Extraction, Physicochemical and Film Properties of Polysaccharides from Highland Barley Bran Fermented by *Aureobasidium Pullulans*

Qin Liu,¹ Pei Liu,¹ You Zhou,¹ Jinyu Chang,¹ Xuan Chen,¹ Qingyun Lyu^{1,2,*} and Gang Liu^{1,2,*}

Abstract

Plastic packaging films are not recyclable, difficult to be degraded and thus threaten the environment and the health of humans and other animals. Therefore, the purpose of the research is to derive edible polysaccharide films from highland barley bran fermented by *Aureobasidium pullulans*. Fermentation conditions using UV-mutated *A. pullulans* were optimized and the rheological, mechanical and barrier properties of edible polysaccharide films were analyzed. The polysaccharides produced by mutagenesis were increased by 8.6% after fermentation. The products before and after fermentation contained α - and β -glycosidic bonds, C-O-C, C=O, O-H, and other characteristic peaks of polysaccharides. Post-fermented polysaccharide films (post-FPF) were more moisture resistant than pre-fermented polysaccharide (pre-FPF) and control group (CGPF). Mechanical properties were better for CGPF than post-FPF, but post-FPF had better water solubility. It was feasible to make an edible polysaccharide film with improved water solubility. Post-fermentation polysaccharide films could help to reduce the cost of edible film production and improve the comprehensive utilization of bran.

Keywords: Edible film; Highland barley bran; Pullulan; Rheological property; UV-mutation.

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1. Introduction

Highland (naked) barley is a major cereal crop in Tibet, Gansu, Yunnan, and other regions in China due to its rich nutritional value conferred by cellulose, protein, vitamins, fat, β -glucan, inorganic salts, and other ingredients.^[1] Highland barley is highly resistant to cold, and has a short growth period, high yield, and strong adaptability.^[2,3] It is also a main source of food, fuel, and a raw material used to produce beer, medicine, and health products.^[4-6] Highland barley bran (HBB), a by-product of barley processing, is mainly used as feed or directly discarded in industrial production. To render HBB into food ingredients would be significantly beneficial to the environment by reducing non-recyclable waste.

Pullulan is a water-soluble, extracellular, amorphous, viscous, and linear polysaccharide secreted by *Aureobasidium pullulans* that comprises basic maltotriose

units connected by α -1, 6 glycosidic bonds. This unique linkage gives pullulan good film-forming properties.^[7] Pullulan is often used as a packaging material as it has good color retention, preservation, and oxidation resistance and has been applied in the food, preservation, pharmaceutical packaging, cosmetics, and petroleum industries because it has good plasticity, as well as film-forming and gas barrier properties.^[8,9] It also has other promising applications, but it has not been produced on a large scale because melanin generated during *A. pullulans* fermentation firmly adheres to pullulan and is difficult to be removed. In polysaccharide decolorization, the product recovery rate is greatly reduced, and the cost is high. The retained melanin affects the color and yield of products; therefore, it must be removed.

Bacterial strains can be manipulated by physical and chemical means, complex mutagenesis, gene shuffling, or genetic engineering to obtain high-yield or low-pigment strains. For example, Imshenetskiĭ and Kondrat'eva treated wild *Pullularia pullulans* strains with 0.3% colchicine, and the amount of pullulan produced by a mutant strain was increased by 7–8 mg/L.^[10] Szymańska and Galas irradiated wild *P. pullulans* strains with ultraviolet to obtain two

¹School of Food Science and Engineering, Wuhan Polytechnic University, Wuhan 430023, China

²Key Laboratory for Deep Processing of Major Grain and Oil, Wuhan Polytechnic University, Ministry of Education, Wuhan 430023, China

* E-mail: lqiny2018@whpu.edu.cn (Q. Lyu); lg820823@163.com (G. Liu)

mutant strains, namely, M-u1 and M-u2, which reduced pigment and increased polysaccharide yields to 10 g/L.^[11] Kang *et al.* generated the F3-2 strain through genome shuffling the *A. pullulans* N3.387 strain, and its pullulan yield reached 20.7 g/L.^[12] Ma *et al.* knocked out the pullulan synthase gene of the original HN6.2 strain to increase polysaccharide yield.^[13] However, the development of the pullulan industry is hindered by many technical problems, such as melanin secretion and low pullulan yield. These technical issues can be solved in several ways, such as medium optimization, fermentation condition control, and mutation breeding. Among these, mutation breeding is the most economical selection.^[14] Mutagenic strains with a high polysaccharide yield and low pigment production have rarely been reported, and polysaccharide extraction and applications have not been studied in details.

The present study aims to promote in-depth research and practical applications of extracellular polysaccharides generated by *A. pullulans* by optimizing the fermentation conditions of less pigmented polysaccharides and identifying the properties of the purified polysaccharide films.

2. Material and methods

2.1. Materials

The following materials were used in this study: HBB (Tibetan Crystal Barley Food Co., Ltd., Tibetan, China); *A. pullulans* As.3.837 strain (Guangdong Provincial Fungus Preservation Center, Guangdong, China); Congo red (Shanghai Yuming Industry Co., Ltd., Shanghai, China); total starch (AA/AMG) assay kits (Megazyme International Ireland Ltd., Bray, Ireland); and other reagents (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China).

2.2. Preparation of seed and fermentation broth

Powdered HBB was mixed with distilled water to prepare a turbid fermentation broth. The seed liquid medium comprised 1000 mL of distilled water containing sucrose 50 g, dipotassium hydrogen phosphate 5 g, sodium chloride 1 g, magnesium sulfate heptahydrate, 0.2 g, ammonium sulfate 0.6 g and yeast extract 3 g. Flat medium comprised 1000 mL of H₂O containing potato 200 g, glucose 20 g and agar 20 g at pH 6.5.

These media were sterilized at 121 °C for 30 min using a YXQ-LS-70A vertical pressure steam sterilization pot (Shanghai Boxun Medical and Instrument Corp., Shanghai, China). A single-cell suspension (1 mL) of *A. pullulans* at the logarithmic growth phase was diluted 1,000-fold and added to sterile culture dishes. Flat medium was inoculated with bacterial solution (0.1 mL) via a coating method, stirred for 1 min under a 15 Watt UV light located 40 cm from the sample on an SW-CJ-1G clean bench (Purification equipment Corporation, Suzhou, China), then incubated at 28 °C for 72 h in a GHP-9050 Water-Jacketed Thermostatic Incubator (Qixin Scientific Instruments Co., Ltd., Shanghai, China). Three rings of *A. pullulans* at the logarithmic growth stage

were inoculated into seed liquid medium and incubated at 28 °C for 36 h to prepare bacterial suspensions.

2.3. Determination of HBB component content

Total fat, total protein, moisture, and β -glucan in HBB were determined using GB/T 5009.6-2015, GB/T 5009.5-2016, GB 5497-1985, and Congo red methods. The total starch content was evaluated using Total Starch HK test kits (Megazyme).

2.4. Optimization of fermentation conditions

2.4.1 Standard curves of β -glucan and pullulan

Phosphate buffer solution (0.1 mol/L, and pH 8.0) was prepared, and then Congo red solution (0.1 mg/mL) was configured with this solution.

Standard curve for β -glucan: Standard β -glucan (10 mg) was dissolved in deionized water (100 mL) in a volumetric flask then 1 mL of standard β -glucan solution was diluted to 2 mL with deionized water and reacted with Congo red (4 mL) at 20 °C for 10 min in darkness (the blank: deionized water (2 mL) and Congo red (4 mL)). The maximum absorption at 545 nm in a wavelength range of 200–700 nm was measured using an Evolution 220 scanning ultraviolet spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Solutions with specific graded concentrations were prepared similarly. Absorbance was measured using an Enspire microplate reader (PerkinElmer Life and Analytical Sciences Inc., Waltham, MA, USA) at the maximum absorption wavelength. Concentration was the abscissa (X), and absorbance was the ordinate (Y) to draw the standard curve. The regression equation of the standard curve was $Y = 0.0103X + 0.0075$ ($R^2 = 0.9972$; linear range: 0–31.25 μ g/mL).

Standard curve for pullulan: The QBT 4485-013 Pullulan method was the reference. Concentration was the abscissa (X), and absorbance was the ordinate (Y) to draw the standard curve. The regression equation of the standard curve was $Y = 3 \times 10^7 X - 2 \times 10^6$ ($R^2 = 0.9931$; linear range: 0–1.00 mg/mL).

2.4.2 Single-factor test

Fermentation broth (30, 50, 100, and 150 mL) at ratios of 1:10, 1:15, 1:20, and 1:25 was placed in 250-mL Erlenmeyer flasks and sterilized at 121 °C for 15 min. The initial pH of the broth mixed with HBB and distilled water was adjusted to 5.5, 6, 7, 8, and 8.5, and the inoculum volume was 2 mL. The mixture was disrupted at 200 rpm using a DDHZ-300 shaker (Suzhou Peiying Experiment Equipment Ltd., Co., Suzhou, China) at a constant fermentation temperature of 28 °C for 1, 2, 3, 4, and 5 days.

Cells in the fermentation broth were pelleted by centrifugation at 4,000 rpm for 10 min. The supernatant mixed with absolute ethanol (2 volumes) was passed through 0.22- μ m filters and incubated at 4 °C overnight to precipitate polysaccharides. Polysaccharide precipitate was obtained, and then residual crude polysaccharides were sedimented by

centrifugation at 4000 rpm for 5 min and then the two parts of polysaccharides were dried at 50 °C. The absorbance of β -glucan was measured using a TU-1900 UV-visible spectrophotometer (Beijing Puxi General Instrument Co., Ltd., Beijing, China), and the peak area of pullulan was measured with an UltiMate-3000 HPLC (Thermo Fisher Scientific Inc.) in crude polysaccharide solution diluted with deionized water.

2.5. Physicochemical properties of polysaccharides before and after fermentation

2.5.1 Purification of polysaccharide

Polysaccharides from bran before fermentation and fermented broth were purified, respectively. After removing the starch and protein using the Sevag method, and salting out method,^[15] the polysaccharides were lyophilized and concentrated in vacuo.

2.5.2 Scanning electron microscopy (SEM)

Pre- and post- fermented bran, pre-fermented (from unfermented bran), and post-fermented (from fermented broth) polysaccharides were dried, and their morphology was assessed using a 160 S-3000N SEM (Hitachi Ltd., Tokyo, Japan). Samples were flattened with double-sided tape, then sputter-coated with gold. Samples were magnified $\times 1000$ at an accelerated voltage of 15 kV.^[16]

2.5.3 Fourier transform infrared spectroscopy

The infrared absorption spectra of samples were determined in the range of 650–4000 cm^{-1} using a Nexus 670 Thermo Nicolet Fourier Transform Infrared Spectrometer (Thermo Fisher Scientific Inc.) under the following conditions: resolution, 4 cm^{-1} ; beam precision, 0.01 cm, number of scans, 16 times; ambient temperature, 25 °C.^[17]

2.5.4 Rheological properties

Polysaccharides (1.0%, 1.5% and 2.0%) were stirred, heated at 60 °C and completely dissolved in distilled water, then viscosity was measured as shear rates using a Kinexus Pro+ rheometer (NETZSCH-Gerätebau GmbH., Selb, Germany). The test conditions were as follows: parallel plate clamp, 40 mm; gap setting, 150 μm ; test temperature, 25 °C; and shear rate range, 1–1000 s^{-1} . Solutions of polysaccharide (1.0%, 2.0%, 2.5%, and 3.0%) were prepared as described above. The dynamic viscoelasticity of each polysaccharide solution was measured as angular frequency using a rheometer under the following test conditions: parallel plate clamp, 40 mm; gap setting, 1 mm; test temperature, 25 °C; angular frequency range, 0.1–100 r/s.^[8,18]

2.6. Properties of polysaccharide films

2.6.1 Preparation of polysaccharide film

Experimental (pre- or post- fermented polysaccharide (0.3 g) with glycerol (0.1 g)) and control (β -glucan (0.08 g), pullulan

(0.22 g), and glycerol (0.1 g)) groups were dissolved in 20 mL of distilled water, then polysaccharide films were prepared as described by Chang *et al.*^[19]

2.6.2 Oxygen barrier properties (OBP)

The conical flasks containing 3 g of vegetable oil were sealed with a film and set in a thermostat at 50 °C for 5 days. The OBP (g/100g) was expressed by the peroxide value of the oil and was calculated according to the method of Chang.^[19] The calculated formula was as follows:

$$OBP = \frac{0.1269(V - V_0)C}{M} \times 100$$

where 0.1269 is mass of iodine equivalent to 1mL sodium thiosulfate standard titration solution; V represents the volume of sodium thiosulfate consumed by the sample (mL); V_0 represents the volume of sodium thiosulfate consumed by the blank sample (mL); C means concentration of sodium thiosulfate standard solution (mol/L) and M is the mass of the sample (g).

2.6.3 Water vapor transmission (WVT)

The WVT ($\text{g}/\text{m}^2 \cdot \text{d}$) of the films was measured in accordance with method of Chang.^[19] The calculated formula was as follows:

$$WVT = \frac{\Delta M}{AT}$$

where ΔM (g) represents the increased mass of the desiccant after 24 hours; A (m^2) means the area of water vapor permeation; T (d) means the interval time.

2.6.4 Tensile strength (TS)

Parameter was measured using a TA.XT2i instrument (Testometric, S.M.S. Corporation, USA). The TS (MPa) of films was measured in accordance with the method of Chang.^[19] Three samples (width of 10 mm and length of 20 mm) cut from each film were evaluated. Test conditions were set as follows: the measurement speed, 10 mm/min; the measurement speed, 1 mm/min; the initial grip interval, 26 mm; the force, 5 g. The calculated formula was as follows:

$$TS = \frac{F}{A}$$

in which, F means the maximum force (N); A represents the cross-sectional area of the sample film (mm^2).

2.6.5 Water dissolution time (WDT)

Film samples (15 mm wide and 15 mm long) were set in boiling water and the time for the films to completely dissolve was recorded.^[19]

2.7. Data analysis

All data are shown as the means $\pm$ SD of three independent replicates per sample. Values with $p \leq 0.05$ were considered statistically significant. Mean values were analyzed by ANOVA using SPSS (v19.0; IBM Corp., Armonk, NY, USA) software.

Fig. 1 Cell morphology of *Aureobasidium pullulans*, colonies, and polysaccharides before and after mutagenesis. (A–C) Cell morphology of pre-mutagenic *Aureobasidium pullulans*, colonies, and polysaccharide produced by fermentation, respectively. (D–F) cell morphology of colonies and polysaccharides respectively produced by fermentation after mutagenesis (A and D 125 × 10 times).

Table 1. Comparison of *Aureobasidium pullulans* before and after UV mutagenesis.

train	Colony color	Colony diameter (mm)	Texture	Fermentation color	Polysaccharide color	Pullulan yield (mg/ml)
Wild type	Brown green	5	Smooth leathery surface; difficult to grasp; produces mycelia	Brownish green	Brownish green	3.4
Mutated	White	3	Smooth, moist surface; easy to grasp; produces sterile silk	Light yellow	White	12

3. Results and discussion

3.1. Content of Highland barley bran components

The content of soluble β -glucan was lower in HBB than in hulled barley (3.65% vs. 4.83%).^[20] The starch, water, protein, and fat contents in HBB were 36.88%, 8.37%, 3.8%,

and 3.93%, respectively. Thus, HBB can provide carbon and nitrogen sources for *A. pullulans* growth.

3.2. Effects of mutagenesis

Fig. 1A shows that wild type *A. pullulans* cells appeared (a) yeast-shaped and (b) expanded, (c) and had chlamydo spores

Fig. 2 Effects of material-to-liquid ratios, initial pH, liquid loading, and duration of fermentation on amount of extracted polysaccharide. Effects of material-to-liquid ratio (a), initial pH of fermentation broth (b) liquid loading (c) and fermentation duration (d) on amounts of extracted polysaccharides.

and (d) tangled hyphae with brownish green cell walls before mutagenesis. *Aureobasidium pullulans* during fermentation produces pullulan and melanin, that firmly adhere to each other.^[21] Mechanical disruption and ultraviolet irradiation caused the bacterial coils to break up into circular single cells (Fig. 1D) with a morphology that was similar to that described by others.^[22] In Fig. 1B and C, the color of the strain changed from brownish green to white after UV mutagenesis.

Fig. 1C and F show that polysaccharides generated by mutagenic strains were whiter than those generated by non-mutagenic strains. The DNA strands of *A. pullulans* were broken by UV light, resulting in mutations^[23] and a loss of melanin function. Black cells turned white and did not produce hyphae. Table 1 shows that the pullulan yield was

increased by 8.6% after mutagenesis and changed the original brownish green color to white.

3.3. Single factor tests

3.3.1 Effects of solid-to-liquid ratios on amounts of extracted polysaccharide

Fig. 2a shows that the polysaccharide content in bran was initially increased, then decreased with increasing the ratio of solid to liquid. The pullulan and β-glucan contents were maximal at a solid-to-liquid ratio of 1:20.

3.3.2 Effect of initial pH of fermentation broth on polysaccharide extraction

The acidity and alkalinity of the fermentation broth affected enzyme activities, as well as bacterial growth and metabolism, thus influencing the yield of polysaccharide. Fig. 2b shows that the amount at pH 5.5–8.5. More β-glucans

were produced when the initial pH of the fermentation broth was < 6.0 , but the pullulan yield was very low. Pullulan production was maximal when the initial pH of the fermentation broth was 7.0. Thus, the initial pH should be 7.0 to ensure the total yield of polysaccharides.

3.3.3 Effects of liquid volume on polysaccharide extraction

Aureobasidium pullulans is an aerobic bacterium, and the volume of liquid affects the contents of dissolved oxygen and substrate. Pullulan production was initially increased, then decreased when liquid volumes ranged from 30 to 150 mL (Fig. 2c). The amount of β -glucan was increased initially, then decreased, and increased again. The value was maximal when the volume was 50 mL. The production of β -glucan decreased substantially at 100 mL because it was utilized by *A. pullulans* in the presence of oxygen under this condition. Very little polysaccharide was produced, because the amount of liquid and substrates that could be utilized by *A. pullulans* was insufficient. As the liquid volume was increased, the amount of substrate and dissolved oxygen became sufficient to promote pullulan production. When the volume of liquid was increased again, the decreased amount of dissolved oxygen per unit volume resulted in the ability of *A. pullulans* to be inhibited.

3.3.4 Effects of duration of fermentation on amount of extracted polysaccharide

The strain started to grow and proliferate at the beginning of fermentation when the amount of substrate was sufficient, and total polysaccharides were increased, reaching the maximum on day 3 (Fig. 2d). The amount of produced polysaccharide was gradually decreased over time, perhaps due to a nutrient deficiency and the breakdown of polysaccharides by bacterial enzymes.^[24]

3.4. Physicochemical properties of polysaccharides before and after fermentation

3.4.1 Scanning electron microscopy

Bran particles became finer with more diamond-shaped fragments after fermentation (Fig. 3a and b). These findings (bran particles with more diamond-shaped fragments) indicated that *A. pullulans* secreted a large amount of enzymes to degrade and utilize the bran during fermentation. Nordlund found using microscopy that the structures of stained cell walls in the aleurone layer of fermented rye bran are almost completely degraded and the cell contents are largely released.^[25]

Fig. 3 Surface morphology bran and saccharides before and after fermentation. Pre-fermented (a) and post-fermented (b) bran. Pre-fermented (c) and post-fermented (d) polysaccharide.

Fig. 3c and d shows that polysaccharides extracted before and after fermentation were agglomerated. However, the surface structures of pre-and post-fermented polysaccharides were loose with many pores, and compact, respectively. These structural differences might be attributed to viscous pullulan, which was added after fermentation and tended to adhere to other polysaccharides.

3.4.2 Fourier transform infrared spectroscopy

Fig. 4a and b shows that the absorption peaks characteristics of purified polysaccharides were similar between before and after fermentation. The peaks in the $1,200 - 800 \text{ cm}^{-1}$ regions are mainly the infrared absorption peaks of polysaccharide. The peaks at 850 and 890 cm^{-1} are respectively characteristic of α - and β -glycosidic bonds.^[26] The peaks at $1,196$ and $1,038 \text{ cm}^{-1}$ are characteristic of C-O-C stretching vibration, whereas that at 1380 cm^{-1} is the deformation vibration of C-H. In addition, the peak at $1,659 \text{ cm}^{-1}$ was characteristic of the stretching vibration of C=O, whereas that at $2,926 \text{ cm}^{-1}$ was characteristic of the C-H stretching vibration. The stretching vibration of polysaccharide O-H was near $3,354 \text{ cm}^{-1}$.^[27] The purified

materials before and after fermentation were polysaccharides.

Fig. 4 Infrared spectra of polysaccharides before and after fermentation. Polysaccharides after (a) and before (b) fermentation.

Fig. 5 Rheological properties of polysaccharides before and after fermentation. Viscosity curves (a) and (c) and $\tan\delta$ - ω curves (b) and (d), before and after fermentation respectively.

Table 2. Properties of polysaccharide films.

Film	OBP (g/100g)	WVT (g/m ² ·d)	TS (MPa)	WDT (s)	β-Glucan content (mg/20 mL)
Pre-FPF	0.271±0.007 ^b	61.543±5.001 ^b	3.365±0.481 ^b	96.333±7.767 ^a	197.49±1.053 ^a
Post-FPF	0.264±0.001 ^b	90.182±3.576 ^a	3.066±0.722 ^b	55.000±5.000 ^c	78.06±0.262 ^b
CGPF	1.124±0.007 ^a	57.706±2.851 ^b	16.347±0.377 ^a	79.020±5.291 ^b	80.00±0.010 ^b

Note: Values are means ± SD. Superscript letters indicate significant differences $p < 0.05$ ($n = 3$). CGPF, control polysaccharide film; Post-FPF, post-fermented polysaccharide films; Pre-FPF, pre-fermented polysaccharide films.

3.4.3 Rheological properties

Rheological properties affect the ability of matter to retain moisture and this determines its potential value to industrial applications.^[28] The viscosity of the system increases with increasing the particle size.^[29] The shear frequency and viscosity curves showed that the viscosity of the polysaccharides before and after fermentation decreased with increasing shear frequency, indicating that they were non-Newtonian fluids. The three-dimensional network structure formed by particles was destroyed by shearing, resulting in decreased viscosity and shearing thinning. The viscosity and $\tan\delta$ - ω curves of the polysaccharides before and after fermentation are shown in Fig. 5a–d. The $\tan\delta$ - ω diagrams show that most $\tan\delta$ values for the pre-fermented polysaccharides were < 1 , and those for 1% and 3% polysaccharide before fermentation were ≤ 1 , and those of 2% and 2.5% polysaccharide were < 1 at low frequencies. At a frequency of > 1 , $\tan\delta > 1$ gradually increased, indicating that the 1% and 3% polysaccharides had essentially gel-like properties, whereas the 2% and 2.5% pre-fermentation polysaccharides gradually changed from gel properties to dilute solutions with increasing vibration frequency. Most $\tan\delta$ of polysaccharides after fermentation at different concentrations was ≥ 1 at increased vibration frequencies, indicating that the 1% to 3% polysaccharides after fermentation were close to the nature of a dilute solution. In summary, the viscosity of the polysaccharide after fermentation was lower than the viscosity of the polysaccharide before fermentation.

3.5. Properties of polysaccharide films

The above test results showed that post-fermented edible polysaccharide composite films would be feasible as inner packaging films. Table 2 shows a better moisture resistance for post-FPF than pre-FPF because of the increased pullulan content and good moisture resistance after fermentation. The β -glucan contents of CGPF and post-FPF did not significantly differ ($p < 0.05$). Oxygen barrier performance was better for CGPF than post-FPF probably because post-FPF contained impurities that affected its structure. Polysaccharide-formed films were

available before and after fermentation. The tensile strength of post-FPF changed little, and post-FPF dissolved faster than pre-FPF. However, compared with CGPF, tensile strength was much lower due to impurities in the extracted polysaccharides. The tensile strength of post-FPF requires further improvement.

4. Conclusions

The optimal conditions for fermenting HBB using *A. pullulans* to obtain substantial amounts of pullulan and β -glucan comprised a material-to-liquid ratio of 1:20, initial pH 7, 50 mL of liquid, and 3 days of fermentation. The surface of polysaccharides before fermentation was porous and loose. The viscosity of pre-fermented polysaccharide was higher than that of post-fermented polysaccharide. The test results of the mixed membranes showed that post-fermented polysaccharide was capable of forming a film. The oxygen barrier properties and tensile strength of post-FPF and pre-FPF were similar. The water solubility of post-FPF increased and the moisture barrier of post-FPF was better than pre-FPF and CGPF, but the tensile strength of post- and pre-FPF was lower than that of CGPF. Post-FPF had distinct barrier properties and tensile strength that would be useful in applications with low requested mechanical properties of edible films, for example, the inner packaging of confectionery. The mechanical properties of post-FPF can be improved by combining it with additives. This method can be used to prepare post-FPF as edible films for food preservation.

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Supporting information

Not applicable

Conflict of interest

The authors have no conflicts of interest to declare.

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Author information

Qin Liu is a master student since 2018 under the supervision of Associate Professor Qingyun Lyu in the college of Food Science and Engineering, Wuhan Polytechnic University (China). She obtained her bachelor's degree in engineering from the School of Biological and Pharmaceutical Engineering, Wuhan Polytechnic University in 2018. Her master project focuses on cereal chemistry.

Qingyun Lyu obtained his PhD from Gifu University (Japan) in 2010. He served as a lecturer in the college of Food Science and Engineering, Wuhan Polytechnic University (China) after graduation and became an associate professor in 2014. His current research focuses on grain processing and cereal chemistry.

Gang Liu is a professor in food Science and Engineering, Wuhan Polytechnic University (China). He obtained his PhD degree from South China University of Technology in 2011, and received a two-year postdoctoral training at the Department of Food Science and Technology, the University of Tennessee (United States). After postdoctoral research, he started his independent academic career in Wuhan Polytechnic University. His research interest focuses on the development of basic and applied research on the chemical properties, molecular modification, and nanostructure-functional properties of biomacromolecules from food, such as protein and saccharides.

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