



Valorization of Stale Bread Through Solid State Fermentation Using *Neurospora intermedia*

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Received: 6 October 2025 / Accepted: 25 February 2026
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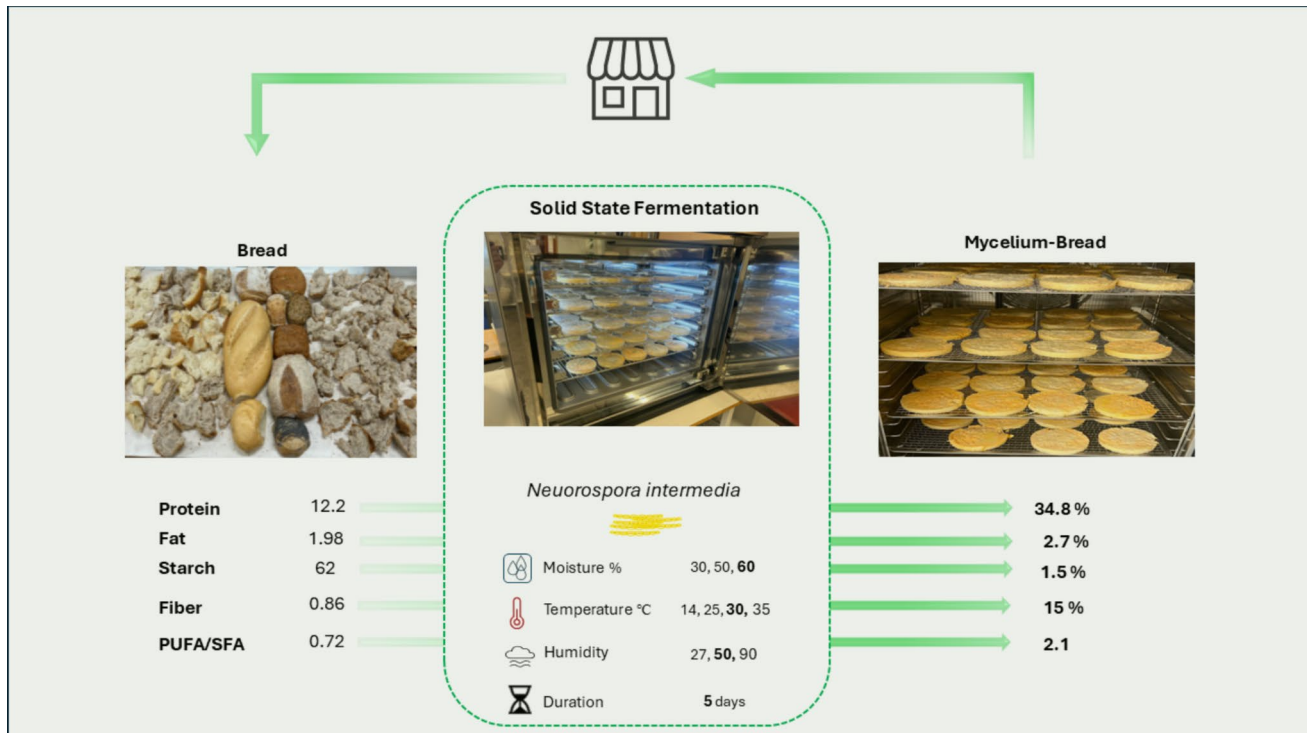
Abstract

Bread waste represents a significant fraction of organic waste in Europe, creating both environmental challenges and opportunities for valorization. Solid-state fermentation (SSF) with filamentous fungi is a proven approach for upcycling such residues into protein-rich biomass. This study aimed to utilize SSF with the edible fungus *Neurospora intermedia* to convert stale bread into a mycelium-bread product with enhanced nutritional qualities for potential applications in both human food and animal feed. A five-day solid-state fermentation was optimized by systematically varying key environmental parameters (temperature, substrate moisture, and relative humidity). The product generated under optimal conditions was then subjected to a comprehensive nutritional analysis, including its amino acid and fatty acid profiles and mineral content, to evaluate its potential for food and feed applications. Under optimal conditions (30 °C, 60% initial moisture and 50% relative humidity, the crude protein content of the mycelium-bread product increased from 12.2 g/100g dry matter (DM) in the initial bread to 34.8 g/100g DM. The nutritional profile of the optimized product was substantially improved, featuring a marked increase in the essential amino acid lysine and the bioconversion of starch (from 61.6 to 1.44%) into 15.2 g/100g DM of functional fungal fiber. Moreover, a substantial improvement was observed in the lipid quality, evidenced by the PUFA/SFA ratio increasing from 0.72 to 2.10 after the fermentation. This study highlights the potential of SSF using *N. intermedia* to upcycle organic residues such as stale bread into a sustainable protein source with multi-nutrient potential for food and feed applications.

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Graphical Abstract



Keywords Filamentous fungi · Sustainable ingredient · Solid-state fermentation · Stale bread

Statement of novelty

This study presents a sustainable solution, using the edible *Neurospora intermedia* to upcycle surplus bread into a high value product named mycelium-bread. Through a solid state fermentation process we identified the ideal conditions to nearly triple the protein content of stale-bread while also converting its starch into healthy fungal fiber and improving its fat quality. This research provides a practical pathway to create a nutrient dense product from common food waste, offering a practical contribution to the circular economy and the development of more sustainable food and feed application.

Introduction

The finite agricultural and natural resources are under significant pressure due to the increasing demand for food, which is estimated to exceed 9 billion by 2050 [1, 2]. This challenge is intensified by the social, economic and environmental impact of food waste generation. The global food waste rate is approximately 1.3 billion tonnes per year, a loss that affects the resources and contributes to 8–10% of

global greenhouse gas emissions [3]. As a major component of this waste stream, stale bread represents both a significant economic loss and environmental challenges [4, 5].

In Sweden, for instance, bread is a primary contributor to this waste stream, with an estimated 80,410 tons lost annually across the supply chain, largely due to its short shelf life [6]. Despite its loss of freshness, stale bread maintains a valuable nutritional profile that is particularly rich in carbohydrates, rendering it an ideal substrate for microbial valorization to a variety of bioproducts [7]. Various microorganisms including yeast [8], bacteria [9] and filamentous fungi [5] have been used for bioconversion of bread waste into single cell protein (SCP) and single-cell fat (SCF) as a strategy to mitigate nutrient deficiencies.

Among microbial candidates, filamentous fungi are exceptionally well-suited for bioconversion due to their unique metabolic flexibility and secretion of effective hydrolytic enzymes [3]. Their fundamental structural units are hyphae which form an interconnected network known as mycelium [10]. A specific edible species such as *Neurospora intermedia* and *Aspergillus oryzae* generally regarded as safe (GRAS) supported by genomic analyses indicating the absence of known mycotoxin biosynthesis pathways [11]. With a protein content of approximately 56% (w/w) [12], *N.*

intermedia represents a viable SCP alternative to conventional sources such as fishmeal or soybean meal. Additionally, the balanced essential amino acid profile has encouraged the use of fungal mycelium in human food applications, particularly in the development of meat analogues [13].

The optimization of total fat content is a critical consideration in SCP development, as higher fat densities improve the caloric potency and functional utility of the final product for industrial feed applications [14]. Fungal mycelium exhibits characteristic lipid profiles enriched with polyunsaturated fatty acids (PUFAs), including linoleic and α -linolenic acids. For instance, the lipid fraction of *N. intermedia* is particularly high in unsaturated fatty acids such as oleic (41.0–59.8%) and linoleic (19.7–33.9%) acids [15]. This composition enhances the ratio of unsaturated to saturated fats, serving as a key indicator for high-quality lipid valorization. Furthermore, fungi utilize specific transporters embedded in their cell membranes to sequester essential minerals, such as iron (Fe), zinc (Zn), and calcium (Ca), from their environment [16]. These nutritional attributes hold significant potential across several feed sectors; for example, in aquaculture, fungal biomass has demonstrated potential as a fishmeal replacement to enhance growth and gut health in species such as rainbow trout [17].

Mycelium production can be achieved through two primary fermentation methods: submerged (SmF) and solid-state fermentation (SSF) [13]. While SmF typically offers superior process control and mass transfer, it is characterized by high water and energy consumption. Conversely, SSF involves the cultivation of microorganisms on solid substrates with low-moisture and minimal free water, significantly reducing resource requirements and wastewater generation [18]. For the nutritional enrichment of solid substrates such as stale bread, SSF is particularly advantageous, enabling the fungus to efficiently utilize available nutrients like starch as a carbon and energy source [19]. Furthermore, because the final SSF product comprises a mixture of fungal mycelium and the unfermented portion of the substrate, it integrates the nutritional benefits of the mycelium with the inherent properties of the original material.

Integrating bread residues into SSF systems facilitates the simultaneous reduction of food waste and the production of nutrient-dense ingredients, thereby directly advancing circular bioeconomy models [20]. However, the primary challenge of SSF with filamentous fungi involves the difficulty of monitoring and controlling critical variables such as biomass, heat, and mass transfer with ensuring uniform fungal growth throughout the substrate [20, 21]. Previous research on bread valorization by *N. intermedia* has primarily focused on combined submerged-solid-state processes for pigment synthesis or SmF for ethanol production [5, 7]. Direct investigations into bread valorization often overlook whole-substrate conversion in favor of liquid metabolite extraction

[22], leaving a significant gap in literature regarding direct SSF for nutritional upcycling. Consequently, a detailed investigation aimed at optimizing a simplified, direct SSF process for the comprehensive nutritional valorization of stale bread remains lacking. To address this, it is necessary to optimize environmental parameters to maximize the protein yield and nutritional quality of the upcycled product.

Therefore, the aim of this study was to produce a mycelium-bread product that has the potential to be applied as sustainable food and feed ingredient from stale bread through solid state fermentation. For this purpose, SSF parameters were optimized to improve the nutritional value of the final mycelium-bread fermented product. Analysis of the nutritional composition of the resulting fermented biomass was carried out focusing on amino acid composition, fat (fatty acid profiles), starch, fiber and mineral content.

Material and Methods

Experimental Design

The study was structured in a two-phase experimental design. The initial phase focused on optimizing the solid-state fermentation process through a systematic screening of environmental parameters. Key variables, including temperature (14, 25, 30, and 35 °C), relative humidity (27, 50, and 90%), and initial substrate moisture content (30, 50, 60%, and 70%), were investigated across 48 distinct treatments. The yield of each treatment was evaluated by measuring the crude protein, total fat, and carbohydrate content of the resulting product. Subsequently, the single set of conditions that yielded the highest crude protein and total fat concentrations was selected as optimal condition. In the second phase, the product from this optimal fermentation was subjected to comprehensive nutritional characterization, including detailed analysis of its amino acid profile, fatty acid composition, and mineral content, to fully assess its potential as a food and feed ingredient.

Fermentation Process

Stale bread was obtained from Steinbrenner & Nyberg (Mölnådal, Sweden) and thoroughly inspected to ensure the absence of mold contamination. The bread was dried in a 70 °C oven for 3 h and subsequently milled to a particle size of 2 mm using a laboratory milling device. Processing stale bread prior to cultivation was conducted following the methods outlined by [7].

In this experiment, *N. intermedia* CBS 131.92 (Centraalbureau voor Schimmelcultures, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) was employed as the edible filamentous fungi. For sporulation, seed culture

Table 1 Substrate and inoculation used for moisture adjustment

Substrate (g)	Moisture content (%)	Water activity (a_w)
10 ± 0.05	30 ± 1.5	0.935 ± 0.005
	50 ± 1.2	0.967 ± 0.004
	60 ± 1.5	0.979 ± 0.005
	70 ± 1.3	0.993 ± 0.003

was inoculated on potato dextrose agar (PDA) plates and incubated for 4 days, following the described method [21]. The inoculum suspension was prepared by adding 20 ml of autoclaved distilled water to the PDA plates. A plastic spreader was then used to disperse and release the spores. The number of spores in the inoculum was counted using a Bürker chamber under a light microscope (Carl Zeiss Axiostar plus, Germany). A 3 ml spore suspension ($\sim 3.2 \times 10^5$ spores/mL) was subsequently added to autoclaved water which was adjusted according to the desired moisture content for each treatment. The moisture content of the substrate was measured and adjusted using a moisture analyzer device (DBS 60–3, KERN & SOHN GmbH, Germany) detailed in Table 1.

The water and spore mixture were added to the substrate and mixed using a sterile plastic spreader. To minimize the risk of contamination, the process was conducted near a flame. The fermentation process was carried out in Petri dishes for a period of 5 days in climate chamber (HPPeco, Memert, Germany) in order to maintain the required relative humidity and temperature. After fermentation, samples were dried at 45 °C in the oven for 24 h according to [7]. Sample weights were recorded for mass balance calculations according to the reference method [21].

Analytical Methods

Crude protein content was determined by calculating the nitrogen content using the Kjeldahl method using an InKiel P digester and a Behrotest 51 distiller (behr Labor-Technik, Germany) according to [23]. Nitrogen to protein conversion factor was considered as 6.25 [23, 24]. The amino acid profile of both the bread and the final product was analyzed by an accredited laboratory (Eurofins, Lidköping, Sweden) using the EU 152/2009 method. Total fat content was measured using a Soxtec manual apparatus (ST243 Soxtec™, FOSS Analytical Co., Ltd., Suzhou, China) with petroleum ether as the solvent in a 26 mm diameter thimble for the extraction process. The extraction of fatty acids was carried out using the two-step transesterification (2-TE) method [25]. Following the lipid extraction, methylation was carried out using the acidic catalyst. The methylated fatty acids were subsequently analyzed by gas chromatography (GC). The analysis was performed on a Clarus 550 gas chromatograph

(Perkin-Elmer, Norwalk, CT, USA) equipped with a J&W DB-wax capillary column (60 m × 0.25 mm × 0.15 μm DB-23) and interfaced with a mass spectrometric detector operating in electron impact mode. A 1 μL sample was injected with a 15:1 split ratio at an inlet temperature of 250 °C. Nitrogen was used as the carrier gas at a constant flow rate of 1 mL/min. The oven temperature program was as follows: an initial hold at 50 °C, a ramp of 11.9 °C/min to 175 °C, a second ramp of 1.9 °C/min to 230 °C, and a final hold for 5 min.

The total starch content before and after fermentation was measured using a standard protocol (AOAC Method 996.11) using thermostable α-amylase and amyloglucosidase to hydrolysis maltodextrins into D-glucose units. The HPLC-system consisted of Waters alliance separation modul2695 (Waters Corporation, Milford, Massachusetts, MA, USA) at a wavelength of 490 nm was conducted to quantification of glucose. The water activity was measured for each moisture treatment using a LabSwift-aw instrument (Novasina AG, Switzerland). Ash content was determined through burning samples in a muffle furnace at 550 °C for 3 h using porcelain crucibles according to [26].

Non-starch polysaccharides including crude fiber (CF), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured using a fiber analyzer apparatus (ANKOM 2000i, ANKOM Technology Co. Ltd., New York, NY, USA) following the filter bag methodology [27]. Furthermore, mineral detection and concentration analysis were performed using a Microwave Plasma Atomic Emission Spectroscopy (MP-AES 4200, Agilent Technologies, Santa Clara, CA, USA). Prior to analysis, the samples were digested using a high-performance microwave digestion device (ETHOS™ UP, Milestone Srl., Italy) in maximum power of 1000W for 32 min in four steps increasing the temperature to 210 °C according to the reference method [28].

Statistical Analysis

All fermentation treatments in the screening phase were performed in triplicate. To ensure analytical precision, measurements for crude protein and total fat were conducted in triplicate for each experimental replicate. The resulting data were analyzed using a one-way analysis of variance (ANOVA), and a response optimizer function was subsequently used to identify the optimal conditions for achieving the maximum theoretical values for both crude protein and total fat. The treatment identified by this optimization was then selected for further analysis, with samples prepared in triplicate for the determination of fatty acid, amino acid, and mineral profiles. Data from treatments with a 70% initial moisture content were excluded from the statistical analysis due to recurrent microbial contamination across all temperatures.

All statistical computations were performed using Minitab® software (Version 22.3.1, Minitab, LLC).

Results and Discussion

This study investigated the valorization of stale bread into a nutritionally enhanced biomaterial using the edible filamentous fungus *N. intermedia*. The primary objective was to determine the optimal solid-state fermentation conditions for maximizing the crude protein content of the final mycelium-bread product. To achieve this, the effects of key physicochemical parameters; temperature, relative humidity, and initial substrate moisture were systematically examined. Following this optimization phase, the mycelium-bread under the most effective conditions was selected for a comprehensive nutritional assessment. The optimized fermented product's amino acid profile, fatty acid profile, and mineral content are compared with those of the original unfermented bread to fully characterize the extent of nutritional changes.

Evaluation of Solid-State Fermentation Conditions

The systematic screening of fermentation parameters revealed that the crude protein and fat content of the final product were distinctly shaped by temperature, initial moisture, and relative humidity. The influence of each parameter, as determined by a Tukey's post-hoc analysis, is detailed in Table 2. All three factors had a pronounced effect on crude protein synthesis. A strong positive correlation was observed with temperature, where the highest protein yields (mean of 24.2–25.0%) were achieved at 30 °C and 35 °C. These levels were substantially higher than those at 25 °C (21.4%) and were markedly greater than at 14 °C, which yielded the lowest protein content (15.7%). Similarly, initial substrate moisture created a clear stepwise improvement, with 60% moisture resulting in the highest protein content (27.4%),

Table 2 Tukey's grouping ($p < 0.05$) for the effects of temperature, moisture, and humidity on crude protein (CP) and crude fat content

Factor	Level	CP (g/100 g DM)	Fat (g/100 g DM)
Temperature (°C)	35	24,9944 ^a	1,38728 ^a
	30	24,2052 ^a	1,4504 ^a
	25	21,3707 ^b	1,45157 ^a
	14	15,6596 ^c	1,27584 ^a
Moisture (%)	60	27,3523 ^a	1,77874 ^b
	50	23,5942 ^b	2,03147 ^a
	30	13,7531 ^c	0,83241 ^c
Humidity (%)	90	22,3403 ^a	1,39705 ^a
	50	22,1739 ^a	1,37722 ^a
	27	20,1583 ^b	1,37654 ^a

which was nearly double the content achieved at 30% moisture (13.8%). Relative humidity also played a role, with the 27% level yielding a lower protein content (20.2%) compared to the 50% and 90% levels (mean of 22.2–22.3%), between which there was no statistical difference.

The ANOVA results indicated that temperature, initial moisture, and relative humidity were all highly significant factors affecting crude protein yield ($P < 0.05$). Furthermore, a significant interaction effect was observed between temperature and moisture content regarding total fat accumulation. To determine the most effective overall fermentation environment, a multi-objective optimization was performed using the Minitab Response Optimizer. The goal was to maximize both crude protein and total fat content simultaneously to produce a high-value, multi-nutrient ingredient. While the absolute highest protein yields occurred at 35 °C, this temperature resulted in a relative decrease in total fat content compared to 30 °C. Consequently, the combination of 30 °C, 60% moisture, and 50% relative humidity was identified as providing the highest overall desirability, representing the optimal balance for creating the mycelium-bread product. These parameters were therefore selected for the production of samples for detailed characterization and nutritional analyses.

Influence of Fermentation Parameters on Crude Protein Content

The crude protein content served as a primary indicator of mycelial growth, increasing from an initial value of 12.25 ± 0.50 g/100 g DM in the stale bread. As illustrated in Fig. 1, the extent of this protein enrichment was highly dependent on the fermentation conditions. At 14 °C, fungal activity was minimal, and the final protein content remained statistically unchanged from the baseline. A significant increase in protein was observed at 25 °C, where levels ranged from 12.94 ± 0.41 to 26.04 ± 1.25 g/100 g DM, depending on moisture and humidity. The highest protein concentrations were achieved at 30 °C and 35 °C, with no significant difference between these two temperatures. Under optimal moisture conditions (60%), protein content at these higher temperatures reached 29.91 ± 0.67 to 34.7 ± 0.8 g/100 g DM.

Temperature is a critical factor in SSF bioprocessing because it regulates fungal metabolic rates and impacts the rate of moisture evaporation from the substrate [29]. Temperature affects the solid substrate's physical properties, including its moisture content and particle size. In SSF, elevated temperatures can adversely impact fungal growth by accelerating moisture loss [30]. Moreover, temperature regulates the expression of genes associated with metabolism and development. A direct correlation exists between temperature, gene expression, and growth performance, as

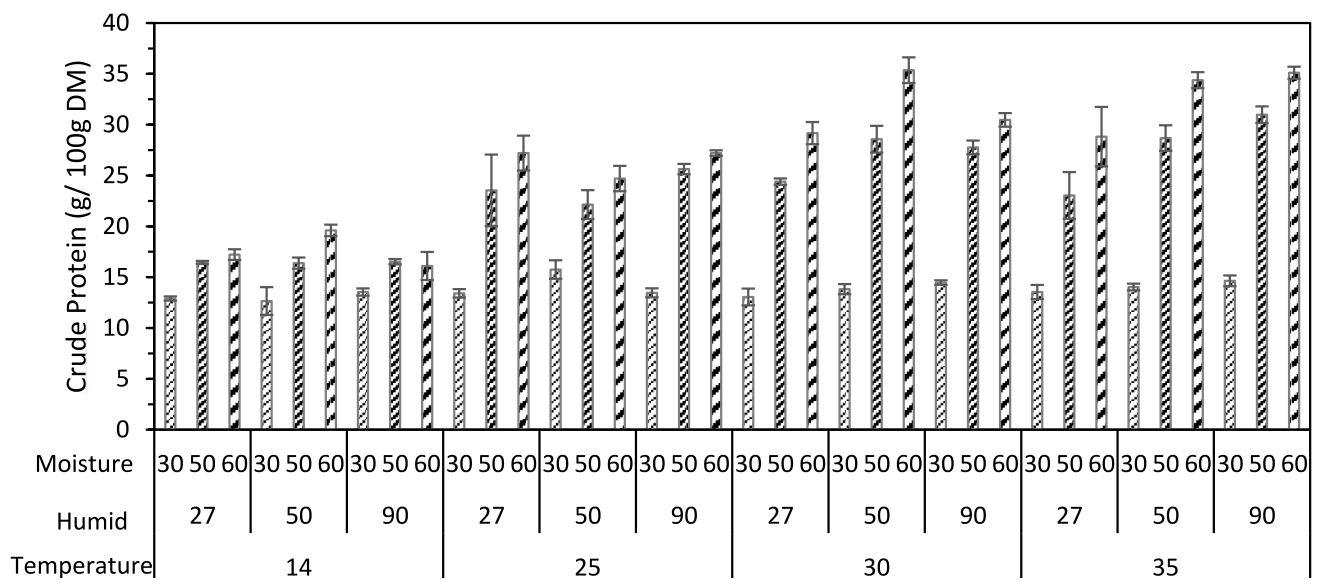


Fig. 1 Crude protein (g/100g DM) of the mycelium-bread product after five days of solid-state fermentation under varying conditions of temperature (°C), initial substrate moisture (%), and relative humidity (%). Error bars represent the standard deviation of the mean

evidenced by the peak growth rates of *Aspergillus niger* observed at 35 °C and 55% moisture content [31]. In the present study, the high protein yield (34.8 ± 0.70 g/100g) achieved at 35 °C with 60% moisture, compared to the low crude protein content observed in the 30% moisture treatment at the same temperature, was attributed to variations in substrate moisture retention. However, fungal growth and protein yield in SSF are driven by the complex interactions between temperature and other physicochemical factors, such as relative humidity and initial moisture content.

One of the most important physicochemical factors in SSF was the initial substrate moisture and its corresponding water activity (a_w) of the substrate. Different water binding characteristics of the substrate determine retention capacity and cause free water to be apparent at different moisture levels. Indeed, different substrates with specified water binding characteristics demonstrate different a_w at the same moisture level. In this study, a low moisture level of 30% (a_w 0.93) severely limited fungal growth, resulting in the lowest protein yields, which were not significantly different from the unfermented bread. Increasing the moisture content to 50% (a_w 0.96) substantially improved protein synthesis, with yields showing a strong temperature dependency, ranging from 15.8 ± 0.5 g/100 g DM at 14°C to 30.9 ± 0.8 g/100 g DM at 35 °C. Optimal results were obtained at 60% moisture (a_w 0.98), which yielded the highest protein concentrations, such as the 34.8 ± 0.70 g/100 g DM observed at 35 °C and 50% relative humidity (RH) as illustrated in Fig. 2.

Although fungi are generally considered as microorganisms that survive in a_w levels below 0.8 but the optimum growth range occurred in higher levels [32]. Therefore,

evaporation of the moisture also may decrease the water activity during 5 days of SSF. Extended lag phase and reduction in specific growth are considered as the main factors affecting low biomass production at low a_w levels. The optimal water activity for bacterial growth typically falls within the range of 0.995 to 1.000 [33], while for most fungal species the optimum level in solid state fermentation is 0.96 [34, 35]. This difference may explain the possible cause of bacterial contamination in *Neurospora* plates as unlike most kinds of fungi, bacteria can dominate at the high moisture levels. This result suggested that 60% moisture content could be considered as a threshold level for contamination-free fungal cultivation in solid state fermentation using *N. intermedia*. Indeed, higher moisture levels may result in reduced substrate porosity and limited oxygen and carbon dioxide transfer within the substrate [35]. Agglomeration of the substrate and limited gas transfer in excessively high moisture levels have negative affect on the fungal growth [36] which adversely impacts protein yield. Therefore, the moisture content of 60% was identified as the point at which the highest protein yield was achieved.

Relative humidity (RH), a critical parameter for maintaining substrate moisture and controlling water activity (a_w), was also found to significantly influence the final protein content. The results indicated that increasing the RH from 27 to 50% substantially improved protein synthesis; for instance, at 35 °C and 60% moisture, the protein content rose from 29.91 ± 0.67 g/100 g DM to 34.79 ± 0.79 g/100 g DM. However, a further increase in RH to 90% did not yield a statistically significant improvement, with the protein content remaining stable at 34.71 ± 0.59 g/100 g DM. This suggests

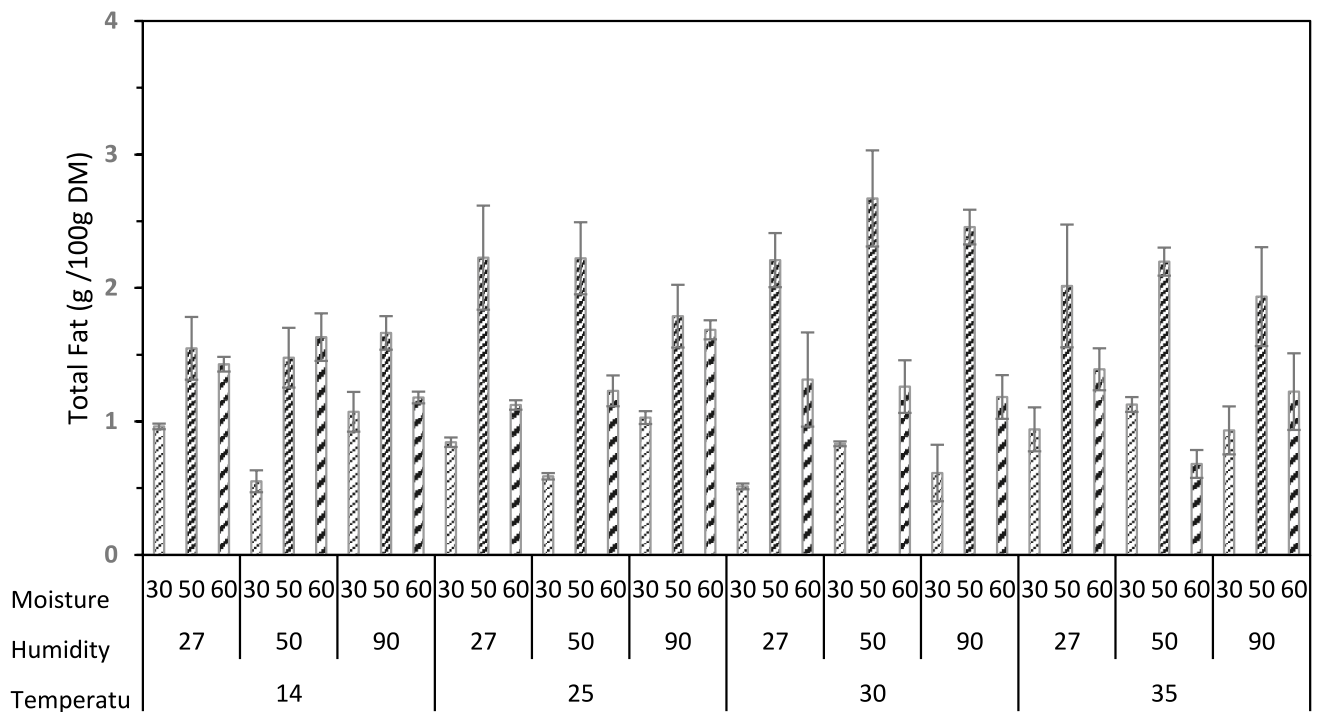


Fig. 2 Total fat content (g/100g DM) of the mycelium-bread product after five days of solid-state fermentation under varying conditions of temperature (°C), initial substrate moisture (%), and relative humidity (%). Error bars represent the standard deviation of the mean

that an RH of 50% is sufficient to prevent excessive moisture loss from the substrate over the five-day fermentation period. The role of RH in SSF is directly linked to its effect on water activity at the substrate surface. At low RH levels, water adsorption is minimal, while at intermediate levels (20–50%), a more stable multilayer of water is deposited on the substrate particles [37]. Therefore, an RH of 50% likely maintains the optimal water activity for fungal metabolism without reaching the high levels (> 80% RH) where excess capillary condensation could hinder gas transfer. These findings underscore the complex interplay between temperature, moisture, and humidity, all of which must be considered in concert to optimize fungal growth in SSF.

Analyzing the amino acid composition aimed at better understanding the protein quality of the fermented product. The total amino acid content nearly doubled during fermentation, increased from 118.2 g/kg in stale bread to 217.5 g/kg in the final mycelium-bread (Table 3). All indispensable amino acids were significantly enhanced, though the degree of improvement varied. The most substantial increase was observed for lysine, which increased from 2.59 to 14.6 g/kg before and after fermentation. Threonine also showed a significant increase from 3.34 to 11.2 g/kg. Other essential amino acids such as arginine (4.94 to 13.8 g/kg), valine (5.07 to 12.2 g/kg), and histidine (2.75 to 6.02 g/kg) demonstrated same patterns in concentration. These findings were in accordance with the previous reports which experienced a

similar increase in essential amino acids both in submerged and SSF processes [24, 38].

Analysis of the amino acid profile demonstrated that the fermentation process significantly addresses the nutritional deficiencies inherent in wheat-based products. Lysine, the primary limiting amino acid in cereal grains [41], increased approximately 5.6-fold. While stale bread falls short of the daily recommended lysine intake 30 mg/kg [42], the fermented mycelium-bread lysine content (42.0 mg/g protein) brings the product much closer to meeting the requirements for a complete protein source. This enhancement is particularly relevant for the development of meat analogues, where high-quality protein with a balanced amino acid profile is essential [43]. Furthermore, the relationship between crude protein (total nitrogen) and true protein (total amino acids) provides insight into the nitrogen distribution. In stale bread, amino acids accounted for over 96% of crude protein, whereas in mycelium-bread, they constituted approximately 63%. This decrease in true protein percentage is attributed to the synthesis of non-protein nitrogenous compounds, specifically chitin and beta-glucans in the fungal cell wall [44]. These fibers act as prebiotics, promoting the growth of beneficial gut microbiota and contributing to overall gastrointestinal health [45].

Table 3 Proximate analysis of fermented biomass and comparison with feed ingredients

		Stale bread	Mycelim-bread	<i>N. intermedia</i> (biomass)[39]	Fish meal [40]	Soybean meal [40]
Crude protein (%)		12.2±0.5	34.8±0.70	57.6	65.3	47.5
Total lipid (%)		1.98±0.77	2.67±0.4	62.2	8.9	1.5
Crude Fiber (%)		0.86±0.1	15.22±0.2	4	–	3.9
Starch (%)		61.6±1.5	1.44±0.1	–	0	0
Energy (kJ/g)		18	19	–	19	17.2
Moisture (%)		7	7	6.5	8	12
Indispensable Amino acids (g/Kg Dry matter)	Argenine	4.94	13.8	33.9	38	37
	Histidine	2.75	6.02	13.2	15	12
	Isoleucine	4.26	8.93	21.5	27	21
	Leucine	8.38	15.2	37	45	36
	Lysine	2.59	14.6	40.2	47	31
	Methyonine	4.4	8.12	9.1	23.3	14
	Phenylalanine	8.97	15.67	20.7	44	42
	Threonine	3.34	11.2	24.3	23	19
	Valine	5.07	12.2	27.2	28	26
Total Amino Acids (g/Kg DM)		118.22	217.52	471.9	565.8	389.1
Macroelements g/Kg	P	1.58±0.05	5.8±0.20	8.9	25.2	6.2
	Ca	0.17±0.00	0.96±0.05	1.7	38.5	3.4
	Mg	0.46±0.00	1.90±0.11	1.1	2.2	2.9
Microelements mg/Kg	Zn	9.53±0.14	63.80±2.70	–	85	47
	Fe	28.44±1.0	122.0±4.10	–	351	178
	Cu	3.04±0.11	13.27±1.10	–	7	17
	Mn	9.36±0.17	46.18±0.50	–	13	34

Total Fat

Biomass derived from submerged fermentation of *N. intermedia* typically contains 4.0–9.0% (of DM) total fat depending on the substrate composition [46]. The presence of initial fat in the substrate (2.5 ± 0.1 g/100g DM) used for SSF allows for a more accurate assessment of fungal metabolic activities. Following the five-day fermentation, the total fat content was influenced primarily by the initial substrate moisture, as detailed in Table 2. The highest concentration of fat, 2.67 ± 0.40 g/100 g DM, was achieved at 30 °C with 50% initial moisture and 50% RH. Under the same moisture and humidity, an increase in temperature to 35 °C led to a lower final fat content of 2.17 ± 0.11 g/100 g DM. The observed changes in fat content reflect the complex metabolism of *N. intermedia*, which involves both the synthesis of fungal lipids and the degradation of lipids present in the bread substrate. This study's findings align with previous research; for instance, Gmoser et al. [38] reported a significant increase in total fat from 2.4% to 10.5% after a 6 day SSF of stale sourdough. In another study by [47] on household fermentation of stale bread, the total fat content reached 5.4% while initial fat was reported as 1.1% (of DM).

The difference of above-mentioned reports can be related to metabolic pathways for fat degradation and glycerol release. The variation in fat content observed at different temperatures suggests a metabolic shift; at higher temperatures, the fungus may prioritize lipid catabolism for energy over lipid synthesis and storage. The final lipid profile is thus a net result of these competing metabolic pathways, which are highly dependent on the specific substrate and process conditions [46].

While the total fat content did not increase significantly under the optimal condition, remaining at 2.06 g/100 g DM compared to the initial 1.98 g/100 g DM in stale bread, the composition of the lipid fraction was substantially altered by fungal metabolism (Table 4). The detailed fatty acid analysis presented in this section corresponds to the biomass produced under the optimal conditions identified previously (30 °C, 60% initial moisture, 50% RH). The most significant change was the enhancement of the polyunsaturated fatty acid (PUFA) content, which increased from 0.70 to 0.95 g/kg DM. Concurrently, both saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) decreased. This metabolic shift resulted in the improvement in the PUFA/SFA ratio, from 0.72 in the initial bread to 2.10 in the

Table 4 Fatty acid profile of bread and mycelium-bread after solid state fermentation (SSF)

Fatty Acids profile	g/Kg of Dry Matter		% of Extracted Lipid Fraction	
	Bread	SSF	Bread	SSF
C8:0	0.01 ± 0.00	0.02 ± 0.00	0.06 ± 0.01	0.09 ± 0.01
C10:0	0.00 ± 0.00	0.34 ± 0.01	0.00 ± 0.00	1.67 ± 0.14
C13:0	0.05 ± 0.01	0.00 ± 0.00	0.25 ± 0.04	00.00 ± 0.00
C15:0	0.01 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	00.39 ± 0.01
C16:0	4.01 ± 0.14	3.14 ± 0.11	20.25 ± 0.06	15.23 ± 0.14
C16:1	0.06 ± 0.00	0.21 ± 0.00	0.34 ± 0.01	1.02 ± 0.04
C17:1	0.00 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.17 ± 0.00
C18:0	0.49 ± 0.01	0.43 ± 0.03	2.47 ± 0.02	2.08 ± 0.05
C18:1	7.31 ± 0.22	6.08 ± 0.62	36.87 ± 0.26	29.39 ± 1.37
C18:2 (LA)	6.08 ± 0.27	7.78 ± 0.17	30.68 ± 0.16	37.72 ± 0.84
C18:3 n6	0.02 ± 0.00	0.02 ± 0.00	0.14 ± 0.03	0.10 ± 0.01
C18:3 n3 (ALA)	0.59 ± 0.01	0.64 ± 0.03	3.00 ± 0.01	3.13 ± 0.03
C20:0	0.06 ± 0.02	0.04 ± 0.01	0.32 ± 0.10	0.21 ± 0.04
C20:1 n9	0.25 ± 0.01	0.15 ± 0.01	1.27 ± 0.03	0.75 ± 0.03
C20:2	0.05 ± 0.00	0.07 ± 0.00	0.29 ± 0.03	0.35 ± 0.02
C20:4 n6	0.05 ± 0.00	0.00 ± 0.00	0.26 ± 0.02	0.00 ± 0.00
C20:3 n3	0.00 ± 0.00	0.06 ± 0.00	0.00 ± 0.00	0.29 ± 0.01
C22:0	0.05 ± 0.01	0.09 ± 0.01	0.29 ± 0.04	0.46 ± 0.05
C20:5 (EPA)	0.03 ± 0.02	0.09 ± 0.01	0.19 ± 0.10	0.44 ± 0.08
C22:1	0.11 ± 0.01	0.10 ± 0.00	0.60 ± 0.10	0.50 ± 0.02
C22:2	0.04 ± 0.00	0.09 ± 0.00	0.22 ± 0.00	0.47 ± 0.05
C23:0	0.11 ± 0.00	0.12 ± 0.00	0.58 ± 0.05	0.60 ± 0.02
C24:0	0.1 ± 0.00	0.20 ± 0.02	0.50 ± 0.01	1.00 ± 0.07
C24:1	0.06 ± 0.00	0.07 ± 0.00	0.35 ± 0.04	0.35 ± 0.01
C22:6 (DHA)	0.06 ± 0.07	0.00 ± 0.00	0.30 ± 0.30	0.00 ± 0.00
Total	1.98 ± 0.77	2.06 ± 0.10	100.00	100.00
SFA	0.49	0.45	24.86	21.85
MUSFA	0.79	0.67	39.62	32.32
PUFA	0.70	0.95	35.52	45.83
PUFA/SFA	0.72	2.10		

mycelium-bread, indicating a notable improvement in the nutritional quality of the fat. This trend is further reflected in the profile of individual fatty acids. A key transformation was the shift in the dominant fatty acid. In stale bread, the monounsaturated oleic acid (C18:1) was most abundant, constituting 36.9% of the lipid fraction. After fermentation, the essential omega-6 fatty acid, linoleic acid (C18:2), became predominant, increasing from 30.7 to 37.7% of the total lipids. This was accompanied by a decrease in the primary saturated fat, palmitic acid (C16:0), from 20.3 to 15.2%. Furthermore, other nutritionally valuable PUFAs, such as α -linolenic acid (ALA, C18:3n3) and eicosapentaenoic acid (EPA, C20:5), also showed slight increases. These results highlight the capability of *N. intermedia* to

actively modify the lipid profile of the substrate, selectively synthesizing valuable PUFAs while catabolizing SFAs and MUFAs, thereby upgrading the overall nutritional value of the final product.

This significant increase in the PUFA/SFA ratio is a critical nutritional indicator. A PUFA/SFA ratio of 1.0–1.5 is indicated to be within a favorable range to reduce the risk of cardiovascular diseases [48]. The bioconversion was driven by a marked decrease in palmitic acid (C16:0), the primary saturated fatty acid, which fell from 20.3 to 15.2% of the total lipids. Simultaneously, linoleic acid (LA, C18:2), an essential omega-6 fatty acid, became the dominant component, increasing from 30.7 to 37.7%. For animal feed applications, this shift is equally vital; linoleic and alpha-linolenic acids are essential for maintaining membrane fluidity and supporting the growth of fish species like rainbow trout [49]. Furthermore, the presence of trace amounts of EPA (C20:5) in the final product suggests that *N. intermedia* possesses the desaturase and elongase enzymatic machinery necessary to synthesize long-chain PUFAs, albeit in limited quantities. Consequently, the fermentation transforms the stale bread lipid profile from a saturated-heavy energy source into a health-promoting fraction rich in essential fatty acids and with a vastly improved lipid quality index.

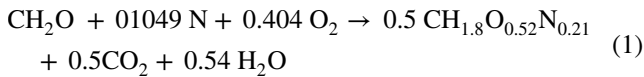
Carbohydrate Composition

The proximate analysis of stale bread revealed that starch is the main carbohydrate component, comprising over 62% of the dry matter. *Neurospora intermedia* is known for its capacity to produce amylolytic enzymes during solid-state fermentation, which enables the efficient utilization of such starch-rich substrates. The primary enzyme, amylase, is crucial for the hydrolysis of starch into simpler sugars [50]. The amylolytic complex of *N. intermedia* likely includes glucoamylase and α -amylase, which are common in starch-degrading fungi [51]. Consequently, in this SSF bioprocess, the enzymatic activity of *N. intermedia* induced a significant reduction in starch levels, specifically in the optimum cultivation condition from 61.6% in the initial substrate to approximately 1.5% in the mycelium-bread.

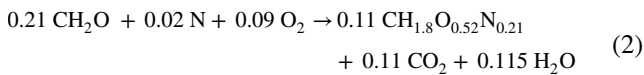
This consumption of starch directly induced the production of fungal biomass, which was reflected in a dramatic increase in crude fiber content. The fiber content rose from 0.86 g/100g DM in the original bread to 15.22 g/100g DM in the final product. This increase is a direct measure of fungal growth, as the fiber being synthesized is composed of the structural polysaccharides of the fungal cell wall. Unlike plant fiber, which is primarily cellulose and hemicellulose, fungal fiber consists mainly of chitin, chitosan, and β -glucans [44]. Therefore, the fermentation process effectively converted simple, digestible starch along with

nitrogen sourced from bread protein into a complex, functional fiber, fundamentally altering the carbohydrate profile of the substrate. This bioconversion not only demonstrates the efficiency of fungal growth but also enhances the potential nutrition values of the final product by enriching it with bioactive fibrous compounds.

Since substrate is the only source of carbon in solid state fermentation process, the potential and deficiencies of the substrate can be evaluated by carbon reaction analysis. According to [21], the biomass yield in SSF can be calculated as 0.5 mol C biomass/mole C substrate in the solid state fermentation of stale bread (Eq. 1):



The current experiment yielded similar results, assuming starch as the only carbon source available to the fungi in SSF and assuming that roughly all the converted nitrogen in the substrate has been turned into protein and not into other metabolites. Hence, non-starch material in the substrate were considered as non-reactants that are not involved in the bioconversion (Table 4) since it was assumed that starch was the carbon source which converted to biomass. Based on starch analysis, *N. intermedia* efficiently consumed nearly all the starch content during the 5 day fermentation period, evidenced by a significant decrease in the amount of starch from 63% to approximately 1.5% of DM. By modifying the mass balance formula and considering the available starch content in the substrate (6.3 g/10 g), the adapted formula has been achieved (Eq. 2).



According to the modified formula, *N. intermedia* in the substrate with starch content of 63% required a nitrogen content of 3.1% to achieve the maximum yield of protein (54.5%). However, since the nitrogen content in the bread was only 1.9%, the mycelium-bread reached the maximum protein yield of 34.7% (± 0.7). This value represents the maximum yield achievable with the given substrate characteristics and nitrogen availability. The mass balance analysis indicated that nitrogen was a limiting factor in the SSF of *N. intermedia* using the stale bread as the available substrate. Additional nitrogen sources need to be added to the substrate

for protein enhancement in solid state fermentation of stale bread.

The adapted formula revealed that 0.21 mol of starch (6.3g) was converted to 0.11 mol (2.6g) of fungal biomass. This transformation during solid-state fermentation was the main cause of changes in protein and fiber content, which attributed to mycelium growth. However, 0.11 mol carbon was emitted as carbon dioxide gas during the fermentation process due to the metabolic activity of filamentous fungi. Although the dry weight of fungal biomass after the fermentation process was 2.6 g (Table 5), the non-starch material (3.3 g), presumed inert in the process, represented unfermented segments of the substrate.

Minerals

The macro and micro element composition of both the initial stale bread and the final fermented product is presented in Table 3. A notable increase in the concentration (g/kg DM) of all measured minerals was observed after fermentation. Specifically, phosphorus increased from 1.58 ± 0.05 g/kg DM to 5.8 ± 0.20 g/kg DM. Similarly, calcium content rose 5.6-fold from 0.17 ± 0.00 g/kg to 0.96 ± 0.05 g/kg, and magnesium showed a 4.1-fold increase from 0.46 ± 0.00 g/kg to 1.90 ± 0.11 g/kg. The concentration of trace minerals also increased markedly: zinc from 9.53 to 63.80 mg/kg, iron by 4.3-fold (from 28.44 to 122.0 mg/kg), copper from 3.04 to 13.27 mg/kg, and manganese from 9.36 to 46.18 mg/kg. This phenomenon is not due to the synthesis of minerals but is a direct consequence of the change in mycelium-bread mass during the fermentation process. As the fungus consumes carbohydrates (primarily starch) and converts them into CO_2 through respiration, and moisture dissipation, the total dry mass of the substrate decreased. As detailed in the mass balance (Table 5), an initial 10 g of dry bread substrate yielded approximately 5.9 g of the final dry fermented product. Since the minerals themselves are not consumed in this process, they become more concentrated in the remaining biomass. Essential minerals, incorporated into the fungal biomass, are present in a bioavailable, organic form. This is often superior to the inorganic mineral salts typically used in feed fortification, potentially leading to better absorption and improved health outcomes, such as enhanced growth rates and feed conversion ratios in livestock and aquaculture [17, 52]. Therefore, the fermentation not only upgrades the protein and fiber content of stale bread but also transforms

Table 5 Changing of substrate composition after solid state fermentation

	Substrate %				After fermentation		
	Initial (g)	Starch	N	Inert	Biomass (g)	% N	Crude protein%
Theoretical	10	63	3.1	33	2.7	8.85	54.5
Experimental	10	63	1.9	33	2.6	5.6	36.02

it into a valuable, multi-nutrient ingredient rich in bioavailable minerals.

Minerals are crucial for the physiological and metabolic activities of filamentous fungi, acting as cofactors for a vast array of enzymes and playing structural roles in cellular components. Key minerals such as iron (Fe), phosphorus (P), potassium (K), magnesium (Mg), and zinc (Zn) are indispensable for fundamental processes including respiration, energy storage (ATP synthesis), protein synthesis, and the maintenance of osmotic balance [53, 54]. For instance, iron is vital for the function of cytochromes in the respiratory chain, while phosphorus is a core component of nucleic acids and energy-carrying molecules [55]. The availability of these minerals in the growth substrate directly influences fungal metabolism, including the production of organic acids and secondary metabolites [56, 57]. The mineral content of fungal biomass is highly dependent on the composition of the substrate, as fungi accumulate minerals from their environment. For example, a study by Li et al. [54] on *Talaromyces flavus* grown on various silicate minerals demonstrated that the fungus specifically tailored its production of organic acids to solubilize and uptake essential elements like Fe and Mg from the mineral matrix. Similarly, research has shown that the mineral profile of *Aspergillus niger* biomass reflects the mineral content of its growth medium, with the fungus efficiently accumulating elements even from complex substrates like agro-industrial wastes [57]. Another study highlighted that nutrient limitations, such as phosphate deficiency, can trigger significant metabolic shifts in fungi, leading to increased excretion of organic acids to mobilize minerals from the environment [56]. This ability to bioaccumulate minerals makes fungal biomass a potentially rich source of essential nutrients.

Conclusion

This study demonstrates the valorization of stale bread through solid-state fermentation with *N. intermedia*, transforming a low-value waste stream into a nutritionally enhanced product. The optimized fermentation process, conducted at 30 °C with 60% initial moisture and 50% relative humidity, resulted in a mycelium-bread with a crude protein content of 34.8 g/100g DM. This protein enrichment was accompanied by a significant improvement in the amino acid profile. Furthermore, the fermentation fundamentally altered the carbohydrate and lipid profiles, converting digestible starch into 15.2 g/100g DM of functional fungal fiber and improving the PUFA/SFA ratio of the fat from 0.72 to 2.10. While the nutritional values of this mycelium-bread product were lower than those of conventional feed ingredients like fish meal and soybean meal, its creation from food

waste highlights its potential as a sustainable component in food and feed value chains. The upcycled biomass, with its enhanced profile of protein, essential amino acids, bioavailable minerals, and functional fibers, presents a promising avenue for circular bioeconomy models. For human consumption, it could be incorporated into foods like meat analogues or baked products to improve their nutritional density. However, to fully realize this potential, further research on optimizing the process for specific nutritional targets, potentially by co-fermenting other nutrient-rich substrates is necessary.

Acknowledgements The authors would like to express their gratitude to the University of Borås for technical and financial support of this work. Moreover, authors would like to thank Dr. Alexandra Leeper from Iceland Ocean Cluster, Iceland, for the scientific support provided in this study.

Funding Open access funding provided by University of Borås. The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Data Availability The data that support the finds of this study are available from the corresponding author, [VA] upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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