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Increased Extracellular Saponin Production after the Addition of Rutin in Truffle Liquid Fermentation and Its Antioxidant Activities

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Abstract: Saponins possess a variety of pharmacological effects and exhibit great potential in the food industry as bioactive substances. In this study, extracellular saponin production via the liquid fermentation of *Tuber melanosporum* occurred with the addition of rutin. For this purpose, medium composition and culture conditions were optimized using single-factor experiments and an orthogonal experiment design. The optimal medium consisted of glucose (43.5 g/L), peptone (6 g/L), KH_2PO_4 (1.15 g/L), NaCl (0.2 g/L), vitamin B_2 (0.082 g/L), vitamin B_6 (0.1 g/L), vitamin C (0.02 g/L), and rutin (4.8 g/L). The culture conditions were as follows: 12.5% (*v*/*v*) inoculation, medium volume of 50 mL/250 mL flask, culture temperature of 24 °C, shaker speed of 190 rpm, initial pH of 5.7, and culture time of 96 h. Finally, a maximal extracellular saponin content of 0.413 g/L was obtained, which was 134.7% higher than that in the base medium. Rutin proved to be an excellent promoter, because the saponin production was increased by 50.2% compared to that in the optimized medium without rutin. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging activity, hydroxyl radical scavenging activity, and ferric reducing antioxidant power of truffle saponins reached 94.13%, 79.26%, and 42.22 mM, respectively. This study provides a useful strategy for fungal bioactive saponin production by liquid fermentation with the addition of flavonoid compounds.

Keywords: saponin; liquid fermentation; antioxidant activities; *Tuber melanosporum*; rutin

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1. Introduction

Saponins are composed of sapogenins and sugars [1]. According to the different carbon skeletons of aglycon, saponins can be divided into two large categories: triterpene saponins and steroidal saponins [2]. Saponins exhibit different bioactivities, such as antitumor properties [3]; immune function regulation [4]; lipid and cholesterol reduction [5]; and anti-aging, antioxidant [6], and antibacterial activities [7]. As such, the comprehensive development and utilization of saponins is worthwhile, since they possess great potential in the food industry as bioactive substances.

The extraction of saponin from plants is the traditional method used for saponin production, involving Soxhlet extraction, refluxing, and dipping [8]. Due to the low content of saponins in natural plants, saponin extraction consumes a lot of natural resources and the extraction cost is high; therefore, researchers have tried different strategies to improve the extraction efficiency of saponin with low energy consumption and reduced costs, such as through method development and process optimization [9–11]. Chemical and semi-chemical synthesis can also be alternatives for saponin production with high synthesis

efficiency; however, by-product production occurs from saponin synthesis, and this method is not environmentally friendly because of the usage of corrosive or toxic reagents.

The biosynthesis of saponin by plants or microbial cells in controllable culture systems is a useful method for saponin production and has great potential in industrial applications. A stable and efficient adventitious root multiplication system has been established for a suspension culture of *Panax notoginseng*, with the ginsenoside Re concentration being 1.8 times higher than in a three-year field cultivation study [12]. The level of saponin secretion has been shown to increase by 5.7 times in the suspension culture of *Calendula officinalis* hairy root using nutrient selection and optimization [13]. Fungi are also good saponin producers. *Dioscorea zingiberensis* C.H. Wright has been isolated and diosgenin has been produced using a combined pH control strategy [14]. In addition, the parameters for the solid-state fermentation of *D. zingiberensis* C.H. Wright have been optimized, obtaining a yield of 2.16% for diosgenin production [15]. Besides the natural saponin producers, the production pathways of triterpene saponins have also been reconstructed in yeast for saponin production [16–18]. The production of ginsenoside Rh2 in a mutant yeast strain has been shown to be more than 900 times higher than that of the original strain by the mutation of the glycosyltransferase gene [17].

Truffle (*Tuber melanosporum*) is a fungus unique to the host of subterranean Ascomycetes. Its symbiosis with the roots of various angiosperms and gymnosperms can form ectomycorrhizas [19]. Black truffles are rich in bioactive and healthy compounds, which are a popular food because of their unique aroma and taste [20]. Truffles are rich in saponins, protein, fat, carbohydrates, fiber, lipids, several minerals, and unsaturated fatty acids, and truffle saponins are some of their main bioactive ingredients [21]. The nutrients in truffles play a great role in supplementing nutrition and improving physical health [22]. These ingredients also provide potential biological properties, including antibacterial, antioxidant, antimutation, liver protection, anti-inflammatory, anticancer, and antiviral activities [23]; however, there are few studies on truffle saponin production via liquid fermentation. In this study, the selection of medium components and the optimization of medium composition and culture conditions were investigated in the liquid fermentation of *T. melanosporum* to increase extracellular truffle saponin production. The antioxidant activity of truffle saponins was also evaluated.

2. Materials and Methods

2.1. Microorganism and Raw Materials

T. melanosporum was kindly provided by Prof. Hailong Li (Zhejiang Subtropical Crops Institute, China). *Sophora japonica* bud, *Pueraria lobata* root, *Scutellaria baicalensis* root, *Viscum coloratum*, *Glycyrrhiza uralensis*, mulberry twig, mulberry bark, safflower, honeysuckle, and Citri Reticulatae Pericarpium were purchased from local Chinese medicine stores, while rutin (≥95%) was purchased from Nanjing Yuanzhi Biotechnology Co., Ltd. (Nanjing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals of analytical grade were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Culture Medium

The components of the liquid seed culture medium were 35 g/L of glucose, 5 g/L of peptone, 2.5 g/L of yeast extract, 1 g/L of KH_2PO_4, and 0.5 g/L of $MgSO_4$ $7H_2O$. The basic fermentation medium was composed of 35 g/L of glucose, 5 g/L of peptone, 1 g/L of KH_2PO_4, 0.5 g/L of $MnSO_4$, and 0.05 g/L of VB_2. After the above medium was prepared, it was sterilized at 121 °C for 20 min.

2.3. Inoculation and Culture

T. melanosporum was maintained on a solid slant medium of potato dextrose agar (PDA). The strain was transferred to the PDA slant, cultured at 27 °C for five days, and then stored in a refrigerator at 4 °C. The activated mycelia in the PDA slant were inoculated

into 250 mL Erlenmeyer flasks containing 100 mL of liquid seed culture medium, then these Erlenmeyer flasks were incubated on a shaker at 25 °C and120 rpm for five days. Ten milliliters of seed liquid culture was inoculated in a 250 mL Erlenmeyer flask containing 100 mL of liquid fermentation medium, then cultivated in an incubator at 25 °C and 120 rpm for six days.

2.4. Experimental Design

2.4.1. Nutrient Selection of the Fermentation Medium

For the screening of carbon sources, glucose, maltose, xylose, fructose, sucrose, glycerin, soluble starch, cellulose, and mannitol were tested in basic fermentation medium with the removal of the carbon source for saponin production. The dosage for the different tested carbon sources was 50 g/L. The basic fermentation medium with the removal of carbon source was set as the control.

For the screening of nitrogen sources, peptone, yeast extract, corn gluten, urea, $(NH_4)_2SO_4$, beef extract, and KNO_3 were tested in basic fermentation medium with the removal of the nitrogen source for saponin production. The dosage for the different added nitrogen sources was 5 g/L. The basic fermentation medium with the removal of nitrogen source was set as the control.

For the screening of inorganic salts, KH_2PO_4, $MgSO_4$, $MnSO_4$, $ZnSO_4$, NaCl, and KCl were tested in basic fermentation medium with the removal of inorganic salts for saponin production. The dosage for the different added inorganic salts was 1 g/L. The basic fermentation medium with the removal of inorganic salts was set as the control.

Many vitamins are coenzymes of enzymes or components of coenzymes. By controlling the type and concentration of vitamins in the fermentation medium, the enzyme activity in microorganisms can be regulated, which affects the cell metabolism in microorganisms. For the screening of vitamins in which the nutrient factors were removed, VB_1, VB_2, VB_4, VB_6, VB_{12}, and VC were tested in the basic fermentation medium with the removal of vitamins for saponin production. The dosage for the different added vitamins was 0.05 g/L. The basic fermentation medium with the vitamins removed was set as the control.

Traditional Chinese medicines and their extracts have exhibited improvements in the production of bioactive substances in the liquid culture of mushrooms. In this study, *Sophora japonica* bud, *Pueraria lobata* root, *Scutellaria baicalensis* root, *Viscum coloratum*, *Glycyrrhiza uralensis*, mulberry twig, mulberry bark, safflower, honeysuckle, and Citri Reticulatae Pericarpium were pulverized into powders of 60 mesh and tested in basic fermentation medium for saponin production. The dosage for the different added traditional Chinese medicines was 0.5 g/L. Because the *Sophora japonica* bud provided the best improvement in saponin production and as rutin was the main bioactive component of *Sophora japonica* bud, 0.5 g/L of rutin was also tested in basic fermentation medium for saponin production. The basic fermentation medium was set as the control.

2.4.2. Orthogonal Experimental Design for Medium Composition Optimization

Based on the above nutrient selection for the fermentation medium, glucose (F_1), peptone (F_2), rutin (F_3), KH_2PO_4 (F_4), NaCl (F_5), VB_2 (F_6), VB_6 (F_7), and VC (F_8) were selected to design an L_{50} (5^8) orthogonal experiment (Table 1), and the influence of various factors on the production of extracellular saponins via the fermentation of *T. melanosporum* was investigated using extracellular truffle saponin content (ETSC; g/L) as an indicator (Table 2).

2.4.3. Orthogonal Experimental Design for Culture Condition Optimization

For the culture condition optimization in truffle fermentation, the amount of inoculum (F_1), temperature (F_2), shaker speed (F_3), medium volume (F_4), initial pH (F_5), and culture time (F_6) were the five factors considered in the design of the $L_{25}(5^5)$ orthogonal experiment (Table 3). Using ESTC as an indicator, the influence of these five factors on the production of extracellular saponins by truffle fermentation was investigated (Table 4).

Table 1. The factors and levels of the orthogonal design for medium composition optimization.

Factor	Level				
	1	**2**	**3**	**4**	**5**
F_1 (g/L)	20	30	40	50	60
F_2 (g/L)	2	3	4	5	6
F_3 (g/100 mL)	0.2	0.4	0.6	0.8	1
F_4 (g/L)	0.2	0.7	1.2	1.7	2.2
F_5 (g/L)	0.2	0.7	1.2	1.7	2.2
F_6 (g/L)	0.02	0.04	0.06	0.08	0.10
F_7 (g/L)	0.02	0.04	0.06	0.08	0.10
F_8 (g/L)	0.02	0.04	0.06	0.08	0.10

Note: F_1, glucose; F_2, peptone; F_3, rutin; F_4, KH_2PO_4; F_5, NaCl; F_6, VB_2; F_7, VB_6; F_8, VC.

Table 2. The designs and results of the orthogonal experiments for medium composition optimization.

RUN	F_1	F_2	F_3	F_4	F_5	F_6	F_7	F_8	ETSC
1	30	6	0.4	0.7	0.2	0.08	0.1	0.06	0.183
2	30	2	0.2	0.2	0.7	0.04	0.06	0.02	0.105
3	30	2	1	0.2	1.2	0.08	0.04	0.1	0.042
4	50	5	0.2	1.2	0.2	0.08	0.06	0.02	0.144
5	60	3	0.2	0.2	0.7	0.06	0.1	0.02	0.102
6	20	2	0.2	0.2	0.2	0.02	0.02	0.02	0.040
7	20	3	0.4	2.2	0.7	0.08	0.02	0.08	0.084
8	20	3	0.8	0.7	1.2	0.02	0.06	0.06	0.037
9	40	3	1	0.7	0.2	0.02	0.02	0.02	0.049
10	30	6	0.2	1.7	0.7	0.02	0.04	0.04	0.126
11	20	3	0.2	0.2	0.2	0.04	0.04	0.08	0.042
12	30	2	0.6	0.7	2.2	0.06	0.06	0.08	0.101
13	20	2	0.6	0.7	0.2	0.04	0.1	0.04	0.032
14	30	3	1	1.7	2.2	0.04	0.08	0.02	0.042
15	30	3	0.4	0.2	0.2	0.02	0.02	0.04	0.079
16	50	4	0.4	0.2	2.2	0.02	0.1	0.1	0.125
17	30	4	0.2	1.2	0.7	0.04	0.02	0.06	0.190
18	50	6	1	0.7	0.7	0.04	0.02	0.08	0.142
19	20	2	0.4	0.7	0.7	0.02	0.04	0.02	0.046
20	40	5	0.2	1.7	1.2	0.02	0.1	0.08	0.123
21	60	6	0.6	0.2	1.2	0.1	0.02	0.02	0.176
22	60	4	1	2.2	0.2	0.02	0.06	0.04	0.104
23	20	6	0.8	1.2	2.2	0.02	0.04	0.02	0.109
24	50	2	0.4	2.2	1.2	0.04	0.04	0.02	0.086
25	60	3	0.2	0.7	2.2	0.08	0.04	0.04	0.070
26	40	2	0.8	0.2	0.7	0.08	0.08	0.04	0.054
27	20	2	1	1.2	0.7	0.1	0.1	0.04	0.055
28	40	2	0.2	2.2	2.2	0.1	0.02	0.06	0.071
29	50	3	0.2	0.7	1.7	0.1	0.04	0.04	0.103
30	30	5	0.6	2.2	0.7	0.02	0.04	0.04	0.109
31	40	6	0.4	0.2	1.7	0.04	0.06	0.04	0.148
32	20	3	0.4	1.7	0.7	0.1	0.06	0.1	0.055
33	20	4	0.6	1.7	1.7	0.08	0.02	0.02	0.072
34	30	5	0.4	0.7	0.2	0.1	0.08	0.02	0.166
35	30	4	0.8	0.2	0.2	0.1	0.04	0.08	0.115
36	30	3	0.4	1.2	1.2	0.06	0.02	0.04	0.076
37	40	3	0.6	1.2	0.2	0.04	0.04	0.1	0.139
38	60	2	0.4	1.2	1.7	0.02	0.08	0.08	0.074
39	60	5	0.8	0.7	0.7	0.04	0.02	0.1	0.156
40	30	2	0.2	0.7	1.7	0.02	0.02	0.1	0.043
41	50	3	0.6	0.2	0.7	0.02	0.08	0.06	0.137
42	60	2	0.4	1.7	0.2	0.04	0.04	0.06	0.061
43	20	6	0.2	2.2	0.2	0.06	0.08	0.1	0.135
44	20	5	0.4	0.2	2.2	0.04	0.02	0.04	0.069
45	30	3	0.8	2.2	1.7	0.04	0.1	0.02	0.088
46	40	4	0.4	0.7	0.7	0.06	0.04	0.02	0.162
47	20	5	1	0.2	1.7	0.06	0.04	0.06	0.063
48	50	2	0.8	1.7	0.2	0.06	0.02	0.04	0.097
49	20	4	0.2	0.7	1.2	0.04	0.08	0.04	0.067

Note: F_1, glucose; F_2, peptone; F_3, rutin; F_4, KH_2PO_4; F_5, NaCl; F_6, VB_2; F_7, VB_6; F_8, VC; ETSC, extracellular truffle saponin content.

Table 3. The factors and levels of the orthogonal design for culture condition optimization.

Factor	Level				
	1	**2**	**3**	**4**	**5**
F_1(v/v, %)	4	7	10	13	16
F_2 (°C)	20	22	24	26	28
F_3 (rpm)	70	100	130	160	190
F_4 (mL)	50	70	90	110	130
F_5	5.0	5.5	6.0	6.5	7.0
F_6 (h)	96	120	144	168	192

Note: F_1, amount of inoculum; F_2, temperature; F_3, shaker speed; F_4, medium volume; F_5, initial pH; F_6, culture time.

Table 4. The designs and results of the orthogonal experiments for culture condition optimization.

RUN	F_1	F_2	F_3	F_4	F_5	F_6	ETSC
1	4	24	100	110	6	5	0.147
2	16	24	190	50	7	7	0.102
3	4	20	70	50	5	4	0.219
4	7	28	190	130	6	4	0.144
5	4	26	190	90	6.5	8	0.092
6	13	24	130	90	5.5	4	0.255
7	16	26	130	130	5	5	0.196
8	13	20	100	130	7	8	0.201
9	7	24	160	70	5	8	0.088
10	10	28	100	90	5	7	0.096
11	10	24	70	130	6.5	6	0.226
12	4	28	130	70	7	6	0.125
13	13	28	160	50	6.5	5	0.126
14	16	28	70	110	5.5	8	0.148
15	16	22	100	70	6.5	4	0.181
16	13	22	190	110	5	6	0.115
17	7	22	70	90	7	5	0.210
18	16	20	160	90	6	6	0.117
19	10	26	160	110	7	4	0.291
20	4	22	160	130	5.5	7	0.096
21	13	26	70	70	6	7	0.163
22	10	22	130	50	6	8	0.096
23	10	20	190	70	5.5	5	0.104
24	7	20	130	110	6.5	7	0.115
25	7	26	100	50	5.5	6	0.091

Note: F_1, amount of inoculum; F_2, temperature; F_3, shaker speed; F_4, medium volume; F_5, initial pH; F_6, culture time; ETSC, extracellular truffle saponin content.

2.5. Analytical Methods

2.5.1. Determination of Extracellular Saponin

The fermentation broth was filtered with filter paper and the filtrate was collected. Then, 25 mL of the filtrate was transferred into a 50 mL Erlenmeyer flask and dried to a constant weight in an oven at 65 °C. After this, 25 mL of ethanol was added to the flask and heated in a water bath at 75 °C for 4 h. Next, the mixture was centrifuged at 5000 rpm for 10 min and the supernatant was collected as an extracellular saponin extract. The saponin content was measured according to the method proposed by Li et al. [24]. Briefly, 1 mL of the supernatant was pipetted into a 10 mL test tube and 0.2 mL vanillin-glacial acetic acid and 0.8 mL of perchloric acid were added. The mixture was heated at 60 °C for 15 min and then cooled to room temperature. Afterward, 3 mL of glacial acetic acid was added into the mixture and mixed thoroughly. The absorbance at 570 nm was measured and the saponin content was calculated based on the standard curve of oleanolic acid. The saponin content mentioned throughout this study refers to extracellular saponin.

2.5.2. Measurement of Dry Biomass

A certain volume of fermentation broth was filtered with a filter cloth. The mycelia on the filter cloth were rinsed with deionized water three times and then scraped into a pre-weighed petri dish. The petri dish with mycelia was dried at 65 °C to constant weight and the dry weight of the mycelia was calculated.

2.5.3. Test of Glucose Content

The glucose content in the fermentation broth was determined using an enzymatic membrane biosensor with a glucose-oxidase-immobilized membrane (SBA-40E, Institute of Biology, Shandong Academy of Sciences, China).

2.5.4. Determination of Antioxidant Activities

The crude saponin extracts from the fermentation broth with and without rutin were lyophilized and used for saponin solution preparation at different concentrations. The DPPH scavenging activity, hydroxyl radical scavenging activity, and ferric reducing antioxidant power (FRAP) of the saponin solutions were measured according to the methods used by Xu et al. [25], Wang et al. [26], and Kumkrai et al. [27], respectively.

3. Results and Discussion

3.1. Effects of Various Carbon Sources on Saponin Production

A carbon source is an essential nutrient element for the growth and metabolism of all microorganisms. A suitable carbon source can improve the production of saponin by the liquid fermentation of *T. melanosporum*. Figure 1 shows the influence of different carbon sources on saponin production using truffle liquid fermentation. The order of saponin production in the fermentation medium with different carbon sources was: glucose > sucrose > soluble starch or maltose > cellulose or glycerol or mannitol. The saponins produced in the medium using glucose as the carbon source were 0.176 g/L, which was significantly higher than that of the control and was the best among all of the tested carbon sources; however, the saponin production was poor when cellulose, glycerol, or mannitol was used as the carbon source, similar to the result in medium without a carbon source. As such, glucose was selected as the best carbon source for truffle liquid fermentation. Carbon sources have also been proven to affect the cell growth and synthesis of the bioactive compounds produced by other edible fungi. Glucose is beneficial for mycelial growth in the liquid fermentation of *Cordyceps militaris* [28]. In the fermentation of *Ganoderma lucidum*, glucose provides better polysaccharide production [29].

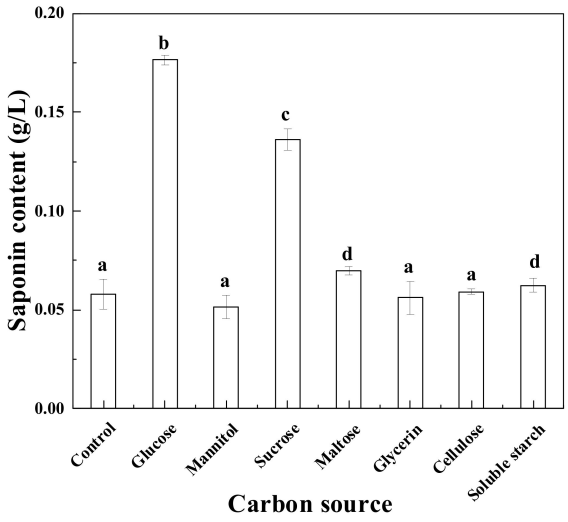

Figure 1. Effects of carbon sources on the saponin production of *T. melanosporum*. (Different letters on the top of column indicate significant differences between means: a, b, c, d, $p < 0.05$).

3.2. Effects of Various Nitrogen Sources on Saponin Production

The production of truffle saponins by liquid fermentation was screened in the medium with the addition of different nitrogen sources. When peptone was used as the nitrogen source in the culture medium, the best saponin production rate was obtained for truffle liquid fermentation (Figure 2); thus, peptone was selected as the nitrogen source for the fermentation medium. In fact, the saponin production in the medium with the addition of nitrogen was obviously higher than that of the control without the addition of nitrogen. This indicates that a nitrogen source is important and beneficial for the production of truffle saponins. When different organic and inorganic nitrogen sources were used as the sole nitrogen source in the fermentation of *G. lucidum*, the organic nitrogen sources exhibited better enhancement of the synthesis of triterpenoids [30]. This may be due to the fact that organic nitrogen sources, in addition to rich proteins, polypeptides, and free amino acids, often contain small amounts of sugars, fats, inorganic salts, vitamins, and growth factors, which can meet the demands of microorganisms for growth and metabolism [31]. In our previous research, the addition of peptone to a truffle culture medium increased the production of polysaccharides [32]. Zhu et al. also reported that the addition of peptone to a culture medium with enzymatic, pretreated *Dioscorea zingiberensis* tubers increased the production of diosgenin by *Trichoderma reesei* [33]; therefore, peptone can be used as a good nitrogen donor for cell growth and saponin production. Moreover, the increased saponin production can be partially attributed to the increased biomass.

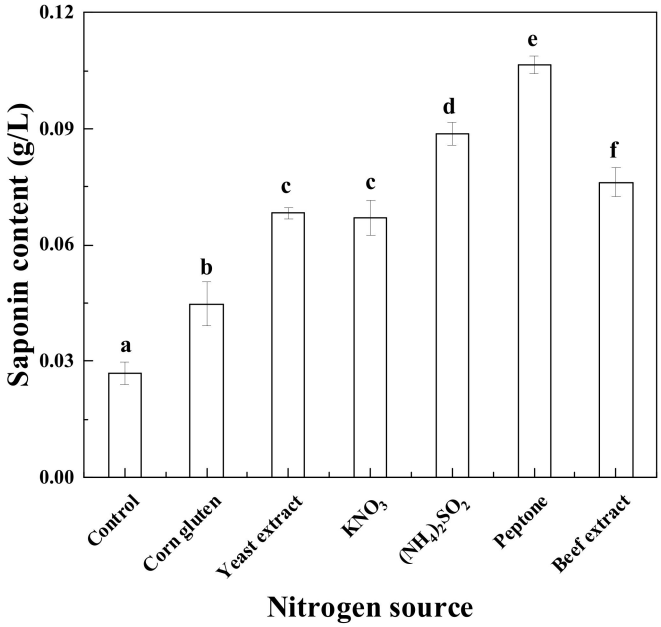

Figure 2. Effects of the nitrogen source on the saponin production of *T. melanosporum*. (Different letters on the top of column indicate significant differences between means: a, b, c, d, e, f, $p < 0.05$).

3.3. Effects of Various Inorganic Salts on Saponin Production

Inorganic salt ions can regulate the permeability of cell membranes and act as an activator of various enzymes in cells, which modulate the growth and metabolism of microorganisms. As indicated in Figure 3, the addition of KH_2PO_4 or NaCl enhanced the truffle saponin production and can be used in the liquid fermentation of *T. melanosporum*; however, the addition of the other inorganic salts brought about decreases in truffle saponin production compared to that of the control without inorganic salt. In the fermentation of *Phellinus igniarius*, KH_2PO_4 promoted the production of exopolysaccharide [34]. Moreover, the addition of KH_2PO_4 accelerated the mycelial growth of *Sparassis latifolia* by submerged fermentation [35].

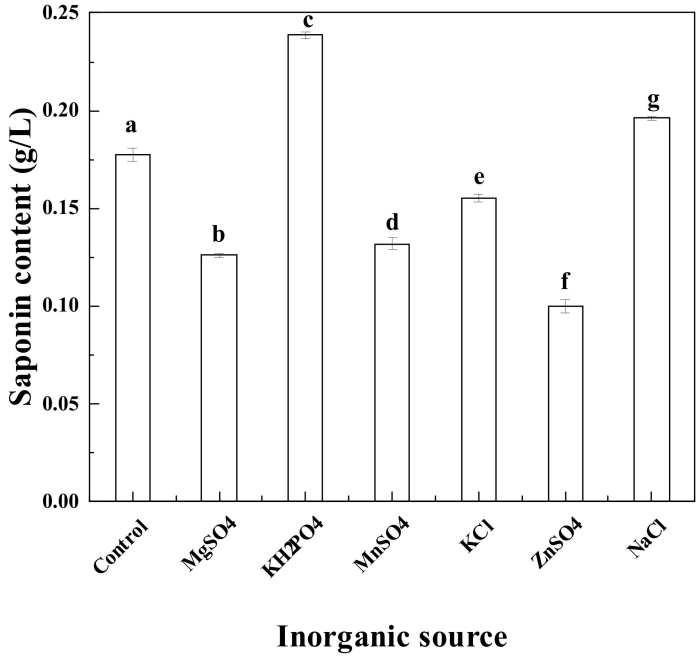

Figure 3. Effects of inorganic salts on the saponin production of *T. melanosporum*. (Different letters on the top of column indicate significant differences between means: a, b, c, d, e, f, g, $p < 0.05$).

3.4. Effects of Various Vitamins on Saponin Production

Vitamins can be used as a coenzyme to regulate microbial growth and the synthesis of metabolites by regulating enzyme activity. For the fermentation of *T. melanosporum*, VB_6, VB_2, and VC significantly increased truffle saponin production and were suitable for use as the supplementary nutrients in truffle fermentation medium (Figure 4). Vitamin B is helpful for the growth of *Cordyceps cicadae* when added into the medium as a nutrient factor [36]. It has also been found that vitamin B1 increases the intracellular adenosine content of *Andrographis paniculate* [37].

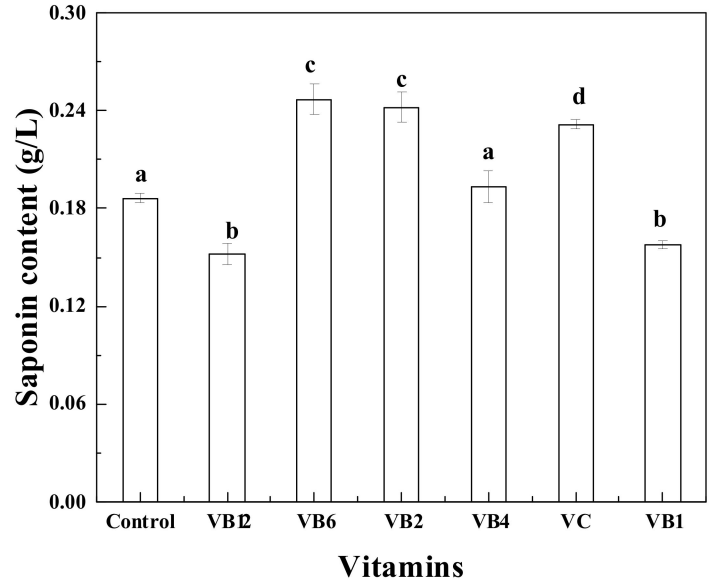

Figure 4. Effects of different vitamins on the saponin production of *T. melanosporum*. (Different letters on the top of column indicate significant differences between means: a, b, c, d, $p < 0.05$).

3.5. Effects of Various Traditional Chinese Medicines on Saponin Production

The effects of 10 kinds of Chinese herbal medicines containing flavonoids on truffle saponin production were investigated, including *Sophora japonica* bud, *Pueraria lobata* root, *Scutellaria baicalensis* root, *Viscum coloratum*, *Glycyrrhiza uralensis*, mulberry twig, mulberry bark, safflower, honeysuckle, and Citri Reticulatae Pericarpium (Figure 5). Among all of the tested groups, *Sophora japonica* bud significantly increased saponin production, obtaining a maximum value of 0.260 g/L in this case. Since rutin is the main bioactive component of *Sophora japonica* bud, the effects of rutin on the synthesis of truffle saponins were also analyzed, and a saponin production rate of 0.241 g/L was achieved with the addition of rutin; therefore, rutin was selected as an important medium component for further study of truffle saponin production because of its clear structure and good performance in enhancing saponin production. It has been reported that flavonoids can improve the permeability of cell membranes [38,39]. This could be one of the reasons for the enhancement of the extracellular saponin content. When rutin was added to a liquid fermentation broth of *Monascus aurantiacus*, the production of citrinin was changed [40]. It has also been found that the production of extracellular polysaccharide significantly increases by adding 5 g/L of dioscin to the liquid fermentation of truffles [32]. In the case of *G. lucidum* fermentation, the biomass has been shown to increase by 130% and the content of ganoderic acid by 7.32% after the addition of rutin [41].

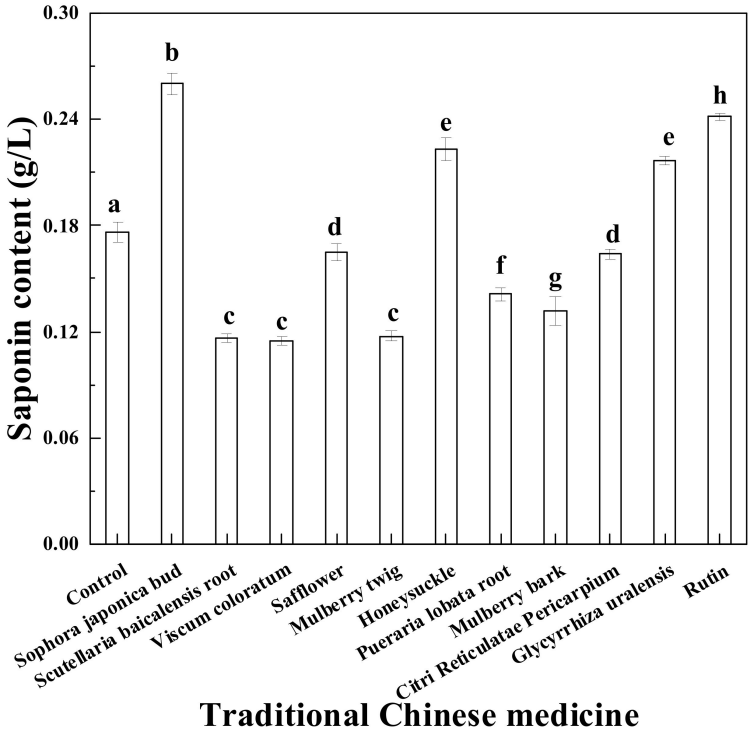

Figure 5. Effects of Chinese medicine on the saponin production of *T. melanosporum*. (Different letters on the top of column indicate significant differences between means: a, b, c, d, e, f, g, h, $p < 0.05$).

3.6. Analysis of the Orthogonal Results of Medium Composition Optimization

After fitting based on the data in Table 2, $F = 6.959$ and $p = 0 < 0.01$, indicating that the model fit was significant and the results obtained were reliable (Table 5). $F_1{}^2$ and F_1 had the highest *t*-values, followed by $F_3{}^2$ and F_3. Glucose showed the greatest effect on saponin content, followed by rutin. NaCl and VB_6 exhibited the lowest effects on the production of saponins. After model fitting, Equation (1) was obtained:

$$\begin{aligned} Y = &-0.008F_1^2 + 0.696F_1 - 0.041F_2^2 + 2.372F_2 - 12.521F_3^2 + 12.082F_3 - 0.731F_4^2 + 1.674F_4 - 0.448F_5^2 \\ &-0.05F_5 - 394.311F_6^2 + 65.102F_6 + 237.123F_7^2 - 20.234F_7 + 304.787F_8^2 - 29.144F_8 - 29.144 \end{aligned} \quad (1)$$

Table 5. Orthogonal experiment analysis of medium composition optimization.

Item	Coefficient	*t*	Significance
(Constant)	−15.222	–2.768	0.009
F_1^2	–0.008	–3.384	0.002
F_1	0.696	3.973	0.000
F_2^2	–0.041	–0.186	0.853
F_2	2.372	1.353	0.185
F_3^2	–12.521	–2.257	0.031
F_3	12.082	1.837	0.075
F_4^2	–0.731	–0.823	0.417
F_4	1.674	0.793	0.433
F_5^2	–0.448	–0.504	0.617
F_5	–0.050	–0.024	0.981
F_6^2	–394.311	–0.711	0.482
F_6	60.102	0.990	0.330
F_7^2	237.132	0.427	0.672
F_7	–20.234	–0.308	0.760
F_8^2	304.787	0.549	0.587
F_8	–29.144	–0.443	0.661
Model: R^2 = 0.777; Adj-R^2 = 0.665; *F*-value = 6.959; p = 0.000			

Note: *t*, t-value of t-test.

The coefficients of F_1^2, F_2^2, F_3^2, F_4^2, F_5^2, and F_6^2 were less than 0, which indicates that the curve for the relationships between Y (extracellular truffle saponin content (ETSP)) and F_1, F_2, F_3, F_4, F_5, and F_6 was inverted U-shaped. Y was derived from F_1, F_2, F_3, F_4, F_5, and F_6, and Equations (2)–(7) were achieved, respectively:

$$Y' = -0.016F_1 + 0.696 \quad (2)$$

$$Y' = -0.082F_2 + 2.372 \quad (3)$$

$$Y' = -25.02F_3 + 12.082 \quad (4)$$

$$Y' = -1.462F_4 + 1.674 \quad (5)$$

$$Y' = -0.896F_5 + 0.05 \quad (6)$$

$$Y' = -788.622F_6 + 65.102 \quad (7)$$

where the following were set: Y' = 0, F_1 = 43.5, F_2 = 28.9, F_3 = 0.48, F_4 = 1.14, F_5 = 0.05, and F_6 = 0.082. Because the curve for the relationships curve between Y and F_1, F_2, F_3, F_4, F_5, and F_6 was inverted U-shaped, the content of saponin was the maximum when F_1 = 43.5 g/L, F_2 = 28.9 g/L, F_3 = 0.48 g/100 mL, F_4 = 1.14 g/L, F_5 = 0.05 g/L, and F_6 = 0.082 g/L; however, the optimal values of peptone (F_2) and NaCl (F_5) obtained from fitting the model were out of the experimental concentration range. According to the data in Table 2, 6 g/L and 0.2 g/L were selected as the concentrations of peptone and NaCl in the fermentation medium, respectively; therefore, within the experimental concentration range, the maximal saponin content was achieved when glucose, peptone, rutin, KH_2PO_4, NaCl, and VB_2 were 43.5 g/L, 6 g/L, 4.8 g/L, 1.14 g/L, 0.2 g/L, and 0.082 g/L, respectively.

The coefficients of F_7^2 and F_8^2 were greater than 0, which indicates that the relationship curve between Y and F_7 and F_8 was U-shaped. When Y was derived from F_7 and F_8, the following Equations (8) and (9) were obtained, respectively:

$$Y' = 474.264F_7 - 20.234 \quad (8)$$

$$Y' = 609.574F_8 - 29.144 \quad (9)$$

where the following were set: Y' = 0, F_7 = 0.043, and F_8 = 0.048. Because the relationship curve of Y with F_7 (VB_6) and F_8 (VC) was U-shaped, the saponin content in the fermentation

broth was at the minimum when F_7 = 0.043 g/L and F_8 = 0.048 g/L. According to the results in Table 2, the saponin content was the highest when VB_6 and VC were 0.1 g/L and 0.02 g/L, respectively. In conclusion, the optimal fermentation medium composition was: 43.5 g/L of glucose, 6 g/L of peptone, 4.8 g/L of rutin, 1.14 g/L of KH_2PO_4, 0.2 g/L of NaCl, 0.082 g/L of VB_2, 0.1 g/L of VB_6, and 0.02 g/L of VC.

3.7. Analysis of the Orthogonal Results of Fermentation Condition Optimization

After fitting based on the data in Table 4, F = 6.266 and p = 0.002 < 0.01, indicating that the model fit was significant and the results obtained were reliable (Table 6). The t-values of F_6^2 and F_6 were the largest, followed by F_5^2 and F_5. The fermentation time had the greatest effect on the saponin content, followed by the initial pH of the medium. The medium volume and shaker speed exerted the least influence on the content of saponins. After model fitting, Equation (10) was obtained:

$$\begin{aligned} Y = -0.045F_1^2 + 1.132F_1 - 0.134F_2^2 + 6.354F_2 + 0.00006566F_3^2 - 0.071F_3 - 0.00009911F_4^2 + 0.0079F_4 \\ +2.949F_5^2 - 33.484F_5 + 1.042F_6^2 - 14.784F_6 + 79.136 \end{aligned} \quad (10)$$

Table 6. Orthogonal experimental analysis of culture condition optimization.

Item	Coefficient	*t*	Significance
(Constant)	79.136	1.062	0.309
F_1^2	–0.045	–1.125	0.283
F_1	1.132	1.391	0.189
F_2^2	–0.134	–1.492	0.162
F_2	6.354	1.467	0.168
F_3^2	0.00006566	0.164	0.873
F_3	–0.071	–0.678	0.511
F_4^2	–0.00009911	–0.110	0.914
F_4	0.0079	0.484	0.637
F_5^2	2.949	2.045	0.063
F_5	–33.483	–1.932	0.077
F_6^2	1.042	2.891	0.014
F_6	–14.784	–3.401	0.005
Model: R^2 = 0.862; Adj-R^2 = 0.725; F-value = 6.266; p = 0.002			

Note: t, t-value of t-test.

The coefficients of F_1^2, F_2^2, and F_4^2 were less than 0, which indicates that the relationship between Y and F_1, F_2, and F_4 was inverted U-shaped. When Y was derived from F_1, F_2, and F_4, the following respective Equations (11)–(13) were achieved:

$$Y' = -0.09F_1 + 1.132 \quad (11)$$

$$Y' = -0.268F_2 + 6.354 \quad (12)$$

$$Y' = -0.00019822F_4 + 0.0079 \quad (13)$$

where the following were set: Y' = 0, F_1 = 12.5, F_2 = 23.7, and F_4 = 39.4. Since the relationship curve between Y and F_1, F_2, and F_4 was inverted U-shaped, the saponin content reached the maximum when F_1 = 12.5%, F_2 = 23.7 °C, and F_4 = 39.4 mL. Considering the experimental setting values for the different conditions in Table 3 and the results in Table 4, the following conditions were selected for the best saponin production rate: 12.5% inoculation, temperature of 24 °C, and medium volume of 50 mL.

The coefficients of F_3^2, F_5^2, and F_6^2 were greater than 0, indicating that the curve of the relationship between Y and F_3, F_5, and F_6 was U-shaped. When Y was derived from F_3, F_5, and F_6, Equations (14)–(16) were achieved, respectively:

$$Y' = 0.0013132F_3 - 0.071 \quad (14)$$

$$Y' = 5.898F_5 - 33.483 \quad (15)$$

$$Y' = 2.084F_6 - 14.784 \quad (16)$$

with $Y' = 0$, $F_3 = 54$, $F_5 = 5.678$, and $F_6 = 7.094$. Since the relationship curve between Y and F_3 (shaker speed), F_5 (initial pH), and F_6 (fermentation time) was U-shaped, the saponin concentration of the fermentation broth was at the minimum when $F_3 = 54$ rpm, $F_5 = 5.678$, and $F_6 = 7.094$ h. Based on the experimental setting values for the different conditions in Table 3 and the results in Table 4, a shaker speed of 190 rpm, an initial pH of 5.7, and a fermentation time of 96 h were selected for truffle saponin production; therefore, the optimal fermentation conditions were as follows: 12.5% inoculation, culture temperature of 24 °C, shaker speed of 190 rpm, medium volume of 50 mL, initial pH of 5.7, and culture time of 96 h.

3.8. Truffle Fermentation under Optimal Medium Composition and Culture Conditions

After the optimization by orthogonal design, the optimal medium composition and fermentation conditions were achieved. The fermentation of *T. melanosporum* was carried out using the optimized medium and conditions (Figure 6). Glucose was consumed with the prolonged culture time and decreased to less than 4 g/L. The biomass increased with the consumption of glucose and reached 8.35 g/L in the optimized medium with the addition of rutin after five days of culture, which was approximately 18% higher than that in the optimized medium without rutin. The production of truffle saponins in the medium with rutin was 0.413 g/L, which was 50.2% higher than that in the optimized medium without rutin (0.275 g/L) and increased by 134.7% compared to that in the base fermentation medium (0.176 g/L). The saponin yield based on biomass with the addition of rutin was 53.3 mg/g dry of biomass but was only 39.6 mg/g of dry biomass without the addition of rutin; therefore, the increased saponin production was mainly attributed to the enhanced saponin yield and partly due to the increased biomass. These results indicate that truffle saponin production was greatly improved after optimization of the medium composition and culture conditions, and that the addition of rutin enhanced the saponin production by *T. melanosporum*.

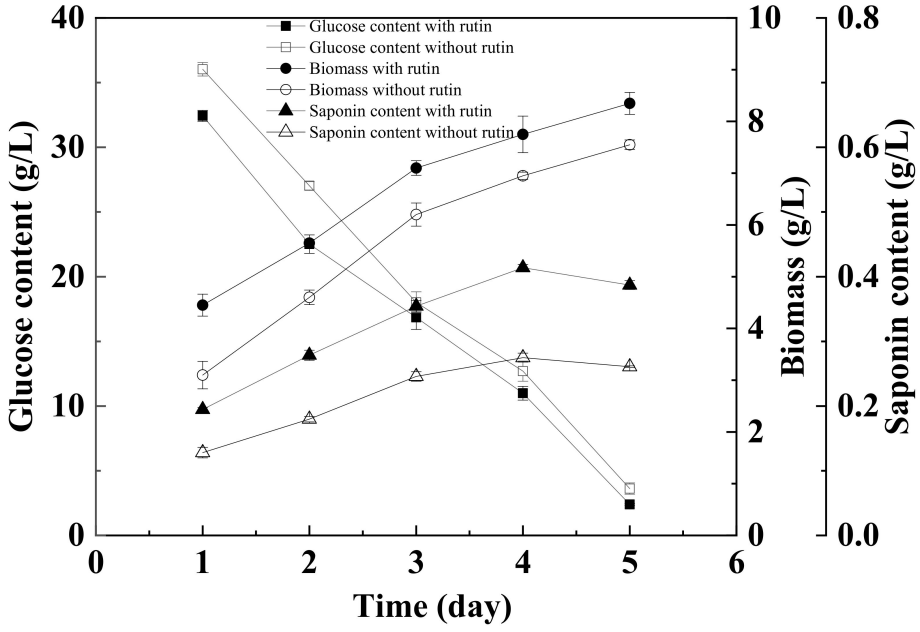

Figure 6. Time profiles of *T. melanosporum* fermentation under the optimized medium and culture conditions with and without rutin.

3.9. Antioxidant Activities of Truffle Saponin

The results for scavenging DPPH• using the crude saponin extract from the optimized medium with rutin (RTS) and the crude saponin extract from the optimized medium without rutin (TS) are shown in Figure 7. With the increased concentration of RTS or TS, their ability to scavenge DPPH• was enhanced. In the case of 2.0 mg/mL, the scavenging rate for DPPH• by RTS was 22.2% higher than that of TS; the scavenging rates for DPPH• by RTS and TS were 93.98% and 76.88%, respectively. When the dosage of saponin extract increased to more than 3.0 mg/mL, the DPPH• clearance rate did not change significantly.

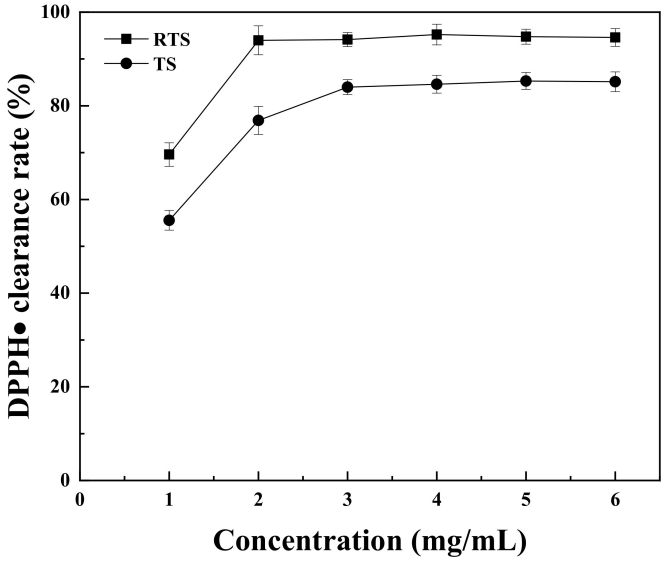

Figure 7. The DPPH scavenging activity levels of RTS and TS.

The inhibition rates for RTS and TS regarding the formation of •OH are shown in Figure 8. The inhibition rates for RTS and TS regarding the formation of •OH were dose-dependent and increased with respect to the saponin extract concentration. At a concentration of 4.0 mg/mL, the inhibition rate for RTS was 79.26%, 15.2% higher than for TS (68.83%). When the RTS concentration was higher than 4.0 mg/mL, the inhibition rate for the truffle saponin extract regarding hydroxyl radical formation was almost unchanged.

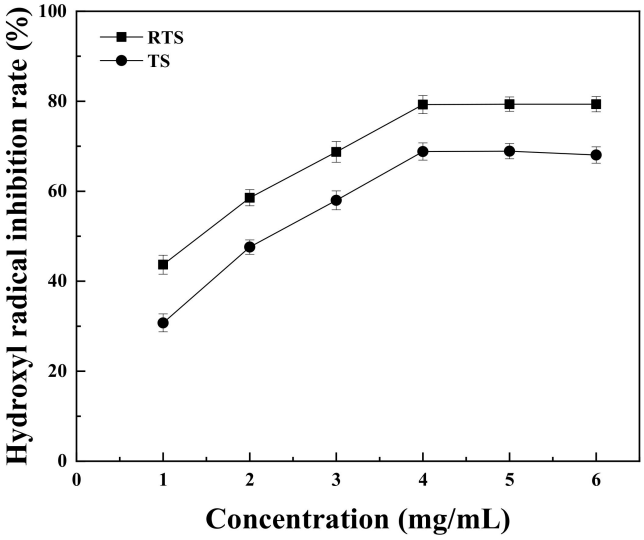

Figure 8. The hydroxyl radical scavenging activity levels of TS and RTS.

The total antioxidant capacity levels of RTS and TS with different concentrations were tested and the results are presented in Figure 9. The total antioxidant capacity of RTS was always higher than that of TS at different concentrations. When the concentration of TS was greater than 4 mg/mL, the total antioxidant activity remained unchanged, indicating that the reaction basically reached the limit. The maximal ferric reducing antioxidant power (FRAP) levels of RTS and TS were 42.22 mM and 38.57 mM, respectively.

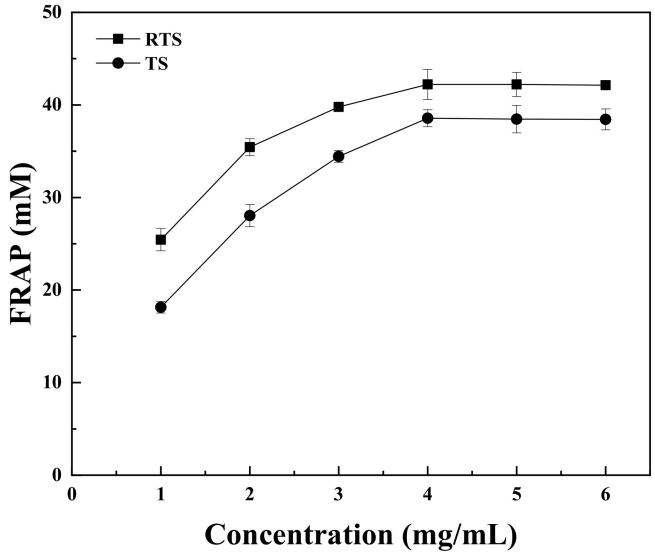

Figure 9. The ferric reducing antioxidant power (FRAP) levels of TS and RTS.

According to the results for the antioxidant activities of the truffle saponin extract, the addition of rutin was beneficial for improvement of the antioxidant activities of the truffle saponin extract. As shown in Table 7, RTS exhibited comparable antioxidant activities. The DPPH and hydroxyl radical scavenging activities of RTS were better than some of the saponins from plant sources, such as *Panax notoginseng*, *Radix trichosanthis*, and *Albuca bracteata* Jacq. Bulb. To analyze the component-based differences of TS and RTS, UPLC–QTOF–MS/MS was performed, with the results shown in the Supplementary Materials. The content of rutin in RTS equaled 1.324 mg/g of crude saponin extract (Figure S1); therefore, the contribution of residual rutin in RTS to the antioxidant activities was very poor and could be omitted. In addition, a new substance, namely azaleatin 3-rutinoside, was identified in RTS, indicating the transformation of rutin in the liquid fermentation of *T. melanosporum* (Figures S2 and S3). It was also found that the saponin of "smilanippin A" was present in the crude saponin extracts with and without rutin addition (Figure S4); therefore, smilanippin A is one of the saponins produced by *T. melanosporum*.

Table 7. Antioxidant activities of the different saponins from plants and fungi.

Source	Preparation Method	Composition	Concentration (mg/mL)	DPPH· (%)	·OH (%)	FRAP (mM)	Reference
Tuber melanosporum broth	Extraction	TS mixture	3	84.00	57.97	26.44	This study
Tuber melanosporum broth	Extraction	RTS mixture	3	94.13	68.75	30.28	This study
Panax ginseng root	Extraction and purification	Ginsenoside-Rg18	0.048	8.76	87.70	–	[42]
		6-acetyl ginsenoside-Rg3	0.057	4.58	87.55	–	
		Ginsenoside-Rs11	0.057	7.37	90.74	–	
		Ginsenoside-Re7	0.049	2.19	89.48	–	

Table 7. *Cont.*

Source	Preparation Method	Composition	Concentration (mg/mL)	DPPH· (%)	·OH (%)	FRAP (mM)	Reference
Ophiopogon japonicus	Extraction and purification	Saponin mixture	3	94.62	83	–	[43]
Panax notoginseng	Extraction	Saponin mixture	5	84.7	50	–	[44]
Radix trichosanthis	Extraction	Saponin mixture	3	70	3	–	[45]
Albuca bracteata Jacq. Bulb	Extraction	Saponin mixture	4	87		–	[46]
Penicillium chrysogenum broth	Extraction	Snef1216	0.5	63.86		–	[47]
White ginseng	Extraction	Saponin mixture	1	65	18.3	–	[48]
Panax ginseng root	Extraction	Saponin mixture AR-5R	0.2	–	–	17.42	[42]
		Saponin mixture AR-4M	0.2	–	–	13.91	
Astragalus psilocentros	Extraction	Astragaloside mixture	2.5	–		43.76	[49]
Alternaria alternata broth	Extraction	Saponin mixture SaF-2	0.5	83.25%	–	1.65	[50]
Fusarium proliferatum broth	Extraction	Saponin mixture SaR-2	0.5	90.14%	–	1.68	[50]
Schizophyllum commune broth	Extraction	Saponin mixture SaR-3	0.5	–	–	0.58	[50]
Trametes hirsuta broth	Extraction	Saponin mixture SaR-6	0.5	–	–	0.51	[50]
Salvia miltiorrhiza Bge root	Extraction	Saponin mixture	0.5	80.23%	–	1.34	[50]
Moringa oleifera root	Extraction	Saponin mixture	0.37	–	–	0.87	[51]

Note: –, not determined; DPPH·, 1,1-diphenyl-2-picrylhydrazyl radical, ·OH, hydroxyl radical, FRAP, ferric reducing antioxidant power.

4. Conclusions

The optimal medium composition for the maximal saponin production by liquid fermentation of *T. melanosporum* was 43.5 g/L of glucose, 6 g/L of peptone, 1.15 g/L of KH_2PO_4, 0.2 g/L of NaCl, 4.8 g/L of rutin, 0.082 g/L of VB_2, 0.1 g/L of VB_6, and 0.02 g/L of VC. The optimized culture conditions of *T. melanosporum* were 12.5% inoculation, medium volume of 50 mL/250 mL flask, culture temperature of 24 °C, shaker speed of 190 rpm, initial pH of 5.7, and culture time of four days. After optimization, the truffle saponin production increased to 0.413 g/L and rutin was identified as a good promoter in saponin production. The extracted saponin from the truffle fermentation broth possessed a strong ability to scavenge DPPH and hydroxyl radicals and showed good FRAP; therefore, enhanced truffle saponin production with good antioxidant activities was shown in this study. Fungi are a potential food source for saponin production by liquid fermentation, and the addition of flavonoid compounds could be a good strategy to improve fungal saponin production.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/fermentation7030103/s1, Figure S1: Diode array analysis for the crude saponin extract at 350 nm, Figure S2: QTOF-MS/MS analysis for the crude saponin extract, Figure S3: The structure and MS/MS spectrum of the new substance in crude saponin extract with rutin addition, Figure S4: The structure and MS/MS spectrum of the possible saponin in crude saponin extract with/without rutin addition (Retention time = 4.29 min).

Author Contributions: Conceptualization, F.W. and L.X.; methodology, Q.S. and L.X.; software, Z.Z. and G.G.; validation, F.W. and Z.Z.; formal analysis, Q.S. and D.H.; writing—original draft preparation, Q.S. and F.W.; writing—review and editing, N.T. and F.W.; supervision, F.W.; project administration, F.W. and L.X.; funding acquisition, F.W. and L.X. All authors have read and agreed to the published version of the manuscript.

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