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Valorization of Bread Waste to Fungal-Based Products for Medical Textile and Food Applications

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Valorization of Bread Waste to Fungal-Based Products for Medical Textile and Food Applications

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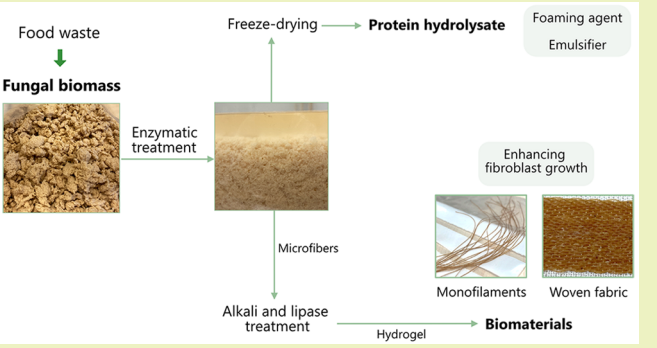
ABSTRACT: The current study aimed at the valorization of bread waste in a fungal biorefinery for the recovery of protein hydrolysate for food applications and monofilaments for medical textile applications. *Rhizopus delemar* was cultivated on bread waste in a 1 m^3 airlift bioreactor to obtain fungal biomass. The protein hydrolysate was isolated as a soluble fraction after a mild enzymatic treatment of fungal biomass with a protease enzyme. The recovered protein hydrolysate was rich in eight essential amino acids and showed foaming and emulsion properties. The fungal microfibers rich in chitin and chitosan were recovered as an insoluble fraction of fungal biomass during the protease treatment process. A hydrogel of the fungal microfibers was wet-spun to monofilaments, which showed high elongation at break. In in vitro scratch assay, the monofilaments demonstrated significant improvements of the rate of cell migration and wound closure compared to viscose fibers (which are commonly used in wound healing dressings). Furthermore, fungal biomaterials in the form of microfibers, hydrogel, and monofilaments showed excellent biocompatibility against fibroblast cells and significantly enhanced cell growth at higher concentrations (above 500 μg/mL). This work suggests a sustainable approach to using abundant food wastes to create value-added products for food and medical textile applications.



KEYWORDS: *chitin/chitosan, food waste, fungal biomaterials, protein hydrolysate, filamentous fungi, biomaterials, wound healing*

INTRODUCTION

There are environmental concerns regarding the use of nonrenewable resources and especially the impact that these materials have on the environment after their use.[1] To replace fossil-based materials, there is a need for new circular approaches to produce sustainable materials. Filamentous fungi can be used in biorefineries valorizing low-cost feedstocks[2] to produce a broad range of products, such as chemicals, enzymes, polymers, and proteins. Often lipids, chitin/chitosan (cell-wall components), and proteins are found in fungal biomass of Zygomycetes.[3,4] The previous taxonomic group Zygomycota has been replaced by several phylum-level clades; in this work, we refer to strains belonging to the taxonomic group Mucoromycota, the largest group of zygomycetes fungi.[5,6] There are mucoromycetes strains used in fermented food products for human consumption, such as *Rhizopus delemar*, which are generally regarded as safe.[3] Therefore, utilizing these strains facilitates the production of food, feed, or materials from filamentous fungi.

There is a need for climate-smart substitutes to animal proteins, and one potential candidate is mycoprotein, a food component with high contents of protein and fiber from fungi.[7] Protein-rich fungal biomass can be used directly as the mycoprotein. Alternatively, the fungal cell wall, rich in nondigestible polysaccharides, can be separated or disintegrated from the fungal cell to obtain a more concentrated extract of intracellular components such as amino acids, peptides, and nucleotides.[8,9] However, studies on the extraction of proteins for food applications from filamentous fungal biomass are scarce. Different treatments can be used to separate and recover fungal proteins from their cell-wall polysaccharides, such as alkaline extraction with isoelectric precipitation,[10] alcohol−alkaline extraction,[11] hydrolysis with proteolytic enzymes, and mechanical treatment.[8] There is interest in the food industry to replace traditional chemical food additives with extracts from natural sources. A potential

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application for protein or other components from fungal biomass in the food industry is using them as foaming agents and emulsifiers.[12] Foaming agents have the role of facilitating the dispersion of a gaseous phase in a product, while emulsifiers can facilitate the mixing of two immiscible liquid phases.[13] Proteins often have both polar and nonpolar amino acids, which can stabilize emulsions by attaching to oil−water interfaces.[14]

The fungal cell wall isolated from fungal biomass containing mainly polysaccharides can be considered for numerous applications. Mucoromycetes fungi can produce chitosan from chitin via an intracellular enzyme (chitin deacetylase), and therefore fungal strains belonging to this group have a high proportion of chitosan in their cell walls.[15] Chitosan has been applied as a wound treatment material because of its biocompatible, antimicrobial, and nontoxic properties.[16] Fungal chitosan is also a promising material for medical applications, but it has been less studied and applied compared with chitin/chitosan from crustacean sources. Fungal chitosan from mucoromycetes fungi such as *Cunninghamella elegans* and *Mucor rouxii* have been reported to show inhibitory activity against bacteria.[17,18]

Cultivation of filamentous fungi can generate several value-added products from low-cost substrates such as food waste. In our previous studies, *R. delemar* grown on bread waste yielded a fungal biomass rich in fibers, proteins, and lipids.[19] Microfibers were isolated from fungal biomass through alkali treatment, and by the addition of lactic acid, a hydrogel was formed. Wet spinning of the hydrogel resulted in the alignment of fungal microfibers and the formation of monofilaments with antibacterial and biocompatible properties.[20] However, in the alkali treatment of fungal biomass, the protein fraction is dissolved in a sodium hydroxide (NaOH) solution while being heated in an autoclave. The harsh conditions involved in this process, including high pH, pressure, and temperature, will deteriorate the protein, which reduces the quality of the recovered proteins and limits their application potential.[21] In addition, the isolated microfibers contained residues from other fractions of the biomass (approximately 4.5 mol % protein and 7.6 mol % lipids), which may affect the properties of the hydrogel formed from the microfibers.

We hypothesized that the quality of the protein fraction would be improved if it was recovered first from fungal biomass using a mild proteolytic enzyme treatment instead of an alkaline treatment. The solid fraction remaining after the protease treatment, containing mainly the cell wall of the fungus, was further subjected to an alkali treatment to prepare the cell-wall material in the form of microfibers. We also hypothesized that removing the residual lipids in the microfibers could facilitate the formation of a hydrogel by increasing the exposure of the amino groups on chitosan to the acid. Therefore, the cell-wall material was also treated with a lipase enzyme before the formation of a hydrogel by the addition of lactic acid. Finally, the wet-spinning procedure used in our previous work was improved to collect longer filaments, with improved flexibility. The produced monofilaments were evaluated for medical textile applications. This approach could enable the recovery of all major components in fungal biomass for the development of multiple value-added products.

EXPERIMENTAL SECTION

Chemicals and Materials. Protease from *Bacillus* sp. (Protamex, P0029), lipase from porcine pancreas (Type II, L3126), L-(+)-lactic acid solution (88−92%), and lysozyme from chicken egg white (≥40000 units/mg of protein) were purchased from Sigma-Aldrich (Germany). Ethanol (EtOH; absolute ethanol) was purchased from VWR (France).

Preparation of the Protein Fraction and Alkali-Insoluble Materials (AIMs). Fungal biomass was obtained from cultivation of *R. delemar* CBS 145940 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) on bread waste in a 1 m^3 airlift bioreactor according to a previous work.[22] Fungal biomass (pressed biomass in wet form with about 20% dry weight) was washed with distilled water and pressed again. Triplicate samples were taken from the biomass and dried in an oven, and the average dry weight of the samples was calculated. The pressed biomass, in wet form, was then subjected to an enzymatic treatment to recover the protein fraction of the fungal biomass (Figure 1). The biomass was dispersed in distilled water to achieve a 3% (w/w) suspension, and the suspension was ground using ultrafine grinding (Suzuku, Japan). The distance between the stones (MKE46 grinding stones) was set to +50 μm, and the suspension was passed through the grinder three times in total at a rotor speed of 2700 rpm. The ground suspension was mixed with the protease (10 mg/g of dry weight biomass), and the treatment was conducted in a rotary evaporator (Laborota 20, Heidolph Instruments, Germany) with total reflux at 60 °C, 60 rpm for 4 h. The liquid fraction after the protease treatment was collected by sieving the suspension through a kitchen sieve with fine mesh and was thereafter freeze-dried (CoolSafe 55 ScanLaf A/S, Lynge, Denmark). The protease-treated biomass was washed with distilled water, pressed, and then treated with a dilute alkali solution to obtain the AIM. The pressed protease-treated biomass was used for the alkali treatment with a ratio of 50 mL of 0.2 M NaOH/g of dry weight of protease-treated biomass, and the treatment was conducted in an autoclave for 20 min at 121 °C. The AIM was separated from the liquid fraction by sieving with a kitchen sieve with fine mesh and washed with distilled water until neutral pH was observed (with a pH paper indicator). The washed AIM was pressed and stored in wet form at 4 °C until use.

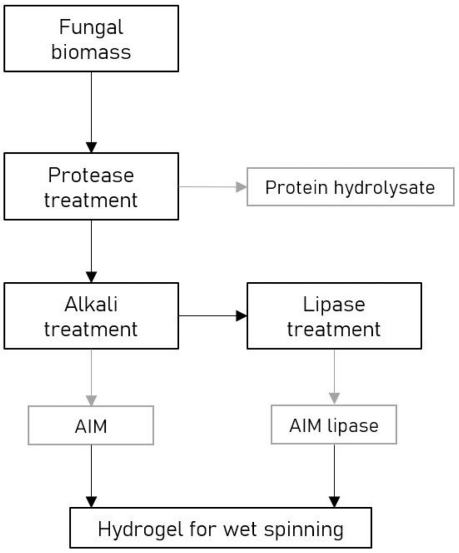

Figure 1. Process from fungal biomass to protein hydrolysate and AIMs.

The AIM was applied to an enzymatic treatment with lipase to remove any fatty acids remaining in the cell-wall material.[20] The AIM was diluted with distilled water to 1% (w/w), and lipase powder (100 mg/g of dry AIM) was mixed with the dispersion for 1 h at 37 °C and pH ∼7.4. Thereafter, the AIM was washed with distilled water twice and pressed in a kitchen sieve with fine mesh. The obtained wet AIM had a dry weight of approximately 6−7% (w/w). Hydrogels with 6% (w/w) dry weight were prepared from the wet forms of the AIM or lipase-treated AIM, by the dropwise addition of a 3.5 M lactic acid

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solution until gelling occurred and a homogeneous hydrogel was obtained. After 1 h of gelation time, the hydrogel was finally centrifugated (1 min, 4000*g*) to remove any air bubbles. The hydrogel was kept in a refrigerator until further use.

Wet Spinning. The prepared hydrogels from the AIMs were subjected to a wet-spinning method previously described,[23] with some modifications. A coagulation bath containing 90/10% (w/w) EtOH/distilled water was used for the wet spinning of the monofilaments. The hydrogel was injected using a syringe pump with a speed of 10 mL/h (WPI, Friedberg, Germany) through a 10 mL syringe and a needle (1.2 mm diameter and 50 mm length). The coagulated monofilaments were taken up on a roll after 2 min of coagulation time (Figure S1) and predried under hot air for 1−2 min. The semidried monofilaments were then transferred to a vertical position, fixed between two points, and allowed to dry completely (>1 h).

Emulsion and Foaming Properties of Fungal Protein Hydrolysate. To measure the emulsification capacity, 30 mg of freeze-dried protein hydrolysate was mixed with 3 mL of deionized water, and the pH of which was adjusted to 3, 5, 7, or 9 with 2 M NaOH or 2 M hydrochloric acid (HCl). The samples were fully dispersed by vertexing for 1 min, then mixed with 1 mL of sunflower oil (edible, purchased from a local supermarket, Sweden), and homogenized at 20000 rpm. After that, the emulsion activity index (EAI) and emulsion stability index (ESI) of fungal protein hydrolysate were measured and calculated according to the method described by Abdollahi et al.[24]

To evaluate the foaming properties, 15 mL of the sample (10 mg of freeze-dried protein hydrolysate/mL, pH 7.0) was transferred to a 100 mL glass beaker with mixing (10000 rpm), and after 2 min of foam formation, the foam was immediately transferred to a 100 mL graduated cylinder. The foam volume was recorded at 0 and 30 min. The foaming capacity (FC) and foaming stability (FS) were determined using eqs 1 and 2, where V_{fi} is the volume of foam immediately after homogenization, V_{li} is the volume of the initial liquid sample, and V_{ft} is the volume of foam remaining after 30 min.

$$\text{FC}\ (\%) = \frac{V_{fi}}{V_{li}} \times 100 \tag{1}$$

$$\text{FS}\ (\%) = \frac{V_{ft}}{V_{fi}} \times 100 \tag{2}$$

Enzymatic Degradation of the Monofilaments. The degradation properties of the monofilaments were tested with an enzymatic degradation test for 28 days. Autoclaved fibers (0.01 g) were mixed with 3 mL of a lysozyme solution (0.8 mg/mL) in phosphate-buffered saline (PBS; $n = 3$). As a reference, fibers in only PBS were tested. The enzyme solution was changed with a fresh enzyme solution (in PBS) every third day to ensure the activity of the enzyme. At days 7, 14, 21, and 28, samples were washed three times with distilled water and then dried at 70 °C overnight to measure the residual weight according to eq 3.

$$\text{residual weight}\ (\%) = \frac{\text{initial weight of fibers}}{\text{final weight of fibers}} \times 100 \tag{3}$$

Cell Culture for a Cellular Biocompatibility Test. Human foreskin fibroblasts (HFF; Royan Institute cell bank) at passage 7 were used for proliferation assay. Dulbecco's modified Eagle's medium (Gibco, 11965092) containing 10% fetal bovine serum (Gibco, 26140079), 2 mM L-glutamine (Gibco, 35050061), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, 15140122) was used to cultivate the cells in 96-well plates. The cells were seeded at 1500 cells/well with 150 μL/well of media. The fungal biomass derivatives AIM, biomass (B), monofilament (F), and gel (G) were autoclaved (121 °C for 20 min) and added to cell culture inserts [96-well plate inserts, polyester (PET) membrane, 1.0 μm pore size, Corning, 10043832]. The inserts with the fungal biomass derivatives were placed onto the plates with the cells. Different concentrations of the fungal biomass derivatives were used: 1, 10, 50, 100, 250, 500, 1000, and 1500 μg/mL. The cells were incubated at 37 °C with 95% humidity and 5% CO_2. The cells without any treatment were considered to be the control group.

Cell Proliferation Assay Based on Cellular Mitochondrial Activity (MTS Assay). The cell mitochondrial activity, which is an indicator of the cell number or cell proliferation rate, was measured by colorimetric assay using MTS/PMS reagent (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega Corp., G5430). MTS/PMS reagent contains a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, MTS] and an electron coupling reagent (phenazine methosulfate, PMS). The cells were exposed to the compounds for 7 days. On day 8, the culture inserts were removed, the medium was replaced with 80 μL/well of a fresh medium, and the cell mitochondrial activity was measured by MTS assay. Assay was done by adding 20 μL of MTS/PMS reagent to each well of a 96-well plate and incubating for 3 h at 37 °C in a CO_2 incubator. The absorbance of the wells was measured using an ELISA microplate reader at a 492 nm reference wavelength and subtracted from the absorbance at a background wavelength at 630 nm (OD 492-630). The experiments were done in triplicate.

In Vitro Scratch Assay Test. In vitro scratch assay was performed to evaluate the potential of wound-healing properties of the monofilament, based on the method presented by Suarez-Arnedo et al.[25] Briefly, human dermal fibroblast cells were cultured in 24-well plates at a concentration of 1×10^5 cells/well until 80% confluency was reached. Unique-size scratches were made on cell cultures with a 200 μL sterile pipette tip using a sterile ruler, and thereafter detached cells were washed away using PBS. Horizontal reference lines were drawn on the bottom of the plate with an ultrafine tip marker to prepare a grid for alignment for image acquisition. The samples (viscose fiber and monofilament) were autoclaved (121 °C, 20 min) and loaded into cell culture inserts (Falcon 24-well plate inserts, PET membrane 1.0 μm pore size, Corning, 353104). Loaded inserts were then placed into the wells of culture plates. The final (w/v) ratios of the samples were 20 mg/mL. For imaging, the cell culture inserts were removed and kept in sterile conditions, and then plates were placed under a phase-contrast microscope using the reference marks as a guide. At 12 and 24 h, images were captured using a Zeiss inverted microscope. The scratch area, wound coverage of the total area, and average scratch width were determined using *ImageJ* software. Finally, the rate of cell migration (RM) and percentage of wound closure were determined.

Characterizations. The contents of *N*-acetylglucosamine (GlcNAc) and glucosamine (GlcN) polymers, as a representative for chitin and chitosan, respectively, in the freeze-dried AIM ($n = 3$) were determined using the method presented by Mohammadi et al.[26] Analysis of arabinan, galactan, glucan, and xylan was performed by neutralization of the sulfuric acid-hydrolyzed AIM samples with calcium carbonate, followed by centrifugation to remove the solids. Finally, the samples were filtered through 0.2 μm filters and analyzed with high-performance liquid chromatography (HPLC) using a lead(II)-based column (Aminex HPX-87P, Bio-Rad, Hercules, CA) operating at 85 °C with 0.6 mL/min ultrapure water as an eluent and a refractive index detector (Waters 2414, Waters Corp., Milford, CT).

The protein content of the protein hydrolysate was calculated based on measurement of the nitrogen content using the Dumas method with a nitrogen analyzer (LECO, St. Joseph, MI) according to Sajib et al.[27] The amino acid composition of fungal protein hydrolysate was analyzed according to the method described by Abdollahi et al.[24] A total of 10 mg of the protein hydrolysate sample was hydrolyzed by adding 4 mL of 6 N HCl and incubating at 110 °C for 24 h. Hydrolyzed samples were then diluted 20 times using 0.2 M acetic acid and automatically injected into an liquid chromatograph/mass spectrometer (Agilent 1100 HPLC, Agilent Technologies, Waldbron, Germany) with a Phenomenex column [C18 (2), 250 μm × 4.6 μm × 3 μm]. The separation was conducted at 0.7 mL/min for 40 min using different ratios of mobile phase A (3% methanol, 0.2% formic acid, and 0.01% acetic acid) and mobile phase B (50% methanol, 0.2% formic acid, and 0.01% acetic acid). The samples were run in duplicate and compared against amino acid

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standards. Tryptophan and cysteine were not measurable with this method.

Scanning electron microscopy (SEM) was used to assess the morphology of the materials and wet-spun monofilaments. Ultrahigh-resolution field-emission SEM (Hitachi S4800) and a 3 kV acceleration voltage were used to obtain the images, and a layer of 2 nm palladium/platinum was coated on the samples. Fourier transform infrared (FT-IR) spectroscopy was used to obtain the spectra of the protein hydrolysate and AIM samples. The freeze-dried AIM samples were briefly dried (105 °C for 1 h) prior to analysis to remove any residual moisture. A Nicolet iS10 FT-IR spectrometer (Thermo Scientific, Waltham, MA) was used to obtain the spectra in absorbance mode (32 scans and a resolution of 4 cm^{-1}). The thermal degradation behavior of the protein hydrolysate was investigated with thermogravimetric analysis (TGA). The protein hydrolysate (10−15 mg, n = 3) was heated in a Q500 TA Instruments (Waters LLC, Hazelwood, MO) from 30 to 700 °C with a rate of 20 °C/min under a N_2 flow. The ash content of the protein hydrolysate was measured after ashing 0.3 g of the sample (n = 3) in a muffle furnace (Gallenkamp, London, U.K.) at 550 °C. The testing of the mechanical properties of the monofilaments was conducted as described in a previous work.[20] Briefly, monofilaments (n = 5) with a diameter of 0.2 mm were tested with a Tinius Olsen tensile tester. The elastic modulus was calculated from the slope of the stress−strain curve between 0.25 and 0.5% strain.

RESULTS AND DISCUSSION

This work aimed at the valorization of food waste by utilizing filamentous fungi to produce several value-added products. Fungal biomass from *R. delemar* grown on bread waste consists of mainly protein, polysaccharides, and lipids.[19] Thereby, bread waste was valorized into two products derived from fungal biomass: a protein fraction in the form of protein hydrolysate and microfibers, which were wet-spun into monofilaments.

Composition of Fungal Protein Hydrolysate. Previously, we investigated the cultivation of *R. delemar* on bread waste in different scales, where the protein content was between 27 and 36% of fungal biomass.[19] In this work, the fungal biomass produced (yield of 0.15 g/g of bread waste) is expected to have a similar composition. The enzymatic treatment with protease yielded a recovered fiber-rich solid fraction of 85 ± 1% (w/w) of fungal biomass; thereby, approximately 15% was extracted as the protein fraction. The protein hydrolysate contained 46.7% (w/w) protein according to the Dumas method (Table 1). Protein extract separated from the cell wall of Brewer's yeast by enzymatic hydrolysis with papain was reported to have a protein content of about 63%.[28] Other fractions of the protein hydrolysate (Table 1) were comprised of 9.8% glucan, 1.8% arabinan, and 10.6% ash.

Table 1. Crude Composition of Freeze-Dried Protein Hydrolysate from Fungal Biomass

component	% of protein hydrolysate
protein	46.7 ± 0.3
glucan	9.8 ± 0.3
arabinan	1.8 ± 0.1
ash	10.6 ± 0.5

In the fungal cell, glycoproteins are located outside the chitin/chitosan-rich layer of the cell wall and soluble proteins in the cytoplasm.[29] Liu et al. recovered protein extracts from yeast cells by breakage of the cell wall in order to recover intracellular cytoplasmic components with high protein content.[31] Cell-wall disruption of a filamentous fungus with highly branched structure and resistant cell walls can be more challenging than that of yeast or bacteria;[30] therefore, to achieve a more complete extraction of protein from filamentous fungal biomass, extensive mechanical disruption of the cell wall is probably required. The recovery of protein from *R. delemar* fungal biomass could possibly be improved by mechanical disruption of the cell wall and a more efficient enzymatic treatment. Zeng et al.[9] reported that the mechanical treatment of fungal biomass followed by isoelectric precipitation to obtain a protein extract yielded a 71% recovery of the initial protein content of the biomass, and the protein extract had a 77% protein content.

In the FT-IR spectrum of the protein hydrolysate in Figure 2a, the main contribution from the protein in the sample can be assigned to the prominent amide I band appearing at 1645 cm^{-1} and amide II at 1516 cm^{-1}.[32] Polysaccharides (mainly glucan) possibly contribute to the band at 1083 cm^{-1} (C−O stretching). The thermal degradation behavior of the protein hydrolysate was investigated by TGA to evaluate different onsets of degradation for components in the material. The initial weight loss of 5% is connected to the residual moisture in the freeze-dried sample (Figure 2b), which is followed by a steep curve from around 190 to 360 °C from the degradation of proteins and polysaccharides.[22] Because the compositional analysis showed around 47% protein in the sample, the degradation peak in the differential thermal gravimetric (DTG) curve at 310 °C is thought to be mainly connected to protein degradation. The final weight loss, seen as the second shoulder in the DTG curve at 390 °C, could possibly be lipid degradation[33] and the residue (27%) left at 700 °C residual char.

Amino Acid Profile and Functional Properties of Protein Hydrolysate. Protein extracts can be used for different food applications, and their functional properties can be measured to evaluate their suitability for specific applications. The amino acid profile can also give an indication of the nutritional value of proteins. Protein hydrolysate contained all eight essential amino acids (Table 2), which were measurable using the acid hydrolysis method. The amino acids with the highest content were glutamic acid and aspartic acid, which comprised 78.6 and 58.7 mg/g protein, respectively. These two amino acids also play an important role in the creation of umami flavor[34] in animal-based food, which can be a potential application for the recovered fungal protein. Thereafter, the essential amino acids isoleucine, leucine, and lysine had yields of 42.4, 40.2, and 37.3 mg/g protein, respectively. The levels of threonine, isoleucine, and phenylalanine in the fungal protein hydrolysate sample are above the daily recommended intake for adults.[35] Similarities can be found in the levels of essential amino acids in the recovered protein in our study compared to the amino acid profile of the product from Quorn.[36] However, the levels of nonessential amino acids were higher in the protein recovered in this study. The amino acid profile of protein hydrolysate aligns with the amino acid profile previously reported for the whole fungal biomass (from *R. delemar* grown on bread waste).[19]

Emulsifiers can be used as food additives, where surface-active properties of the emulsifier promote droplet dispersion, which, in turn, increases the stability of the emulsion, the shelf-life, and the textural properties of the product.[37] Protein hydrolysate's potential as an emulsifier was assessed by measuring the EAI and ESI at different pH values. The

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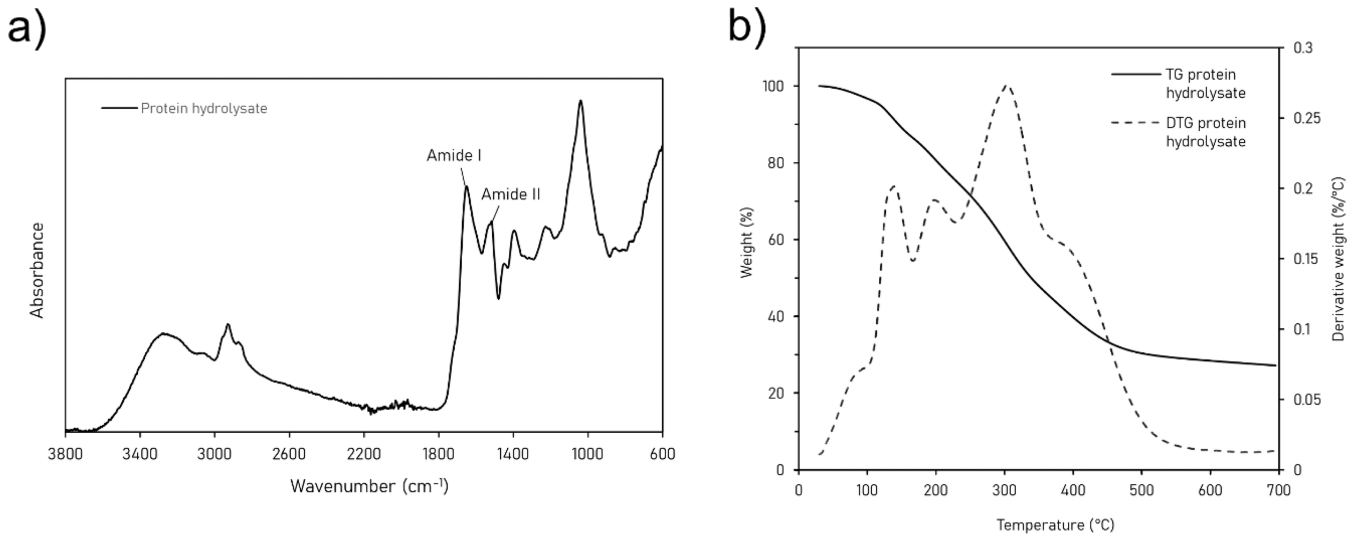

Figure 2. (a) FT-IR spectrum and (b) TGA thermogram of fungal protein hydrolysate.

Table 2. Amino Acid Composition of Fungal Protein Hydrolysate

amino acid	mg/g protein (this work)	mg/g mycoprotein[36]	FAO/WHO adult (mg/g protein)[35]
valine[a]	24.9 ± 0.9	28	39
threonine[a]	31.4 ± 1.1	25	23
isoleucine[a]	42.4 ± 0.2	24	30
leucine[a]	40.2 ± 0.9	39	59
lysine[a]	37.3 ± 1.3	38	45
methionine[a]	3.4 ± 0.4	10	17
histidine[a]	1.5 ± 0.8	16	15
phenylalanine[a]	19.4 ± 0.2	23	19
glycine	20.1 ± 0.5	20	
serine	34.1 ± 0.7	23	
alanine	32.2 ± 2.0	28	
arginine	10.2 ± 1.1	33	
aspartic acid	58.7 ± 0.3	46	
glutamic acid	78.6 ± 1.4	56	
proline	21.5 ± 0.3	20	
tyrosine	17.7 ± 0.8	18	
EAAs[b]	200.5 ± 1.4	209	
NEAAs[c]	273.1 ± 2.8	246	

[a]Essential amino acid. [b]EEAs: essential amino acids. [c]NEAAs: nonessential amino acids.

emulsifying activity of protein hydrolysate was between 320 and 1450 m^2/g, with the highest acitivity at pH 7 and the lowest at pH 9 (Figure 3). The emulsion stability was highest at pH 9 (ESI = 300 min), and from pH 3 to 7, it ranged between 30 and 40 min. The emulsion properties of an unexploited coproduct after RNA-reduction treatment on the fermented product Quorn (*Fusarium venenatum*) containing residual biomass, carbohydrates, nucleotides, and proteins with 57% nitrogen-containing material showed an EAI of 15 m^2/g and an ESI of 120 min at pH 7,[12] indicating that recovered proteins from fungal biomass have improved emulsion properties compared to residual streams with a mixture of cell-wall components and protein. This could be due to either the higher purity of the proteins or their partial hydrolysis improving their solubility and interfacial properties.

Protein hydrolysate was analyzed for its foaming properties. Protein can be applied as a foaming agent in the food industry,

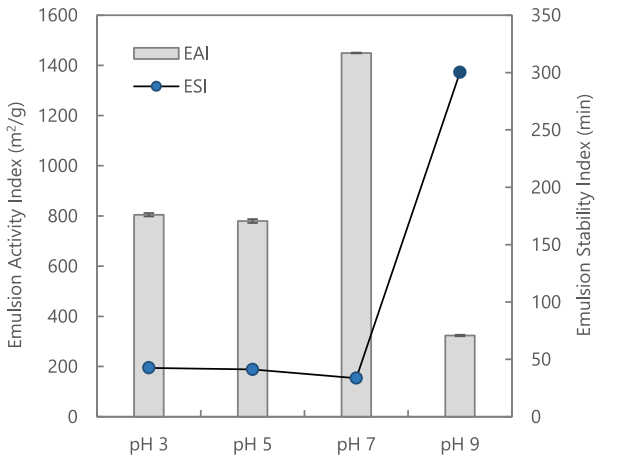

Figure 3. EAI and ESI of protein hydrolysate at pH 3−9.

providing texture in aerated food products.[38] Protein hydrolysate showed a FC of 26.7% and a FS of 62% at pH 7, as presented in Table S1. Moura et al.[10] reported high FC (120%) and FS (91%) at pH 7 of protein isolate from *Pencillium maximae* fungal biomass. Protein isolate was obtained by alkaline extraction followed by isoelectric precipitation, and a relatively low yield of protein isolate was achieved (3.5% mass recovery).

AIM Composition. Preliminary experiments indicated that the gelling ability of the microfibers first appeared after the alkali treatment with dilute NaOH, indicating that the use of only an enzymatic treatment to separate the protein from the cell wall was not enough to obtain the gelling properties of the microfibers. Therefore, both protease and alkali treatments were applied in combination to achieve a hydrogel from the microfibers. Compositional analysis showed that, after lipase treatment, the content of chitin/chitosan in the AIM was slightly increased (Table 3), which can explain the improved gelling ability and spinnability in the wet-spinning procedure. Therefore, the AIM after lipase treatment was used for monofilament production. The compositions of the AIM resemble our previous findings;[20] however, here the glucan amount is lower, which indicates that any bread residue was removed during the protease and alkali treatments, and this is

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Table 3. Composition of AIMs

	AIM (g/g)	AIM lipase (g/g)
GlcN polymer[a]	0.319 ± 0.011	0.350 ± 0.008
GlcNAc polymer[b]	0.217 ± 0.006	0.234 ± 0.002
glucan	0.015 ± 0.004	0.014 ± 0.001
arabinan	0.013 ± 0.001	0.014 ± 0.001
galactan	0.018 ± 0.002	0.014 ± 0.002
xylan	0.016 ± 0.004	0.012 ± 0.002

[a]Chitosan. [b]Chitin.

also supported by the 10% glucan present in protein hydrolysate. Early on, glucans were thought to be present in the spores of zygomycete fungi but not in the hyphae.[39] Minor amounts of β-1,3-glucan in the vegetative phase of mucormycotina fungi have been indicated.[40,41] The structure of the materials was confirmed with FT-IR. The high contents of chitin/chitosan in the AIM samples are confirmed by the typical bands in Figure S2 appearing around 3287 cm^{-1} (O−H stretching), 2919 cm^{-1} (C−H stretching), 1625 cm^{-1} (amide I), 1559 cm^{-1} (amide II), and 1310 cm^{-1} (amide III).[42]

Morphology of the Materials. In Figure 4a, the protease-treated fungal biomass is shown. The microfiber diameter was in the range of 5−10 μm, which is consistent with our previous findings.[23] The microfibers in Figure 4a−c became gradually thinner, indicating that, after alkali treatment with NaOH, the microfibers (Figure 4b) had been separated from the residues of the protein (which were not isolated during protease treatment) and, after enzymatic treatment with lipase, the residues of lipids had possibly been removed. The addition of lactic acid to the lipase-treated AIM caused swelling of the microfibers, and an interconnected structure was formed (Figure 4d), thereby forming a gel-like material. The gel formation was better for the AIM after lipase treatment; thereby, the hydrogel of AIM lipase (Figure 4d) was chosen to produce monofilaments with the wet-spinning procedure, where the microfibers were aligned along the axis of the coagulated monofilament (Figure 4e). In preliminary experiments, using pure EtOH resulted in brittle coagulated filaments with white swollen surfaces because of the formation of porous areas, which can be caused by rapid dehydration of the filament.[43] To avoid the surface irregularities caused by pure EtOH, the coagulation bath was adjusted to a ratio of 90/10% of EtOH/water. The monofilaments had a tensile strength of 30 ± 3 MPa and exhibited high flexibility, having an elongation of break of 28 ± 5% (Table S2), which could be assigned to the predrying step. The monofilaments were demonstrated for their flexibility by weaving a structure with viscose yarn (Figure S4). The filaments could easily be integrated into the structure of the textile without major breaking of the filaments (Figure 4f).

Enzymatic Degradation of Fungal Monofilaments. The wet-spun monofilaments were evaluated for their degradation properties by incubation with lysozyme. The monofilaments had residual weights of 68 and 63% after 1 week of incubation in a lysozyme solution and PBS, respectively (Figure 5). The higher residual weight for the monofilaments in an enzyme solution could be assigned to the entrapment of enzyme inside the structure of the fibers, which was impossible to wash off. The initial weight loss after 1 week indicates that components of the cell-wall material with higher solubility have been dissolved or possibly smaller pieces of microfibers have loosened from the surface of the monofilaments. It has been suggested that, with increasing amounts of *N*-acetyl groups present in chitosan fibers, the degradation rate is expected to increase.[44] The results indicated that the high fraction of GlcN in the material entailed a low affinity for the lysozyme to break down the monofilaments; therefore, no effect of enzymatic degradation could be seen after 4 weeks,

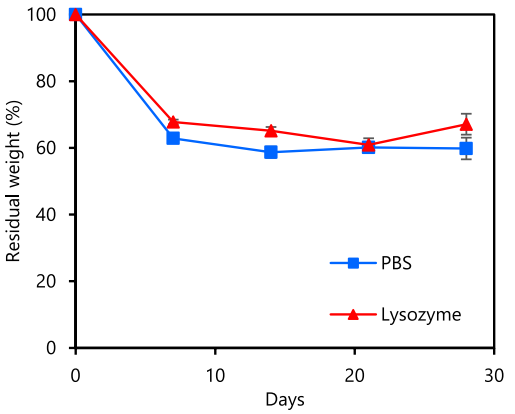

Figure 5. Degradation behavior of the monofilaments in PBS and a 0.8 mg/mL lysozyme solution (in PBS) after 28 days.

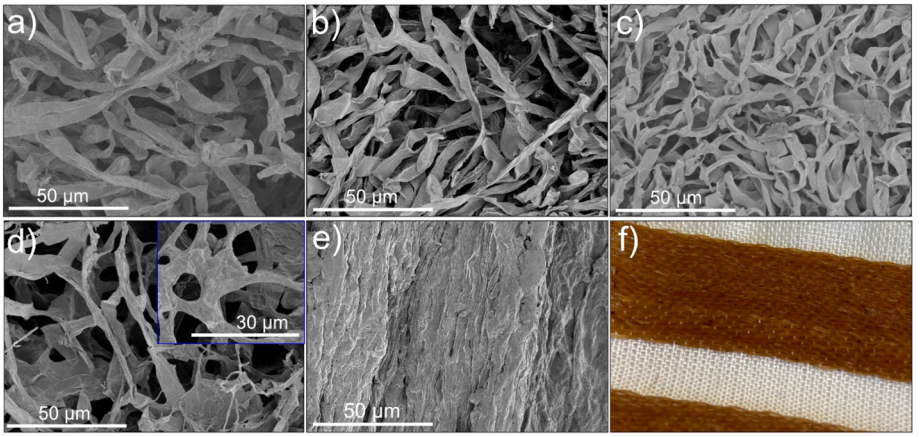

Figure 4. SEM images of (a) protease-treated biomass, (b) AIM, (c) AIM lipase, (d) hydrogel of AIM lipase, and (e) surface of the monofilament. (f) Photograph of woven fabric.

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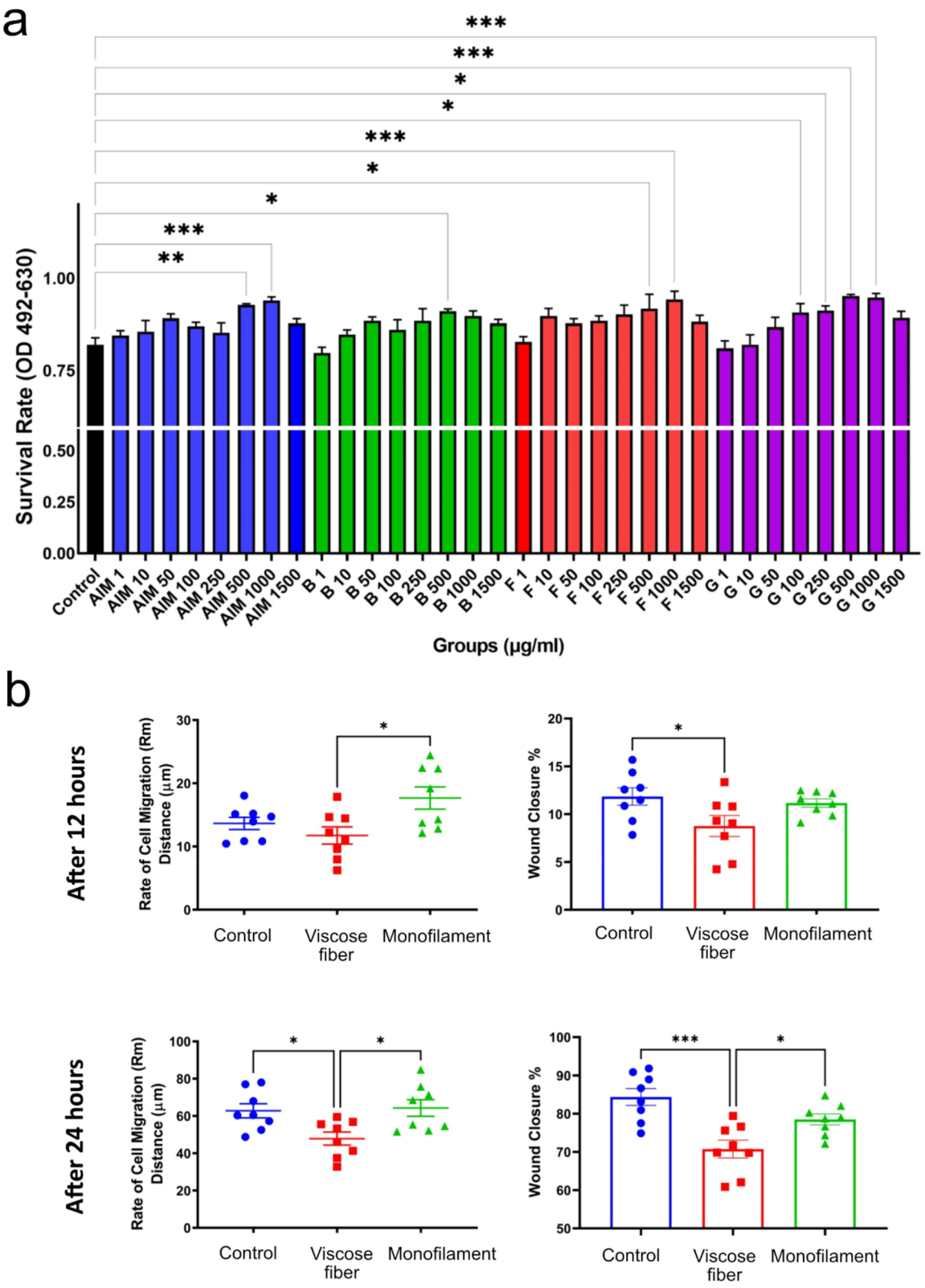

Figure 6. (a) Possible cytotoxic or proliferative effects of fungal biomass derivatives (AIM = alkali-insoluble material, B = biomass, F = fungal monofilament, and G = fungal hydrogel) at different concentrations (1−1500 μg/mL) on HFF. (b) In vitro scratch assay with the RM and percentage of wound closure for the viscose fiber, monofilament, and control samples at 12 and 24 h. Significant differences are represented using *, **, and *** symbols, which respectively signify $P < 0.05$, $P < 0.01$, and $P < 0.001$.

where the residual weight was stable from week 1 to 4. In addition to the low affinity of the lysozyme for the amino groups, the structure of the monofilaments may hinder the transportation of the enzyme inside the monofilament. Figure 4e shows the surface of the wet-spun filament, where the fungal microfibers are aligned in a tightly packed manner. To assess

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the complete degradation of the monofilaments, studies with longer incubation time are needed (more than 4 weeks). Chitosan materials with a more porous structure could promote degradation. Chitosan scaffolds have a porous structure, enabling the enzyme to be transported to the structure of the material as well as allowing any degradation products to diffuse from the structure.[45] The degradation of wet-spun chitosan fibers showed that chitosan with a higher degree of deacetylation had a lower degradation speed, and the lowest residual ratio achieved after 5 weeks was around 85%.[46] Wet-spun chitin fibers, on the other hand, were reported to have a higher degradation rate, reaching down to 53% residual mass after 15 days in a lysozyme/PBS solution.[47]

Biocompatibility of Fungal Biomaterials toward Fibroblast Cells and the Wound-Healing Properties of Monofilaments. The biocompatibility of the fungal materials was evaluated to assess the possible medical applications of the products. A thorough investigation of the cytotoxicity was conducted throughout the whole process of fungal monofilament production, including fungal biomass, AIM, fungal hydrogel, and monofilaments, at a wide range of concentrations (Figure 6a). There were no cytotoxic effects in any of the groups and a higher survival rate than the reference was observed above 500 μg/mL in all groups. The results from the 500 and 1000 μg/mL groups indicated that at these concentrations fungal biomaterials could support the proliferation of cells; furthermore, for the hydrogel, this proliferative effect was even initiated at lower concentrations of 100 and 250 μg/mL. Fungal biomass-derived biomaterials created a suitable niche for the cells and increased cell attachment, which stimulated cell growth at higher concentrations. Therefore, these biomaterials may be promising candidates for the development of scaffolds for tissue engineering applications. The results were consistent with our previous findings on the biocompatibility and cell proliferation of wet-spun monofilaments[20] and confirmed the safety and potential of these materials for medical applications.

Viscose fibers are commonly used in wound dressings, while compounds such as chitosan have been incorporated in such materials to enhance the wound-healing properties.[48,49] The results of in vitro scratch assay with human dermal fibroblast cells demonstrated significant improvements in the RM and percentage of wound closure (Figure 6 b) in both the monofilament and control groups compared to the viscose fiber group after 24 h of treatment ($P < 0.05$). This suggests that the viscose fiber group experienced a delayed healing process during scratch assay, while the healing process in the monofilament group closely resembled that of the control group (shown in the 24-h period images, Figure S6). Additionally, significant differences in the RM (monofilament vs viscose fiber) and percentage of wound closure (control vs viscose fiber) were observed (Figure 6b) as early as 12 h of cell culture ($P < 0.05$). The scratch assay results indicate that the monofilament group demonstrated superior outcomes, suggesting that the filaments in this group are capable of effectively restoring the normal healing process. Conversely, the viscose fiber group exhibited a delay in the healing process during in vitro scratch assay.

CONCLUSIONS

Bread waste was successfully valorized into valuable products (protein hydrolysate and monofilaments) in a fungal biorefinery concept. Fungal protein hydrolysate exhibited promising foaming and emulsifying properties and contained several essential amino acids. All fractions obtained in the process of conversion of fungal biomass to fungal monofilaments showed biocompatibility against fibroblast cells and improved cell viability with increased concentrations of the materials. The in vitro scratch assay test indicated a higher wound-healing capacity of fungal monofilaments compared to viscose fibers, which often are used to make wound-healing dressings. This work represents a scalable method in which, through the valorization of 1 kg of bread waste, 150 g of fungal biomass was obtained, which subsequently was used for the production of 22.5 g of fungal protein hydrolysate and 37.5 g of fungal microfibers for the formation of hydrogel and subsequent monofilament production, for potential applications in food industry and medical textiles, respectively.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssusresmgt.3c00021.

Foaming properties of protein hydrolysate, schematic picture of the wet-spinning setup, FT-IR spectra of AIM and AIM after lipase treatment, tensile properties of monofilaments, stress−strain curves from tensile testing, description of woven fabric, phase-contrast images from cell cultures prior to MTS assay, and in vitro scratch assay images (PDF)

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Notes

The authors declare no competing financial interest.

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