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Review

Green Technology for Fungal Protein Extraction—A Review

Tanvir Ahmed [1], Md Suzauddula [2], Khadiza Akter [3], Monir Hossen [3] and Md Nazmul Islam [3,*]

[1] Department of Food Engineering and Tea Technology, Shahjalal University of Science and Technology, Sylhet 3114, Bangladesh; tanvirsust2011@gmail.com
[2] College of Agriculture and Natural Resources, National Chung Hsing University, Taichung 402, Taiwan; mdsuzauddula@gmail.com
[3] Department of Microbiology, Noakhali Science and Technology University, Noakhali 3814, Bangladesh; khadizaakter01799@gmail.com (K.A.); monir.mbg@gmail.com (M.H.)
* Correspondence: mdinazmul@gmail.com

Abstract: Fungal proteins are highlighted for their nutritional value and bioactive properties, making them a significant alternative to traditional protein sources. This review evaluates various green extraction technologies, including enzymatic-, ultrasound-, higher-pressure homogenization-, microwave-assisted, pulsed electric fields-, and supercritical fluid-assisted extraction, focusing on their effectiveness in disrupting fungal cell walls and preserving protein integrity. The findings indicate that these technologies could have the potential to improve protein yield and quality, addressing the challenges posed by fungal cell walls' complex and resilient structure. The review also underscores the bioactivities of fungal proteins, including antifungal, antibacterial, antioxidant, and anticancer properties. The conclusion emphasises the need for further optimisation and scaling of these technologies, as well as exploring a wider range of fungal species to fully understand their potential as sustainable protein sources. Future research directions include refining extraction methods, integrating multiple approaches, and utilising novel green solvents to maximise efficiency and yield.

Keywords: processing technologies; extraction; fungi; protein; bioactivity; sustainability

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1. Introduction

Fungi have emerged as a significant source of alternative proteins, presenting a sustainable and versatile option for addressing global food and nutritional security challenges. Unlike traditional protein sources, such as meat and legumes, fungi-based proteins offer unique benefits due to their nutritional profiles, environmental sustainability, and potential for widespread cultivation. Fungi, a diverse kingdom of organisms distinct from plants and animals, encompasses many species, including mushrooms, yeasts, and molds. These organisms are renowned for their role in nutrient cycling and ecosystem symbiotic relationships. Beyond their ecological roles, fungi have been utilised in food and medicine for centuries, with mushrooms being a popular culinary choice and yeasts essential in baking and brewing [1]. The interest in fungi as a source of alternative protein stems from several key attributes. Firstly, fungi are highly efficient in converting substrates into biomass, requiring less water, land, and energy than conventional animal protein sources. This efficiency is critical in the context of increasing environmental concerns and the need to reduce the carbon footprint of food production [2]. Additionally, fungi can thrive on various organic waste materials, transforming low-value byproducts into high-quality protein, thereby contributing to circular economy principles. Nutritionally, fungi-derived proteins are of high quality, containing essential amino acids required by humans [3]. This is particularly significant as many plant-based protein sources often lack one or more essential amino acids. Moreover, fungi are rich in vitamins, minerals, and other bioactive compounds, such as beta-glucans, which have been shown to have immunomodulatory and health-promoting properties [4]. The unique umami flavour of fungi also enhances the

palatability of food products, making them an appealing option for consumers seeking to reduce their meat intake.

Fungal cell walls comprise polysaccharides like chitin, glucans, mannans, proteins, and other minor components like lipids and pigments. This complex matrix provides mechanical strength and protection and is crucial in cell signalling and environmental interaction. The heterogeneity and resilience of the fungal cell wall pose the first significant challenge for protein extraction: accessibility. The cell wall must be breached or disrupted to efficiently extract proteins without significantly damaging the proteins of interest [5]. This requires a delicate balance of physical, chemical, and enzymatic methods tailored to the specific fungal species and the target protein's characteristics. Physical disruption methods, such as mechanical grinding, sonication, or pressure homogenisation, can be effective but often lead to the denaturation or degradation of sensitive proteins [6]. Chemical methods, including detergents or solvents, can solubilise cell wall components but may interfere with protein activity or purity [7]. Enzymatic methods, which use specific enzymes to degrade cell wall polysaccharides, offer a more targeted approach but require a deep understanding of the cell wall's composition and the action of these enzymes [8]. Another significant challenge is the variability of fungal cell walls between species and even within different strains of the same species. This variability affects the efficiency of cell wall disruption and protein extraction methods, making it challenging to develop a one-size-fits-all approach.

Green extraction technologies have emerged as a transformative approach in biotechnology, particularly in addressing the challenges of fungal cell wall disruption for efficient protein production and extraction. One of the critical advantages of green extraction technologies, specifically supercritical fluid extraction (SFE), ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurised liquid extraction (PLE), subcritical water extraction (SWE), and green solvent extraction, in the context of fungal protein extraction, is their ability to preserve proteins' structural integrity and functionality [9]. Unlike conventional methods that might denature proteins due to extreme pH, temperature, or mechanical forces, green techniques employ milder conditions that maintain protein activity [10]. Amid the dynamic landscape of green extraction, this review outlines the challenges in fungal protein extraction and the progress of green extraction technologies in disrupting fungal cell walls and extracting proteins. The objective is to critically assess the efficacy of these techniques and identify potential solutions to current challenges. By synthesising existing literature, this review endeavours to enhance the understanding of green extraction's role in fungal protein production and stimulate further research in this essential field.

2. Fungal Proteins and Their Bioactivities

The bioactivity of fungal proteins, particularly those derived from edible macro-fungi and yeast, is of significant interest due to their diverse health benefits. Edible macro-fungi, which include various species of mushrooms, are renowned for their rich composition of bioactive constituents. These constituents encompass polysaccharides, proteins, vitamins, minerals, and dietary fibre, all contributing to their nutritional and medicinal value. Among these, the antifatigue properties of edible macro-fungi stand out, making them popular as health foods and supplements [11]. The antifatigue effects are primarily attributed to the presence of specific polysaccharides and proteins that enhance physical endurance and reduce fatigue. Fungal proteins exhibit a wide range of bioactivities that are crucial for numerous biological processes. One notable category is fungal immunomodulatory proteins (FIPs), which have garnered attention for their ability to modulate the immune system [12]. These proteins can enhance the body's immune response, providing protective effects against various pathogens and diseases. The immunomodulatory properties of FIPs make them potential candidates for developing therapeutic agents aimed at boosting immune function and treating immune-related disorders. In the context of plant–fungal interactions, fungal proteins play a crucial role. Fungi secrete effector proteins that interact with plant hosts to modulate their responses and facilitate colonisation [13]. These effector

proteins are essential for the establishment and maintenance of symbiotic relationships, as well as for pathogenic infections. By manipulating plant signalling pathways, these fungal effectors help fungi evade plant defences and establish a successful infection or symbiosis. The fungal cell wall, a complex structure composed of glucans, chitin, and glycoproteins, is another critical area where proteins are involved. Proteins associated with the cell wall influence the virulence of fungi and their interactions with host organisms. For instance, enzymes that degrade cell wall components can affect the structural integrity of the cell wall, impacting the fungus's ability to invade host tissues. Additionally, cell wall proteins play roles in the adhesion of fungi to host cells, a crucial step in the infection process [14]. Table 1 in the referenced material provides a detailed overview of the bioactive properties of proteins detected in various fungal species. This table highlights the diversity of bioactivities exhibited by fungal proteins, including antihypertensive, antibacterial, antifungal, antioxidant, antiviral, and anticancer.

One study investigated the antihypertensive potential of protein extracts from mushrooms, specifically *Pleurotus fastidious* (E1Pc and E5Pc) and *Agaricus bisporus* (E1Ab and E3Ab). Protein extracts from these mushrooms exhibited high antihypertensive activity, particularly fractions E5PcF3, and E3AbF6, obtained through reverse-phase high-performance liquid chromatography (RP-HPLC). The results suggest that the antihypertensive activity in these mushroom species is likely due to proteins with molecular masses ranging from 3 to 10 kDa [15]. Another study identified L-amino acid oxidases (LAOs) from the fruiting bodies of *Amanita phalloides* and *Infundibulicybe geotropa*, which exhibit broad substrate specificities and significant antibacterial activity, particularly against Gram-negative bacteria. These LAOs, along with a similar enzyme from the mycelia of *I. geotropa*, effectively inhibited *Ralstonia solanacearum* and other phytopathogenic bacteria in vitro, with CgLAO and CgmycLAO also demonstrating in vivo efficacy in tomato plants. The findings highlight the potential of fungal LAOs as novel biological agents for plant disease management, sourced from the fruiting bodies of fungi [16]. In addition, the enzyme D-amino acid oxidase (DAAO) from the yeast *Rhodotorula gracilis* showed its potential as a biological antimicrobial agent in the food industry [17]. DAAO demonstrated significant antibacterial activity against both Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*) bacteria, primarily through hydrogen peroxide (H_2O_2) generation during the oxidative deamination of D-amino acids. The enzyme effectively reduced bacterial growth in various foodstuffs, such as grated cheese, without exogenous D-amino acids, highlighting its potential to enhance food safety and stability by preventing spoilage. The antifungal proteins (AFPs) from the fungus *Penicillium expansum*, identify that only PeAfpA is naturally produced and shows strong antifungal activity against various plant and human pathogens. Biotechnological production of PeAfpB and PeAfpC was achieved in *Penicillium chrysogenum*, revealing PeAfpB's moderate antifungal activity and no detectable activity for PeAfpC. PeAfpA demonstrated significant protection against fungal infections in tomato leaves and oranges, highlighting its potential as a non-cytotoxic antifungal agent for agriculture, medicine, and food preservation [18]. In another study, an antifungal protein, trichogen, was isolated from the mushroom *Tricholoma giganteum* var. golden blessings. The study showed that it exhibited antifungal activity against *Physalospora piricola*, *Mycosphaerella arachidicola*, and *Fusarium oxysporum*. Also, trichogin inhibited HIV-1 reverse transcriptase with an IC_{50} of 83 nM [19].

The yeast Tsa1 peroxiredoxin, a 2-Cys peroxiredoxin, has dual roles as a peroxidase and a molecular chaperone, with its peroxidase activity essential for ribosomal function and protection against translation inhibitors [20]. Tsa1's antioxidant activity protects actively translating ribosomes from endogenous reactive oxygen species (ROS), and its loss leads to ribosomal protein aggregation, indicating its crucial role in maintaining translation apparatus integrity. In thioredoxin system mutants, deregulated Tsa1 promotes translation defects, including hypersensitivity to inhibitors, increased error rates, and protein aggregation, suggesting its broader implications in stress and growth control. Another study highlights Tsa1's function as a specific antioxidant protecting cells from

oxidative stress caused by nascent-protein misfolding and aggregation [21]. Yeast mutants lacking TSA1 are sensitive to the proline analogue azetidine-2-carboxylic acid (AZC), which induces protein aggregation and ROS production, primarily from mitochondria. This sensitivity can be mitigated by generating [rho(0)] cells lacking mitochondrial DNA or inhibiting nascent-protein synthesis, confirming the role of protein aggregation in ROS generation. Tsa1 localises to protein aggregation sites adjacent to mitochondria, indicating its protective role against ROS generated due to protein misfolding and aggregate formation. Moreover, the Ski2p protein from *Saccharomyces cerevisiae* has been identified for its antiviral activity, forming a complex with Ski3p and Ski8p to exert its antiviral effects. Research has shown that Ski2p, Ski3p, and Ski8p can coimmunoprecipitate as a heterotrimeric complex, indicating their role in antiviral defence mechanisms. The Ski antiviral system in *Saccharomyces cerevisiae*, involving Ski2p, Ski3p, and Ski8p, acts by blocking the translation of viral mRNA, providing insights into the molecular mechanisms underlying the antiviral activity of these proteins [22,23].

Lectins in Agaricales and other medicinal fungi have demonstrated therapeutic properties against various cancers in animal and clinical studies. However, the precise anti-cancer mechanisms of these lectins are not yet fully understood. What is known is that lectins preferentially bind to sugars on cancer cell membranes, leading to cytotoxicity and apoptosis. Additionally, lectins can influence the production of interleukins, thereby affecting the immune system. They can also bind to ribosomes, which modulate the cell's proteome and inhibit protein synthesis. Moreover, the antiproliferative effect of lectins in *Agaricus bisporus* is believed to stem from the inhibition of protein uptake into the nucleus. This occurs after the lectins traffic to the nuclear periphery, where they block sequence-dependent protein import. Recently, a lectin-like protein from *A. bisporus* was found to bind mannose in MCF7 cellular membranes. This binding induces cell death at high concentrations (100 µg/mL), while it causes cell growth arrest at lower concentrations [24]. A novel lectin isolated from the wild mushroom *Paxillus involutus* was characterized, revealing a molecular mass of 28 kDa composed of four identical subunits and a unique N-terminal amino acid sequence. This lectin exhibited hemagglutinating activity, inhibited by inulin and certain metal ions, and showed poor thermostability but high tolerance to NaOH. Biologically, the lectin demonstrated significant antiphytovirus activity against tobacco mosaic virus (TMV) but lacked inhibitory effects on pathogenic fungi, HIV-1 reverse transcriptase, and displayed antiproliferative activity against lung and colon cancer cell lines [24]. Furthermore, a new fungal immunoregulatory protein, FIP-bbo, was identified from *Botryobasidium botryosum* and produced using an optimised *E. coli* expression system. FIP-bbo demonstrated broad-spectrum immunomodulatory and anticancer activities, similar to LZ-8, by inhibiting various cancer cell lines (Hela, Spac-1, and A549) at low concentrations. However, FIP-bbo was found to be less potent than LZ-8. Molecular dynamics simulations and predicted point mutations suggested potential improvements in the thermal stability and anticancer activity of FIP-fve and FIP-bbo, presenting new candidates for the development of anticancer adjuvants [25].

Table 1. Fungal proteins and their bioactivities.

Fungal Protein and Enzyme	Fungal Strains	Bioactivities	Experimental Model	Key Findings	Refs.
E5PcF3, E3AbF6	*Pleurotus cystidiosus*, *Agaricus bisporus*	Anti-hypertensive	In silico	The antihypertensive activity in the two mushroom species could be due to proteins with molecular masses ranging from 3 to 10 kDa.	[15]

Table 1. *Cont.*

Fungal Protein and Enzyme	Fungal Strains	Bioactivities	Experimental Model	Key Findings	Refs.
L-amino acid oxidases (LAOs)	*Amanita phalloides*, *Infundibulicybe geotropa*	Antibacterial	In vitro and In vivo	The in vitro and in vivo antibacterial efficacy of LAOs against various bacterial species (*R. solanacearum* and other plant pathogenic Bacteria) highlights their potential as new biological phytoprotective agents.	[16]
D-amino acid oxidase (DAAO)	*Rhodotorula gracilis*	Antibacterial	In vitro	DAAO reduced bacterial growth on various foodstuffs, with 10-fold fewer colonies on grated cheese after 16 h at 37 °C when 0.01 mg (1.2 units) of DAAO was added.	[17]
PeAfpA	*Penicillium expansum*	Antifungal	In vitro	PeAfpA was demonstrated to efficiently protect against fungal infections caused by *Botrytis cinerea* in tomato leaves and *Penicillium digitatum* in oranges.	[18]
Trichogin	*Tricholoma giganteum*	Antifungal	In vitro	Trichogin exhibited antifungal activity against *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola*. It also inhibited HIV-1 reverse transcriptase with an IC50 of 83 nM.	[19]
Tsa1	*Saccharomyces cerevisiae*	Antioxidant	In vitro	Deregulated Tsa1 promotes translation defects, including hypersensitivity to inhibitors, increased error rates, and protein aggregation, suggesting its broader implications in stress and growth control.	[20]
Lectins	*Paxillus involutus*	Antiphytovirus activity	In vitro	The *Paxillus involutus* lectin possesses antiphytovirus activity against tobacco mosaic virus with 70.6% inhibition at a concentration of 200 μg/mL.	[24]
Ski2	*Saccharomyces cerevisiae*	Antiphytovirus activity	In vitro	Ski2, a cytoplasmic RNA helicase with a broad RNA-binding specificity and distinct structural features, functions with the exosome in mRNA turnover and quality control, suggesting its potential antiviral activity through the degradation of viral RNA in the cytoplasm.	[22,23]

Table 1. *Cont.*

Fungal Protein and Enzyme	Fungal Strains	Bioactivities	Experimental Model	Key Findings	Refs.
Lectins	*Agaricus bisporus*	Anticancer	In vitro	Mannose impeded lectin-like protein orf239342's ability to inhibit the proliferation of the MCF-7 breast cancer cells, providing further evidence for the mannose-binding onto the protein.	[24]
FIP-bbo	*Botryobasidium botryosum*	Anticancer	In vitro	Anti-proliferation, pro-apoptosis, and inhibiting migration experiments on Hela, Spca-1 and A549 showed that rFIP-bbo has anticancer activity. The anticancer activity of the rFIP-bbo lies between that of rLZ-8 and rFIP-fve.	[25]

3. Challenges in Fungal Protein Extraction and Maintenance Quality

Extracting proteins from fungi presents several challenges owing to fungal cell walls' unique composition and resilience. These challenges can be summarised as follows:

3.1. Cell Wall Structure

Fungi are characterised by unique cell wall structures that are integral for maintaining cellular integrity, resilience against environmental adversities, and facilitating interactions within their surroundings. These walls are primarily composed of glucans (diverse polysaccharides composed of glucose monomers) chitin (consisting of β-1,4-linked N-acetylglucosamine units) chitosan (a heterogeneous polymer of β-1,4-linked glucosamine and N-acetylglucosamine), and proteins [5]. However, the composition of fungal cell walls varies significantly among species, even within different developmental stages of the same species. For example, the cell walls of *Saccharomyces cerevisiae*, a model yeast, are primarily composed of β-glucans and mannoproteins, while those of filamentous fungi like *Aspergillus* spp. contain additional components such as chitin and galactomannoproteins [26]. The combined presence of these constituents strengthens cell wall rigidity and resilience against disruption or lysis, thereby complicating the extraction of proteins from fungal cells.

3.2. Extraction Methods

Various extraction methods have been applied for fungal protein, each with advantages and limitations. The choice of method depends on factors such as the fungal species, the target protein, and the downstream applications. Mechanical and non-mechanical methods are the two main disruption approaches. Mechanical methods, like bead milling, ultrasonication, and high-pressure homogenisation, are non-selective but offer scalability and cost-effectiveness [27,28]. Bead milling, while effective, may cause protein modification due to the high-speed grinding action [29]. Ultrasound-assisted extraction, despite its efficiency, may result in incomplete cell lysis, leading to lower protein yields [30]. High-pressure homogenisation, although environmentally safe, may require specific equipment and additional steps to break emulsions, potentially increasing complexity. High-speed homogenisation, while enhancing productivity, could also cause protein modification due to shear forces [31]. Microwave-assisted extraction, despite its rapidity, may entail high maintenance costs for commercial-scale operations [32]. On the other hand, non-mechanical

methods, including electrical, physical, chemical, and enzymatic approaches, are more selective and less energy-intensive but may be limited in scale-up potential. Enzymatic treatment is a promising method for protein extraction from fungus, offering simplicity and avoidance of harsh chemicals, but it requires further optimisation [33]. Acid treatment, using acids like HCL and H_2SO_4, shows efficiency but may lead to gel formation or require additional agitation during scaling up. Base treatment proves less effective for protein recovery [34]. Osmotic shock, though simple, results in mild disruption and requires lengthy treatment, limiting scalability [35]. These limitations underscore the need to carefully consider the method's suitability based on factors such as protein integrity, yield, and scalability.

3.3. Protein Solubility

Fungal proteins exhibit diverse solubility properties influenced by factors like amino acid composition, post-translational modifications, and structural conformation [36]. While some proteins readily dissolve in aqueous buffers, others require denaturing agents, chaotropic reagents, or organic solvents to overcome insolubility barriers. Hydrophobins, unique proteins from filamentous fungi, offer versatile applications in fields such as food technology, pharmaceuticals, cosmetics, and materials science due to their amphiphilic nature and remarkable surface activity, but their hydrophobic nature complicates solubilisation and extraction process [37]. Krishnaswamy et al. [38], showed that proteins extracted using TE (Tris + EDTA) and yeast-breaking buffer yielded concentrations exceeding 2 μg/mL, indicating insufficient extraction. In contrast, the Tris-$MgCl_2$ buffer system produced the highest protein levels from lyophilised *Aspergillus terreus* biomass. The challenge of poor protein solubility can significantly impact the achievement of maximum protein yield and quality. Therefore, customising extraction buffers and conditions such as pH, buffer composition, and temperature to align with the solubility characteristics of the target proteins is essential for maximising yield and purity.

3.4. Contaminant Removal

After protein extraction, removing detergents and contaminants is essential for obtaining pure and homogeneous fungal protein samples. Detergents can interfere with enzymatic digestion and various separation techniques, including reverse-phase separations and mass spectrometry, potentially damaging instruments and columns irreversibly. Contaminants such as polysaccharides, lipids, nucleic acids, and secondary metabolites may co-purify with the target proteins, impeding accurate protein quantification, identification, and functional assay outcomes [39]. Multiple approaches, such as centrifugation, filtration, precipitation, and chromatography, are utilised for detergent and contaminant removal, each with limitations and challenges [40]. Centrifugation may not efficiently remove all contaminants, especially those with similar densities to fungal proteins, and prolonged centrifugation can lead to protein denaturation or aggregation. Filtration methods can remove larger contaminants but may not effectively eliminate smaller molecules or aggregates, and clogging of the filter can occur, reducing efficiency. Precipitation methods (chloroform-methanol mixture, acetone, or trichloroacetic acid precipitation) remove contaminants but may co-precipitate proteins, posing challenges in sample dissolution. Vigorous techniques like vortexing or sonication aid in pellet suspension but require caution to prevent protein loss. Chromatographic methods offer high specificity and resolution but can be time-consuming, require specialised equipment, and may suffer from non-specific binding or column degradation, impacting sample purity. In summary, while these methods are valuable for contaminant removal, careful optimisation and considering their limitations are necessary to obtain pure and homogeneous fungal protein samples.

3.5. Protein Stability

Preserving the stability of fungal proteins during extraction is essential for maintaining their native structure and biological activity. Temperature, pH, ionic strength, and the

presence of proteases are critical factors influencing protein stability [41]. For instance, fluctuations in pH disrupt electrostatic interactions, while temperature changes can induce unfolding or aggregation. In addition, alterations in ionic strength affect protein solubility, and protease activity can lead to protein degradation [42]. Therefore, it is imperative to control these factors during extraction to preserve protein integrity. Buffering solutions maintain pH stability, low temperatures reduce protease activity, and additives like salts, protease inhibitors, osmolytes, and reducing agents shield proteins from environmental stressors [43]. By carefully managing these factors, successful protein extraction can yield active proteins suitable for downstream applications.

3.6. Quantification and Quality Assessment

Accurately quantifying and assessing the quality of extracted proteins presents challenges, particularly in interfering substances such as polysaccharides, secondary metabolites, proteases, lipids, and cellular debris, which can significantly impact protein quality assessment [44]. Detergents like SDS and contaminants such as lipids, nucleic acids, salts, and phenolic compounds hinder protein migration in isoelectric focusing (IEF), while the presence of precipitation solution and dithiothreitol (DTT) can disrupt protein movement during IEF [45]. Salt and contaminants in protein samples also pose challenges to quantification and electrophoresis, interfering with chemical reactions and protein migration, leading to inaccurate measurements and distorted band patterns [46]. High salt concentrations may cause protein aggregation, complicating sample preparation. On the other hand, polysaccharides from fungal cell walls, secondary metabolites like pigments, proteases, lipids, and cellular debris can interfere with protein analysis, impacting quantification assays and disrupting spectrophotometric measurements [47]. Managing these interferences is essential for accurate protein quality assessment. Advanced techniques like mass spectrometry (MS) and chromatography are integral to overcoming challenges in protein analysis. MS analyses ion mass-to-charge ratios with high sensitivity and specificity, while chromatography separates protein mixtures based on physicochemical properties. Despite their resource-intensive nature, MS and chromatography provide crucial accuracy and specificity in protein analysis, making them indispensable for complex sample assessment [48].

4. Cell Wall Disruption by Green Extraction Technologies

Disrupting the fungal cell wall is a crucial step in protein production because it allows for the efficient extraction and recovery of valuable proteins. The fungal cell wall, composed mainly of chitin, glucans, and various proteins, is a robust barrier that protects the cell's contents. While protective of the fungus, this structure poses a significant challenge for biotechnological applications where access to intracellular enzymes and proteins is necessary. Traditional methods for cell wall disruption, such as mechanical grinding, sonication, or harsh chemicals, can be effective but often lead to protein denaturation and reduced yield. Consequently, there has been a growing interest in developing green extraction technologies that are more sustainable and gentler, yet effective, in breaking down the fungal cell wall to enhance protein production. Green extraction technologies aim to reduce environmental impact and improve the efficiency of extracting bioactive compounds, including proteins, from biological sources. These methods are characterised by using sustainable solvents, lower energy consumption, and preserving the extracted compounds' biological activity. These technologies have shown significant promise in the context of fungal cell wall disruption.

4.1. Enzyme-Assisted Extraction

Enzyme-assisted extraction (EAE) is a method that utilises enzymes to break down plant cell walls, facilitating the release of bioactive compounds from various natural sources. This technique is environmentally friendly due to its mild extraction conditions and minimal environmental impact. EAE has improved the extraction efficiencies of antioxidant compounds such as phenolics, flavonoids, anthocyanins, and carotenoids while preserving

their functional properties [49]. EAE involves using enzymes to catalyse the breakdown of plant cell walls, facilitating the release of bioactive compounds such as proteins (Figure 1). Studies have shown that EAE can result in higher protein recovery than other extraction methods, such as alkaline extraction [50]. Furthermore, EAE has been demonstrated to be effective in extracting proteins from different plant materials, including Moringa seeds, where commercial enzyme mixtures were utilised for simultaneous oil and protein extraction [51]. The basic principle of EAE involves using enzymes as catalysts to hydrolyse plant cell walls under optimal conditions, releasing intracellular components [52]. EAE involves well-defined steps to extract and purify proteins from biological materials efficiently. The process begins with carefully selecting appropriate enzymes, such as proteases like papain, bromelain, trypsin, and pepsin, known for their ability to cleave peptide bonds in proteins. Before enzyme treatment, the biological material undergoes pretreatment processes to enhance enzyme accessibility, including grinding, homogenisation, freeze–thaw cycles, or heat treatment to disrupt cell structures. Subsequently, the pretreated material is mixed with an optimised enzyme solution, considering factors like enzyme concentration, temperature, pH, and incubation time to ensure effective protein hydrolysis. As the enzymes act on the proteins, breaking down their structures, they are solubilised and released into the extraction solution. This solution, often containing buffers for pH maintenance and stability, is then separated from solid residues through filtration or centrifugation. The crude protein extract obtained undergoes further purification steps, such as precipitation, chromatography, or membrane filtration, to remove impurities and concentrate the proteins. Finally, the extracted and purified proteins are characterised using analytical techniques like SDS-PAGE, Western blotting, mass spectrometry, and functional assays to assess their purity, molecular weight, structure, and biological activities, ensuring the quality and integrity of the extracted proteins [53].

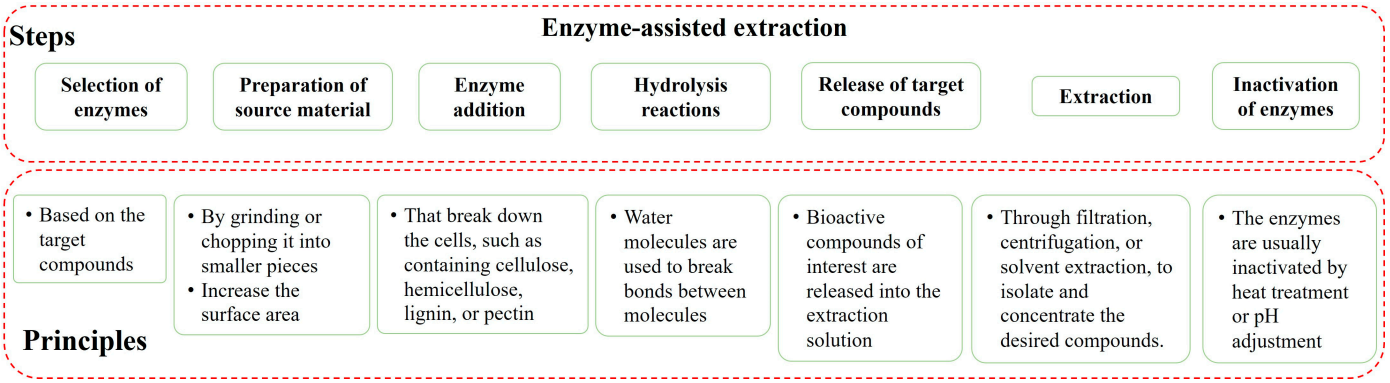

Figure 1. Schematic representation of enzyme-assisted extraction process.

4.2. Mild Mechanical Methods

Mild mechanical methods for cell disruption and protein extraction are gentle techniques that aim to break down cell structures while preserving the integrity of intracellular components. These methods are crucial for extracting proteins without denaturing them and are commonly used in various research fields. Among the mild mechanical methods used for cell disruption and protein extraction, bead milling, ultrasonication, and high-pressure homogenisation are effective techniques.

4.2.1. Bead Milling

Bead milling, or bead beating, is a mechanical technique for cell disruption that utilises shear forces to break down cellular structures (Figure 2). This disruption is caused by the shear forces generated as the cells and beads move and rotate and as cells are ground between the beads [54]. Typically, a bead mill includes components such as a cooling system, a rotating shaft with impellers, a milling chamber, beads, a wire mesh, a location for adding the cell suspension, and an outlet for the disrupted cells. A biomass paste must

first be prepared to initiate the extraction of proteins from cells using this method. This paste is then resuspended in an appropriate buffer. The solution is subsequently introduced into the bead mill's chamber. Within this chamber, beads are set in motion by the rotary shaft, and their speed is increased by an accelerator. As the beads move or rotate, the cell is positioned between two beads in a 'bead-cell-bead' configuration. The intense force exerted in this setup breaks and disrupts the cell wall. The diameter and density of the beads must be optimised based on the size of the target cells. Additionally, to achieve optimal yield from bead millings, factors such as bead type, agitation speed, and lysis agents must be carefully optimised. Throughout this process, the temperature in the chamber is regulated by a continuous cold-water flow through the cooling jacket.

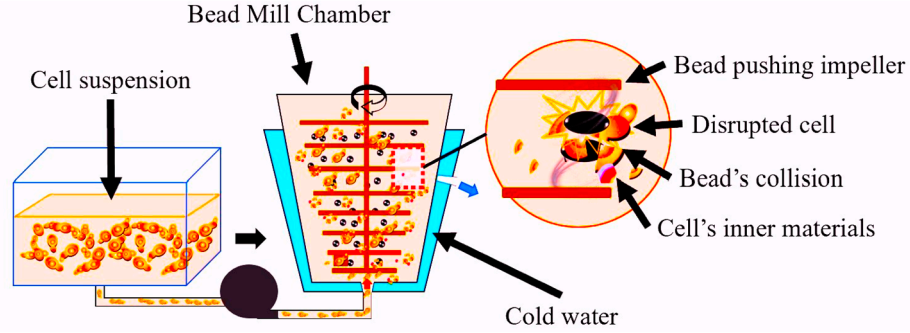

Figure 2. Schematic representation of the bead milling-assisted cell disruption process.

4.2.2. Ultrasonication

Ultrasonication is a powerful technique for protein extraction, utilising mechanical effects such as cavitation, mechanical agitation, and thermal effects to enhance the transport and release of proteins from the extracted matrix (Figure 3). Additionally, ultrasonication can promote the disaggregation and dispersion of proteins in aqueous solutions, resulting in increased extraction yields [55]. Ultrasound, a form of mechanical wave, necessitates an elastic medium for transmission and boasts a wide array of applications. The categorisation of ultrasound technology is typically based on the frequency and energy levels at which the ultrasound waves propagate through the medium, characterized by parameters such as sound power (W), sound intensity (W/m^2), or sound energy density (W/m^3). Ultrasound applications are generally divided into two main groups: low-intensity ultrasound, which operates at high frequencies (100 kHz–1 MHz) and low power levels (typically < 1 W/cm^2), and high-intensity ultrasound, which functions at lower frequencies (16–100 kHz) and higher power levels (typically 10–1000 W/cm^2) [56]. High-frequency ultrasound is predominantly utilised in non-destructive applications, whereas low-frequency ultrasound is commonly employed for inducing physical or chemical modifications in material properties. Ultrasound-assisted extraction (UAE) involves the transmission of acoustic waves into a mixture comprising raw material and a liquid solvent. The mechanism of UAE is characterised by acoustic cavitation, thermal effects, and mechanical actions [57]. When ultrasonic waves are applied, the material undergoes expansion and compression cycles. During expansion, bubbles may form in the liquid solvent due to internal negative pressure, while compression leads to the collapse of these bubbles, inducing cavitation. The collapse of bubbles in proximity to the cell walls of the raw material causes cell disruption, facilitating solvent penetration and enhancing mass transfer. Besides cell disruption, UAE also triggers additional phenomena such as fragmentation, localised erosion, pore formation, increased absorption, and swelling index within the solid matrix of the raw material. To prevent overheating, ultrasonic treatment is administered to a sample immersed in an ice bath. The frequency of ultrasound plays a crucial role in the yield and quality of the target compound.

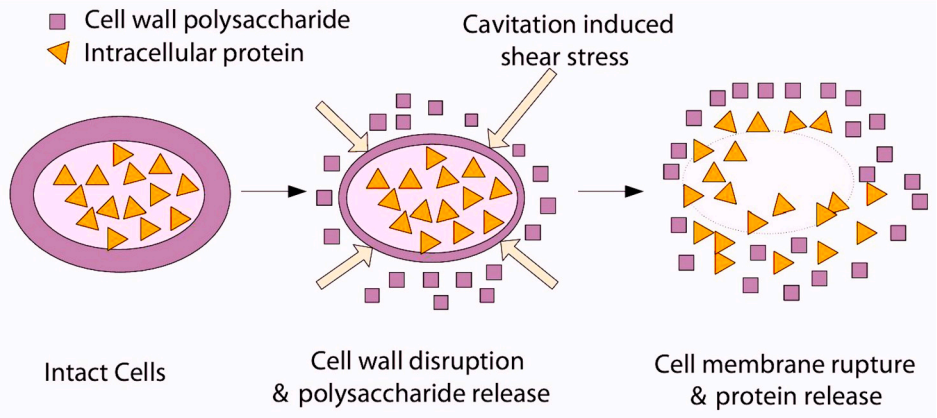

Figure 3. Schematic representation of the ultrasound-assisted cell disruption process (Figure was modified from [58]).

4.2.3. High-Pressure Homogenisation

High-pressure homogenisation is a mechanical method that accomplishes cell disruption by shearing cellular jets at high velocities against solid surfaces (Figure 4). The process involves pumping the cell suspension into a compression chamber, which is pressurised to high levels [59]. In preparation for cell disruption using a high-pressure homogeniser, cells are initially suspended in a liquid medium, such as a buffer or saline solution. This suspension is subsequently directed into the high-pressure chamber of the homogeniser, where it encounters intense pressure and shear forces. The precise regulation of pressure and flow rate is crucial to ensure optimal cell disruption while safeguarding the integrity of the intracellular components. Upon traversing the narrow orifice or valve of the homogeniser, the high-pressure environment induces cell rupture, releasing cellular contents into the surrounding liquid medium. These liberated components can then be gathered and further processed as required.

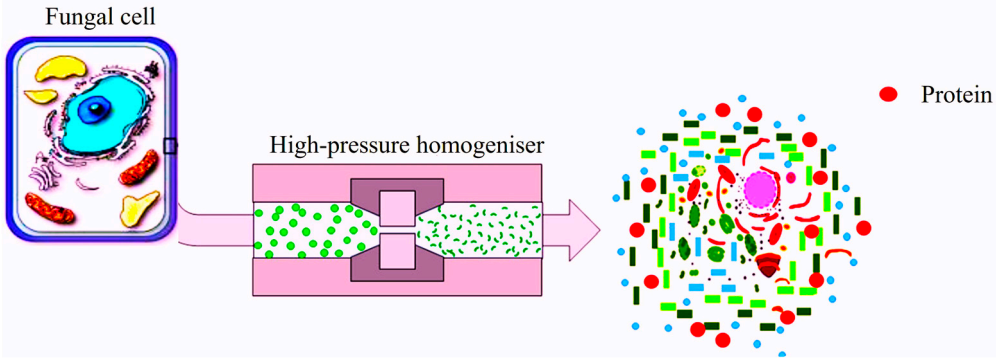

Figure 4. Schematic representation of the high-pressure homogenisation-assisted cell disruption process (Figure was modified from [60]).

4.3. Pulsed Electric Fields

The pulsed electric fields (PEF) method is employed to achieve cell disruption for protein extraction. PEF involves the application of short, high-voltage electric pulses to cells, forming nanopores in the cell membrane and allowing the extraction of intracellular components (Figure 5). This technique, known as electroporation, has been widely used for disrupting various types of cells, including microalgae and bacteria [61,62]. PEF has been shown to induce multiple physiological phenomena, such as irreversible electroporation and reversible electro-permeabilisation. Furthermore, PEF has been found to be effective in accelerating the extraction of bioactive compounds, such as anthocyanins and ginsenosides, from plant sources [63,64]. The process involves applying high-voltage direct-current pulses intermittently for very short durations (microseconds to milliseconds) through

a product positioned between two electrodes. This voltage creates an electric field, the strength of which is determined by the distance between the electrodes and the applied voltage. When the electric field reaches a certain intensity, it induces electroporation, increasing the permeability of the cytoplasmic membrane to the passage of ions and macromolecules. Consequently, PEF treatment promotes the migration of compounds from the cell's cytoplasm through the membrane, as the membrane loses its selective permeability following the treatment. PEF has been shown to disrupt fungal cells, such as *Candida albicans* [65]. Additionally, PEF-assisted autolysis has been explored as a method for the nondestructive extraction of intracellular molecules from yeast, indicating the disruptive potential of PEF on fungal cells [66].

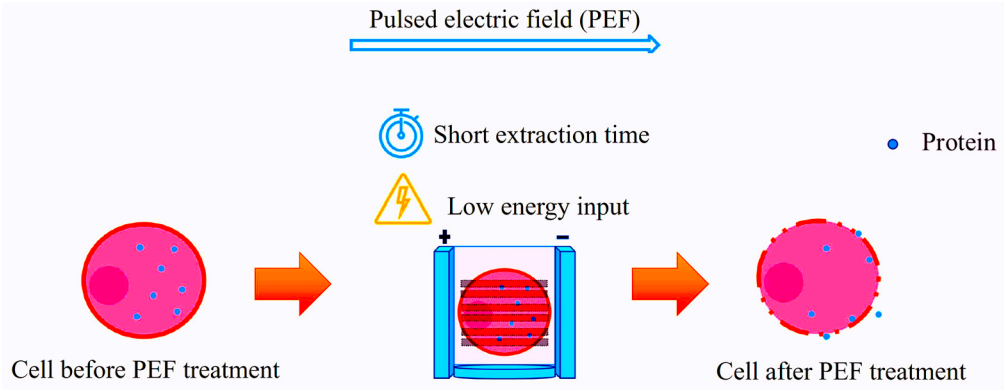

Figure 5. Schematic representation of the pulsed electric field-assisted cell disruption process (Figure was modified from [67]).

4.4. Microwave-Assisted Extraction

Microwave-assisted extraction (MAE) is a sophisticated technique that leverages the unique properties of microwaves to enhance the extraction of proteins from various biological matrices. Microwaves are electromagnetic fields with a frequency range from 300 MHz to 300 GHz, or wavelengths between 1 cm and 1 m, consisting of perpendicular electric and magnetic fields [68]. This method is particularly effective due to its ability to rapidly heat and break down cellular structures, facilitating the release of proteins. The core mechanism of microwave heating involves converting electromagnetic energy into thermal energy through two primary processes: ionic conduction and dipole rotation [69]. Ionic conduction generates heat as the movement of ions through a medium is impeded, leading to friction and subsequent heat production (Figure 6). In contrast, dipole rotation involves the alignment of molecules with a dipole moment to the fluctuating electric field. This alignment causes rotational motion and friction among molecules, releasing heat energy. Specifically, microwave heating disrupts weak hydrogen bonds in biological molecules through the dipole rotation of water and other polar molecules, enhancing the breakdown of cellular components. When microwaves interact with a sample, their energy absorption is determined by the dielectric constants of the sample components. An intriguing aspect of MAE is that microwaves can penetrate the solvent and directly heat the solid sample without significant absorption by the solvent itself. This leads to rapid heating of the moisture within the solid sample, which then evaporates, creating high vapour pressure. This elevated vapour pressure exerts force on the cell walls, causing them to rupture and release their cellular contents, including proteins, into the solvent. The MAE process can be broken down into three sequential steps [70]. The first step involves the separation of solutes (proteins and other bioactive compounds) from the sample matrix under the influence of increased pressure and temperature. This separation is facilitated by the rapid and uniform heating provided by the microwaves, which destabilises the cell wall and other binding structures. The second step is the diffusion of the solvent into the sample matrix. The high temperature and pressure help the solvent permeate the matrix more effectively, dissolving the solutes. The final step is the release of these solutes from the

matrix into the solvent, which is expedited by the continuous agitation and heat provided by the microwave energy.

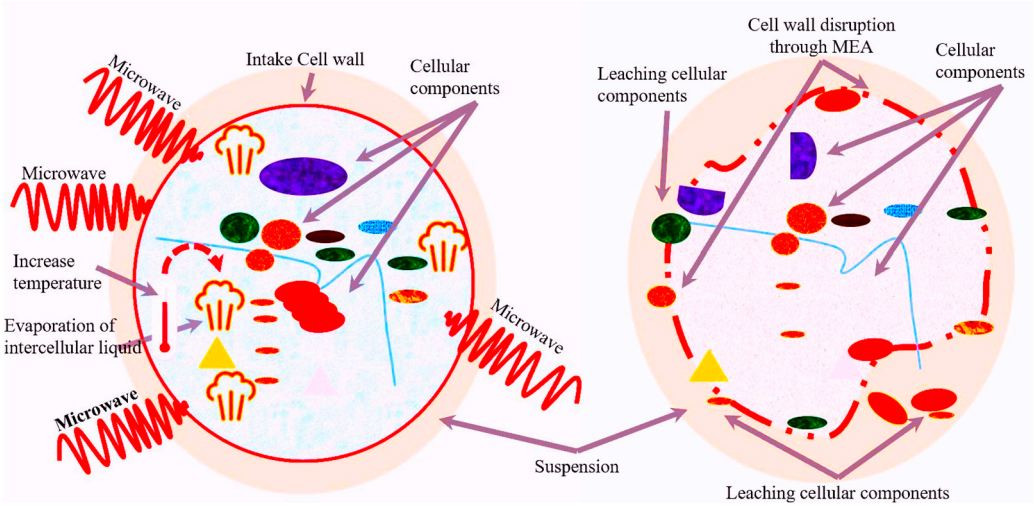

Figure 6. Schematic representation of the microwave-assisted cell disruption process.

4.5. Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) is an innovative method extensively used for extracting valuable compounds from various matrices, including the disruption of cell walls for protein production [71]. This technique primarily leverages supercritical fluids, most commonly carbon dioxide (CO_2), due to its moderate critical temperature and pressure, making it suitable for extracting heat-sensitive biological molecules like proteins without denaturing them (Figure 7). The mechanism begins when CO_2 is pressurised and heated above its critical point, exhibiting unique properties of gases and liquids [72]. At this supercritical state, CO_2 has a gas-like diffusivity and viscosity and a liquid-like density, enabling it to penetrate cellular structures more effectively than either state alone. This penetration is crucial for disrupting cell walls, especially in robust cells like microalgae, fungi, or yeast, which are typical sources of valuable proteins. The supercritical CO_2 is pumped into an extraction vessel containing the biomass during extraction. As it permeates the cell walls, it causes the walls to swell and rupture due to the pressure differential and the solvation effects of the supercritical fluid. This rupture releases intracellular contents, including proteins, into the surrounding fluid. The solubility of proteins in supercritical CO_2 is generally low, which aids in their separation. As the fluid containing the disrupted cellular components flows out of the extraction vessel, it is depressurised and returned to a gaseous or liquid state. The rapid change in conditions causes the proteins and other cellular components to precipitate out, as they are not soluble in non-supercritical CO_2. This phase separation allows for the collection of proteins in a relatively pure form. Furthermore, the selectivity of supercritical CO_2 can be enhanced by adjusting temperature and pressure or by adding co-solvents like ethanol or water. These modifications can target specific cellular components or improve the efficiency of cell wall disruption and protein release. For instance, higher pressures generally increase the extraction yield by enhancing the fluid's density and solvation power.

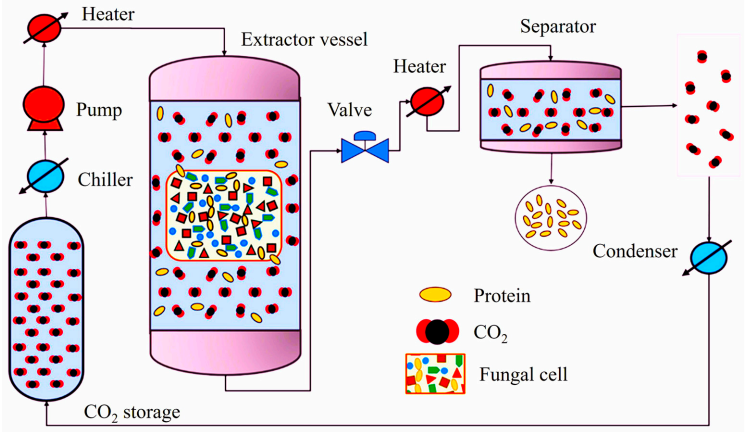

Figure 7. Schematic representation of the supercritical fluid extraction-assisted cell disruption process (Figure was modified from [72]).

4.6. Innovative Solvent Use

Green solvents, including deep eutectic solvents (DES) and natural deep eutectic solvents (NADES), represent a revolutionary shift in protein extraction due to their environmentally friendly properties and effectiveness [73]. Deep eutectic solvents are a class of solvents formed by mixing a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA) at a specific molar ratio. This leads to a significant depression in the mixture's melting point [74]. This unique property allows DES to remain liquid at room temperature, facilitating the extraction of proteins under mild conditions. The mechanism by which DES and NADES extract proteins is multifaceted [75]. First, these solvents disrupt the hydrogen bonding network within proteins and between proteins and water molecules. This disruption weakens the structural integrity of the protein, making it more soluble in the solvent. Additionally, DES's low viscosity and high solvation power enhance solvent molecules' diffusion into the protein matrix, leading to more efficient extraction. NADES, a subset of DES, comprises natural components like organic acids, sugars, and amino acids. These solvents leverage their constituents' biocompatibility and non-toxic nature to extract proteins in a manner that is not only effective but also environmentally benign. The natural components of NADES often interact with protein molecules via hydrogen bonding and hydrophobic interactions, further facilitating the dissolution and extraction of proteins. The efficacy of DES and NADES in protein extraction is also influenced by their tunable properties. By adjusting the ratio of HBD and HBA or choosing different natural components for NADES, the polarity, hydrophobicity, and other solvent characteristics can be optimised for specific proteins. This tunability allows for the selective extraction of target proteins or the removal of unwanted contaminants, enhancing the purity and yield of the final product. Moreover, the low volatility of DES and NADES contributes to reduced environmental impact compared to traditional volatile organic solvents [76]. This aspect minimises the release of harmful vapours into the atmosphere and reduces the risk of fire and health hazards in the laboratory or industrial setting.

5. Application of Green Extraction Technologies for Fungal Protein Extraction

While green extraction technologies have significantly advanced protein extraction from various food matrices, their application in extracting proteins from fungi remains underexplored. This oversight is notable given the increasing interest in fungal proteins for their potential health benefits and sustainability. Among the innovative techniques that show promise for fungal protein extraction are enzyme-, ultrasound-, pulsed electric field-, and high-pressure-assisted extraction techniques (Table 2).

One study utilised yeast extract enzyme and alkaline protease to extract protein from regular brewer's yeast and selenium-rich brewer's yeast, which contained a high selenium concentration of 1900 μg/g [77]. This process yielded eight distinct peptide fractions.

In vitro experiments revealed that a specific peptide fraction from the selenium (Se)-rich yeast, with a molecular weight of less than 1 kDa, exhibited protective effects against skin damage induced by UVB radiation. It achieved this by boosting aquaporin-3 expression and obstructing detrimental signalling pathways, thus alleviating oxidative stress in skin cells. In a separate investigation, researchers extracted three ACE inhibitor peptides from *Agaricus bisporus* using protease and alkaline protease [78]. These peptides demonstrated an average activity of 80.68%. Their strong resistance to temperature, pH, and digestive enzymes in the gastrointestinal tract suggests that they could be helpful in the development of blood pressure-lowering medications.

One study demonstrated that ultrasound-assisted enzymatic extraction (UAEE) effectively extracted 73.94% of water-soluble protein and 61.24% polypeptide from waste beer yeast [79]. This method proved more efficient and time-saving than traditional techniques, achieving higher protein yields compared to other sources: 39.12% from *Moringa oleifera* seeds [80] and only 8.5% of *Ulva rigida* macroalgae following alkaline pretreatment [81]. Another investigation employed UAEE to extract proteins and polypeptides from *Cordyceps militaris* [82]. This approach surpassed heated reflux extraction in protein yield, suggesting its potential to enhance the extraction of chemical contents. Repeated extractions under optimal conditions increased the protein yield to 45.06%, a 1.22-fold improvement over the control. The highest polypeptide yield reached was 16.42%. Additionally, polypeptides (<3000 Da) showed significant antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*, with inhibition zones of 12.08 ± 0.22, 6.67 ± 0.12, and 10.32 ± 0.23 mm, respectively. Anticancer tests on osteosarcoma SAO-S cells and bladder cancer T24 cells revealed IC_{50} values of 0.49 mg/L and 0.23 mg/L, indicating the polypeptide's potent effect against these cancer lines.

Traditional mechanical rupture methods disrupt cells, releasing compounds and micronising cellular debris, complicating downstream separation [83]. On the other hand, PEF uses electroporation selectively and efficiently, releasing cytoplasmic compounds from microbial cells like yeast or microalgae without damaging the cell wall [84]. This technique has been applied to extract a range of valuable biomolecules from *S. cerevisiae* yeast biomass, reducing waste and efficiently recovering protein at 187.82 mg/g dry weight. In another study, a PEF-assisted pressure technique, ranging from 100–1000 V/cm, was employed to extract polysaccharides, proteins, and terpenoids from *Agaricus bisporus*. Parniakov et al. [85] utilised this technique along with a solvent method to extract proteins from *Agaricus bisporus* at an electric field strength of 800 V/cm. The maximum yield of protein extraction using pressure extraction (PE) alone was approximately 0.26. However, when combined with PEF-assisted extraction, the yield increased to approximately 0.42. Additionally, protein extracted from water extraction at 70 °C for 2 h and ethanol extraction at 25 °C for 24 h were cloudy and contained impurities. In contrast, extracts from the combined PE + PEF method were clear. Furthermore, another study highlighted that the combination of PEF with PE produced extracts of edible fungi with higher yields of polysaccharides and proteins compared to using PE alone [86].

Table 2. Fungal protein extraction using different green technology.

Green Extraction Technology	Fungi Species	Experimental Conditions	Protein Yield	Protein Type	Key Findings	Refs.
Ultrasound-assisted extraction	*Saccharomyces cerevisiae*	Ultrasonic conditions: power-250, 300, 350, 400, and 450 W; pH-5.5, 6.5, 7.5, 8.5, and 9.5; Solid–liquid ratio: 6%, 8%, 10%, 12%, and 14% Enzyme used: trypsin	73.94%	Antioxidant	The polypeptide's scavenging activity against hydroxyl radical, DPPH radical, and ABTS radical reached 95.10%, 98.37%, and 69.41%, respectively.	[79]

Table 2. *Cont.*

Green Extraction Technology	Fungi Species	Experimental Conditions	Protein Yield	Protein Type	Key Findings	Refs.
Enzymatic-assisted extraction	*Cordyceps militaris*	Ultrasonic conditions: temperature: 25 °C; power: 100 W; pH: 8.0, 8.5, and 9.0; Solid–liquid ratio: 1:25, 1:28, and 1:30; time: 3.0, 3.2, and 3.5 h Enzymes used: alkaline protease, neutral protease, papain, trypsin, and pepsin	45.06%	Antimicrobial and anticancer polypeptides	Polypeptides (<3000 Da) showed good antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*, with inhibitory zones of (12.08 ± 0.22), (6.67 ± 0.12), and (10.32 ± 0.23) mm, respectively.	[82]
	Agricus bisporus	Alkaline protease, pH: 8.43, enzymolysis temperature: 44.32 °C, and enzymolysis time: 3.52 h	6.678%.	ACE inhibitor	The average activity of the three novel ACE inhibitory peptides was 80.68%, and the IC_{50} value was 0.9 mg/mL.	[78]
	Se-rich brewer's yeast	Alkaline protease, pH: 11, temperature: 60 °C, enzyme to substrate ratio: 6000 U/g.	100-fold	Se-rich peptide fraction	In vitro free radical scavenging and lipid peroxidation inhibition assays showed that Se-rich peptide fractions with lower MW of <1 kDa had the highest antioxidant activity compared with Se-rich peptide fractions with higher MW of <3 kDa or normal peptide fractions.	[77]
Pulsed electric fields-assisted extraction	*Saccharomyces cerevisiae*	10, 15, and 20 kV/cm, 39.8 and 159.3 Hz, 50–200 µs	187.82 ± 3.75 mg/g dry weight (protein)	Antioxidant	84–89% of the total antioxidant activity	[87]
Pressure extraction (PE) assisted by pulsed electric field (PEF)	*Agaricus bisporus*	100–1000 V/cm, 5 bar, 0.4 s	The maximum protein yield using PE alone was about 0.26, but with PEF extraction, it increased to around 0.42.	Polyphenol-enriched protein	The PE + PEF method gave a higher ratio of nucleic acid/proteins in comparison with the PE method.	[85]

Abbreviation: DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), ACE: angiotensin-converting Enzyme, MW: molecular weight.

6. Conclusions and Future Perspectives

This review underscores the potential of green extraction technologies in enhancing the efficiency and sustainability of protein production from fungi. The increasing global demand for sustainable and high-quality protein sources necessitates innovative approaches, and fungi, with their unique nutritional profiles and ecological benefits, present an excellent alternative. However, the challenge lies in efficiently disrupting the robust fungal cell walls and extracting proteins without compromising their integrity. Green extraction technologies have shown promise in addressing these challenges. These methods employ milder

conditions compared to conventional techniques, thereby preserving the structural integrity and functionality of proteins. The review has highlighted the specific advantages of each method in terms of efficiency. For instance, EAE uses specific enzymes to degrade cell wall components, offering a targeted approach that minimises protein damage. Similarly, UAE and MAE employ physical methods that enhance solvent penetration and mass transfer, leading to higher yields of bioactive compounds. Using PEF and supercritical fluids also demonstrates the potential for efficient cell disruption and protein extraction, with the added benefit of being environmentally friendly. The review also addresses fungal proteins' bioactivities, including antifatigue, immunomodulatory, and various therapeutic properties such as antihypertensive, antibacterial, antifungal, antioxidant, antiviral, and anticancer activities. These bioactivities make fungal proteins a valuable nutritional source and a promising component in pharmaceutical and nutraceutical applications.

Despite the advancements in green extraction technologies, several challenges remain. The variability in fungal cell wall composition across different species and strains necessitates tailored extraction approaches. Additionally, the scalability of these technologies from laboratory to industrial scale requires further optimisation. The stability and solubility of extracted proteins are also critical factors that need to be managed to ensure the quality and efficacy of the final product [88]. Advancements in green extraction technologies will likely centre around refining existing methods and integrating multiple approaches to maximise efficiency and yield. For instance, combining EAE with other mild mechanical methods such as ultrasonication and high-pressure homogenisation could enhance cell wall disruption while preserving protein integrity. This hybrid approach could leverage the benefits of each method, resulting in higher protein recovery rates and better preservation of bioactive compounds. The exploration and application of novel green solvents, particularly DES and NADES, represent a cutting-edge area for future research [89]. These solvents, composed of environmentally benign components, can dissolve a wide range of biomolecules, including proteins. Future studies should identify the optimal combinations of hydrogen bond donors and acceptors in DES/NADES to enhance protein solubility and extraction efficiency. Additionally, understanding the interactions between these solvents and fungal cell wall components will be crucial for developing tailored extraction protocols. Furthermore, the design of bioreactors and extraction processes for green extraction technologies will be critical for scaling up production [90]. Future research should investigate the integration of continuous-flow systems and in situ extraction methods to streamline the process and reduce energy consumption. Bioreactors equipped with real-time monitoring and control systems can optimise extraction conditions dynamically, ensuring consistent product quality and maximising yield. Most current research focuses on a limited number of fungal species. Expanding the scope to include a broader diversity of fungi, particularly those with unique metabolic and structural properties, will uncover new opportunities for protein extraction. Future research should explore underutilised fungal species and their potential as alternative protein sources, leveraging their unique biochemical pathways and ecological roles.

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