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Comparison of immunomodulatory activity of polysaccharides and soluble dietary fibers and adsorption capacities of insoluble dietary fibers extracted from *Lentinus edodes* stipes

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Abstract

Stipes are the major waste from the processing of *Lentinus edodes*. To make full use of *L. edodes* stipes (LES), different fractions of LES polysaccharides (LESPs) were first obtained by water extraction and gradient ethanol precipitation. Afterwards, the LES residues were treated with an optimal combination of *Aspergillus niger* and *Saccharomyces cerevisiae* for the preparation of soluble/insoluble dietary fibers (LESS/LESI) using the response surface methodology and the Box-Behnken design. Subsequently, the in vitro immunomodulatory activity of LESPs and LESS, as well as the adsorption capacities of LESI were evaluated. The results showed that LESPs were neutral polysaccharides, mainly containing glucose. The optimal parameters for modifying the residues of LES were the followings: 4% (*w/w*) *A. niger*, 8% (*w/w*) *S. cerevisiae*, 31 ℃, 3 d, and a solid–liquid ratio of 1:12.5 in a yield of 14.73%/82.45% of LESS/LESI, respectively. The in vitro immunomodulatory activity assays revealed that LESPs and LESS had potent immunostimulatory activity to increase phagocytosis, acid phosphatase activity and nitric oxide production of RAW264.7 murine cell macrophages. The evaluation of adsorption capacities revealed that LESI owned stronger water holding capacity, oil holding capacity and water swelling capacity. This research could provide an effective way to fully utilize discarded *L. edodes* stipes with high added-value.

Keywords *Lentinus edodes* stipes, Polysaccharide, Dietary fiber, Immunomodulatory activity, Adsorption capacities

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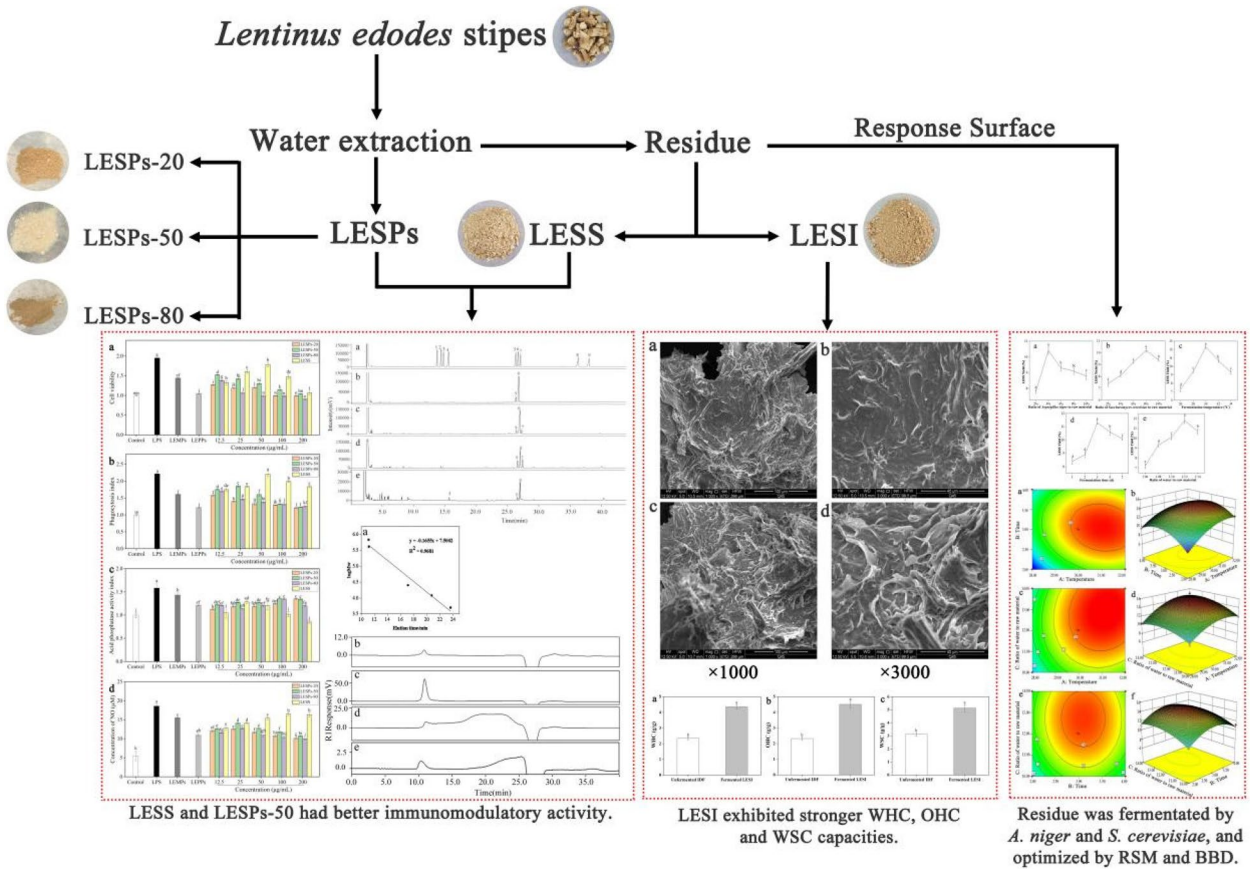
Graphical Abstract

An effective strategy was built to fully utilize discarded *L. edodes* stipes by preparing polysaccharides (LESPs-20, LESPs-50 and LESPs-80), soluble and insoluble dietary fibers (LESS/LESI). Their products showed immunomodulatory activity and adsorption capacities.



Introduction

Lentinus edodes (Berk.), also known as Shiitake mushroom, is the most widely consumed edible mushroom in the world due to its good taste, nutritional benefits and medicinal values (Roszczyk et al., 2022). *L. edodes* has been cultivated in China and Japan for about 2000 years, and is also cultivated in Asia, Europe, North America and Australia nowadays (Sheng et al., 2021). The fruit body of *L. edodes* is made up of pileus and stipes, accounting for approximately 75% and 25% of the whole fruit body on a dry basis (Li et al., 2018). In China, *L. edodes* has a huge annual production of more than 11.88 million tons (Zhu et al., 2023). During the processing of *L. edodes*, especially in drying processes, a large number of stipes are residual and discarded. According to statistics, nearly 3 million tons of stipes are discarded and wasted every year merely in China (Lu et al., 2023). With the rapid development of the edible fungi industry in the world, the waste of by-products has led to a series of economic and environmental problems. Therefore, *L. edodes* stipes (LES) deserve more attention for rational utilization toward value-added products (Tian et al., 2022; Zhu et al., 2023). Compared to the pileus of *L. edodes*, stipes are of low commercial use, due to their high crude fiber content and poor palatability (Zhang et al., 2012). However, LES still preserve the appropriate nutritional ingredients, which are primarily used for animal feed and composting (Chou et al., 2013). Besides, few studies are applied LES to prepare shiitake sauce, bread, noodle, bread and biscuit (Lin et al., 2008; Wang et al., 2020). Therefore, it is very important to find more alternative strategies for the use of valuable compounds (and/or nutrients) present in LES.

Since LES still preserve essential nutrients, which should not be underestimated or discarded directly (Li et al., 2018). Previous studies have extracted umami compounds, polysaccharides and insoluble dietary fibers (IDF) from LES (Harada-Padermo et al., 2020; Jiao et al., 2018; Tian et al., 2022). Compared to *L. edodes* pileus, LES have significantly lower water, protein and ash content, while possessing markedly higher carbohydrate and fiber, accounting for 439.56 g / kg of carbohydrates and 82.94 g / kg of fibers, respectively (Li et al., 2018). Therefore, it is essential to make full use of carbohydrates and fibers in LES.

β-Glucans are the main polysaccharides in the fruit body of *L. edodes*, exhibiting various biological activities, such as immunomodulatory, anti-oxidant, anti-tumor, anti-diabetic, anti-aging and anti-viral activities, etc. (Roszczyk et al., 2022; Sheng et al., 2021; Yehia, 2022). Several studies have reported that stipes contain higher amounts of β-glucans than pileus, suggesting that stipes are more nutritional than pileus in some respects (Bak et al., 2014; Shimizu et al., 2003; Vetter, 2023). It is noteworthy that β-glucans are potent immunological stimulators, and some kinds of them have been used clinically in China and Japan (Chou et al., 2013). However, there are rarely studies on the immunomodulatory activity of polysaccharides isolated from LES.

In addition, the extraction of polysaccharides from LES (LESPs) could still produce a large amount of insoluble residues, which are rich in IDF. Therefore, the further recycling of the residues plays an important role in promoting the effective and reasonable utilization of biomass resources, and improving the economic benefits of edible fungi enterprises (Tian et al., 2022). Dietary fibers (DF), known as the seventh nutrient, have many benefits for human health, such as improving the intestinal flora and increasing its diversity and function, increasing fecal volume, promoting bowel movement, lowering blood glucose, and decreasing the probability of diabetes mellitus, obesity, cardiovascular and cancer diseases, etc. (Makki et al., 2018; Shah et al. 2020; Tian et al., 2022). Considering the solubility of DF, they can be divided into soluble dietary fibers (SDF) and IDF, of which SDF are more important with respect to physiological and functional perspectives than IDF (Bader et al., 2019; Gan et al., 2021). Various modification methods have tried to convert IDF into SDF, including physical methods utilizing blasting, extrusion, ultrafine comminution, high-pressure microfluidization, high hydrostatic pressure, high-pressure homogenization, cavitation jet processing, chemical methods utilizing alkaline hydrogen peroxide, acid treatment, alkali treatment, carboxymethylation treatment, biological methods utilizing specific enzymes or microorganisms to enzymatic hydrolysis or ferment raw materials, physical method combined with physical method, chemical method or biological method, and biological method combined with biological method, etc. (Gan et al., 2021; Park et al., 2013; Wang et al., 2021; Yu et al., 2018; Zhang et al., 2020a, 2020b).

Among the above modification methods, biological methods received the most attention due to their advantages of milder processing conditions, stabler processing process, higher processing efficiency, less operational risks, higher purity of processing products, and friendlier to the environment (Gan et al., 2021). Fermentation by *Bacillus natto*, *Monascus anka*, *Trichoderma viride* and *Trichoderma harzianum* has been commonly used to increase the content of SDF, which can produce various enzymes that could hydrolyze complex carbohydrates, such as hemicellulose, cellulose, etc. (Chen et al., 2020a, 2020b; Chu et al., 2019; Jia et al., 2019; Sun et al., 2020). *Aspergillus niger* has been used by industries due to its strong growth ability, wide selection of substrates, and high efficiency in the secretion of cellulolytic enzymes (Ma et al., 2021; Zhang et al., 2017). *Saccharomyces cerevisiae* can secrete hydrolase to accelerate hydrolysis, improving the biodegradability of hemicellulose, cellulose, lignin, etc. (Zhao et al., 2020). These two fungi are mainly used to produce organic acids, such as citric acid, lactic acid, methane, oxalic acid, etc. (Ma et al., 2021; Roukas & Kotzekidou, 2020; Wang et al., 2022; Zhao et al., 2020). However, few studies have used *A. niger* or *S. cerevisiae* as a modification method to increase the content of SDF (Xu et al., 2023), and no studies focused on the production of SDF by their co-fermentation.

Together, this study was aimed to explore an efficient strategy to make full of LES. The process to prepare LESPs and SDF from LES is shown in Fig. 1. In the first stage, different LESPs (LESPs-20, LESPs-50, LESPs-80) were obtained by water extraction and gradient ethanol precipitation. Afterwards, the LES residues were adjusted to prepare soluble/insoluble dietary fibers (LESS/LESI), through single-factor experiments and the response surface methodology (RSM) based on a Box-Behnken design (BBD) by fermentation with *A. niger* and *S. cerevisiae*. Subsequently, the in vitro immunomodulatory activity of LESPs and LESS, as well as the adsorption capacities of LESI were further evaluated. This study will provide new ideas for the utilization of LES of high economic value.

Materials and methods

Materials

The dried LES were collected from an edible fungus factory in Longxian County (Shaanxi, China). *A. niger* was purchased from Hezhong Kangyuan Biotechnology Co., Ltd (Zibo, Shandong, China). *S. cerevisiae* was purchased from Angel Yeast Co., Ltd (Yichang, Hubei, China).

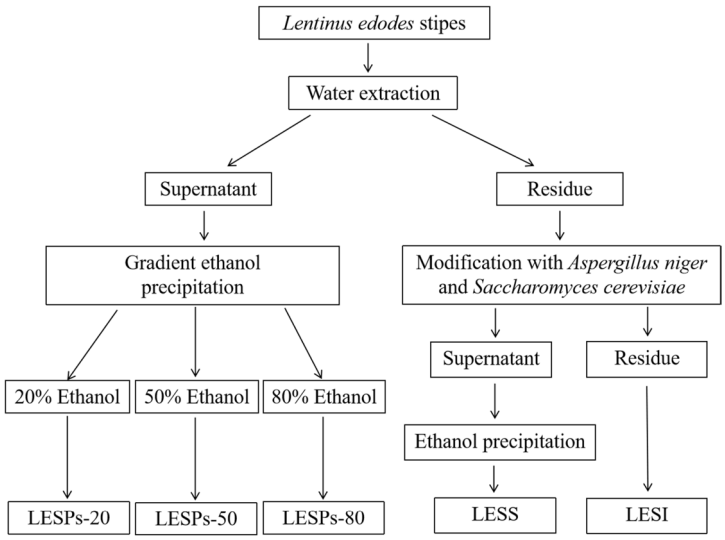


Fig. 1 Flowsheet for the process to prepare LESPs and LESS/LESI from LES

Soybean oil was purchased from Luhua Group Co., Ltd (Laiyang, Shandong, China). Monosaccharide standards, including rhamnose (Rha), fucose (Fuc), arabinose (Ara), xylose (Xyl), mannose (Man), glucose (Glc), galactose (Gal), galacturonic acid (GalA) and glucuronic acid (GlcA); lipopolysaccharides (LPS), thiazolyl blue (MTT), neutral red staining solution, p-nitrophenyl phosphate, Triton X-100, Griess reagent, penicillin and streptomycin were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Dextran standards with molecular weights of 5, 12, 25, 410 and 670 kDa, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Murine monocyte-macrophage RAW264.7 cells were obtained from the Institute of Cell Biological, Chinese Academy of Sciences (Shanghai, China). DMEM medium and fetal bovine serum (FBS) were purchased from HycloneTM by GE Healthcare Life Science (Logan, Utah, USA). *L. edodes* mycelia polysaccharides (LEMPs) tablet was purchased from Hubei Guangren Pharmaceutical Co., Ltd (Suizhou, Hubei, China). All other chemicals were of analytical grade.

Extraction of LESPs

The dried LES were powdered and then put through a 40-mesh sieve. The final powders were collected and used for polysaccharide extraction by our previous study with minor modifications (Sun et al., 2018a, 2018b). Briefly, LES powders (50 g) were treated with 2000 mL of deionized water at 60 °C for 4 h (twice). The extraction solution was then concentrated and precipitated with 20%, 50% and 80% (*v/v*) of ethanol at 4 °C for 24 h, respectively. The precipitate was centrifuged, deproteinated, dialyzed and lyophilized to obtain LESPs, named LESPs-20, LESPs-50 and LESPs-80, respectively. The left residues were lyophilized for the following modification.

Modification of the LES residues with *A. niger* and *S. cerevisiae*

To investigate the optimal conditions for modification of the LES residues with *A. niger* and *S. cerevisiae*, the most favorable process parameters were determined by single-factor experimental design and response surface optimization. The yield of LESS was the detection index.

Single-factor experimental design

(1) Effect of adding ratio of *A. niger* and *S. cerevisiae* to raw material for the production of LESS: 10 g of dried LES residues were added to 100 mL of deionized water at 30 °C for 3 days, the adding ratios were adjusted to 2%, 4%, 6%, 8% and 10% (*w/w*).
(2) Effect of fermentation temperature, time and ratio of water to raw material for the production of LESS: After determining the most favorable ratio of *A. niger* and *S. cerevisiae* to raw material, fermentation temperature, time and ratio of water to raw material were evaluated under the optimal adding ratio. The fermentation temperatures were tested of 26 °C, 28 °C, 30 °C, 32 °C and 34 °C. The fermentation times were set at 1 day, 2 days, 3 days, 4 days and 5 days. The ratios of water to raw material were adjusted to 6%, 8%, 10%, 12% and 14% (*v/w*).

Experimental design of response surface optimization

After obtaining the appropriate range of the variables through single-factor experiments, three variables (fermentation temperature, time and ratio of water to raw material) with three levels were selected for further optimization of LESS production (Feng & Zhang, 2020).

Preparation of LESS and LESI

After fermentation, the whole supernatant was collected, concentrated and precipitated with a fourfold volume of 95% (*v/v*) ethanol at 4 °C overnight. The precipitate was then collected and lyophilized to produce LESS, while the fermented residues were freeze-dried to produce LESI (Xie et al., 2017). The yield of LESS/LESI (%) = Ms / Mr; Where Ms is the dried weight of LESS/LESI, and Mr is the dried weight of the raw materials.

Measurements of structural characteristics

The total sugar content of LESPs and LESS was determined according to the phenol–sulfuric acid method using Glc as the standard (Dubois et al., 1956).

The monosaccharide composition of LESPs and LESS was analyzed by gas-chromatography (GC), according to our previous study with Rha, Fuc, Ara, Xyl, Man, Glc, Gal, GlcA and GalA as standards (Sun et al., 2022).

The molecular weight of LESPs and LESS was analyzed by high performance gel-permeation chromatography (HPGPC), according to our previous study with different molecular weights of dextrans (5, 12, 25, 410 and 670 kDa) as standards (Sun et al., 2022).

The microstructure and morphology of LESI were observed by scanning electron microscopy (SEM), according to our previous study (Sun et al., 2018a, 2018b).

The in vitro immunomodulatory activity analysis of LESPs and LESS

RAW264.7 cells were pre-cultured in a DMEM medium supplemented with 1% (*v/v*) penicillin and streptomycin, and 10% (*v/v*) fetal bovine serum at 37 °C in a 5% CO_2 incubator. When the cell growth reached 70–80% of the bottom of culture box, a sub-culture was carried out for the assays of cell viability, phagocytosis, acid phosphatase activity and NO production based on our previous study (Sun et al., 2018a, 2018b). The DMEM medium in the absence of polysaccharides was used as a blank control. LPS of 10 μg/mL, *L. edodes* mycelia polysaccharides (LEMPs) purchased from Pharmaceutical Co., Ltd of 200 μg/mL, and *L. edodes* pileus polysaccharides (LEPPs) extracted by the above method and precipitated with 80% (*v/v*) ethanol of 200 μg/mL were used as positive controls.

The adsorption capacities analysis of LESI

Water holding capacity (WHC)

LESI (0.5 g, M_1) were mixed with 10 mL of deionized water at 26 °C for 1 day. After centrifugating for 8 min at 8000 × *g*, the supernatant of the mixture was discarded, while the sediment was collected and weighted as M_2 (Wang et al., 2021; Xu et al., 2023). The WHC was calculated by the following equation: WHC (g/g) = (M_2-M_1)/M_1.

Oil holding capacity (OHC)

LESI (1.0 g, M_1) were maintained with 20 mL of soybean oil at 26 °C for 1 day. After centrifugating for 8 min at 8000 × *g*, the sediment was collected and weighted as M_2 (Wang et al., 2021; Xu et al., 2023). The OHC was calculated by the following equation: OHC (g/g) = (M_2-M_1)/M_1.

Water swelling capacity (WSC)

LESI (1.0 g, M_1) were mixed with 20 mL of deionized water at 26 °C for 2 h. The sample volume before and after expansion was measured as V_1 and V_2, respectively (Gan et al., 2020; Xu et al., 2023). The WSC was calculated by the following equation: WSC (g/g) = (V_2-V_1)/M_1.

Statistical analysis

All the experiments were carried out in triplicate and averaged. All the data are expressed as means ± standard deviation (SD) with significant analysis after passing an LSD test. Statistical analyses were processed with SPSS Statistics (SPSS 20.0 software, IBM Inc., Chicago, IL, USA).

Results and discussion

The preparation and characterization of LESPs

Three LESPs (LESPs-20, LESPs-50 and LESPs-80) were obtained from LES by fractional precipitation with 20%, 50% and 80% (*v/v*) of ethanol, yielding 11.42% ± 0.72%, 2.51% ± 0.98% and 1.42% ± 0.42% of dry weight, respectively. The total sugar content of LESPs-20, LESPs-50 and LESPs-80 was 65.82% ± 3.23%, 84.31% ± 4.21% and 71.21% ± 3.23%, suggesting that LESPs-50 had the highest polysaccharide content ($p < 0.05$).

The monosaccharide composition analysis of LESPs showed that LESPs-20, LESPs-50 and LESPs-80 were neutral polysaccharides and mainly consisted of Glc, but varied in minor differences. As shown in Fig. S1b-S1d, LESPs-20 contained a small amount of Man in a ratio of 1.00 (Man): 5.09 (Glc); LESPs-50 and LESPs-80 contained a small amount of Man and Gal in a ratio of 1 (Man): 8.39 (Glc): 1.01 (Gal) and 1 (Man): 4.36 (Glc):

1.72 (Gal), respectively. Glucans are the most important and abundant constituents in *L. edodes*. Their basic unit is Glc, however, other monosaccharides (e.g. Man and Gal) are generally found in the mushroom (Vetter, 2023; Wang et al., 2017; Zhao et al., 2018).

The average molecular weight of LESPs was calculated by the calibration curve ($y=-0.1655x+7.5002$, $R^2=0.9681$), showing that LESPs-20, LESPs-50 and LESPs-80 was 484.97 ± 18.85 kDa, 477.52 ± 25.34 kDa and 470.60 ± 11.01 kDa, respectively (Fig. S2b-S2d). The result is consistent with previous studies, which have demonstrated that the molecular weight of glucan fractions from *L. edodes* varies between 300 and 800 kDa, with an average of 500 kDa (Vetter, 2023; Zhang et al., 2011).

The preparation and characterization of LESS and LESI

After the polysaccharides were extracted from LES, there were still left a large amount of insoluble residues. To further utilize the residues, *A. niger* and *S. cerevisiae* were employed to modify and improve the residues.

Single-factor experimental analysis

The yields of LESS under different adding ratios of *A. niger* to raw material are shown in Fig. 2a. The results were evaluated by sequentially setting the ratio at 2%, 4%, 6%, 8% and 10% (*w/w*) with the fermentation temperature, time and the ratio of water to raw material at 3 days, 30 °C and 10% (*v/w*). The yield of LESS reached a maximum level of 10.04% ± 0.24%, when the *A. niger* adding ratio was 4%. According to the results, performing fermentation with *A. niger* higher or lower than 4% significantly suppressed the yield of LESS. Therefore, 4% was chosen as the optimal point for subsequent experiments.

The yields of LESS under different adding ratios of *S. cerevisiae* to raw material are shown in Fig. 2b. Similarly, the effects were investigated by sequentially setting the ratio at 2%, 4%, 6%, 8% and 10% (*w/w*) with the fermentation temperature, time and the ratio of water to raw material at 3 days, 30 °C and 10% (*v/w*). The yield of LESS achieved the highest value of 10.02% ± 0.16% when the *S. cerevisiae* adding ratio was 8%. According to the results, fermentation with *S. cerevisiae* higher or lower

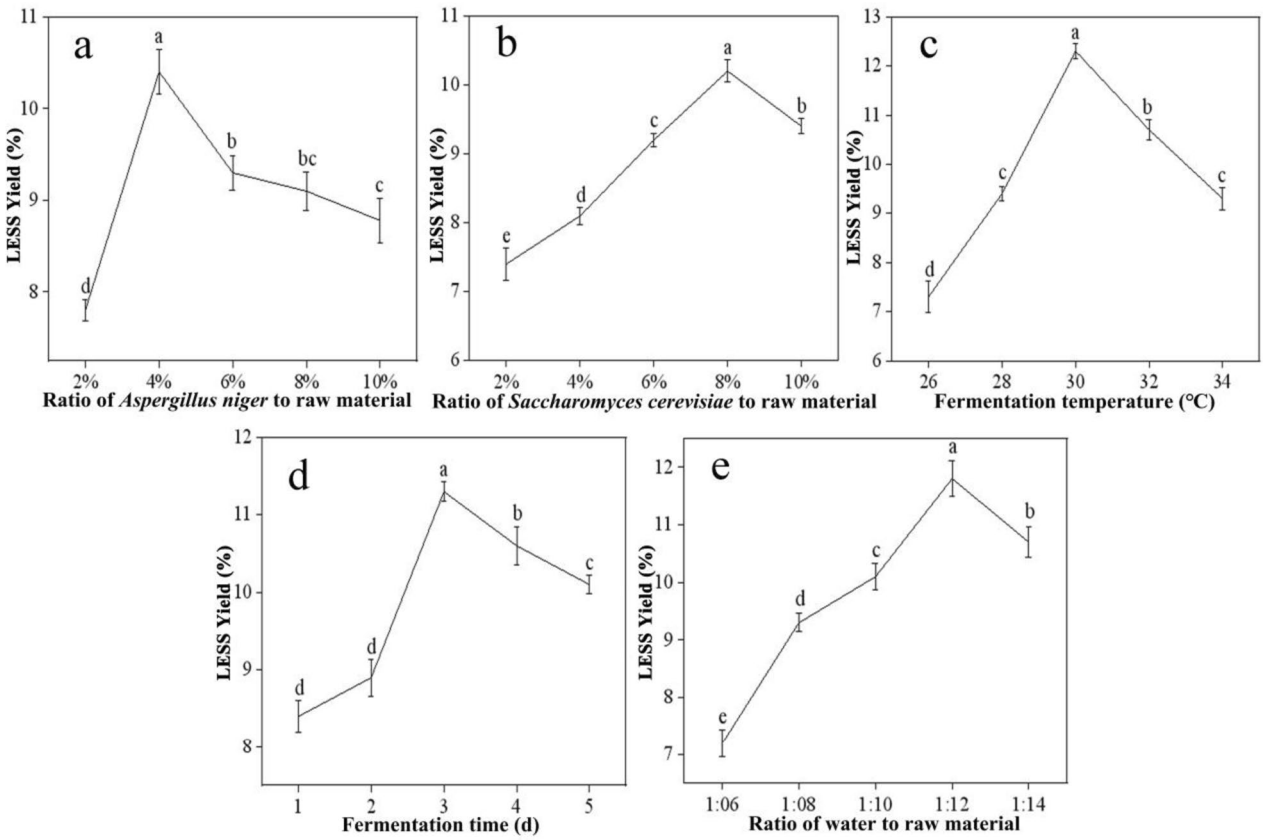


Fig. 2 Effect of (**a**) ratio of *Aspergillus niger* to raw material, (**b**) ratio of *Saccharomyces cerevisiae* to raw material, (**c**) fermentation temperature, (**d**) fermentation time and (**e**) ratio of water to raw material on the yield of LESS. Data are expressed as means ± SD (n = 3). The graph points marked with different letters on top represent statistical significances ($p < 0.05$) by an LSD test, whereas points marked with the same letter correspond to results without significant differences

than 8% significantly suppressed the yield of LESS. Therefore, 8% was chosen as the optimal point for subsequent experiments.

The yields of LESS under different fermentation temperatures are shown in Fig. 2c. The results were evaluated by sequentially setting the temperature at 26 °C, 28 °C, 30 °C, 32 °C and 34 °C with the adding ratio of *A. niger* and *S. cerevisiae* to raw material, fermentation time and the ratio of water to raw material at 4%, 8%, 3 days and 12%. The yield of LESS reached a maximum level at a temperature of 30 °C. During the growth and metabolism of *A. niger* and *S. cerevisiae*, a large number of enzymes are produced and some enzymatic reactions occurred to hydrolyze cellulose, pectin and other macromolecules (Hao et al., 2020). Their growth and enzymatic hydrolysis largely depend on temperature, hence higher or lower temperature might suppress the yield of LESS.

The yields of LESS under different fermentation times are shown in Fig. 2d. The results were evaluated by sequentially setting the time at 1 day, 2 days, 3 days, 4 days and 5 days with the adding ratio of *A. niger* and *S. cerevisiae* to raw material, fermentation temperature and the ratio of water to raw material at 4%, 8%, 30 °C and 12%. The yield of LESS reached a maximum level in 3 days. During the second day of fermentation, the mycelium of *A. niger* and *S. cerevisiae* grew and began to produce spores. The yield of LESS increased rapidly from the 2nd-3rd day, possibly due to the increased enzymatic activity of secretions. As time increased, the yield of LESS was decreased, which might result from some microbial cells beginning to die or some generated LESS continuing to degrade. A similar finding has been reported in the previous study (Hao et al., 2020).

The yields of LESS under different ratios of water to raw material are shown in Fig. 2e. The results were evaluated by sequentially setting the ratios at 6%, 8%, 10%, 12% and 14% (*v/w*) with the adding ratio of *A. niger* and *S. cerevisiae* to raw material, fermentation temperature and time at 4%, 8%, 30 °C and 3 days. The yield of LESS reached a maximum level at the water to raw material ratio of 12%. A greater contact surface area between the solid and liquid phases could better access the solvent into intracellular active substrates (Zhao et al., 2018), but the excess water could lead to the dilution of enzyme content, cause a larger humidity in the residues, and bring a poor gas exchange in the system, thus causing a decrease in LESS production.

Response surface analysis

RSM is an effective statistical method used to develop and optimize multivariable problems, which aids in the design of tests and uses multiple quadratic regression equations to fit functional models between factors and response values (Hao et al., 2020; Zhao et al., 2018). Since temperature, time and the ratio of water to raw material are important for microbial fermentation (Feng & Zhang, 2020; Xu et al., 2023), these three parameters were further optimized for response surface optimization, choosing the fermentation temperature, time and the ratio of water to raw material at 30 °C, 3 days and 12% as the center points for subsequent experiments. The levels and codes of fermentation variables used in the BBD are shown in Table 1. The BBD design and the results of LESS yield are shown in Table 2. Based on the experimental data of 17 test points obtained by the far regression method, the following quadratic regression equation can be used to explain the yield of LESS (Y, %) and test component variables:

$$Y = 14.32 + 1.69*A + 0.43*B + 0.80*C - 0.43*A*B + 0.40*A*C - 0.032*B*C - 1.63*A^2 - 2.20*B^2 -$$

Table 1 The levels and codes of fermentation variables used in Box-Behnken design (BBD)

Variables	Coded symbols	Coded levels		
		-1	0	1
Fermentation temperature (°C)	A	28	30	32
Fermentation time (d)	B	2	3	4
Ratio of water to raw material (%)	C	10	12	14

Table 2 The BBD experimental design and the results for LESS yield

Runs	A	B	C	LESS yield (%)
1	30	4	10	11.16
2	28	3	10	9.13
3	30	4	14	12.21
4	30	3	12	14.12
5	28	2	12	8.32
6	32	4	12	11.82
7	30	3	12	13.91
8	30	3	12	14.61
9	28	3	14	10.42
10	30	3	12	14.7
11	32	3	10	11.94
12	30	3	12	14.26
13	32	3	14	14.82
14	32	2	12	12.31
15	28	4	12	9.53
16	30	2	14	10.91
17	30	2	10	9.73

A, B and C is fermentation temperature, time and ratio of water to raw material, respectively

$1.12*C^2$; Where A, B and C is fermentation temperature, time and the ratio of water to raw material, respectively.

The variance analysis of the BBD experimental results is displayed in Table 3. The *p*-value of the quadratic regression equation was 0.0001, which demonstrated that the model was significant. The lack-of-fit was not significant ($p=0.1405$), demonstrating that the model used for the regression had a fair degree of fitting and a small amount of experimental error, thus the real value could be substituted for it when analyzing the findings. Furthermore, the high value of R^2 ($R^2=0.9791$) indicated that 97.91% of the variability and accuracy of the response were adequate and could be explained in the model; The $R^2_{Adj}=0.9522$ implied that the model is in reasonable agreement with the R^2 value (Feng & Zhang, 2020). The linear coefficients (A, B and C) and quadratic coefficients (A^2, B^2 and C^2) showed significances, while other regression coefficients were not significant. According to the significance of the regression coefficients, fermentation temperature (A, $p<0.0001$) exerted the greatest influence on the yield of LESS, followed by the ratio of water to raw material (C, $p=0.0019$) and then fermentation time (B, $p=0.0347$).

As presented in Fig. 3, the 2D contour plots and the 3D response showed the combined effects of fermentation temperature, time and the ratio of water to raw material on the yield of LESS. Figure 3a and b showed the effects of fermentation temperature and time, Fig. 3c and 4d showed the effects of fermentation temperature and the ratio of water to raw material, and Fig. 3e and 4f showed the effects of fermentation time and the ratio of water to raw material. The optimal fermentation conditions obtained by the regression model were a fermentation temperature of 30.79 °C, a fermentation time of 3.10 days, and a ratio of water to raw material of 12.37. Under the optimal condition, the predicted value of LESS yield was 14.91%. In view of the operability in actual production, the fermentation process can be modified by the fermentation temperature to 31 °C, the fermentation time to 3 days and the water to raw material to 12.5. Three verification tests were conducted under the modified parameters, a final LESS yield of 14.73% ± 0.21% was obtained, which was quite agreed with the predicted value of the model. The result was also confirmed the accuracy and reliability of the regression model by the response surface optimization.

Structural characterization of LESS and LESI

The total sugar content of LESS was 67.92% ± 3.23%. As shown in Fig. S1e, monosaccharide composition analysis of LESS showed that it was also consisted of Man and Glc, which was similar to that of LESPs-20. But after fermentation, the content of Xyl was increased in LESS, exhibiting a ratio of Xyl, Man and Glc as 0.89: 1: 6.29. IDF is usually composed of lignin, hemicellulose and cellulose, etc. (Xu et al., 2023). Thus, the release of SDF with Xyl, Man and Glc monosaccharides might be attributed to the decomposition of LES during fermentation. According to the previous studies, during the fermentation of *A. niger* and *S. cerevisiae*, a number of enzymes are produced, and some enzymatic reactions occur (Hao et al., 2020). *A. niger* can secrete amylase, cellulase, glucosidase, endoglucanase, lignin peroxidase and xylanase,

Table 3 Variance analysis of the BBD experimental results

Variables	Sum of squares	df	Mean square	F-value	*p*-value	Prob. > F
Model	71.38	9	7.93	36.43	< 0.0001	***
A	22.75	1	22.75	104.5	< 0.0001	***
B	1.49	1	1.49	6.83	0.0347	*
C	5.12	1	5.12	23.52	0.0019	**
AB	0.72	1	0.72	3.32	0.1113	
AC	0.63	1	0.63	2.9	0.1322	
BC	0.00423	1	0.00423	0.019	0.8931	
A^2	11.12	1	11.12	51.08	0.0002	***
B^2	20.38	1	20.38	93.62	< 0.0001	***
C^2	5.26	1	5.26	24.16	0.0017	**
Pesidual	1.52	7	0.22	-	-	
Lack of fit	1.08	3	0.36	3.28	0.1405	
Pure error	0.44	4	0.11	-	-	
Cor total	72.9	16	-	-	-	

$R^2=0.9791$; $R^2_{Adj}=0.9522$

A, B and C is fermentation temperature, time and ratio of water to raw material, respectively. The significances are presented as $*p<0.05$, $**p<0.01$ and $***p<0.001$

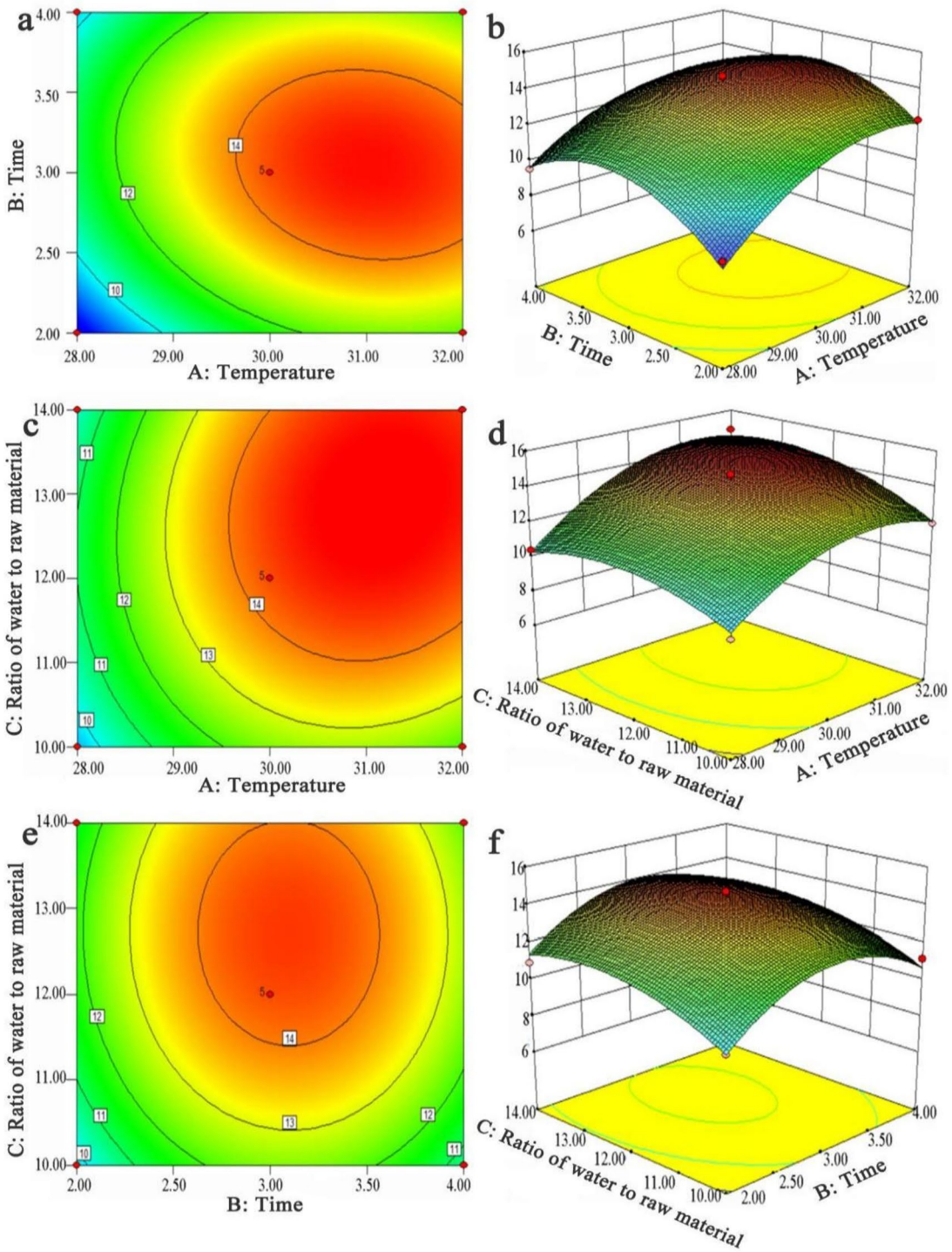


Fig. 3 Response surface plots showing effects of fermentation temperature, time and ratio of water to raw material on the yield of LESS. (**a**, **b**) Interaction of fermentation temperature and time; (**c**, **d**) Interaction of fermentation temperature and ratio of water to raw material; (**e**, **f**) Interaction of fermentation time and ratio of water to raw material

and *S. cerevisiae* can secrete various glycoside hydrolases (e.g. cellulase, hemicellulase, xylanase), improving the biodegradability of starch, lignin, hemicellulose, cellulose and other macromolecules (Hao et al., 2020; He et al., 2021; Ma et al., 2021; Wang et al., 2022; Zhao et al., 2020). Besides, as shown in Fig. S2e, the average molecular weight of LESS was 607.40 ± 41.11 kDa, which was significantly higher than LESPs ($p < 0.05$).

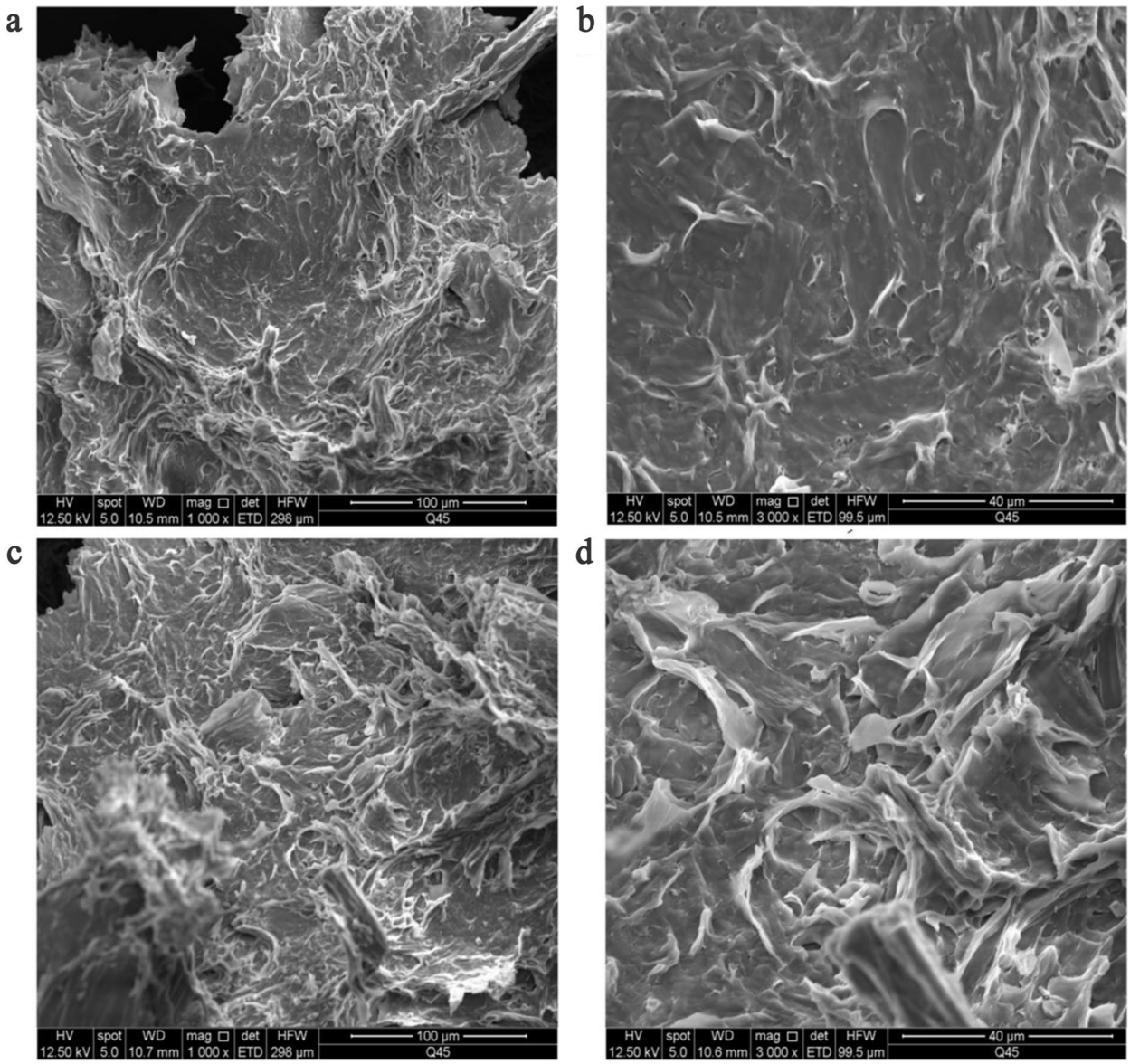


Fig. 4 SEM micrographs of (**a**, **b**) unfermented IDF from LES residues and (**c**, **d**) LESI prepared after fermentation

The microstructure of LESI was monitored by SEM at 1000 × and 3000 × magnifications. As shown in Fig. 4, compared with unfermented IDF from LES residues, LESI had more rough, irregular and complicated structures, with many looser folds on its surface. The irregular surface of LESI might be caused by the degradation of some fibers and semi-fibers, and the formation of a discontinuous loose surface of the fiber during the fermentation (Zhang et al., 2020a, 2020b). Changes in the external and internal structures could impact the absorption abilities of dietary fibers, improving the WHC, OHC and WSC of dietary fibers (Wang et al., 2021).

The in vitro immunomodulatory activity of LESPs and LESS

Macrophages are important immune cells in animals, exhibiting many immunomodulatory functions. RAW264.7 is a mouse peritoneal macrophage, and is usually used for the in vitro study of cell phagocytosis and cell immunity (Akhtar et al., 2020). Herein, cell viability, phagocytosis, acid phosphatase activity and NO production were measured to evaluate the immunomodulatory activity of LESPs and LESS. Besides, to better compare the immunomodulatory activity of LESPs and LESS, *L. edodes* mycelia polysaccharides, named LEMPs, as well as *L. edodes* pileus polysaccharides, named LEPPs, were employed as positive controls.

Effects of LESPs and LESS on cell viability of RAW264.7

The cell viability assay depends on the potential of viable cells to metabolize a water-soluble tetrazolium salt into a water-insoluble formazan product (Akhtar et al., 2020). The effects of LESPs-20, LESPs-50, LESPs-80 and LESS on the proliferation rate of RAW264.7 cells were measured by the MTT method. As shown in Fig. 5a, compared to the control group, LESPs had significant proliferation effects under low-concentration treatments ($p < 0.05$), showing that LESPs-20 enhanced the RAW264.7 cell viability at the concentrations of 12.5–50 μg/mL, LESPs-50 improved the cell viability at the concentrations of 12.5–100 μg/mL, and LESPs-80 enhanced the cell viability at the concentrations of 12.5–25 μg/mL, respectively. When comparing LESPs-20, LESPs-50 and LESPs-80, LESPs-50 at a concentration of 12.5 μg/mL exhibited the best effect on the proliferation of RAW264.7 cells. LESS had significantly increased impacts on the cell viability of RAW264.7 cells with all tested concentrations ranging from 12.5–200 μg/mL, also showing a significantly higher effect than that of LESPs under the same treatment concentration from 25–200 μg/mL ($p < 0.05$). In addition, compared to LEMPs, LESS of 25–50 μg/mL and LESPs-80 of 12.5 μg/mL showed higher impacts ($p < 0.05$); Compared to LEPPs, LESS and LESPs-50 of 12.5–100 μg/mL, LESPs-20 of 12.5–50 μg/mL and LESPs-80 of 12.5 μg/mL showed higher impacts ($p < 0.05$).

Effects of LESPs and LESS on phagocytosis of RAW264.7

Phagocytosis of macrophages plays a vital role in immune function, which is described as the first and determining phase in the immune response (Akhtar et al., 2020). The effects of LESPs-20, LESPs-50, LESPs-80 and LESS on the phagocytosis of RAW264.7 cells were measured with the neutral red assay. As shown in Fig. 5b, compared to the control group, the three purified fractions of LESPs and LESS could significantly enhance phagocytosis under the entirely tested concentrations from 12.5–200 μg/mL ($p < 0.05$). When comparing LESPs-20, LESPs-50 and LESPs-80, LESPs-50 at a concentration of 50 μg/mL exhibited the best effect on the phagocytosis of RAW264.7 cells. Among the concentrations of 50–200 μg/mL, LESS exhibited much stronger phagocytic activity than LESPs ($p < 0.05$). Furthermore, compared to LEMPs, LESS of 12.5–200 μg/mL, LESPs-50 of 12.5–25 μg/mL and LESPs-80 of 12.5 μg/mL showed higher effects ($p < 0.05$); Compared to LEPPs, LESS of 12.5–200 μg/mL, LESPs-20, LESPs-50 and LESPs-80 of 12.5–100 μg/mL showed higher impacts ($p < 0.05$).

Effects of LESPs and LESS on acid phosphatase activity of RAW264.7

Acid phosphatase is a signal enzyme for macrophage activation that functions as a lysosomal marker enzyme. The activity level of acid phosphatase directly reflects the immunocompetence of macrophages (Akhtar et al., 2020). As shown in Fig. 5c, compared to the control group, LESPs-20, LESPs-50 and LESPs-80 showed increased acid phosphatase activity at the concentrations of 12.5–200 μg/mL ($p < 0.05$), whereas LESS showed enhanced acid phosphatase activity at the concentrations of 25–50 μg/mL ($p < 0.05$). When comparing LESPs-20, LESPs-50 and LESPs-80, LESPs-50 at the concentration of 100–200 μg/mL, as well as LESPs-20 at the concentration of 200 μg/mL and LESPs-80 at the concentration of 100 exhibited the best effect on acid phosphatase activity of RAW264.7 cells. Moreover, compared to LEPPs, LESS of 25 μg/mL, LESPs-20 of 200 μg/mL, LESPs-50 of 100–200 μg/mL and LESPs-80 of 100 μg/mL showed greater effects ($p < 0.05$).

Effects of LESPs and LESS on NO production of RAW264.7

NO is well known as a signaling molecule associated with the cytolytic action of RAW 264.7, and is essential for battling pathogens, parasites and cancer cells (Zhang et al., 2020a, 2020b). As shown in Fig. 5d, compared to the control group, the three purified fractions of LESPs and LESS could significantly activate macrophages to release NO under the entirely tested concentrations from 12.5–200 μg/mL ($p < 0.05$). When comparing among LESPs-20, LESPs-50 and LESPs-80, LESPs-50 at a concentration of 25 μg/mL exhibited the best effect on NO secretion of RAW264.7 cells. Among the concentrations of 50–200 μg/mL, LESS exhibited much higher NO secretion than LESPs ($p < 0.05$). Furthermore, compared to LEMPs, LESS of 100–200 μg/mL had higher effects ($p < 0.05$); Compared to LEPPs, LESS of 12.5–200 μg/mL, LESPs-20 of 12.5–50 μg/mL, LESPs-50 of 12.5–100 μg/mL and LESPs-80 of 25 μg/mL showed higher impacts ($p < 0.05$).

Thus, comprehensively considering the effects of LESPs and LESS on cell viability, phagocytosis, acid phosphatase activity and NO production of RAW264.7, LESS had better immunomodulatory activity than LESPs, and LESPs-50 was more potent than LESPs-20 and LESPs-80. These findings might be due to their structural characteristics. Various studies have reported that the biological activities of polysaccharides depend on their structural features, such as monosaccharide composition, molecular weight, functional group, glycosidic branching, etc. (Akhtar et al., 2020). Monosaccharide composition is an important characteristic of polysaccharides (Wang et al., 2020). Polysaccharides bind immune cells via membrane receptors,

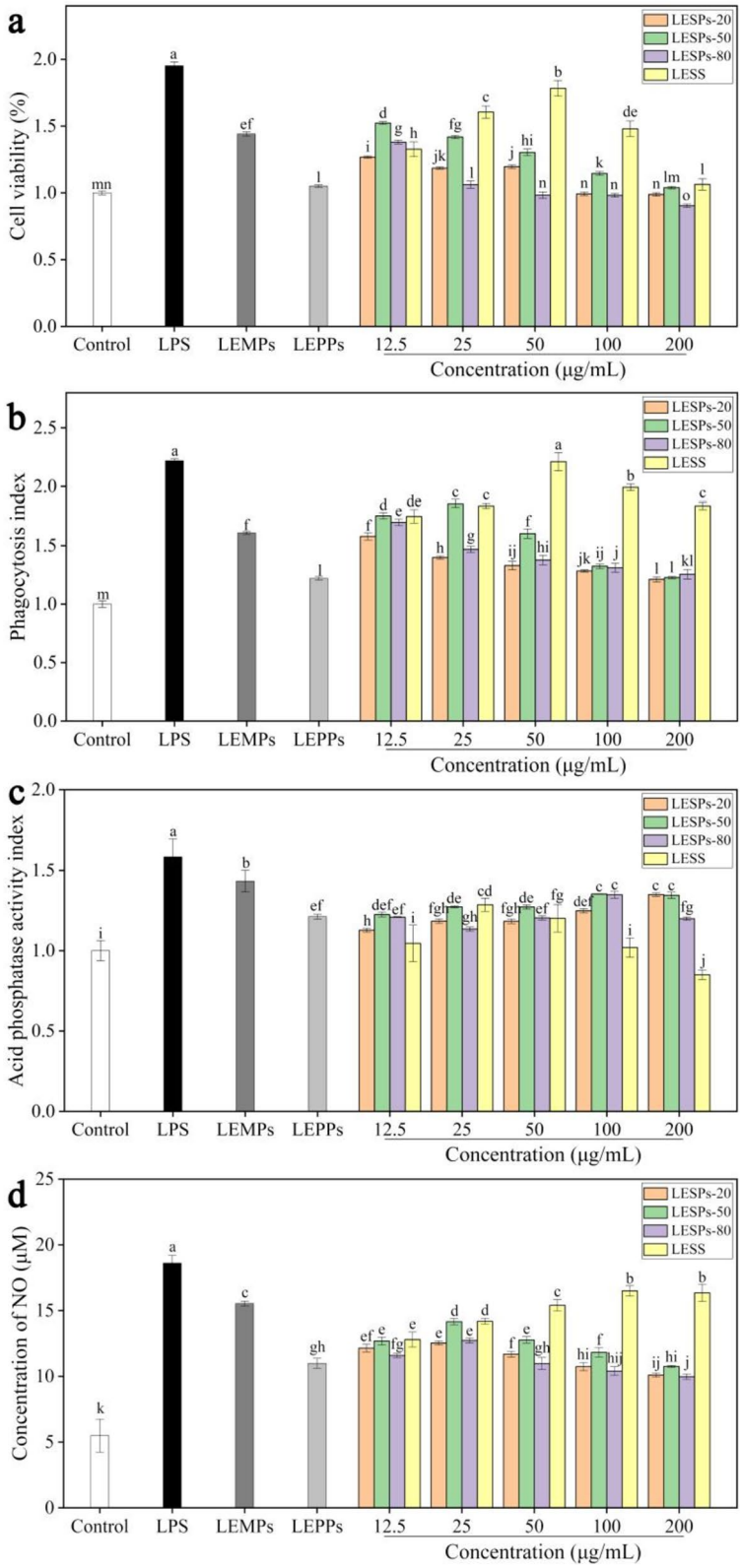


Fig. 5 Effects of LESPs and LESS on (**a**) cell viability, (**b**) phagocytosis, (**c**) acid phosphatase activity and (**d**) NO production of RAW 264.7 macrophages in vitro. Data are expressed as means ± SD (n = 6). The graph bars labeled with different letters on top represent a statistical significance ($p < 0.05$) by an LSD test, whereas bars marked with the same letter correspond to results without significant differences

resulting in the stimulation of intracellular signaling cascades for immunological responses. The stimulating activities of polysaccharides triggered by the recognition of the cell receptors depend on monosaccharide composition (Huang et al., 2014). Generally, *L. edodes* polysaccharides are primarily composed of Glc, Man and Gal, but some other monosaccharides (e.g. Xyl, Fuc and Ara, etc.) are also detected with minor content (Tang et al., 2020; Vetter, 2023; Zhao et al., 2018). Glc, although present as a major monosaccharide component in fungi and presumably forming the backbone of polysaccharides, is not a major determinant factor for in vitro macrophage stimulatory activities. Previous studies have reported that Xyl, Man and Gal played an important role in the stimulation of macrophage (Lo et al., 2007; Wang et al., 2017; Yin et al., 2019). It is also interesting that the polysaccharides most rich in Xyl have higher immunomodulatory activity, suggesting that the presence of Xyl may be important for monocyte/macrophage immunomodulatory activity (Schepetkin et al., 2023; Zhang et al., 2014). Herein, LESS showed the strongest immunomodulatory activity might be attributed to its unique composition of Xyl monosaccharide residues. More detailed information on the mechanism behind the immunomodulatory activity of the Xyl monosaccharide residues will be explored in our future study.

Molecular weight also plays an important role in the functional properties of polysaccharides, but the differences in molecular weight could be not as relevant as other structure features (Ferreira et al., 2015). Some researchers have found that polysaccharides with a lower molecular weight have better immunomodulatory effects, but some studies have revealed that high molecular weight polysaccharides exhibit an immunoregulating effect, while the fractions of low molecular weight did not have an effect (Chen et al., 2020a, 2020b). Herein, the molecular weight of LESS was higher than that of LESPs, which might contribute to the immunomodulatory activity of LESS. Low molecular weight polysaccharides do not easily form a triple-helical conformation, therefore significantly influencing their bioactivity (Chen et al., 2009). However, single-helix glucans or heteroglucans without helical conformation also displayed stimulatory activity in immune cells (Ferreira et al., 2015). It has been suggested that the presence of other monosaccharide residues surpasses the requirement of helical conformations for the exhibition of immunomodulatory activity.

In addition, LESPs-50 with the highest purity showed a better immunomodulatory effect than LESPs-20 and LESPs-80, but not stronger than LESS. Therefore, duo to the complexity and variety of structural features in polysaccharides, further studies are needed to determine which factor finally decides the immunomodulatory effect to affect their application in the fields of functional food and drug development.

The adsorption capacities of LESI

Adsorption capacities, such as WHC, OHC and WSC, are important indexes for evaluating the quality of dietary fibers. The WHC represents the capacity of the most material in water retention when an external compression or centrifugal gravity force is applied, such as hydrodynamic water, physically trapped water and linked water (Wang et al., 2021). High WHC can prevent the shrinkage of foods and alter their viscosity (Elleuch et al., 2010). As shown in Fig. 6a, the WHC value of fermented LESI was up to 4.36 ± 0.17 g/g, which was 1.86 times to unfermented IDF from LES residues. The improvement might be associated with the diverse dietary fibers surface areas, structures and densities, as well as the raised hydrophilic site chemical nature and quantity (Xie et al., 2016).

The OHC plays a vital role in various food processing processes, which is used to assess the ability of dietary fibers to prevent oil loss, such as preventing fat losses in the case of cooking, or removing excessive fat from high-fat foods (Raghav, 2018; Song et al., 2018). As shown in Fig. 6b, the OHC value of fermented LESI was up to 4.51 ± 0.27 g/g, which was 1.94 times to unfermented IDF from LES residues. The changes might depend on the hydrophobicity, hydrocolloid surface property and overall electrical charge density (Jia et al., 2019; Yu et al., 2018).

The WSC is significantly affected by the bonded micellar networks, wrinkled surface and the fiber molecular structure (Jia et al., 2019). As shown in Fig. 6c, the WSC value of fermented LESI was up to 5.13 ± 0.36 g/g, which was 1.64 times to unfermented IDF from LES residues. The increase might be attributed to an increase in the amount of short chains and the surface area of dietary fibers caused by the fermentation (Chen et al., 2014; Zhang et al., 2019).

Generally, after fermentation, the WHC, OHC and WSC of LESI were significantly higher than those of unfermented IDF ($p < 0.05$), suggesting that the fermentation of *A. niger* and *S. cerevisiae* probably loosened the structure of dietary fibers with a rougher surface so that more polar and nonpolar groups as well as short chains were exposed, which could be an effective method applied for the modification of IDF in LES residues. The generated LESI could be a good dietary resource for related food products, which avoid water syneresis in formulated foods, act as an emulsifier for foods with a high fat content and improve mouth-feel experienced by consumers.

In total, it is of great research value and practical significance to extract active polysaccharides and functional

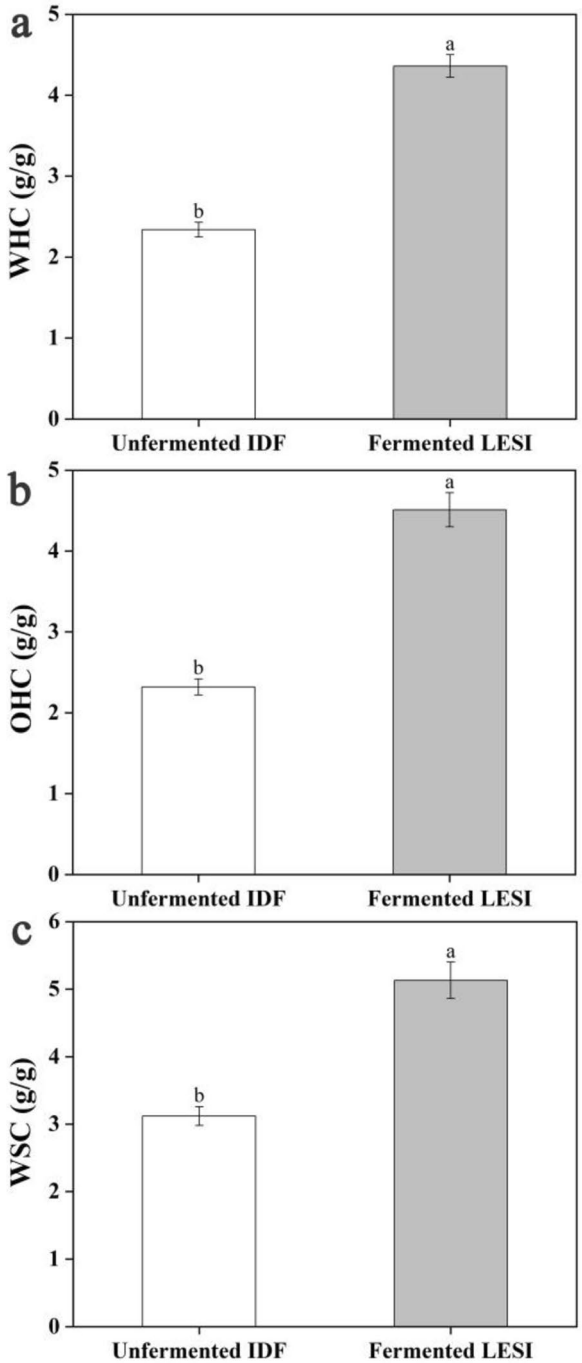


Fig. 6 The (**a**) water holding capacity (WHC), (**b**) oil holding capacity (OHC) and (**c**) water swelling capacity (WSC) of unfermented IDF from LES residues and LESI prepared after fermentation

dietary fibers from inexpensive *L. edodes* stipes. LESPs and LESS with potent immunomodulatory effects could be considered as potential immunoenhancers as food supplements for hypoimmunity (Li et al., 2018). Additionally, LESI have been found to effectively improve the gel properties of pork myofibrillar gels, due to the high WHC capacity of LESI (Lu et al., 2023). The fermented LESI have greater WHC, OHC and WSC capacities than LESI, which might have a wide application in the processing of meat, baking, beverages and dairy products, to improve their stability, water absorption capacity and emulsion strength (Yu et al., 2018).

Conclusions

This study built an efficient strategy to make full of LES. Three polysaccharide fractions (LESPs-20, LESPs-50, LESPs-80) were first obtained by water extraction and gradient ethanol precipitation. The soluble/insoluble dietary fibers (LESS/LESI) were then prepared by *A. niger* and *S. cerevisiae* fermentation, and their optimized conditions consisted of *A. niger* set at 4% (*w*/*w*), *S. cerevisiae* at 8% (*w*/*w*), fermentation temperature at 31 °C, fermentation time at 3 d, the material-to-liquid ratio at 1:12.5. LESS with unique Xyl monosaccharide residues and higher molecular weight showed stronger in vitro immunomodulatory activity than LESPs. LESPs-50 with the highest purity showed a better immunomodulatory effect than LESPs-20 and LESPs-80. Besides, LESS related to more rough, irregular and complicated structures showed improved WHC, OHC and WSC capacities. Since the large amount of *L. edodes* stipes being discarded every day, this study could provide new ideas for the fully utilization of the waste, generating such by-products with great potential to be used as functional additives in the food industry.

Abbreviations

LES	*L.edodes* Stipes
LESPs	*L.edodes* Stipes polysaccharides
LESS	*L.edodes* Stipes soluble
LESI	*L.edodes* Stipes soluble insoluble
RSM	Response surface methodology
BBD	Box-Behnken design
NO	Nitric oxide
WHC	Water holding capacity
OHC	Oil holding capacity
WSC	Water swelling capacity
DF	Dietary fibers
IDF	Insoluble dietary fibers
SDF	Soluble dietary fibers
Rha	Rhamnose
Fuc	Fucose
Ara	Arabinose
Xyl	Xylose
Man	Mannose
Glc	Glucose
Gal	Galactose
GalA	Galacturonic acid
GlcA	Glucuronic acid
LPS	Lipopolysaccharides
MTT	Thiazolyl blue
DMSO	Dimethyl sulfoxide
FBS	Fetal bovine serum
DMEM	Dulbecco's modified eagle medium

LEMPs	*L.edodes* Mycelia polysaccharides
LEPPs	*L.edodes* Pileus polysaccharides
GC	Gas-chromatography
HPGPC	High performance gel-permeation chromatography
SEM	Scanning electron microscopy

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s43014-024-00240-w.

Additional file 1: Fig. S1. Monosaccharide composition of LESPs and LESS. (a) Monosaccharide standards analysis of (1) Rha, (2) Fuc, (3) Ara, (4) Xyl, (5) Man, (6) Glc, (7) Gal, (8) GlcA and (9) GalA; (b) LESPs-20; (c) LESPs-50; (d) LESPs-80 and (e) LESS. **Fig. S2.** HPGPC chromatogram of LESPs and LESS. (a) The calibration curve of 5, 12, 25, 410 and 670 kDa dextrans; (b) LESPs-20, (c) LESPs-50, (d) LESPs-80; (e) LESS.

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Not applicable.

Authors' contributions
Yujiao Sun: Conceptualization, Project administration, Resources, Supervision, Project administration, Writing-original draft and Writing-review & editing; Baobao Li & Yuanye Xue: Data curation, Formal Analysis, Investigation, Methodology and Visualization; Jiankang Wang & Bingbing Miao & Yang Liu: Methodology and Investigation, Investigation and Validation; Yungang Cao & Yanjun Li: Resources; Dawei Chang: Supervision.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Dr. Jiankang Wang is a member of Editorial Board of *Food Production, Processing and Nutrition* and he was not involved in the journal's review of, or decisions related to this manuscript.

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