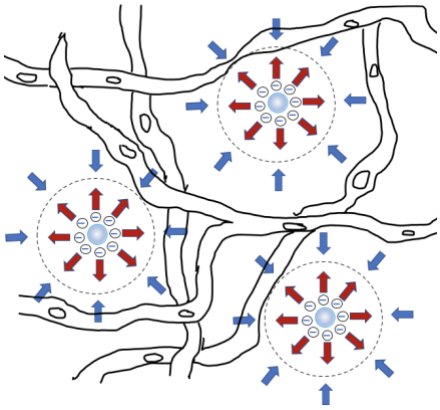


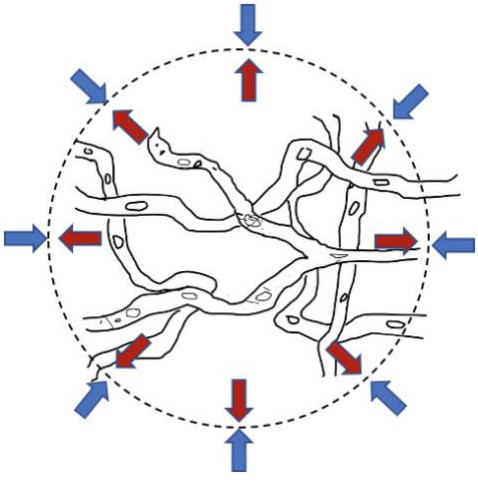
Figure 3-12 Effects of 25%-NBW on the ROS production in macrophage cells. The cells were treated with various concentrations of 25%-NBW for 24 h.



Cohesion was the major force in the mycelia liquid cultivation system (without NBW).



Nanobbubles provide a surface negative charge against cohesion force in mycelia liquid cultivation system (with addition NBW).



The New balance was built in mycelia liquid cultivation system.

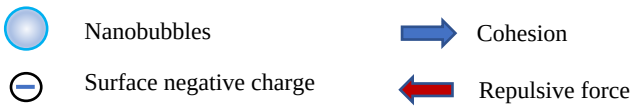


Figure 3-13 The possible mechanisms for mycelia under NBW addition.

Chapter 4. Conclusions and perspectives

In the present study, the crude polysaccharides from 25% of NBW supplementation were harvest maximum production and mainly analyzed, the *in vitro* immunomodulatory activities were evaluated with RAW 264.7 cell model.

4.1. Conclusions

The main results could be summarized as follows.

(1) The supplementation with KF 1.0 mg/L during the *Cordyceps militaris* fermentation benefited the crude polysaccharide accumulation, achieving the highest mycelium concentration of 3.4 mg/mL. The crude polysaccharide extracts exhibited higher antioxidant and immunomodulatory activities. The extraction yield was higher from the A1(NH_4F 10 mg/L) and B3 (KF 0.1 mg/L) groups. All the test groups showed a low SOD inhibition activity and higher DPPH inhibition rate, especially for B2 (KF 1.0 mg/L) group. In the immunomodulatory assay, B2 (KF 1.0 mg/L) at higher concentration (100 μg/mL) exhibited significant macrophage cell proliferation ability.

There are three major fractions (CMPF-1, CMPF-2, and CMPF-3) were purified from crude polysaccharide induced by fluoride by using Sephacryl S-400 chromatography. CMPF-1, CMPF-2, and CMPF-3 were the typical pectic polysaccharides, among which CMPF-3 reflected the strongest antioxidant and immunomodulatory activities and a higher SOD activity. The hydroxyl radical scavenging results suggested that the CMPF-2 and CMPF-3 had higher scavenging ability against hydroxyl radical than CMPF-1and B2 (KF 1.0 mg/L).

(2) 25%-NBW addition could achieve the highest biomass accumulation of 3.90 mg/mL and crude polysaccharides extraction yield of 12.76%. All the polysaccharides from the NBW groups possess higher antioxidant abilities at the tested concentrations when compared with the control group. The 50%-NBW group could harvest 60% of total sugar content and 33.62 mgGAE of total phenolic content, higher than other test conditions. This observation shows that 25%-NBW addition might be beneficial for polysaccharide accumulation, owing to the enhanced mass transfer, high mobility of water molecules, suitable NB size and concentration. The polysaccharides from 25%-NBW group exhibited excellent radicals scavenging ability, reaching 100% in the DPPH assay when the test concentration > 1.2 mg/mL. The 75%-NBW group had a slightly higher SOD activity while the 100%-NBW group might not benefit the SOD activity. The ABTS radical scavenging assay showed the 25%-NBW group had the lowest IC_{50} value of 1.2 mg/mL, while the 75%-NBW group showed a higher ABTS scavenging

activity. The broad and strong peak between 3,392 and 3,410 cm^{-1} was observed in the extracted polysaccharides, which was ascribed to the stretching vibration peak of O-H bond in the sugar molecule. The peaks observed around 2,975-2,988 cm^{-1} were surmisable with C-H bond. The absorption occurring at around 1,638-1,647 cm^{-1} and 1,406-1,418 cm^{-1} supporting the appearance of COO^-, the strong absorption peaks at 1,640 cm^{-1}were signals of C=O carbonyl stretching vibration. 25%-NBW extracts have obvious positive effect on viability of RAW264.7 cell, the viability reached highest at 6.25 ug/mL, achieving the highest viability of $123.6 \pm 6.9\%$ achieved at 6.25 ug/mL. Besides, the proliferation effect increased at all test concentrations, in comparison to the control group (0%-NBW). The G2 phase was significantly increased in the 25%-NBW group, reaching the maximum percentage of 9.67%, which also IL-2, IL-4, and IL-10 expression achieved a higher promotion after supplemented with 25%-NBW extracts. The 25%-NBW group obtained the highest IL-2, IL- 4, and IL-10 expression production, about 180.4, 86.7, and 152.2 pg/mL, respectively, which were significantly higher than the control and 0%-NBW groups. The fluorescence intensity in cells in the 25%-NBW group was noticed to significantly increase in a dose-dependent manner, demonstrating that 25%-NBW supplementation could mediate the upregulation of intracellular ROS production.

As a summary, the polysaccharides extracts from *C. militaris* induced by fluoride and nanobubbles water exhibited a positive effect on immunomodulatory activity *in vitro*. In particular, nanobubbles water treatment technology which is a safe and convenient treatment method could obtain a larger number and more efficiency *C. militaris* production. There will be more potential could be developed in the future.

4.2. Future research

(1) The chemical composition, and the structure of CMPF-3 would be analyzed. The surface structure would be analyzed by SEM. The immunomodulatory mechanism of CMPF-3 on macrophage cell is to be investigated.

(2). Although the extracts treated by 25% NBW were proved to benefits for the cell cycle, cytokines generation and ROS production, the more mechanisms of 25%-NBW on macrophages cell proliferation would be discussed in future study. The monosaccharide content would be detected using HPLC method as well.

(3). The experiments *in vivo* scale of immunomodulatory is necessary for identification of

25%-NBW.

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