

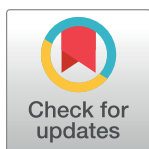
RESEARCH ARTICLE

Production, purification, characterization, and biological properties of *Rhodospiridium paludigenum* polysaccharide

Mengjian Liu¹, Wenju Zhang^{1*}, Jun Yao¹, Junli Niu¹

College of Animal Science and Technology, Shihezi University, Shihezi, Xinjiang, China

* These authors contributed equally to this work.

* zhangwj1022@sina.com

Abstract

The yield of marine red yeast polysaccharide (MRYP) obtained from *Rhodospiridium paludigenum* was increased by optimizing fermentation conditions, and the pure polysaccharide was extracted by column chromatography. The molecular weight of pure MRYP and the ratio of mannose to glucose in components of MRYP were determined. Antioxidant and anti-bacterial abilities of MRYP were investigated *in vitro* and *in vivo*. The optimal fermentation parameters were as follows: Medium 4, pH = 6.72, temperature = 30.18°C, blades speed = 461.36 r/min; the optimized yield reached 4323.90 mg/L, which was 1.31 times the original yield. The sequence of factors that affected the MRYP yield was the blades speed > pH > temperature. The main components of MRYP were MYH-1 and MYH-2. The molecular weights of MYH-1 and MYH-2 were 246.92 kDa and 21.88 kDa, respectively; they accounted for 53.60% and 28.75% of total polysaccharide. In MYH-1 and MYH-2, the proportion of glucose and mannose accounted for 46.94%, 38.46%, and 67.10%, 7.17%, respectively. *In vitro*, the ability of scavenging DPPH•, •OH, and •O₂⁻ radical was 32.26%, 24.34%, and 22.09%; the minimum inhibitory concentration (MIC) of MRYP was 480 µg/mg. *In vivo*, MRYP improved the lambs' body weight, antioxidant enzyme activity, and the number of probiotics, but it reduced the feed/gain (F/G) ratio and the number of pathogenic bacteria in 60-days-old lambs.

OPEN ACCESS

Citation: Liu M, Zhang W, Yao J, Niu J (2021) Production, purification, characterization, and biological properties of *Rhodospiridium paludigenum* polysaccharide. PLoS ONE 16(1): e0246148. <https://doi.org/10.1371/journal.pone.0246148>

Editor: Chenyu Du, University of Huddersfield, UNITED KINGDOM

Received: March 20, 2020

Accepted: January 14, 2021

Published: January 29, 2021

Peer Review History: PLOS recognizes the benefits of transparency in the peer review process; therefore, we enable the publication of all of the content of peer review and author responses alongside final, published articles. The editorial history of this article is available here: <https://doi.org/10.1371/journal.pone.0246148>

Copyright: © 2021 Liu et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper.

Funding: National Key R&D Program of China (2018YFD0502100) <https://service.most.gov.cn/>

Introduction

Marine red yeast *Rhodospiridium paludigenum* is a kind of single-celled aquatic organism, which is widely distributed in the sea and various rivers. Recent studies have shown that marine microorganisms have superior characteristics compared to their terrestrial counterparts. First of all, marine yeasts showed higher tolerance to all the inhibitors tested than terrestrial yeasts, and many reports have revealed that most of marine yeasts strains showed excellent fermentation capability compared with the reference terrestrial yeast and other different yeasts species [1]. Besides, marine microorganisms, including marine yeasts, live in extreme environments, and this provides a unique potential for the synthesis of functional

Competing interests: The authors have declared that no competing interests exist.

biomolecules. Recent research showed that marine yeasts have several unique and promising features over the terrestrial yeasts, for example higher osmosis tolerance, higher special chemical productivity and ability to produce industrial enzymes. These indicate that marine yeasts have a great potential to be applied in various industries [2]. Last but not least, it has been reported that marine yeasts are able to produce many bioactive substances, such as amino acids, glucans, glutathione, toxins, enzymes, phytase, and vitamins with potential application in the food, pharmaceutical, cosmetic, and chemical industries as well as for marine culture and environment protection [3].

Compared to the marine red yeast, a crude polysaccharide of *Rhodospiridium paludigenum* (MRYP) is more widely used as a probiotic for immune-enhancement and growth-promotion in local farms, such as pig farms, layer farms, and cattle farms, which are situated in Xinjiang, China. This implies that MRYP might be a prebiotic, which plays an important role in biological activities and has potential developmental prospects. Several studies have reported that yeast polysaccharides have various bioactive properties, including antibacterial, antioxidant, and anti-inflammatory activities [4]. The biological effect of MRYP might be as follows: 1. The yeast polysaccharide lowers attachment ability of pathogens in the intestine [5]; 2. Innate immunity of intestinal surface is regulated through pathogen-associated molecular patterns of yeast polysaccharide [6]; 3. The immune overreaction and inflammation caused by a lipopolysaccharide (LPS) in the intestinal region is inhibited and relieved by MRYP [7]. Besides, the antigen-presenting cells (APC) such as dendritic cells and macrophages are affected by the yeast polysaccharide [8]. Therefore, we deduce that MRYP is much better than the ocean red yeast.

In theory, the molecular weight is one of important indicators of bioactivity of yeast polysaccharide; the molecular weight of 50–200 kDa indicates a high biological activity [9]. Therefore, the bioactivity of MRYP should be evaluated by determining its molecular weight. Mannosan and β -glucan were the most important bioactive components in the yeast polysaccharide [10]. Glucan increased the phagocytic activity of biologically active compounds, such as TNF- α , IL-1, and NO [11]. Mannosan inhibited colonization of pathogenic bacteria and also increased the number of probiotics in the intestine [12, 13]. To indicate the bioactivity of MRYP components, we determined the proportion of mannose to glucose in MRYP.

In the past years, several studies have been undertaken to determine the microbial characteristics, bacteriostatic ability, and immunogenicity of oceanic red yeast. To the best of our knowledge, no previous study has examined the fermentation, characterization, and biological function of MRYP. In this study, firstly, we used single factor tests and Box–Behnken design to optimize the fermentation conditions. Secondly, MRYP was extracted and purified by DEAE-52 cellulose and Sephadex G-100 column chromatography. The molecular weight of MYH-1 and MYH-2, which were the components of MRYP, were determined by liquid chromatography. The ratio of mannose and glucose was also determined by liquid chromatography (mannose and glucose were the components of MYH-1 and MYH-2). Finally, the antioxidant and antibacterial activities of MRYP were evaluated *in vitro* and *in vivo*. This work provides a theoretical foundation to a wide range of possible application of MRYP in the industrial production, design, and operation.

Materials and methods

Animals and strains

The test lambs: One hundred newborn Suffolk-Kazakh lambs (aged 32 days, 2:1 male: female ratio) were chosen as experimental animals in Asar farming cooperatives, Solbastow, Changji Hui Autonomous Prefecture, China. All experimental lambs in this research have been

approved by the Animal Welfare Committee of Shihezi University (Xinjiang, China) with the ethical code: A2019-156-01.

In the antioxidant enzyme activity test, three blood samples (10 mL each) were collected randomly from marginal ear vessels of 10 lambs in each group without anesthesia, euthanasia, or any kind of animal sacrifice in this part of the study.

The experimental strain of marine red yeast *Rhodospiridium paludigenum* was obtained from the Yarkand River, Maigaiti, Kashi, China. The strain was isolated with a YPD solid medium with addition of sugar-cane molasses and identified by multiple sequence alignment (26S rRNA D1/D2) and phylogeny analysis.

The test strains for the antibacterial activities experiment: The Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli* and *Salmonella enteric* bacteria strains were purchased from the College of Animal Science and Technology, Shihezi University, Shihezi, China.

Optimization of fermentation medium. The fundamental fermentation parameters were as follows: pH = 7, 32°C, 300 r/min, and aerobic incubation for 72 h [14]. Each of the fermentation media was used as a single factor to produce MRYP. An automatic fermentation system with a 12 L fermentor was used as the experimental equipment.

Six different fermentation media were used as follows: (1) The yeast peptone dextrose adenine (YPDA) medium: yeast extract 10 g, peptone 20 g, glucose 20 g, and distilled water 1 L [15]; (2) Czapek medium: sucrose 30 g, sodium nitrate 3 g, dipotassium phosphate 1 g, potassium chloride 0.5 g, magnesium sulfate 0.5 g, ferrous sulfate 0.01 g, and distilled water 1 L [16]; (3) Medium 1: sucrose 50 g, soybean peptone 40 g, yeast extract 30 g, glycerin 16 g, and distilled water 1 L [17]; (4) Medium 2: sucrose 50 g, glucose 20 g, yeast extract 50 g, ammonium sulfate 10 g, potassium dihydrogen sulfate 3 g, glycerol 2 g, calcium chloride 1 g, and distilled water 1 L [18]; (5) Medium 3: sucrose 60 g, yeast paste 40 g, ammonium sulfate 10 g, potassium dihydrogen sulfate 3 g, glycerin 10 g, and distilled water 1 L [19]; (6) Medium 4: sucrose 50 g, glucose 20 g, peptone 60 g, ammonium sulfate 30 g, glycerin 3 g, calcium chloride 3 g, magnesium sulfate 1 g, calcium D-pantothenate 0.6 g, and distilled water 1 L. All the media were sterilized at 121°C in an autoclave sterilizer for 30 min.

Optimization of fermentation pH, temperature, blades speed. pH was used as a single factor in this study. In the primary screening test, the medium pH was set to 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0. In the second screening test, based on the primary pH scope, the medium pH was set to 5.0, 5.4, 5.8, 6.2, 6.6, and 7.0. All the processes were repeated three times.

Optimization of fermentation temperature. The temperature, which was used as a single factor, was set to 24, 26, 28, 30, 32, and 34°C. All the processes were repeated three times.

Optimization of fermentation blades speed. The blades speed was used as a single factor in this study and set to 200, 300, 400, 500, 600, and 700 r/min [20]. All the processes were repeated three times.

Box-Behnken design of fermentation conditions. Based on the single factor experiments, we developed a Box-Behnken design (BBD) of three factors and three levels. Our main goal was to further optimize fermentation conditions. Three independent variables, i.e. fermentation pH, temperature, and blades speed, were designated as X_1 , X_2 , and X_3 , respectively [21]. As shown in Table 1, the ranges of values were based on the results of preliminary experiments. The response variables indicated the polysaccharides yield.

Validation of the mathematical model. Based on the optimum fermentation conditions, triplicate fermentation experiments were performed to verify accuracy and availability of the mathematical model representing MRYP production. Moreover, the relative error between the actual and theoretical yield was calculated [22].

Table 1. Independent variables and levels in Box-Behnken design.

	A	B	C
Coding value	pH	Temperature/°C	Blades speed/(r/min)
-1	5.8	28	500
0	6.2	30	600
1	6.6	32	700

<https://doi.org/10.1371/journal.pone.0246148.t001>

Extraction of MRYP. The fermented liquor was centrifuged in a high-speed refrigerated centrifuge with 6000 r/min for 10 min at 4°C, and the supernatant was removed [23]. Three volumes of cold (20°C) distilled water were added to dissolve precipitates within 30 min of agitation. Neutral protease (300 IU/g) was added into the slurry, and it was incubated at a constant temperature of 40°C for 36 h [24]. After centrifugation (6000 rpm, 10 min), the sediment was collected and treated with sodium hydroxide (2%) for 3 h, and then it was neutralized with hydrochloric acid (5%) [25]. Two volumes of ethyl alcohol were added to wash the sediment, and this process was repeated thrice. Finally, the crude polysaccharides were obtained after freeze-drying the sediment for 48 h and grinding it to a powder [26]. The crude polysaccharides were weighed accurately with an electronic balance in three repeated trials.

Purification of MRYP. Two volumes of acetone were added to the crude MRYP, which was extracted by the method above. The mixture was agitated vigorously for 4 hours, and then it was centrifuged at 6000 r/min for 10 min. The supernatant was removed. The sediment was collected, and then it was deproteinated with Savage reagent (1-butanol/chloroform, v/v = 1:4) at 4°C for 24 h. The sediment was collected [27], added into a dialysis bag (3500 Da, 0.22 µm) and mixed with 5 volumes of distilled water for 48 h [28]. Finally, the sediment was lyophilized at -80°C for 48 h, and the pure MRYP was obtained.

Isolation of MRYP. Pure MRYP (200 mg) was dissolved in 10 mL of distilled water, and 2 mL of MRYP solution was passed through an anion exchange column (2.6 cm×50 cm) with DEAE-52 cellulose. The equilibrium liquid was a borate solution (0.05 mol/L, pH = 9.18). The eluent was a gradient sodium chloride solution at concentrations of 0.1, 0.5, 0.9, 1.3, and 1.7 mol/L [29]. In the anion exchange column, the flow velocity was 1 mL/min. Fractions of pure MRYP were eluted with different concentrations of NaCl. The eluents were collected in 6 mL aliquots and desalted for further purification on the Sephadex G-100 column, with distilled water as the equilibrium liquid at 0.5 mL/min [30]. The major polysaccharide fractions were collected, concentrated, and lyophilized at -80°C for 48 h, and all fractions of pure MRYP were obtained [31].

Determination of the molecular weight. The molecular weight of MRYP fractions was determined by high-performance gel permeation chromatography (HPGPC) with an Agilent 1200 HPLC system, which was equipped with a TSKgel-G3000-PWXL (7.8 mm×300 mm) column. Standard dextrans (404 kDa, 212 kDa, 112 kDa, 47.3 kDa, 22.8 kDa, 11.8 kDa, 5.9 kDa, and 2.5 kDa) were used for obtaining the standard curve [32]. The polysaccharide sample solution, which had a concentration of 5 mg/mL, was prepared by mixing the pure polysaccharide with the mobile phase [33]. Furthermore, 20 µL of polysaccharide sample solution was injected into the sample system, and the operation conditions were as follows: sample size was 20 µL; mobile phase was 0.1 mol/L sodium sulfate; column temperature was 35°C; flow velocity was 0.5 mL/min; and the maximum pressure was 25 bar [34].

Analysis of monosaccharides composition. According to the analysis method reported by Xutingting [35], the content of mannose and glucose in MRYP components MYH-1 and MYH-2 was determined by high-performance liquid chromatography (HPLC). Firstly, pure mannose and glucose were weighed precisely and mixed with distilled water separately. The

concentration of the standard solution was diluted down to 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL, and 1000 µg/mL. We injected 50 µL of each standard solution into the sample inlet, and the regression equation was obtained accordingly. Secondly, each sample of MRYP was weighed precisely and mixed with distilled water and injected into the sample inlet. The operation conditions of HPLC were as follows: the mobile phase was deionized water; flow velocity was 0.5 mL/min; column temperature was 85°C; and the sample size was 50 µL [36].

Antioxidant activity of MRYP. 1) *Scavenging of DPPH radical (DPPH•)*. The polysaccharide solution was diluted down to 100 µg/mL, 200 µg/mL, 400 µg/mL, 600 µg/mL, 800 µg/mL, 1000 µg/mL, and 1200 µg/mL. Then 2 mL of polysaccharide solution and the control sample were separately mixed with 2 mL of ethanolic DPPH• (1,1-diphenyl-2-picrylhydrazyl) (5 mmol/L) [37]. The mixture was shaken violently and incubated in the dark at 25°C for 30 min. The absorption of the samples was measured with an ultraviolet spectrophotometer at $\lambda = 517$ nm [38]. Each processing was repeated three times, and the average scavenging activity was calculated by the method explained in paragraphs (2) and (3).

The scavenging activity was calculated as follows:

$$\text{Scavenging activity} = [1 - (A_0 - A_1)/A_2] \times 100\%$$

Where A_0 is the absorbance of the mixture of DPPH solution and the sample; A_1 is the absorbance of ethanolic DPPH• mixed with deionized water; and A_2 is the absorbance of DPPH solution mixed with deionized water.

2) *Scavenging of hydroxyl radical (•OH)*. The FeSO_4 solution (1.0 mL, 9.0 mmol/L) was mixed with H_2O_2 (1.0 mL, 0.06%), deionized water (5 mL), sodium salicylate (1.0 mL, 9.0 mmol/L), and 1.0 mL of sample polysaccharide solution of above concentrations (100–1200 µg/mL), and then the mixture was incubated at 37°C for 1 h [39]. The absorption of the samples was measured with a microplate spectrophotometer at $\lambda = 517$ nm [40].

The antioxidant activity was calculated by using the following equation:

$$\text{Scavenging activity} = [1 - (A_0 - A_1)/A_2] \times 100\%$$

Where A_0 is the absorbance of the sample as mentioned above; A_1 is the absorbance when the H_2O_2 was replaced by the same volume of deionized water; and A_2 is the absorbance when the sample polysaccharide solution was replaced by the same volume of deionized water.

3) *Scavenging of superoxide anion free radical (•O₂⁻)*. The Tris-hydrochloric acid (3 mL, pH = 8.2, 25°C) was mixed with pyrogallol (3 mL) and precisely incubated at 25°C for 4 min. Then the incubation was terminated by mixing with hydrochloric acid (1 mL, 19 mmol/L). The absorption of the samples was measured on the microplate spectrophotometer at $\lambda = 420$ nm [41]. The scavenging activity was calculated from the following equation:

$$\text{Scavenging activity} = [1 - (A_0 - A_1)/A_2] \times 100\%$$

Where A_0 is the absorbance of the sample mentioned above; A_1 is the absorbance when the sample polysaccharide solution was replaced by the same volume of deionized water; and A_2 is the absorbance when the Tris-hydrochloric acid was replaced by the same volume of deionized water.

Antibacterial activity of MRYP. Each culture suspension (200 µL) of bacteria (*Salmonella*, *Escherichia coli* and *Staphylococcus aureus*) was inoculated onto the surface of Luria-Bertani agar medium. The polysaccharide solution was diluted down to 30 µg/mL, 60 µg/mL, 120 µg/mL, 240 µg/mL, 480 µg/mL, 1000 µg/mL, 1200 µg/mL, and the positive control sample of amoxicillin (200 µg/mL) was prepared [42]. Then 50 µL of diluted polysaccharide solution and positive control sample were loaded into holes (9 mm in diameter), which have been

punched in the agar layer. The agar plates were kept at 4°C for 1 h and then incubated at 37°C for 24 h [43]. The antimicrobial activity was evaluated by determining the zone of growth inhibition (diameter expressed in centimeter) around the holes and the minimum inhibitory concentration was obtained [44]. "-" represents no antibacterial activity [45]. Each test was repeated three times.

Effects of MRYP on lambs *in vivo*. The experiments were conducted in the Asar farming cooperatives (Changji, China). The experiments period was 28 days from 24th December 2019 to 20th January 2020.

One hundred lambs were used in a 28-day randomized, complete block design experiment. The experimental lambs were split into five groups, and there were twenty lambs in each group. Following a week of adaption, the lambs were fed artificially. The feed period was 28 days. At the beginning of the experiment, the lambs were 32 days old. At the end of the experiment, the lambs were 60 days old. The groups were designed as follows: control group (milk replacement), antibiotic group (milk replacement + 0.005% gentamicin), MRYP 0.1% group (milk replacement + 0.1% MRYP), MRYP 0.3% group (milk replacement + 0.3% MRYP), and MRYP 0.5% group (milk replacement + 0.5% MRYP). The only additive in lamb feed is the single variable in this experiment.

- a. *Body weight test.* The total feed intake of every group was counted from the 17th to 60th day. At the end of the feeding experiment, each experimental lamb was weighed, and the feed conversion ratio (F/G) was calculated by using the following equation [46]:

$$F/G(\%) = [Averagedailyfeedintake(Kg)/Averagedailygain(Kg)] \times 100\%$$

- b. *Antioxidant enzyme activity test.* At the end of the feeding experiment, three blood samples (10 mL each) were collected randomly from marginal ear vessels of 10 lambs in each group. For determining the antioxidant activity of GSH-Px, T-SOD, MAD, and T-AOC in the serum, we used an assay kit [47].
- c. *Bacteria quantity test.* The feces were collected randomly in the morning of the 60th day. The number of bacteria in feces was counted by the plate counting method. The culture media were as follows: MRS for Bifidobacterium and Lactobacillus, VRBGA for Escherichia coli, LB for Salmonella and Staphylococcus aureus [48]. The Bifidobacterium was incubated in a hostile anaerobic environment of 37°C for 72 h [49]. Other bacteria were incubated in an aerobic environment with the same culture conditions. An automated bacterial colony counter was used to count the number of bacterial colonies.

Statistical analysis

In this study, all values were expressed as the mean ± standard deviations, and the differences between means were considered significant at $p < 0.05$ by SPSS 18.0 software version 18 (SPSS Inc., New York, NY, USA). The differences among groups were analyzed by Duncan's multiple range test [50]. Design-Expert Software (Version 8.0.6, State Ease Inc., Minneapolis, NE, USA) was used to analyze the variance (ANOVA) and the parameters of the response equation. The goodness of fit of the polynomial model was evaluated by the coefficient of determination R_2 and Fisher's F-test [51]. Surface and contour plots illustrated the fitted polynomial equation, which showed the optimal fermentation conditions and the relationship between independent levels.

Table 2. Effect of five fermentation media on the MRYP yield (mg/L).

Culture medium	YPDA medium	Czapek medium	Self-1 matching medium	Self-2 matching medium	Self-3 matching medium	Self-4 matching medium
Yield of polysaccharide	2511.42±30.21a	2212.93±22.86a	2880.04±20.01b	2915.68±22.97b	2927.07±19.75b	3301.21±12.93c

Values are means ± standard deviations. In the same row, values with different small letter superscripts mean significant difference ($P < 0.05$), different capital letter superscripts mean significant difference ($P < 0.01$). The same as below.

<https://doi.org/10.1371/journal.pone.0246148.t002>

Results

Here, we investigated the effects of the fermentation medium, pH, blades speed, and temperature on the MRYP yield. Then we determined the optimal fermentation conditions for MRYP in Box–Behnken design. Besides, the molecular weight and monosaccharides composition were analyzed by HPGPC and HPLC, respectively. At last, the antioxidant and antibacterial activity were evaluated in *vitro* and in *vivo*.

Effect of medium on the yield of MRYP

[Table 2](#) presents the yields of MRYP in each medium. Based on the preliminary single-factor experiments, the yield in Medium 4 was 3301.21 mg/L, which was significantly higher than others ($p < 0.05$).

This result indicated that the ingredients of Medium 4 might have a promoting effect on the production of MRYP. Therefore, Medium 4 was chosen for subsequent experimental optimization.

Effect of pH on the yield of MRYP

pH of the fermentation medium is a crucial factor in the production of yeast polysaccharides. This is because pH affects yeast metabolite production and physiology. Besides, potentials of both inner and outer membranes are affected by the pH of fermentation medium.

[Table 3](#) shows the yields of MRYP at different pH values. In the primary screening test, the yield of MRYP increased with a rise in pH until it reached its peak value (3746.32±74.10 mg/L) at pH = 6.0, which was significantly greater than others ($p < 0.05$). Then the yield of MRYP declined until the end, that is, until the pH value reached 8.0. As seen in [Table 3](#), the yield of MRYP was significantly higher than others when the pH range was from 5 to 7 ($p < 0.05$). Therefore, the pH range of 5–7 was chosen for the second screening test.

In the second screening test, the yield of MRYP was significantly higher than others ($p < 0.05$) when the pH range was between 5.8 and 6.6, and the peak value was 4102.18±76.66 mg/L when the pH was 6.2. Therefore, pH = 5.8, 6.2, and 6.6 were chosen for the fermentation medium.

Table 3. Effect of different pH on the yield of MRYP (mg/L).

Preliminary screening	3.0	4.0	5.0	6.0	7.0	8.0
Yield of polysaccharide	1142.51±71.76a	2723.73±61.18c	3385.38±67.41e	3746.32±74.10f	3138.24±63.94d	2488.03±75.66b
Secondary screening	5	5.4	5.8	6.2	6.6	7
Yield of polysaccharide	3431.71±66.08c	3618.82±58.66d	3644.78±55.71c	4102.18±76.66e	3931.34±89.48d	3073.46±65.31a

<https://doi.org/10.1371/journal.pone.0246148.t003>

Table 4. Effect of different blades speed on the yield of MRYP (mg/L).

Blades speed (r/min)	200	300	400	500	600	700
Yield of polysaccharide	1970.08±13.72a	3061.33±49.30b	3535.75±32.56d	3775.36±63.08e	3647.10±53.83e	3291.62±56.83c

<https://doi.org/10.1371/journal.pone.0246148.t004>

Effect of blades speed on the yield of MRYP

Table 4 shows the yield of MRYP with respect to the different blades speed. As shown in Table 4, both too high and too low values of blades speed led to a lower yield of MRYP. The reasons for this phenomenon are as follows: firstly, a low blades speed causes a low oxygen content in the fermentation liquid, which cannot afford enough oxygen for breeding and growing of yeast. Secondly, a high blades speed causes a huge relative speed between blades and the fermentation liquid, which destroys yeast cells because of the additional shear forces. As shown in Table 4, 300–500 r/min was the appropriate range of blades speed because the MRYP yield was significantly higher than others ($p < 0.05$) in this range. The peak yield was 3775.36 mg/L when the blades speed was 500 r/min. Therefore, the blades speed of 400, 500, and 600 r/min were selected for the response surface experiment.

Effect of temperature on the yield of MRYP

As shown in Table 5, the yield of MRYP increased with a rise in temperature until it reached the peak value (3585.21±87.36 mg/L) at 30°C, which was significantly higher than others ($p < 0.05$). Then the yield of MRYP declined until the end, that is, until the temperature reached 34°C. Therefore, the temperature of 28, 30, and 32°C were selected for the response surface experiment.

Optimization by response surface methodology

In this study, response surface methodology (RSM) was used for precise optimization of fermentation conditions: pH (A), blades speed (B), fermentation temperature (C). According to the results of single-factor experiments (section 2.1 to 2.4), three independent variables were set for three selected variables (−1, 0, +1). Table 6 shows the combinations of different variables and their MRYP yields. Table 7 shows that the experimental outcomes corresponded to every independent variable, and we calculated the coefficients of the regression equation. Finally, by using multivariate linear regression analysis, we developed a mathematical model to determine the optimum fermentation conditions.

Mathematical model building and significance testing. Based on the preliminary single-factor experiments, the following conditions were chosen for the RSM experiments: fermentation pH (A) 5.8, 6.2, 6.6; blades speed (B) 400, 500, 600 r/min, and fermentation temperature (C) 28, 30, and 32°C. As shown in Table 6, when the fermentation pH was 6.2, temperature = 30°C, and blades speed = 500 r/min, the yield of MRYP reached its peak value of 4440.81±141.02 mg/L, which was significantly higher than others ($p < 0.05$).

Multiple regression analysis was used to develop a mathematical model that determined the optimum fermentation conditions and to study the relationship between the response variable

Table 5. Effect of different temperature on the yield of MRYP (mg/L).

Temperature(°C)	24	26	28	30	32	34
Yield of polysaccharide	2162.83±86.68a	3052.78±59.65c	3118.66±66.87c	3585.21±87.36e	3423.06±15.00d	2736.28±104.34b

<https://doi.org/10.1371/journal.pone.0246148.t005>

Table 6. Response surface test of fermentation conditions.

No.	A pH	B Temperature (°C)	C Blades speed (r/min)	Yield of polysaccharide (mg/L)
1	5.8	32	500	3031.54±102.03abcd
2	5.8	28	500	3281.38±146.31cd
3	6.6	32	500	2909.65±185.02abc
4	6.6	28	500	3162.01±153.66bcd
5	5.8	30	600	4159.37±110.74e
6	6.2	30	500	4440.81±141.02e
7	6.2	32	600	2713.69±151.29ab
8	6.2	30	500	4175.04±130.20e
9	6.2	28	600	4271.13±152.86e
10	6.2	30	500	3451.09±157.22d
11	6.6	30	400	2713.88±123.38ab
12	5.8	30	400	3057.63±154.04abcd
13	6.2	30	500	2840.01±136.11abc
14	6.2	32	400	3040.75±140.81abcd
15	6.6	30	600	4213.62±150.51e
16	6.2	28	400	2691.53±133.36a
17	6.2	30	500	3167.20±128.79bcd

<https://doi.org/10.1371/journal.pone.0246148.t006>

and the test variable. The second-order polynomial equation is as follows:

$$(Y) = 4241.40 + 125.81 \times A - 42.82 \times B + 106.60 \times C - 116.28 \times AB + 237.26 \times AC + 138.71 \times BC - 634.68 \times A^2 - 627.73 \times B^2 - 592.25 \times C^2$$

Variance analysis. As shown in Table 7, the analysis of variance, adequacy, and goodness-of-fit of the regression model was performed with Design-Expert software (Version 8.0.6, Stat-

Table 7. Variance analysis of the quadratic model by the response surface test of fermentation parameters.

Item	Quadratic sum	Degree of freedom	Mean square deviation	F-value	P-value
Model	598905.40	9	66545.30	487.02	< 0.0001
A-pH	12667.21	1	12667.21	92.70	0.0187
B-temperature	1463.23	1	1463.25	10.78	0.3352
C-blades speed	9089.48	1	9089.40	66.51	0.0365
AB	5411.85	1	5411.82	39.62	0.0869
AC	22527.73	1	22527.08	164.90	0.0048
BC	7696.98	1	7696.93	56.37	0.0494
A ²	169639.90	1	169639.93	1241.63	< 0.0001
B ²	165919.0	1	165919.10	1214.42	< 0.0001
C ²	147708.46	1	147708.41	1081.10	< 0.0001
Residual	9564.11	7	1366.34		
Lack of fit	3812.20	3	1270.71	0.88	0.5213
Net error	5751.93	4	1438.32		
Total error	608469.66	16			
R ²	0.9661		0.62		
R ² _{adj}	0.9548		0.71		

Note: p<0.05, significant difference; p<0.01, very significant difference.

<https://doi.org/10.1371/journal.pone.0246148.t007>

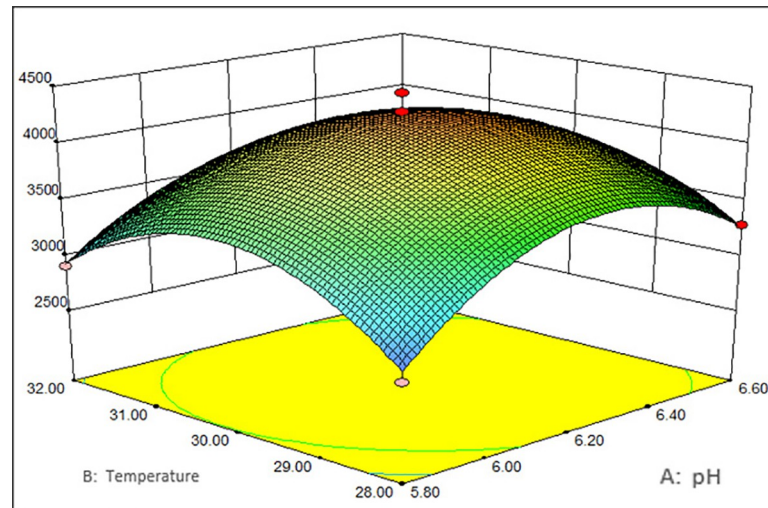


Fig 1. Interaction of temperature and pH vs. the yield of MRYP.

<https://doi.org/10.1371/journal.pone.0246148.g001>

Ease, Inc., Minneapolis, NE, USA). The model was highly statistically significant because of a low P-value ($P < 0.0001$) and a high F-value (48.70). The high R_2 value of 0.9548 and the high R_{2adj} value of 0.9661 indicate that the model was applicable and accurate. It also reveals that the interactions of pH and temperature, pH and blades speed, temperature and blades speed were insignificant ($p > 0.05$).

Response surface analysis. As shown in Figs 1–3, the corresponding three-dimensional response surfaces of MRYP yield and the different independent parameters were plotted, which elucidated the interaction of variables with the uttermost response. The middle values of the three parameters (pH value of 6.2 (A), blades speed of 500 r/min (B), temperature of 30°C (C)) were used to create the graphs. The sequence of factors that affected the MRYP yield was the blades speed > pH > temperature. While one factor was maintained at a fixed value, the effects of the other two factors on the polysaccharide yield were shown.

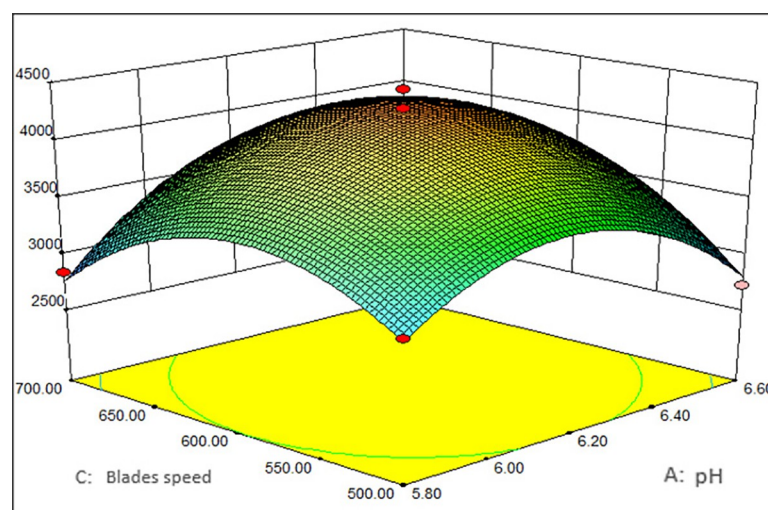


Fig 2. Interaction of blades speed and pH vs. the yield of MRYP.

<https://doi.org/10.1371/journal.pone.0246148.g002>

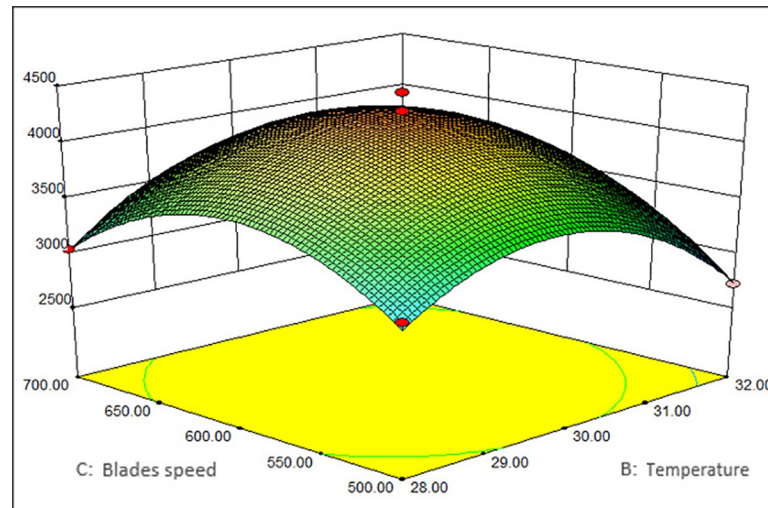


Fig 3. Interaction of temperature and blades speed vs. the yield of MRYP.

<https://doi.org/10.1371/journal.pone.0246148.g003>

Fig 1 illustrates the polysaccharide yield in response to pH and temperature with a fixed blades speed of 500 r/min. The MRYP yield increased with an increase in pH and temperature until it reached its peak. After attaining the peak value, a further increase in pH and temperature led to a decreased polysaccharide yield. The effect of pH and blades speed on the polysaccharide yield is illustrated in Fig 2. When the temperature was 30°C, an increase in both pH and blades speed led to an increase in the polysaccharide yield at the beginning. After reaching the peak yield, the response surface decreased, and similar trends were observed in the effect of blades speed and temperature (Fig 3). Besides, the response surface analysis showed that pH, blades speed, and temperature showed significant interactive effects. According to the model, the maximum yield of MRYP was 4876.80 mg/L when the values of corresponding variables were pH = 6.72, temperature = 30.18°C, and blades speed = 461.36 r/min.

Validation test. The optimal fermentation conditions had been obtained in the prediction model, but we had to validate precisely how well the parameters work in practice. In view of the operating convenience, the optimal fermentation parameters were set to pH = 6.8, temperature 30°C, and blades speed 460 r/min.

The predicted MRYP yield was verified by validation tests after three replications. As shown in Fig 4, the average MRYP yield was 4323.90 mg/L, with a relative error of 11.34%. Thus, the average MRYP yield was close to the predicted yield. This result indicated that the model can be used to guide actual practice and production. The optimal yield of MRYP was 1.31 times higher than the yield before optimization, which was 3300.00 mg/L.

Determination of the molecular weight

As shown in Fig 5, the pure polysaccharide was fractionated by using a DEAE-52 column and Sephadex G-100 column. The two main components of MRYP fractions were eluted, and each component produced a single and symmetrical sharp peak. The two main components were named MYH-1, MYH-2. Then the components were vacuum lyophilized and weighed precisely. The proportions of MYH-1 and MYH-2 in pure MRYP were 53.60% and 28.75%, respectively.

The molecular weight of MYH-1 and MYH-2 was determined by high-efficiency gel permeation chromatography (HPGPC). As shown in Fig 6, two single and symmetrical peaks were

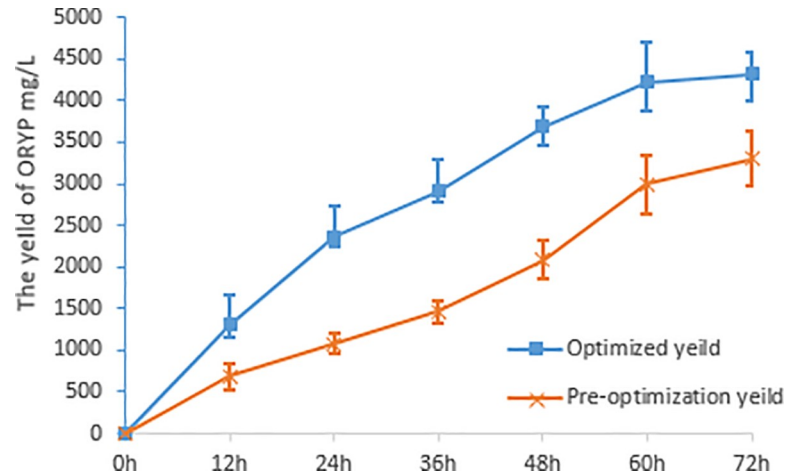


Fig 4. The MRYP yields of optimized conditions and pre-optimization.

<https://doi.org/10.1371/journal.pone.0246148.g004>

observed in the HPGPC chromatogram. This result indicated that the components MYH-1 and MYH-2 were pure enough for the next analysis. The retention time of MYH-1 and MYH-2 was 14.432 min and 20.587 min, respectively. Fig 7 shows the standard curve of molecular weight. The retention time of MYH-1 and MYH-2 was included in the regression equation, and the molecular weight of MYH-1 and MYH-2 was found to be 246.92 kDa and 21.88 kDa.

Analysis of the monosaccharides composition

The retention time and the standard curve of MYH-1 and MYH-2 were obtained by HPLC. Fig 8 shows the standard curve of glucose and mannose. As shown in Figs 9 and 10, the prominent peak retention time of glucose and mannose standard samples was 26.384 min and 30.217 min, respectively. Figs 11 and 12 show the chromatographic peak areas of glucose and mannose in MYH-1 and MYH-2 components, respectively. According to the regression equation, the concentration of glucose and mannose was determined. The glucose and mannose content in MYH-1 and MYH-2 was 938.72 $\mu\text{g/mL}$, 769.15 $\mu\text{g/mL}$ and 1341.97 $\mu\text{g/mL}$,

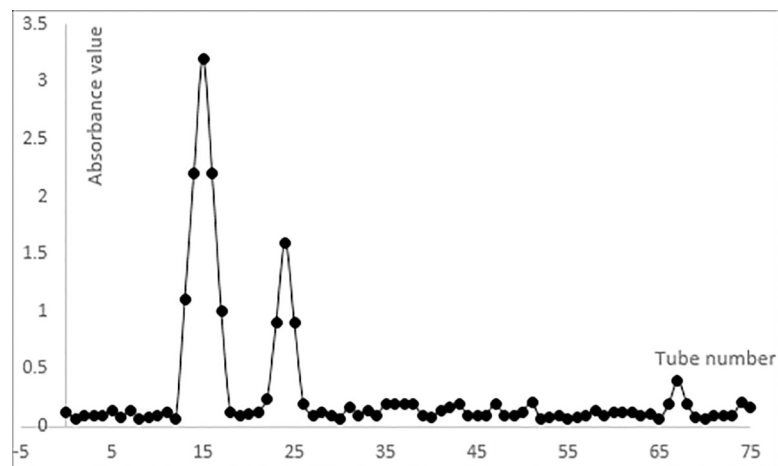


Fig 5. Linear elution of pure polysaccharides.

<https://doi.org/10.1371/journal.pone.0246148.g005>

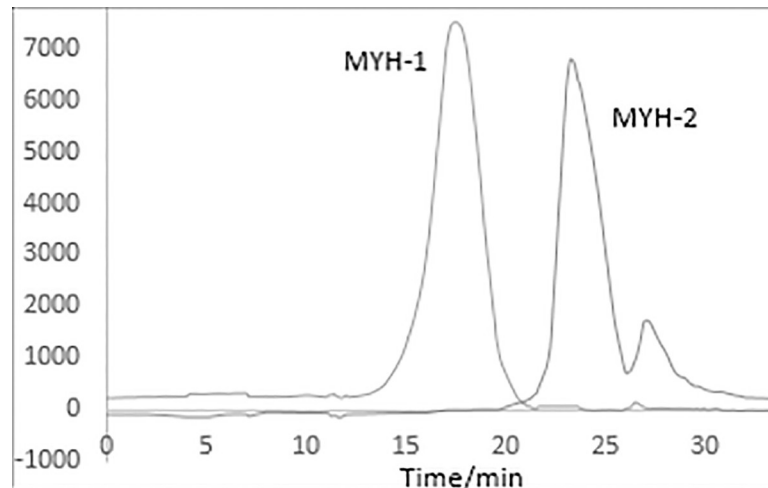


Fig 6. HPGPC chromatograms of MYH-1 and MYH-2.

<https://doi.org/10.1371/journal.pone.0246148.g006>

143.46 $\mu\text{g}/\text{mL}$, respectively. The proportion of glucose and mannose in MYH-1 and MYH-2 was 46.94%, 38.46%, and 67.10%, 7.17%, respectively.

***In vitro* tests**

Antioxidant activity. Fig 13 shows the antioxidant ability of MRYP towards DPPH, OH, and $\cdot\text{O}_2^-$. It shows that there was an obvious dose-response relationship with the antioxidant ability of MRYP. The antioxidant ability increased gently until the concentration of MRYP approached 300 $\mu\text{g}/\text{mL}$. Then the antioxidant ability sharply increased and reached a peak while the concentration of MRYP was 1000 $\mu\text{g}/\text{mL}$. Besides, the antioxidant ability of MRYP towards DPPH \cdot displayed higher values than others, and the clearance rate of MRYP to $\cdot\text{DPPH}$, OH, and $\cdot\text{O}_2^-$ was 32.26%, 24.34%, and 22.09%, respectively.

Antibacterial activity. The antibacterial activity of MRYP involved resistance to three kinds of pathogenic bacteria, namely, *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus*, as shown in Table 8.

The growth of the three pathogenic bacteria was inhibited when the concentration of MRYP mixtures was 480 $\mu\text{g}/\text{mg}$. The antibacterial activity of MRYP on *Salmonella* was more

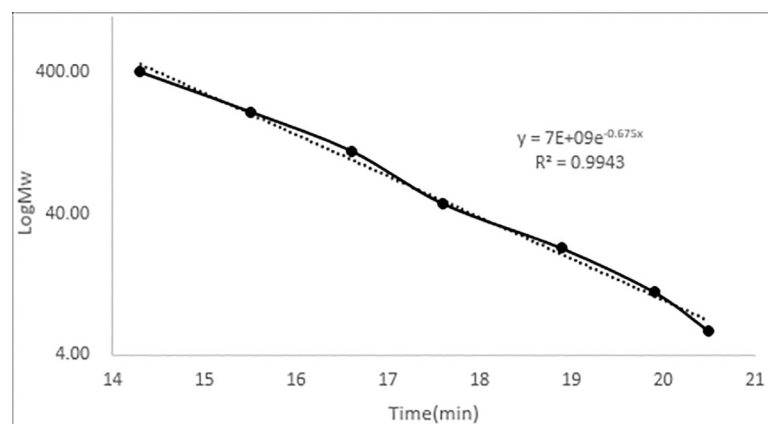


Fig 7. Standard curve of polysaccharide of the standard molecular weight.

<https://doi.org/10.1371/journal.pone.0246148.g007>

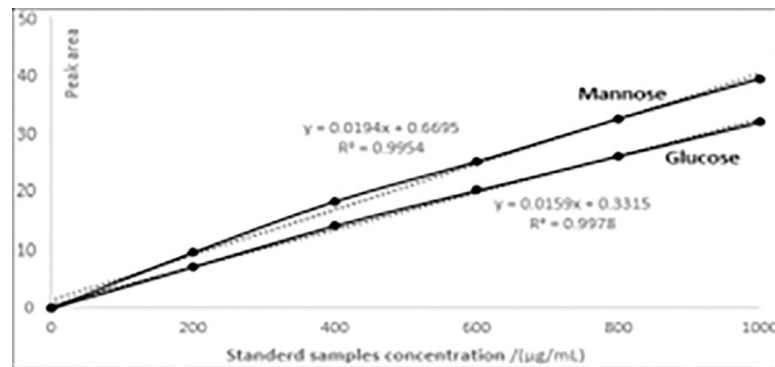


Fig 8. Standard curve of glucose and mannose.

<https://doi.org/10.1371/journal.pone.0246148.g008>

effective than that on *Escherichia coli* and *Staphylococcus aureus*, with the inhibition zone diameters being 14.54 ± 0.85 mm, 10.40 ± 2.25 mm, and 9.53 ± 0.11 mm. It did not show a bacteriostatic effect on any pathogenic bacteria when the mixture concentration was below $480 \mu\text{g}/\text{mg}$. In this test, amoxicillin ($200 \mu\text{g}/\text{mL}$) showed a high antibacterial activity on the three pathogenic bacteria, and the inhibition zones diameters of *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* were 24.74 ± 2.36 mm, 24.67 ± 2.43 mm, and 24.43 ± 2.44 mm, respectively. This result indicated that MRYP exhibits antibacterial activity on common pathogens, but the bacteriostatic effect of MRYP is weaker than that of antibiotics.

***In vivo* tests**

The objective of these experiments was to investigate the effects of MRYP on the growth, serum antioxidant enzyme activity, and feces bacteria in growing lambs.

Effect of MRYP on lamb growth. Every experimental lamb was taken good care of by technicians, and no mortality was observed in the whole period. As shown in Table 9, 32-days-old lambs were weighed individually, and there was no significant difference in the weight of 32-days old lambs included in this study. At the end of the experiment, 60-days-old lambs were weighed individually again, and the weight of lambs in the antibiotic group (14.21 ± 0.33 kg) was significantly greater than those of other groups. However, the weight of MRYP 0.3% group (13.43 ± 0.20 kg) and MRYP 0.5% group (13.37 ± 0.13 kg) was significantly greater than that of the control group (11.44 ± 1.10 kg). This indicates that lamb's weight increased when MRYP was used as a feed additive. Besides, the standard deviation in the weight of MRYP 0.3% group and MRYP 0.5% group was lower than those of other groups, which implied that a

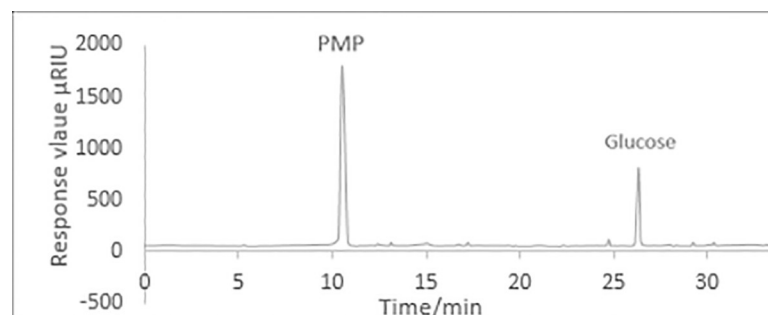


Fig 9. HPLC chromatogram of glucose.

<https://doi.org/10.1371/journal.pone.0246148.g009>

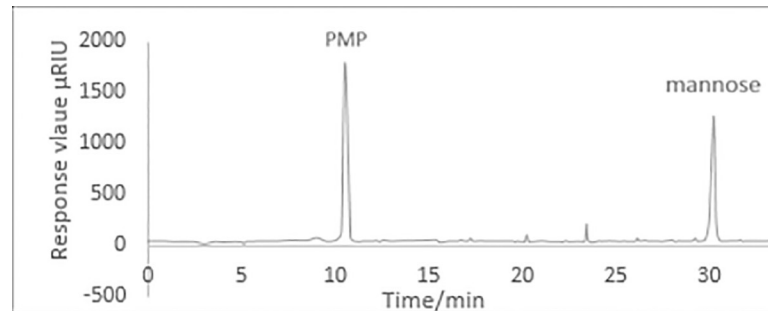


Fig 10. HPLC chromatogram of mannose.

<https://doi.org/10.1371/journal.pone.0246148.g010>

certain amount of MRYP improved uniformity in the weight of lambs. In addition, the F/G of MRYP 0.3% was lower than the others, which indicates that MRYP might improve the production efficiency of lamb breeding.

Effect of MRYP on the antioxidant activity of lamb serum. Table 10 shows the antioxidant activity of 60-days-old lamb serum. Four antioxidant enzymes did not show a significant difference in all the groups. But the antioxidant activity of GSH-Px, T-SOD, and T-AOC in MRYP 0.3% group had higher values than in other groups, which indicates that MRYP increased the antioxidant enzyme activity to a certain extent. Besides, the content of MAD in MRYP 0.3% was lower than in the control group, which indicates that MRYP can prevent damage to cell membranes. The result agreed with the conclusion of antioxidant activity *in vitro* test.

Effect of MRYP on bacteria quantity in lamb feces. Table 11 shows the number of bacteria in lamb faeces. It was obvious that the number of *Bifidobacterium* and *Lactobacillus* in MRYP 0.3% group was significantly higher than in other groups. This indicates that MRYP increased the number of probiotics in lamb's intestines. The number of *Escherichia coli* and *Salmonella* was significantly lower in MRYP 0.3% group than in the control group, which indicates that MRYP inhibited the growth of pathogens in lamb's intestines. This result was approximately the same as in the *in vitro* experiment of antibacterial activity.

Discussion

In this paper, we proved that Medium 4 was the optimal medium for fermentation, but the formulation was different from common media for yeast, such as PDA, YPD, and malt extract medium. This indicates that the nutritional requirement was different in different kinds of

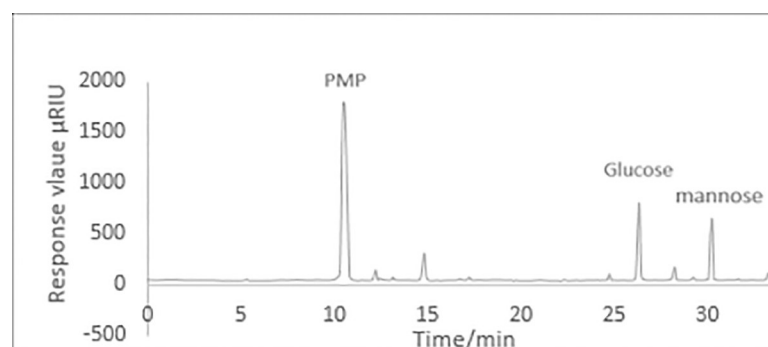


Fig 11. HPLC chromatogram of the MYH-1 hydrolysate.

<https://doi.org/10.1371/journal.pone.0246148.g011>

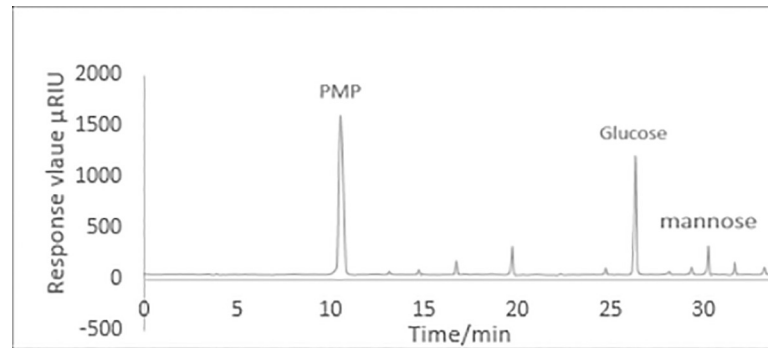


Fig 12. HPLC chromatogram of the MYH-2 hydrolysate.

<https://doi.org/10.1371/journal.pone.0246148.g012>

yeast strains and fermentation products. Furthermore, the optimal fermentation parameters for MRYP were pH = 6.72, temperature = 30.18°C, blades speed = 461.36 r/min, and the optimized yield reached 432.39 mg/100 mL, which was 1.31 times the original yield. There are some reports concerning the optimal fermentation parameters for yeast polysaccharides. It has been reported that a 0.20 g L⁻¹ h⁻¹ polysaccharide that was composed of 18.57% (w/w) kefiran was obtained by using optimal fermentation parameters (whey supplemented with 15% (w/v) glucose and fermented at 30°C for 10 h) [52]. The fermentation conditions of red yeast *Rhodotorula* were optimized as follows: 0.5% yeast powder, 2% molasses, 50 mL of a medium in a 250 mL flask, and temperature 20°C, initial pH = 3.0, cultivation for 96 h [53]. The optimal fermentation conditions of *Monascus* sp. were determined as follows: yeast extract powder 45 g/L, sucrose 45 g/L, MgSO₄·7H₂O 0.85g/L, KH₂PO₄·3H₂O 35 g/L, and inoculation volume 75%, seed culture age 30 h, initial pH 5.75, and fermentation for 84 h. Under the optimal fermentation conditions of red yeast *Rhodotorula*, the highest yield of polysaccharide was 999.8 mg/L and improved by 46.1% compared with the original yield of 684.2 mg/L [54]. In this experiment, the sequence of factors (blades speed > pH > temperature) was different from that in the pilot experiment (temperature > blades speed > pH). This is because the optimal pH scope was minimized in the primary screening test, which led to a pH-insensitive yield of MRYP. Furthermore, the oxygen content in the fermentation solution was different in a fermenter with rotating blades and a shaking flask in the pilot experiment. Because the optimized yield was

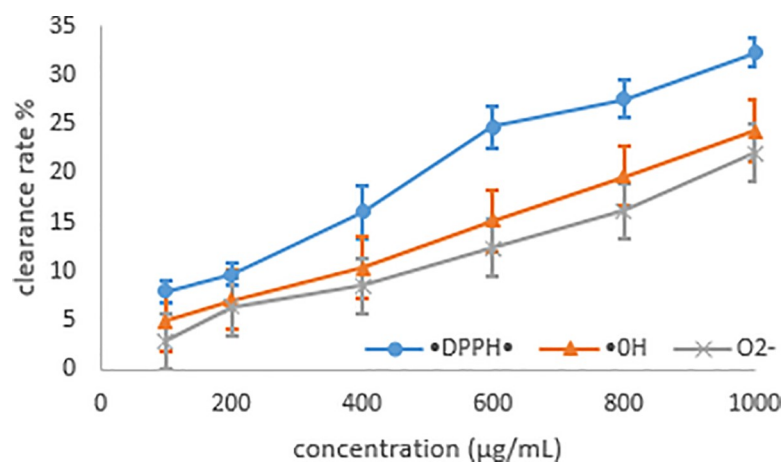


Fig 13. Scavenging capacities of MRYP for DPPH, OH, and O_2^- .

<https://doi.org/10.1371/journal.pone.0246148.g013>

Table 8. The average diameter of the inhibition zone of 3 pathogenic bacteria (mm).

Test strain	MRYP ($\mu\text{g}/\text{mg}$)					Amoxicillin	Sterile water
	30	60	120	240	480		
<i>Escherichia coli</i>	-	-	-	-	10.40 \pm 2.25	24.74 \pm 2.36	-
<i>Salmonella</i>	-	-	-	-	14.54 \pm 0.85	24.67 \pm 2.43	-
<i>Staphylococcus aureus</i>	-	-	-	-	9.53 \pm 0.11	24.43 \pm 2.44	-

<https://doi.org/10.1371/journal.pone.0246148.t008>

below expectations, the screening of high-MRYP-yield yeast strain and genetically engineered yeast was included in our next project. The optimal temperature in this experiment was higher than that reported in previous research studies [55]. This is because the experimental marine red yeast *Rhodospiridium paludigenum* strain was collected from Yarkand River in Kashi region, which is a parched area that supports the thermophilic feature of *Rhodospiridium paludigenum* strain.

As is well known, the biological properties of polysaccharides depend on the molecular weight, chemical composition, and configuration. The molecular weight as the most fundamental indicator should be investigated to indicate the bioactivity of polysaccharides. The higher molecular weight of acidic polysaccharides, the higher activity in the biological complement system the polysaccharides have. It has been reported previously that only polysaccharides with a molecular weight of 50.0 kDa to 200.0 kDa have biological activities, normally [9]. Besides, there is a report suggesting that the polysaccharides with the molecular weight in the range from 4.0 kDa to 100.0 kDa expressed a better antioxidant activity. In addition, there are some differences in the structural characterization and chemical composition of polysaccharides, which may be related to the yeast species and fermentation conditions. Some studies have proved that glucose and mannose are the major bioactive components in polysaccharides, which widely exist in yeast, bacteria, algae, and plants and have been well known for their bioactivities including wound healing, antibacterial, enhancing immunity, and antioxidant activities [56]. In this study, the molecular weights of MYH-1 and MYH-2 were 246.92 kDa and 21.88 kDa, respectively, and they accounted for 53.60% and 28.75% of total polysaccharide. Besides, the proportion of glucose and mannose accounted for 46.94%, 38.46%, and 67.10%, 7.17% in MYH-1 and MYH-2, respectively. Therefore, the MRYP probably expresses some biological activities. It has been reported that the GIP-II was the main fraction in polysaccharides from *Gleostereum incarnatum* and was composed of mannose, glucose, galactose, and xylose [57]. In another study, the major purified polysaccharides in a mulberry leaf polysaccharide were MLP-1 and MLP-2, which were composed of glucose, mannose, galactose, rhamnose, and arabinose. The molecular weights of MLP-1 and MLP-2 were 222.0 kDa and 93.1 kDa, respectively [58]. The polysaccharides from chestnut (*Castanea mollissima* Bl.) kernel were extracted by using successive ultrasound-assisted-extraction, and the major fractions UEP1-1 and UEP2-1 were obtained. The molecular weights of UEP1-1 and UEP2-1 were 74.5

Table 9. Effect of MRYP on body weight.

Items	MRYP level (%)				
	Control group	Antibiotic group	MRYP 0.1%	MRYP 0.3%	MRYP 0.5%
32-days old weight	7.67 \pm 1.38	8.89 \pm 1.22	8.31 \pm 1.42	8.36 \pm 1.26	8.23 \pm 1.11
60-days old weight	11.44 \pm 1.10a	14.01 \pm 0.73c	12.86 \pm 0.51a	13.43 \pm 0.20b	13.37 \pm 0.13b
Average daily gain(g)	134.64 \pm 16.19a	182.86 \pm 15.88c	162.5 \pm 13.42b	181.07 \pm 11.41c	183.57 \pm 12.52c
Average daily feed intake (g)	373.50 \pm 23.51a	405.27 \pm 19.54b	396.21 \pm 16.46b	398.79 \pm 11.15b	429.53 \pm 12.40b
F/G	2.77	2.21	2.44	2.20	2.34

<https://doi.org/10.1371/journal.pone.0246148.t009>

Table 10. Effect of MRYP on the serum antioxidant enzymes activity of lambs.

Items	MRYP levels (%)		
	Control group	Antibiotic group	MRYP 0.3%
GSH-Px ($\mu\text{mol/L}$)	15.45 \pm 1.16	14.83 \pm 0.31	15.79 \pm 0.95
T-SOD(U/mL)	1.34 \pm 0.43	1.38 \pm 0.45	1.42 \pm 0.56
MAD (nmol/mL)	2.57 \pm 0.11	2.46 \pm 0.43	2.33 \pm 0.65
T-AOC(U/mL)	2.47 \pm 0.31	2.50 \pm 0.63	2.61 \pm 0.42

<https://doi.org/10.1371/journal.pone.0246148.t010>

kDa and 59.9 kDa, respectively, and the monosaccharide composition of UEP1-1 and UEP2-1 was glucose, mannose, rhamnose, galactose, and arabinose.

The studies have proven that the antioxidant and antibacterial activities mainly depend on physicochemical features including the molecular weight and monosaccharide composition [59]. The $\cdot\text{DPPH}$, $\cdot\text{OH}$, and $\cdot\text{O}_2^-$ scavenging percentages were used to indicate the antioxidant ability of polysaccharides *in vitro*. The free radical activities were increased by reduction of antioxidant enzymatic activities, therefore, antioxidant enzymatic activities could indicate the radical activities and oxidative stress *in vivo*. *In vitro*, the antioxidant activity of MRYP was weak and far below that of vitamin C. The same result was found *in vivo*, MRYP did not result in a significant difference in the antioxidant activity of GSH-Px, T-SOD, and T-AOC in the lamb serum. This implies that the antioxidant activity is not the main biological function of intestinal tract, and MRYP should not be used as an antioxidant in lambs.

However, *in vitro*, MRYP strongly inhibited common pathogens, especially *Salmonella*. This result was approximately the same as the result of effect of MRYP on bacteria quantity in lamb feces according to which the supplementation of 0.3% MRYP significantly increased the number of *Bifidobacterium* and *Lactobacillus* but decreased the number of *Escherichia coli* and *Salmonella*. This is a meaningful result to encourage the microflora balance of intestines and to promote the healthy function of the intestinal system in non-reactive breeding grounds. It has been reported that association of mannose and NAC therapy resulted similarly in antibiotic therapy in preventing UTIs in patients submitted to urodynamic examination [60]. D-mannose, N-acetylcysteine, and *Morinda citrifolia* fruit extract showed a greater efficacy in reducing urinary discomfort and urinary tract infections with respect to antibiotic use only in breast cancer survivor women affected by genitourinary discomfort [61]. It has been also reported that β -glucan has lots of biological activities, such as antimicrobial, antioxidative, cardioprotective, immunomodulatory, and hepatoprotective activities [62]. The reports showed that β -glucan extracted from yeast cell walls had obvious hydroxyl radical, superoxide anion scavenging activities and anti-lipid peroxidation effect [63].

It has been proved that oxidative stress is harmful for body, and over-produced reactive oxygen species (ROS) cannot be scavenged by the antioxidant system in our body, which can result in enteritis, diarrhea, and cardiovascular diseases. On the other hand, antibiotic resistance is becoming a global problem, and about 70% of known pathogenic bacteria are resistant

Table 11. Effect of MRYP on five kinds of bacteria in lamb feces (log CFU/g).

Items	MRYP levels (%)		
	Control group	Antibiotic group	MRYP 0.3%
<i>Bifidobacterium</i>	6.26 \pm 0.15b	5.55 \pm 0.27a	6.84 \pm 0.13c
<i>Lactobacillus</i>	7.34 \pm 0.28a	6.54 \pm 0.20a	7.78 \pm 0.29b
<i>Escherichia coli</i>	6.73 \pm 0.08b	5.62 \pm 0.25a	6.03 \pm 0.14a
<i>Salmonella</i>	4.77 \pm 0.35c	3.34 \pm 0.41a	3.93 \pm 0.56b
<i>Staphylococcus aureus</i>	4.62 \pm 0.80	4.09 \pm 0.97	4.51 \pm 0.91

<https://doi.org/10.1371/journal.pone.0246148.t011>

to at least one antibiotic [64]. As is well known, *Bifidobacterium* and *Lactobacillus* are probiotics in intestines, but *Escherichia coli* and *Salmonella* can cause diarrhea and enteritis. *In vivo*, the lambs of the MRYP-trial group were fed a certain MRYP amount instead of antibiotics. All the lambs were physically fit, and none of them died in the whole experiment, which proves that MRYP is safe enough to be used in farming production. Besides, a lower F/G in lambs of MRYP-trial group than in the control group indicates that the production efficiency in sheep breeding could be improved partially. However, further research studies must be conducted to verify the conclusions of this experiment. In addition, there was not a significant difference in antioxidant activities of lamb serum.

The process of extraction and purification played an important role in the yield and biological function of MRYP. Based on the data of this experimental study, there is a close relationship between extraction conditions and the yield and biological functions of MRYP. These factors must be explored for providing a theoretical foundation for possible application in industrial production.

Conclusion

In this study, the optimal fermentation cultivation medium and parameters of the production of MRYP were as follows: Medium 4, pH = 6.72, temperature 30.18°C, and blades speed 461.36 r/min. The optimized yield of MRYP was 4323.90 mg/L. The main components of MRYP were MYH-1 and MYH-2. The molecular weight of MYH-1 and MYH-2 was 246.92 kDa and 21.88 kDa. The proportion of glucose and mannose in MYH-1 and MYH-2 was 46.94%, 38.46%, and 67.10%, 7.17%, respectively.

In vivo, the MRYP increased the growth of lambs. The antioxidant activity of MRYP was weak, but the antibacterial activity of MRYP strongly inhibited the three common pathogens both *in vitro* and *vivo*.

This work provides a theoretical foundation to a wide range of possible application of MRYP in the industrial production, design, and operation.

Subsequent work should investigate the effect of MRYP on the intestinal flora in lambs by genomics. The results will provide a theoretical basis for the production and application of MRYP to enhance productivity in animal industries.

Author Contributions

Data curation: Mengjian Liu, Junli Niu.

Formal analysis: Mengjian Liu, Jun Yao.

Funding acquisition: Mengjian Liu.

Investigation: Mengjian Liu.

Methodology: Mengjian Liu, WenJu Zhang.

Project administration: WenJu Zhang.

Supervision: WenJu Zhang.

Writing – original draft: Mengjian Liu.

References

1. Zaky A.S., et al., The establishment of a marine focused biorefinery for bioethanol production using seawater and a novel marine yeast strain. *Scientific Reports*, 2018. 8(1): p. 12127. <https://doi.org/10.1038/s41598-018-30660-x> PMID: 30108287

2. Greetham D., Zaky A.S., and Du C., Exploring the tolerance of marine yeast to inhibitory compounds for improving bioethanol production. *Sustainable Energy & Fuels*, 2019. <https://doi.org/10.1039/C9SE00029A>
3. Zaky A., et al., A New Isolation and Evaluation Method for Marine Derived Yeast spp with Potential Applications in Industrial Biotechnology. *Journal of microbiology and biotechnology*, 2016. 26(11): p. 1891–1907. <https://doi.org/10.4014/jmb.1605.05074> PMID: 27435537
4. TIAGO FC, MATINS FS, SOUZA EL. Adhesion to the yeast cell surface as a mechanism for trapping pathogenic bacteria by *Saccharomyces* probiotics. *J Med Microbiol*. 2012; 61(9): 194–207. <https://doi.org/10.1099/jmm.0.042283-0> PMID: 22580913
5. BUTS JP, DEKEYSER N, STILMANT C. *Saccharomyces boulardii* produces in rat small intestine a novel protein phosphatase that inhibits *Escherichia coli* endotoxin by dephosphorylation. *Pediatric Research*. 2006; 60(1): 24–29. <https://doi.org/10.1203/01.pdr.0000220322.31940.29> PMID: 16690953
6. Castex M, Lemaire P, Wabete N, Chim L. Effect of probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress of *Litopenaeus stylirostris* under *Vibrio nigripulchritudo* challenge. *Fish Shellfish Immunol*. 2009; 28: 622–631. <https://doi.org/10.1016/j.fsi.2009.12.024> PMID: 20045735
7. Bhosale SH, Patil KB, Parameswaran PS, Naik CG, Jagtap TG. Active pharmaceutical ingredient (api) from an estuarine fungus, *microdochium nivale* (fr.). *Journal of Environmental Biology*. 2011; 32 (5): 653–658. <https://doi.org/10.1007/s11852-010-0106-3> PMID: 22319884
8. Ren Z, He CH, Fan YH, Si HM, Wang YW, Shi ZY, et al. Immune-enhancing activity of polysaccharides from *Cyrtomium macrophyllum*. *Int. J. Biol. Macromol*. 2014; 70: 590–595. <https://doi.org/10.1016/j.ijbiomac.2014.07.044> PMID: 25101877
9. Cordero RJB, Frases S, Guimares AJ, Rivera J, Casadevall A. Evidence for branching in cryptococcal capsular polysaccharides and consequences on its biological activity. *Molecular microbiology*. 2011; 79(4), 1101–1117. <https://doi.org/10.1111/j.1365-2958.2010.07511.x> PMID: 21208301
10. Kogan G. (1–3, 1–6)- β -d-glucans of yeasts and fungi and their biological activity. *Studies in Natural Products Chemistry*. 2000; 23(00): 107–152. [https://doi.org/10.1016/S1572-5995\(00\)80128-3](https://doi.org/10.1016/S1572-5995(00)80128-3)
11. Sandula J, Kogan G, Kacurakova M, Machova E. Microbial (1→3)- β -d-glucans, their preparation, physico-chemical characterization and immunomodulatory activity. *Carbohydrate Polymers*. 1999; 38 (3), 247–253. <https://doi.org/10.1063/1.4754510>
12. Li C, Li X, You L, Fu X, Liu RH. Fractionation, preliminary structural characterization and bioactivities of polysaccharides from *sargassum pallidum*. *Carbohydrate Polymers*. 2017; 155(Complete): 261–270. <https://doi.org/10.1016/j.carbpol.2016.08.075> PMID: 27702511
13. Venugopalan A, Potunuru UR, Dixit M, Srivastava S. Effect of fermentation parameters, elicitors and precursors on camptothecin production from the endophyte *Fusarium solani*. *Bioresour Technol*. 2016; 206:104–111. <https://doi.org/10.1016/j.biortech.2016.01.079> PMID: 26851893
14. Zhang Q, Li YJ, Xu FX, Zheng MM, Xi XZ, Zhang XL. Optimization of Submerged Fermentation Medium for Matrine Production by *Aspergillus terreus*, an Endophytic Fungus Harboring Seeds of *Sophora flavescens*, Using Response Surface Methodology. *The Korean Society of Mycology*. 2017; 45(2): 90–96. <https://doi.org/10.5941/MYCO.2017.45.2.90> PMID: 28781541
15. Gu D, Jiao YC, Wu JN. Optimization of EPS Production and Characterization by a Halophilic Bacterium, *Kocuria rosea* ZJUQH from Chaka Salt Lake with Response Surface Methodology. *Molecules*. 2017; 22: 814. <https://doi.org/10.3390/molecules22050814> PMID: 28509876
16. Lei F, Yin JY, Nie SP, Wan YQ, Xie MY. Fractionation, physicochemical property and immunological activity of polysaccharides from *Cassia obtusifolia*. *Int. J. Biol. Macromol*. 2016; 91: 946–953. <https://doi.org/10.1016/j.ijbiomac.2016.05.030> PMID: 27177462
17. Chen XL, Sheng ZC, Qiu SL. Purification, characterization and in vitro and in vivo immune enhancement of polysaccharides from mulberry leaves. *PLoS ONE*. 2019; 14 (1): e0208611. <https://doi.org/10.1371/journal.pone.0208611> PMID: 30601811
18. Cai SQ, Hu CQ, Ren CH. An analysis of biochemical compositions of three marine yeasts. *Journal of Tropical Oceanography*, 2009; 28(2): 62–65. [https://doi.org/10.1016/S1003-6326\(09\)60084-4](https://doi.org/10.1016/S1003-6326(09)60084-4)
19. YANG YY, CHENG YQ, YANG K, et al. Study on fermentation conditions for Marine red yeast *rhodospiridium Paludigenum* strain rh1. *South China Fisheries Science*. 2012; 08: 67–74. <https://doi.org/10.3969/j.issn.2095-0780.2012.01.011>
20. Blandón, Lina M, Noseda MD, Islan GA, Castro G, Pereira M, Gilberto Vinícius, et al. Optimization of culture conditions for kefir production in whey: the structural and biocidal properties of the resulting polysaccharide. *Bioactive Carbohydrates and Dietary Fibre*, 2018; S2212619818300135. <https://doi.org/10.1016/j.bcdf.2018.02.001>

21. Jia J, Yang X, Wu Z, Zhang Q, Lin Z, Guo H, et al. Optimization of fermentation medium for extracellular lipase production from *Aspergillus niger* using response surface methodology. *Biomed Res Int* 2015; 2015:497462. <https://doi.org/10.1155/2015/497462> PMID: 26366414
22. Luciane MC, Andreiza LP, Silvia B. Surface response methodology for the optimization of lipase production under submerged fermentation by filamentous fungi. *Brazilian Journal of Microbiology*, 2016; 47(2). <https://doi.org/10.1016/j.bjm.2016.01.028> PMID: 26991270
23. Yang SP, Wu ZH, Jian JC, Zhang XZ. Effect of marine red yeast *Rhodospiridium paludigenum* on growth and antioxidant competence of *Litopenaeus vannamei*. *Aquaculture*. 2010; 309 (1–4): 0–65. <https://doi.org/10.1016/j.aquaculture.2010.09.032>
24. Meng X, Che CC, Zhang JM. Structural characterization and immunomodulating activities of polysaccharides from a newly collected wild *Morchella sextelata*. *International Journal of Biological Macromolecules*. 2019; 1(226): 608–614. <https://doi.org/10.1016/j.ijbiomac.2019.01.226> PMID: 30771397
25. Tang M, Hou F, Wu Y, Liu Y, Ouyang J. Purification, characterization and tyrosinase inhibition activity of polysaccharides from chestnut (*Castanea mollissima* Bl.) kernel. *International Journal of Biological Macromolecules*. 2019; 3(65): 309–314. <https://doi.org/10.1016/j.ijbiomac.2019.03.065> PMID: 30872058
26. Hindu SV, Thanigaivel S, Vijayakumar S, Chandrasekaran N, Mukherjee A, Thomas J. Effect of micro-encapsulated probiotic *Bacillus vireti*, 01-polysaccharide extract of *Gracilaria folifera*, with alginate-chitosan on immunity, antioxidant activity and disease resistance of *Macrobrachium rosenbergii*, against *Aeromonas hydrophila*, infection. *Fish & Shellfish Immunology*. 2018; 73, 112. <https://doi.org/10.1016/j.fsi.2017.12.007> PMID: 29208500
27. Tian SY, Hao CC, Xu Gk. Optimization conditions for extracting polysaccharide from *Angelica sinensis* and its antioxidant activities, *Journal of Food and Drug Analysis*. 2016. <https://doi.org/10.1016/j.jfda.2016.08.012> PMID: 28987352
28. Pu JB, Xia BH, Hu YJ. Multi-Optimization of Ultrasonic-Assisted Enzymatic Extraction of *Atractylodes macrocephala* Polysaccharides and Antioxidants Using Response Surface Methodology and Desirability Function Approach. *Molecules*, 2015; 20 (12): 22220–22235. <https://doi.org/10.3390/molecules201219837> PMID: 26690404
29. Li TT, Yang Y, Chen LJ. Effects of grinding methods on physicochemical properties of crude polysaccharides from *Phellinus baumii*. *Natural Product Research and Development*. 2019; 78, 02004. <https://doi.org/10.1051/e3sconf/20197802004>
30. Abdelmohsen U R, Pimentel S M, Hanora A. Isolation, Phylogenetic Analysis and Anti-infective Activity Screening of Marine Sponge-Associated Actinomycetes. *Marine Drug*. 2010; 8(2): 399–412. <https://doi.org/10.3390/md8030399> PMID: 20411105
31. Xu TT, Liu Y, Zhang ZJ. Determination of mannan and β -glucan in yeast cell wall by HPLC. *China Brewing*. 2016; 35(7): 180–183. (*in Chinese*)
32. Sun HH, Mao WJ, Chen Y, Guo SD, Li HY, Qi XH, et al. Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus *Penicillium* sp. f23-2. *Carbohydrate Polymers*. 2009; 78(1), 117–124. <https://doi.org/10.1016/j.carbpol.2009.04.017>
33. Tang M, Hou F, Wu Y, Liu Y, Ouyang J. Purification, characterization and tyrosinase inhibition activity of polysaccharides from chestnut (*Castanea mollissima* Bl.) kernel. *International Journal of Biological Macromolecules*. 2019; 3(65): 309–314. <https://doi.org/10.1016/j.ijbiomac.2019.03.065> PMID: 30872058
34. Zhang HJ, Mao WJ, Fang F, Li HY, Sun HH, Chen Y, et al. Chemical characteristics and anticoagulant activities of a sulfated polysaccharide and its fragments from *Monostroma latissimum*. *Carbohydrate Polymers*. 2008; 71(3), 428–434. <https://doi.org/10.1016/j.carbpol.2007.06.012>
35. Jin M, Zhao K, Huang Q, Xu C, Shang P. Isolation, structure and bioactivities of the polysaccharides from *Angelica sinensis* (Oliv.) Diels: a review, *Carbohydr. Polym.* 2012; 89 (2012): 713–722. <https://doi.org/10.1016/j.carbpol.2012.04.049> PMID: 24750855
36. Guo SD, Mao WJ, Han Y. Structural characteristics and antioxidant activities of the extracellular polysaccharides produced by marine bacterium *Edwardsiella tarda*. *Bioresource Technology*. 2010; 101 (12): 4729–4732. <https://doi.org/10.1016/j.biortech.2010.01.125> PMID: 20156684
37. Brand W B, Cuvelier M E, Berset C, et al. Use of a Free Radical Method to Evaluate Antioxidant Activity. *LWT-Food Science and Technology*. 1995; 28(1): 25–30. [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5)
38. Sellimi S, Kadri N, Barragan-Montero V, Laouer H, Hajji M, Nasri M. Fucans from a Tunisian brown seaweed *Cystoseira barbata*: structural characteristics and antioxidant activity, *Int. J. Biol. Macromol.* 2014; 66: 281–288. <https://doi.org/10.1016/j.ijbiomac.2014.02.041> PMID: 24582933
39. Sun HH, Mao WJ, Chen Y, Guo SD, Li HY, Qi XH, et al. Isolation, chemical characteristics and antioxidant properties of the polysaccharides from marine fungus *Penicillium* sp. f23-2. *Carbohydrate Polymers*. 2009; 78(1):117–124. <https://doi.org/10.1016/j.carbpol.2009.04.017>

40. Abdelhedi O, Nasri R, Souissi N, Nasri M, Jridi M. Sulfated polysaccharides from common smooth hound: extraction and assessment of anti-ACE, antioxidant and antibacterial activities, *Carbohydr. Polym.* 2016; 152 (2016): 605–614. <https://doi.org/10.1016/j.carbpol.2016.07.048> PMID: 27516310
41. Park YS, Namiesnik J, Vearasilp K, et al. Bioactive Compounds and the Antioxidant Capacity in New Kiwi Fruit Cultivars. *Food Chemistry.* 2014; 16(5): 354–361. <https://doi.org/10.1016/j.foodchem.2014.05.114> PMID: 25038687
42. Chokboribal J, Tachaboonyakiat W, Sangvanich P, Ruangpornvisuti V, Jettanacheawchankit S, Thunyakitpisal P. Deacetylation affects the physical properties and bioactivity of acemannan, an extracted polysaccharide from *Aloe vera*. *Carbohydrate Polymers.* 2015; 133: 556–566. <https://doi.org/10.1016/j.carbpol.2015.07.039> PMID: 26344314
43. Zhao Y, Selvaraj JN, Xing F, Zhou L, Wang Y, Song H, et al. Antagonistic action of *Bacillus subtilis* strain sg6 on *Fusarium graminearum*. *PLOS ONE.* 2014; 9. <https://doi.org/10.1371/journal.pone.0092486> PMID: 24651513
44. Sharma S, Khanna PK, Kapoor S. Optimised isolation of polysaccharides from *Lentinula edodes* strain ncbi jx915793 using response surface methodology and their antibacterial activities. *Natural Product Research.* 2016; 30(5): 616–621. <https://doi.org/10.1080/14786419.2015.1030741> PMID: 25868404
45. Alves MJ, Ferreira C, Dias J, Teixeira V, Martins A, Pintado MA. A review on antimicrobial activity of mushroom (*Basidiomycetes*) extracts and isolated compounds. *Planta Med.* 2012; 78:1707–1718. <https://doi.org/10.1055/s-0032-1315370> PMID: 23023950
46. Fu RQ, Chen DW, Tian G, Zheng P, Yu B. Dietary supplementation of *Bacillus coagulans* or yeast hydrolysates on growth performance, antioxidant activity, cytokines and intestinal microflora of growing-finishing pigs. *Animal Nutrition* (4). 2019. <https://doi.org/10.1016/j.aninu.2019.06.003> PMID: 31890913
47. Shi MJ, Wei X, Xu J, Chen BJ, Zhao DY, Cui S, et al. Carboxymethylated degraded polysaccharides from *Enteromorpha prolifera*: preparation and in vitro antioxidant activity. *Food Chemistry.* 2017; 215: 76–83. <https://doi.org/10.1016/j.foodchem.2016.07.151> PMID: 27542452
48. Anjum MF, Duggett NA, Abuoun M, Randall L, Nunez-Garcia J, Ellis RJ, et al. Colistin resistance in *Salmonella* and *Escherichia coli* isolates from a pig farm in Great Britain. *Journal of Antimicrobial Chemotherapy.* 2016; 71(8): 2306–2313. <https://doi.org/10.1093/jac/dkw149> PMID: 27147305
49. Sayaka T, Fumito M, Yoshitoshi O, Atsushi T, Tetsuya H, Moriya O, et al. Genomic characteristics of *Bifidobacterium thermacidophilum* pig isolates and wild boar isolates reveal the unique presence of a putative mobile genetic element with *tetW* for pig farm isolates. *Frontiers in Microbiology.* 2017; 8: 1540–1559. <https://doi.org/10.3389/fmicb.2017.01540> PMID: 28861055
50. Talanta. Experimental design and multiple response optimization. Using the desirability function in analytical methods development. *Talanta.* 2014; 124(124): 123–138. <https://doi.org/10.1016/j.talanta.2014.01.034> PMID: 24767454
51. Vichasilp C, Nakagawa K, Sookwong P, Suzuki Y, Kimura F, Higuchi O, et al. Optimization of 1-deoxy-*nojiromycin* extraction from mulberry leaves by using response surface methodology. *Bioscience, Biotechnology and Biochemistry.* 2009; 73(12): 2684–2689. <https://doi.org/10.1271/bbb.90543> PMID: 19966480
52. Blandon, Lina M, Noseda MD, Islan GA, et al. Optimization of culture conditions for kefiran production in whey: The structural and biocidal properties of the resulting polysaccharide. *Bioactive Carbohydrates and Dietary Fibre.* 2018; S2212619818300135. <https://doi.org/10.1016/j.bcdf.2018.02.001>
53. Wang XF, Shi CJ, Kan GF, et al. Optimal cultivation of Antarctic red yeast and its effect on sea cucumber components. *Chinese Journal of Polar Research.* 2018; 30(01), 50–56. (in Chinese)
54. WU JJ, WANG CL, CHEN MH, et al. Optimization of Liquid Fermentation Conditions of Polysaccharide by *Monascus* sp. *Journal of Food Science and Technology.* 2012; 30(6), 57–62. (in Chinese)
55. Beltran G, Novo M, José M, et al. Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *International Journal of Food Microbiology.* 2008; 121 (2), 69–177. <https://doi.org/10.1016/j.ijfoodmicro.2007.11.030> PMID: 18068842
56. Qiang W, Xiao JS, Aimin S, et al. β -Glucans: Relationships between Modification, Conformation and Functional Activities. *Molecules.* 2017; 22(2), 257. <https://doi.org/10.3390/molecules22020257> PMID: 28208790
57. Zhang ZF, Lv GY, Jiang X, et al. Extraction optimization and biological properties of a polysaccharide isolated from *Gleostereum incarnatum*. *Carbohydrate Polymers.* 2015; 117, 185–191. <https://doi.org/10.1016/j.carbpol.2014.09.059> PMID: 25498624
58. Xiaolan Chen, Zhicun, et al. Purification, characterization and in vitro and in vivo immune enhancement of polysaccharides from mulberry leaves. *PLOS ONE.* 2019; 14(1), e0208611 <https://doi.org/10.1371/journal.pone.0208611> PMID: 30601811

59. Liu J, Bai R, Liu Y, et al. Isolation, structural characterization and bioactivities of naturally occurring polysaccharide–polyphenolic conjugates from medicinal plants—A review. *International Journal of Biological Macromolecules*, 2017; S0141813017324303. <https://doi.org/10.1016/j.ijbiomac.2017.10.097> PMID: 29051102
60. Palleschi G, Carbone A, Zanella PP, et al. Prospective study to compare antibiosis versus the association of N-acetylcysteine, D-mannose and *Morinda citrifolia* fruit extract in preventing urinary tract infections in patients submitted to urodynamic investigation *Archivio Italiano di Urologia e Andrologia*, 2017; 89(1). <https://doi.org/10.4081/aiua.2017.1.45> PMID: 28403598
61. Marchiori D, Zanella PP, Marchiori D, et al. Efficacy of N-acetylcysteine, D-mannose and *Morinda citrifolia* to Treat Recurrent Cystitis in Breast Cancer Survivals. *In Vivo*, 2018; 31(5). <https://doi.org/10.21873/invivo.11149> PMID: 28882961
62. Khan AA, Gani A, Khanday FA, et al. Biological and pharmaceutical activities of mushroom β -Glucan discussed as a potential functional food ingredient. *Bioactive Carbohydrates and Dietary Fibre*, 2017; S2212619817300402. <https://doi.org/10.1016/j.bcdf.2017.12.002>
63. Tang Q, Huang G, Zhao F, et al. The antioxidant activities of six (1 \rightarrow 3)- β -D-glucan derivatives prepared from yeast cell wall. *International journal of biological macromolecules*, 2017; 98, 216–221. <https://doi.org/10.1016/j.ijbiomac.2017.01.132> PMID: 28161534
64. Yılmaz Ç, Özcengiz G. Antibiotics: pharmacokinetics, toxicity, resistance and multidrug efflux pumps. *Biochemical Pharmacology*. 2017; 133, 43–62. <https://doi.org/10.1016/j.bcp.2016.10.005> PMID: 27765485