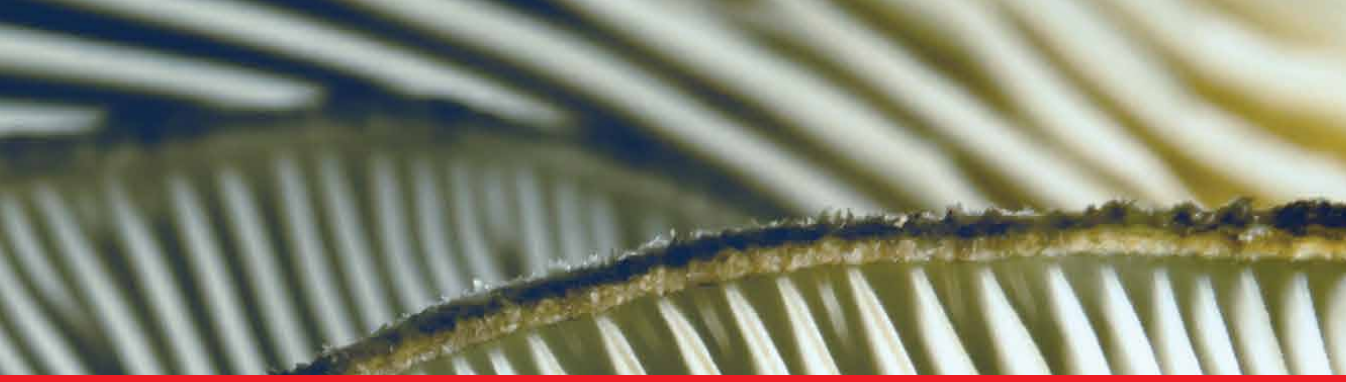


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Advances in Functional Foods

New Perspectives and Sustainable Practices

Edited by Bilge Sayın



Advances in Functional Foods - New Perspectives and Sustainable Practices

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Food Science and Nutrition

Volume 23

Aims and Scope of the Series

The significance of food is undeniable, especially in light of the impending challenge facing humanity: ensuring there will be enough food to meet the basic needs of a population expected to reach approximately 10 billion by 2050. These food-related challenges align with some of the United Nations' sustainable development goals, with a target to achieve them by 2030. One thing is certain: food should be not only nourishing and safe but also tailored to the diverse needs of individuals throughout their lifetimes, all while meeting consumers' sensory expectations. Understanding the diverse chemical composition of food, often referred to as biodiversity, and how these components can contribute to human health by considering factors like bioaccessibility, bioavailability, and bioactivity at the organ level, is crucial for grasping and promoting a healthy diet. Thanks to the continuous evolution of analytical methods and interdisciplinary research, significant strides have been made in the field of food science and nutrition.

Meet the Series Editor



Maria Rosário Bronze has been working in Analytical Chemistry since 1986. Her Ph.D. in 1999 contributed to the study of food products using capillary electrophoresis. The main goal of her research since 1999 has been focused on Analytical Chemistry applied mainly to the analysis of foods and by-products of food industry. She conducted research in collaboration with national and international research groups, at iBET and ITQB Technology Division. From 2017 until 2021 she was head of Food & Health Division at iBET and head of the Food Functionality and Bioactives Laboratory. MR Bronze has been an Associate Professor at the Pharmacy Faculty of Lisbon University and head of the Structural Analysis Laboratory since 2012. As a researcher, MR Bronze is a Senior Scientific Advisor at Food & Health Division at iBET and Head of Food Functionality and Bioactives Laboratory at the same Institute, Collaborator at iMED and Researcher at ITQB NOVA. Her current research is focused on quality and beneficial health effects of food components. Gas and liquid chromatography associated with mass spectrometry are used by MR Bronze in the characterization of samples. Sensory evaluation is also an important area of her research. The main food products studied by her are olive tree products (olive, olive oil, leaves), cereals such as maize, legumes (faba bean, pea, chickpea, lentils) fruits (apple, grapes, opuntia ficus), fruit juices and wine, among others. More recently her interests have also involved biodiversity, bioaccessibility, and bioavailability studies on food products and their components, mainly phytochemicals as phenolic compounds, using different analytical tools such as mass spectrometry. As a senior scientific advisor at Food & Health Division at iBET she is involved in different areas: (i) isolation, characterization and formulation of bioactive and functional compounds or extracts from natural sources and wastes from food and other related industries; (ii) pre-clinical assays to provide support to understand health claims related with the beneficial effects of food nutrients/bioactive components; (iii) establishment of analytical methodologies including mass spectrometry state-of-the-art to fully characterize different matrices, from food products, natural extracts or biological fluids (Food Functionality and Bioactives Laboratory).

Meet the Volume Editor



Dr. Bilge Sayın is an Assistant Professor in the Department of Gastronomy and Culinary Arts at Ardahan University, Türkiye. She received her BSc degree in Food Engineering from Atatürk University, followed by an MSc degree from Ankara University and a Ph.D. degree in Food Engineering from Atatürk University. Her academic training and professional experience have provided a strong foundation in food science and biotechnology. Dr. Sayın's research focuses on food biotechnology, bioprocess optimization, food safety, and functional foods, with particular emphasis on the biotechnological valorization of food and agro-industrial wastes. Her studies aim to convert low-cost and residual substrates into value-added bioproducts such as citric acid and single-cell oil, thereby contributing to sustainable production systems and circular bio-economy principles. She has published extensively in peer-reviewed international journals and contributed to scientific books on green chemistry, fermentation technology, and sustainable food processing. Dr. Sayın has also been actively involved in international scientific conferences, serving as a member of the scientific committee, a session chair, and an invited speaker. In addition to her research activities, Dr. Sayın teaches undergraduate and graduate courses in functional foods, fermentation technology, food microbiology, food biotechnology, and the valorization of food-industry waste. Her ongoing research interests include the scale-up of sustainable bioprocesses and the development of innovative biotechnological solutions for food, pharmaceutical, and cosmetic applications.

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Preface

The rapid evolution of food science and technology has expanded the concept of food beyond basic nutrition, positioning functional foods as key elements in health promotion and disease prevention. At the same time, growing global challenges, including population growth, environmental pressures, food security concerns, and public health demands, have intensified the search for innovative, scientifically grounded approaches that support both human health and sustainable food systems. Within this framework, *Advances in Functional Foods – New Perspectives and Sustainable Practices* brings together contemporary research integrating food science, biotechnology, nutrition, and sustainability. This edited volume offers a multidisciplinary perspective by showcasing emerging bioactive compounds, advanced processing technologies, and novel production systems that contribute to health-promoting foods while encouraging resource-efficient and responsible food production.

The first chapter, "Bioactive Peptides: Functional Roles, Mechanisms, and Applications in Food and Health," provides a comprehensive overview of bioactive peptides derived from food proteins and their functional significance in human health. By addressing their biological activities, mechanisms of action, and applications in food systems, this chapter demonstrates the growing importance of bioactive peptides in functional foods, nutraceuticals, and disease prevention.

The second chapter, "Optimizing Enzymes for Dairy Processing: A Protein Engineering Perspective," focuses on recent advances in protein engineering strategies to improve enzyme performance in dairy processing. This chapter illustrates how tailored enzyme design can enhance processing efficiency, product quality, and sustainability, supporting innovation within the dairy industry.

The third chapter, "Enzymatic Biotransformations via the Recombinantly Produced Enzymes in the Production of Functional Foods," explores the role of recombinant enzymes in driving targeted biotransformation processes. By showcasing enzyme-assisted modification of food components, this chapter demonstrates how modern biotechnology can be used to improve nutritional value, bioavailability, and functional properties of foods.

The fourth chapter, "How Cold Plasma Processing Shapes the Volatile Compound Profile of Fruit Juices," examines the influence of cold plasma technology on the volatile composition and sensory characteristics of fruit juices. This chapter offers insights into non-thermal processing approaches that preserve quality while modulating aroma profiles, contributing to the development of minimally processed and consumer-acceptable beverages.

The fifth chapter, "Valorization of Grape Waste for Sustainable Mushroom Cultivation," addresses the conversion of agro-industrial by-products into value-added

resources. Focusing on grape waste as a substrate for mushroom cultivation, this chapter presents sustainable waste-management practices and circular-economy principles within food production systems.

The sixth chapter, "The Utilization of Cassava Leaves as a Functional Food Based on Local Wisdom," highlights the nutritional and functional potential of cassava leaves by integrating traditional knowledge with scientific evaluation. This chapter underscores the role of underutilized plant resources in improving food security, dietary diversity, and sustainable nutrition.

The final chapter, "Engineered Microbial Factories: Next-Generation Food Production Systems for Emergency and Military Applications," explores engineered microorganisms as innovative and resilient platforms for food production. Addressing the challenges of food supply in emergency and resource-limited contexts, this chapter outlines the strategic potential of microbial systems for future military and humanitarian nutrition solutions.

In this context, the book is intended as a comprehensive resource for researchers, graduate students, food technologists, nutrition scientists, and professionals seeking insights into emerging trends and practical approaches in the field of functional foods.

I want to express my sincere appreciation to all contributing authors for their valuable scientific contributions and collaborative efforts. Gratitude is also extended to the publishing team for their support throughout the editorial process. It is hoped that this book will serve as a useful reference and inspire further research and innovation in the field of functional foods.

I would also like to extend my deepest gratitude to my father, Serdar Sayın, and my mother, Saadet Sayın, whose support and encouragement played a pivotal role in my journey into academic life and enabled me to pursue the work I love with dedication and enthusiasm.

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Chapter 1

Bioactive Peptides: Functional Roles, Mechanisms, and Applications in Food and Health

Na Dong and Chenyu Xue

Abstract

Bioactive peptides (BPs), predominantly generated from food-derived proteins *via* enzymatic hydrolysis and microbial fermentation, have demonstrated multifunctional biological activities. These include antimicrobial, immunomodulatory, antioxidant, and anticancer effects, which are often mediated through mechanisms such as enzyme inhibition, metal chelation, and modulation of cellular signaling pathways. Owing to these properties, BPs have attracted considerable attention for the development of functional foods, dietary supplements, and therapeutic agents. This chapter provides a comprehensive overview of their physiological functions, molecular mechanisms of action, and production methods, including enzymatic hydrolysis, microbial fermentation, membrane separation, and emerging omics-based strategies. Key challenges in bioavailability, stability, and large-scale applications are discussed, along with recent innovations and future perspectives for the integration of BPs into health-promoting food systems.

Keywords: bioactive peptides, immunomodulatory, antimicrobial, antioxidant, functional foods, fermentation technology, purification technology, industrialization of production

1. Introduction

Bioactive peptides (BPs) have gained significant scientific interest due to their roles in health management and disease prevention/treatment [1]. Distinguished from conventional pharmaceuticals, these food-derived molecules exhibit lower toxicity, superior biocompatibility, and potent biological activities, positioning them as viable candidates for functional foods, nutraceuticals, and therapeutics [2]. Notably, the integration of multi-omics technologies (e.g., transcriptomics, peptidomics) and advanced delivery systems (e.g., self-assembled nanoparticles) is now accelerating the discovery and application of novel peptides with targeted functions [3–5].

Despite these technological gains, clinical translation remains constrained by three primary barriers:

- *Poor oral bioavailability*, owing to rapid enzymatic degradation in the gastrointestinal tract [6]. *In vivo*, BPs are exposed to a cascade of proteases (pepsin in the stomach; trypsin, chymotrypsin, and brush-border peptidases in the small intestine) that cleave peptide bonds within minutes of ingestion. This results in very low systemic exposure—often <1% of the administered dose reaches the bloodstream intact—making it difficult to achieve therapeutic plasma concentrations *via* oral routes;

- *Structural instability*, resulting in loss of activity during processing and storage [7]. Many BPs possess flexible, unstructured regions that are highly sensitive to pH shifts, elevated temperatures, and shear forces encountered during spray-drying, lyophilization, or food matrix incorporation. Such conditions can trigger unfolding, aggregation, or deamidation, all of which abolish sequence-specific interactions with biological targets;

- *Economic inefficiency*, in scaled enzymatic or fermentation-based manufacturing [8]. Enzyme-catalyzed hydrolysis requires expensive proteases at high loadings, and downstream purification (e.g., ultrafiltration, chromatography) further drives up costs. Fermentation systems often yield low peptide titers (<100 mg/L) and demand complex media and lengthy culture times, making large-scale production cost-prohibitive without significant process intensification.

Emerging strategies are addressing these barriers:

- *Nano-delivery systems*: Lipid-peptide hybrid nanoparticles, such as CD47-mimetic peptide-decorated lipid nanoparticles (LNPs), enhance systemic circulation and target specificity [9]. These carriers encapsulate BPs within a protective lipid bilayer, shielding them from proteolytic enzymes and preventing premature degradation. Surface decoration with "self" peptides (e.g., CD47 motifs) further reduces opsonization and clearance by the mononuclear phagocyte system, extending half-life and enabling tissue-specific delivery;

- *Omics-driven discovery*: Integrated multi-omics screening coupled with molecular docking has yielded higher hit rates for novel DPP-IV inhibitory peptides from cannabis seed proteins compared with traditional methods [10]. By layering transcriptomic expression profiles, peptidomic mass spectrometry data, and in silico binding-energy predictions, researchers can rapidly prioritize candidates with optimal sequence motifs and binding pockets—bypassing the trial-and-error of one-by-one isolation and sustainable production;

- *CRISPR-engineered microbial chassis*: For example, expressing rice-bean peptides in *Bacillus subtilis* significantly boosts peptide yields and reduces production costs [11]. Metabolic rewiring—such as deletion of competing proteases, overexpression of secretion pathways, and tuning of precursor amino acid pools—can raise titers into the gram-per-liter range. Coupled with low-cost fermentation media and streamlined downstream capture, these platforms approach the economics required for industrial-scale BP manufacture.

Against this backdrop, this chapter systematically reviews BPs peptides, covering their definition, classification, key functions, underlying mechanisms, production

technologies, and current/future applications in food, pharmaceutical, and related fields. Analyzes cutting-edge applications in food/pharma, focusing on nanocarrier-enhanced delivery and multi-omics screening platforms; evaluates industrialization challenges and future directions shaped by bioengineering advances. It also critically examines challenges hindering industrialization and outlines promising research directions.

2. Functions of BPs

In recent years, the value of food proteins has been increasingly recognized due to rapidly expanding knowledge regarding physiologically active peptides. Depending on their amino acid composition and sequence, BPs exhibit diverse biological activities, including antimicrobial, immunomodulatory, antioxidant, and anticancer properties, among others (**Figure 1**).

2.1 Antimicrobial function

BPs exhibit broad-spectrum antimicrobial properties, making them promising alternatives to conventional antibiotics [12]. Notably, these peptides reduce the likelihood of antimicrobial resistance development, which is a growing concern in public health. For example, BCp12, derived from buffalo casein, demonstrates potent bacteriostatic effects with low cytotoxicity [13]. Similarly, bovine casein-derived peptides, such as KNR50, disrupt bacterial membrane integrity and avoid resistance induction [14]. Additionally, peptides from Arsenura armida proteins and Sargassum angustifolium exhibit antimicrobial actions, particularly against Gram-positive pathogens and *Salmonella typhimurium* (*S. typhimurium*) [15]. These antimicrobial peptides are increasingly utilized in food preservation due to their biocompatibility and safety. Together, their multifaceted modes of action and minimal propensity to induce resistance make these peptides especially attractive for enhancing food safety, extending shelf life, and developing next-generation antimicrobial agents.

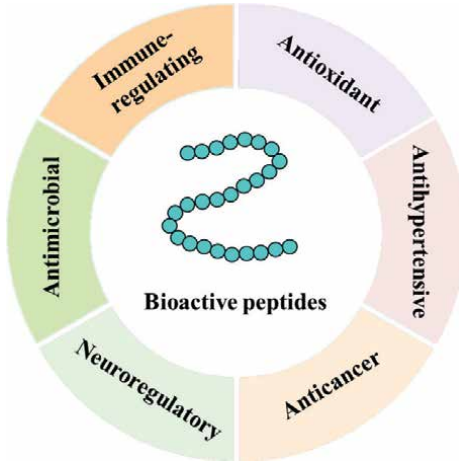


Figure 1.
Functions of bioactive peptides.

2.2 Immune-regulating function

BPs play a crucial role in modulating the immune system, enhancing immune competence through mechanisms such as boosting macrophage phagocytosis, stimulating lymphocyte proliferation, and modulating cytokine production. Several food-derived peptides, such as PSP-5 from porcine spleen protein, have been shown to improve macrophage viability and prevent immune suppression in mice [16]. Peptides derived from sturgeon cartilage, quinoa, and rice seeds also enhance macrophage activity and activate systemic immunity [17–19]. Furthermore, BPs can inhibit inflammation by modulating inflammatory pathways, such as the NF-κB signaling pathway. Examples include peptides from bovine milk osteopontin and fish collagen peptides that reduce inflammation and promote intestinal barrier repair [20, 21]. Some peptide-based nanomaterials also have immunomodulatory activities. For example, active peptide LL37 and CpG nucleic acid self-loaded nanoparticles can increase the secretion of TNF-α by 3.5-fold by activating macropinocytosis and membrane penetration pathways [22]. These peptides demonstrate remarkable versatility in fine-tuning the immune system, capable of both boosting defense mechanisms (phagocytosis, lymphocyte activity) and suppressing excessive inflammation. This balanced immunomodulation is central to their therapeutic potential for managing immune-related disorders and enhancing overall immune competence.

2.3 Antioxidant function

Oxidative stress, caused by an imbalance of reactive oxygen species (ROS) and antioxidant mechanisms, can lead to chronic diseases and accelerate aging [23]. BPs serve as antioxidants, scavenging free radicals, inhibiting lipid peroxidation, and chelating metal ions. Peptides derived from venison, mackerel muscle protein, and Sargassum angustifolium exhibit strong antioxidant properties, including 2,2-Diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and superoxide dismutase-like activity [24, 25]. Peptides from Bombyx mori and other sources activate antioxidant pathways, such as the Nrf2/HO-1 signaling axis, enhancing antioxidant enzyme expression [26, 27]. These peptides play a significant role in mitigating oxidative damage associated with chronic diseases and the aging process.

2.4 Other functions

Beyond core functions, BPs demonstrate clinically relevant activities, including:

- Antihypertensive effects: Legume-derived angiotensin-converting enzyme (ACE)-inhibitory peptides (e.g., lunasin) [28];
- Anticancer activity: Camel lactoferrin peptides inducing apoptosis in HepG2 cells [29];
- Neuroregulation: Beta-casomorphin-10 modulating opioid receptors to alleviate pain [30].
- Synergistic multi-functionality: Camel milk hydrolysates concurrently regulating immunity, blood pressure, and glucose metabolism [31].

This functional diversity, coupled with their generally high biosafety profile, underscores their vast potential as multifunctional ingredients for health promotion and disease management, warranting continued exploration.

3. Mechanisms of BPs

3.1 Antimicrobial mechanisms

BPs exert antimicrobial effects primarily through membrane disruption or interference with intracellular targets (**Figure 2**).

3.1.1 Membrane targeting

Many cationic BPs (e.g., KNR50 derived from bovine casein) leverage a two-step mechanism to disrupt microbial membranes [33]:

- Electrostatic attraction: Initial binding to negatively charged surface components (lipopolysaccharide in Gram-negatives; teichoic acids in Gram-positives).
- Hydrophobic insertion and disruption: Peptides then insert hydrophobic domains into the lipid bilayer *via* one of four canonical models:
- Barrel-stave model: Formation of transmembrane pores (e.g., magainin) [34].
- Toroidal-pore model: Induction of continuous lipid curvature around the peptide pore (e.g., LL-37) [35].
- Carpet model: Surface accumulation followed by detergent-like solubilization of the bilayer (e.g., cecropins) [36].
- Aggregate model: Local peptide aggregates that create transient membrane defects [37].

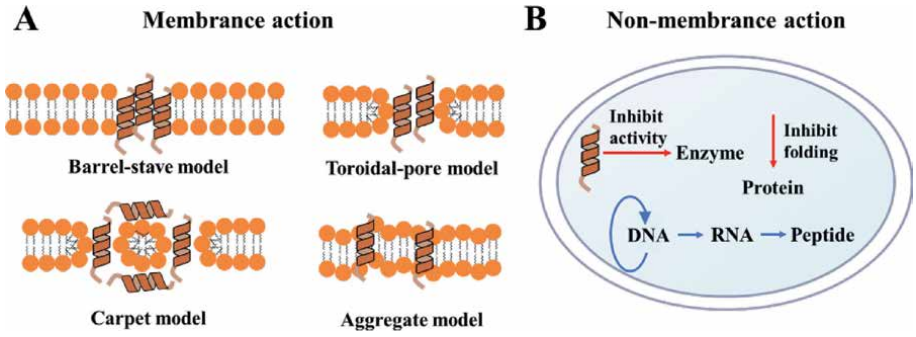


Figure 2.
Antimicrobial mechanisms of bioactive peptides [32]. (A) Membrane-targeting mechanisms, further classified into four models: the barrel-stave model, toroidal-pore model, carpet model, and aggregate model. (B) Non-membrane-targeting mechanisms, primarily involving inhibition of essential cellular processes.

This disruption increases membrane permeability, resulting in uncontrolled ion flux, loss of membrane potential, and ultimately microbial cell lysis.

3.1.2 Non-membrane targeting

Some BPs act as antimicrobials through a variety of nontargeting membrane mechanisms, including inhibition of nucleic acid synthesis (e.g., DNA binding by indoleins), protein synthesis (by targeting ribosomes or key enzymes), and cell wall synthesis (by interfering with enzymes such as transpeptidases) [32, 38]. In addition, some peptides inhibit important metabolic enzymes, such as those from the Arsenura armida protein, which complement their membrane targeting against *S. typhimurium* by affecting metabolic pathways [15]. Many peptides, like LEAP2 and Cath-LW, use a combination of these mechanisms, offering low resistance development, making them promising candidates for antimicrobial agents in food preservation and medicine [39, 40]. Alternatively, electrostatic coassembly of cationic peptides such as LL37 and anionic oligonucleotides forms programmable nanoparticles that exploit charge-dependent cell entry while enhancing endosomal escape and membrane disruption [22]. The efficacy of BPs stems from their ability to exploit fundamental differences in microbial vs. host cell targets (e.g., membrane charge) and utilize diverse, often combinatorial, mechanisms (membrane disruption, intracellular inhibition). This mechanistic complexity contributes significantly to their reduced risk of inducing resistance compared to single-target antibiotics.

3.2 Immune-regulating mechanisms

BPs modulate immune responses through intricate interactions with immune cells and signaling pathways, primarily by regulating cytokine expression and immune cell function (**Figure 3**).

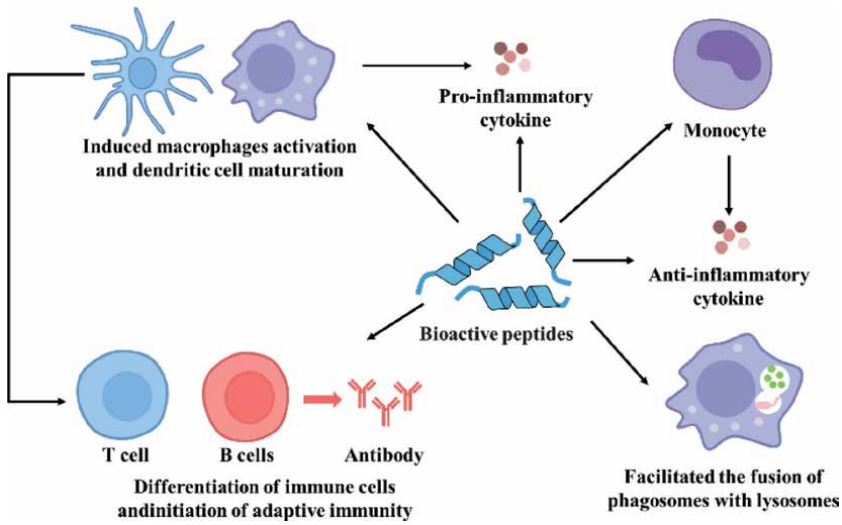


Figure 3.
Immunomodulatory mechanisms of bioactive peptides.

3.2.1 Receptor binding and signaling pathway activation

Peptides like soybean-derived peptides can bind directly to TLRs (e.g., TLR2/TLR4) on immune cells (macrophages, dendritic cells) [41]. This binding recruits adaptor proteins (e.g., MyD88), leading to activation of the IκB kinase (IKK) complex. IKK phosphorylates IκBα, targeting it for degradation and releasing NF-κB (p50/p65). NF-κB translocates to the nucleus and induces transcription of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-12), chemokines, and co-stimulatory molecules [42, 43]. BPs also can modulate signaling downstream of cytokine receptors (e.g., IL-2R, IFNγR), often involving JAK-STAT pathways, influencing T cell proliferation and differentiation [44]. Some peptides may interact with G-protein coupled receptors (GPCRs) or scavenger receptors, affecting cell signaling and immune responses [45]. According to one study, novel peptides from Crocodylus *siamensis* head protein hydrolysates were shown to promote upregulation of immune-related genes (Acod1, Ccl9, COX2, CD40, NF-κB1, IKKε, NF-κB2) in macrophages, confirming NF-κB pathway activation [46].

3.2.2 Immune cell functional modulation

BPs boost macrophage phagocytosis, increase macrophage viability and phagocytic activity, enhance natural killer (NK) cell cytotoxicity, and promote neutrophil recruitment and activation [47]. Peptides like LL-37 can inhibit p38 MAPK phosphorylation, downregulating IL-4 and IL-13 production in Th2 cells, thereby reducing allergic inflammation [48]. Some peptides promote the generation or function of Treg cells (expressing FoxP3), which secrete anti-inflammatory cytokines like IL-10 and TGF-β, maintaining tolerance and preventing autoimmunity [49].

3.2.3 Cytokine network regulation

Activation of pathways like NF-κB drives the production of TNF-α, IL-1β, IL-6, IL-12, and IFN-γ, crucial for initiating and amplifying immune responses against pathogens [50, 51]. For example, the PEP1 peptide enhances IL-2 and IFN-γ secretion *via* NF-κB modulation, promoting adaptive immunity [52]. BPs can simultaneously or selectively enhance the production of IL-10 and TGF-β. These cytokines suppress excessive inflammation, inhibit pro-inflammatory cytokine production, promote tissue repair, and contribute to immune resolution and homeostasis [53, 54]. BPs also demonstrate the ability to inhibit IL-6 and TNF-α, preventing immune overactivation [55]. This balanced regulation ensures effective defense while minimizing collateral tissue damage. At the molecular level, BPs orchestrate immune responses primarily by modulating key signaling hubs (e.g., NF-κB, MAPK pathways) downstream of receptors like TLRs and cytokine receptors. This enables precise control over immune cell activation (macrophages, T cells) and cytokine production, achieving a crucial balance between effective defense and prevention of harmful inflammation, which is fundamental to their therapeutic application.

3.3 Antioxidant mechanisms

The antioxidant activity of BPs is achieved through a multistage synergistic mechanism, mainly including metal chelation prevention, direct free radical scavenging, and boosting endogenous defenses (**Figure 4**).

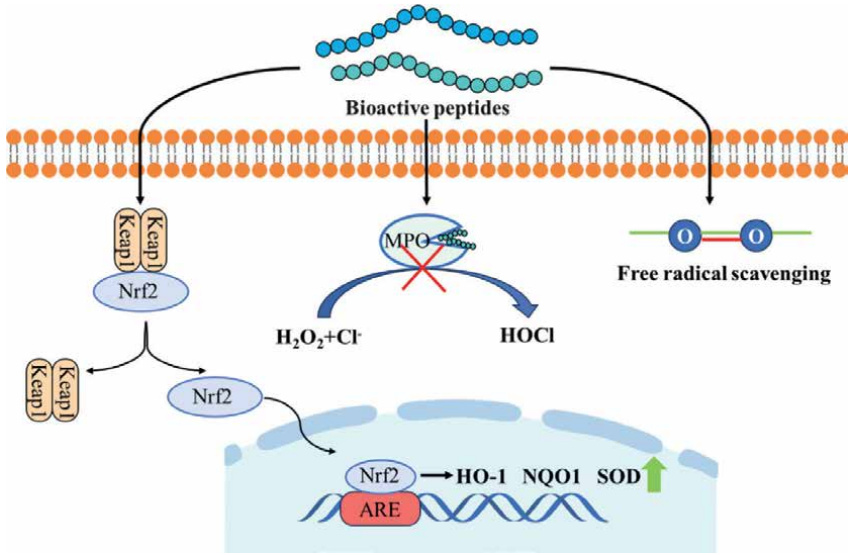


Figure 4.
Antioxidant mechanisms of bioactive peptides [56].

3.3.1 Preventive action (metal chelation)

At the preventive level, the peptide chelates transition metal ions (Fe^{2+}/Cu^{2+}) by residues such as histidine and cysteine, blocking their ability to catalyze the Fenton reaction and inhibiting the generation of hydroxyl radicals from the source [57]. Typical peptides such as lactoferricin significantly reduce lipid peroxidation by chelating Fe^{3+} with histidine imidazolyl groups [58]; similarly, soybean peptide lunasin reduced ROS production by chelating Cu^{2+}/Fe^{2+} with acidic amino acids [59].

3.3.2 Direct radical scavenging

At the level of direct neutralization, peptides act as hydrogen donors (HAT) or electron donors (SET) to quench the existing free radicals. Tyrosine phenolic hydroxyl and tryptophan indole groups provide hydrogen atoms to terminate the chain reaction. For example, the KNR50 peptide reduces the ROS induced by H_2O_2 by 40% with five tyrosine and one tryptophan residues [14]; however, the sulfhydryl group of glutathione (GSH) cleanses •OH by electron transfer, and its oxidation product glutathione disulfide (GSSG) can be reduced and regenerated [60].

3.3.3 Induction of endogenous antioxidant defenses

At the long-term defense level, BPs activate the Nrf2 pathway by modifying the cysteine residues of the Keap1 protein, promoting its nuclear translocation and binding to antioxidant response elements (AREs), and up-regulating the expression of SOD, CAT, HO-1, and other enzymes. For example, milk-derived casein phosphopeptide increased HO-1 expression [61]. Soybean peptide increased the activity of quinone oxidoreductase NQO1 [62]. GSH serves dual roles: as a direct scavenger (SET) and as an essential cofactor for GPx, which reduces lipid hydroperoxides and

hydrogen peroxide [63]. Lunasin has both the ability of metal chelation and Nrf2 activation. This multi-mechanism cooperation makes BPs show significant potential in the prevention and treatment of oxidative stress-related diseases, such as arteriosclerosis and neurodegeneration [64]. The antioxidant power of BPs lies in their synergistic action across multiple defense lines: preventing radical formation (metal chelation), directly neutralizing existing radicals (HAT/SET *via* specific residues), and bolstering the cell's own long-term antioxidant capacity through transcriptional regulation (Nrf2/ARE pathway). This integrated defense strategy offers robust protection against oxidative stress.

4. Classification of BPs

According to the classification criteria defined by the International Union of Food Science and Technology (IUFoST), food-derived BPs are primarily classified into three categories based on their sources: plant-based, animal-based, and microorganism-derived peptides (**Table 1**). This classification not only highlights the biological traits of the source materials but also aligns closely with the mechanisms underlying their functional activities.

4.1 Plant-derived active peptides

Plant-derived BPs are primarily extracted from food sources such as soybeans, wheat germ, seaweeds, microalgae, and nuts. These peptides possess significant health benefits, including immune regulation, antioxidant properties, and disease prevention.

- *Soybean active peptides*: Rich in high-quality protein (about 40%), comparable to animal-based proteins but free from cholesterol and saturated fatty acids, soybeans have become an essential component in combating obesity and metabolic diseases [83, 84]. The fermentation of soybeans with Bacillus species enhances their health benefits by producing specific enzymes and compounds that improve gut health and help fight pathogen infections [85].

- *Wheat-source active peptides*: Wheat germ is a highly nutritious source of protein (up to 30%), containing all essential amino acids and boasting a favorable amino acid profile [86]. It is particularly rich in lysine, isoleucine, and leucine. Wheat germ peptides have demonstrated antioxidant, cholesterol-lowering, and anti-inflammatory effects, making them valuable additives in functional food production [87, 88]. They also exhibit beneficial physicochemical properties like emulsification, foam formation, and water retention.

- *Algal active peptides*: Seaweeds and microalgae are marine organisms capable of photosynthesis. These algal foods possess immune-regulating properties, and their hydrolysates serve as a significant source of immunomodulatory peptides [89]. For example, these peptides can enhance macrophage phagocytosis and stimulate the immune response of animals and reduce the production of cytostatic factors, showing immunomodulatory effects [90, 91]. Despite the great potential of algal peptides, their research is still limited and requires further exploration.

Peptide	Source protein	Mode of production	Function	References
Peptide extracts	Indigenous marine diatoms	Enzymolysis	Antihypertensive/antioxidant	[65]
Dolastatin 10	Indian Ocean mollusk Dolabella auricularia	Enzymolysis	Anticancer	[66]
Codfish protein	Gadus morhua	Subcritical water extraction	Anti-inflammatory	[67]
Kidney bean protein	Kidney bean	HPP pretreatment and enzymatic hydrolysis	Antioxidation	[68]
Tachyplesin I	Scorpion venom	Chemical synthesis	Antibacterial	[69]
VPP	Milk protein	Microbial fermentation	ACE inhibitory	[70]
PASTGAAK	Taihe black-boned silky fowl muscle	Enzymolysis	Anti-osteoporosis	[71]
Phycobiliproteins	Cyanobacterium	Freeze-thawing technique	Anticancer	[72]
PGCPS	Peanuts	Microbial fermentation	Antioxidant and neuroprotective	[73]
	Rice and black gram	Microbial fermentation + pulsed electric	Antioxidation	[74]
RPLY, PGPR	Macadamia nuts	Computer simulation of protease hydrolysis	Inhibits xanthine oxidase	[75]
YFPHF	Duck blood	Enzymolysis	Inhibits xanthine oxidase	[76]
D8K, V9G	Sunflower seed	Enzymolysis + chemical synthesis	Antioxidant and anti-inflammatory activity	[77]
DMP, FFI	Buffalo and camel milk	Microbial fermentation	Anti-diabetes mellitus	[78]
	Chia	Chemical extraction	Antioxidation	[79]
DN8, YR9	Buffalo whey protein	Enzymolysis	Anti-inflammatory	[80]
Lentil peptides	Lentil	Hydrolysis and sonication	Antioxidation and anti-high blood pressure	[81]
DG, ES, and DV	Boneless cured hams	Computer analysis and chemical extraction	Antioxidation and anti-high blood pressure	[82]

Table 1.
Function of origin of BPs.

- *Nut-source active peptides*: Nuts like peanuts, walnuts, pine nuts, and cashews contain protein hydrolysates that yield bioactive peptides with diverse properties, including anticoagulant, antioxidant, and antihypertensive effects. For instance, peptides from peanut Alcalase hydrolysates show anticoagulant activity similar to low-concentration heparin, while walnut peptides exhibit strong antioxidant capacity [92, 93]. Pine nut peptides are effective in inhibiting ACE activity, making them valuable for managing hypertension [94].

4.2 Animal-derived active peptides

Animal-derived BPs, especially those derived from milk and deer antler, are gaining attention due to their immunomodulatory and health-promoting properties.

- *Milk protein peptides*: Milk proteins, particularly whey protein, are a rich source of immunomodulatory peptides [95]. Studies have shown that milk-derived peptides, such as Tyr-Gly and Tyr-Gly-Gly, can enhance immune responses in humans, including acquired immune deficiency syndrome (AIDS) patients, by stimulating the immune system [96]. Additionally, peptides derived from bovine lactoferrin have demonstrated immune-regulating properties, contributing to immune enhancement [97]. These peptides are important due to their low toxicity and high biocompatibility, although more *in vivo* studies are needed to understand their clinical applications.

- *Deer antler peptides*: Deer antler, particularly the young, undeveloped antlers of male deer, is a traditional Chinese medicine known for its health benefits. Enzymatic hydrolysis of deer antler extracts has yielded peptides that exhibit immunomodulatory effects, such as promoting the proliferation of splenic lymphocytes [98]. Additionally, these peptides possess antioxidant properties, including scavenging harmful free radicals and mitigating oxidative stress caused by environmental factors like ultraviolet radiation [99]. The production of deer antler peptides is optimized through enzymatic processes involving papain, which releases peptides that show promise in enhancing immune responses and offering protective effects against oxidative damage [100].

4.3 Microbial-derived active peptides

Microbial-derived bioactive peptides are mainly produced by bacteria, *yeast,* and *actinomycetes* [101]**.** They have become the most well-developed peptides for industrial application due to their high yield (fermentation titer can reach 5–10 g/L), structural diversity (such as cyclic and thioether bond modification), and unique mechanism of action [102]. Among bacterial-derived antimicrobial peptides, lactococcin can effectively inhibit the growth of *Escherichia coli* ATCC 25922 by binding to the precursor lipid II of the Gram-positive bacterial cell wall with high affinity to form transmembrane pores, and the efficiency is about 50 times that of traditional preservatives [103]. As a two-component antimicrobial peptide, plantaricin EF secreted by lactic acid bacteria (LAB) can prolong the shelf life of cheese by ≥14 days by destroying the cell membrane potential [104]. On the other hand, *Actinomycetes* metabolites such as actinomycin D, which blocks transcription by embedding in double-stranded DNA (melting temperature increased by 15°C), have been clinically used in choriocarcinoma to achieve a complete remission rate of >80% [105].

Although microbial peptides have significant advantages, such as the thermal stability of bacteriocins at 121°C, they still face the industrial bottlenecks of low fermentation potency and low extraction purity. Current research focuses on the rational design of non-ribosomal peptide synthetases, aiming to build a modular biosynthetic chassis for the development of next-generation precision peptide drugs [106].

5. Production process of BPs

The production of BPs mainly relies on enzymatic hydrolysis and fermentation technologies [107]. In recent years, genetic engineering, chemical synthesis, and new high-efficiency purification technologies have also begun to play a role in the production of BPs [108, 109]. **Figure 5** summarizes conventional and novel processing methods for the production of BPs. Although the current production technology has made significant progress, there are still many challenges in scale production, cost control, purification efficiency, and product stability [110]. Therefore, the development of more efficient, low-cost, and environmentally friendly production processes has become one of the hotspots in the research of BPs.

5.1 Enzymatic hydrolysis technology

Enzymatic hydrolysis is the most common method for producing BPs. It involves the use of proteases (e.g., trypsin, pepsin, alkaline protease) to cleave protein bonds, releasing active peptides [111]. The advantages of this method include mild reaction conditions, high safety, and easy absorption. For example, when rapeseed protein was hydrolyzed by alkaline protease, the yield of peptides reached 53% [112]. The use of combined commercial enzymes, such as alkaline protease + papain + trypsin, can improve the hydrolysis efficiency and reduce the bitterness, but the cost of enzymatic

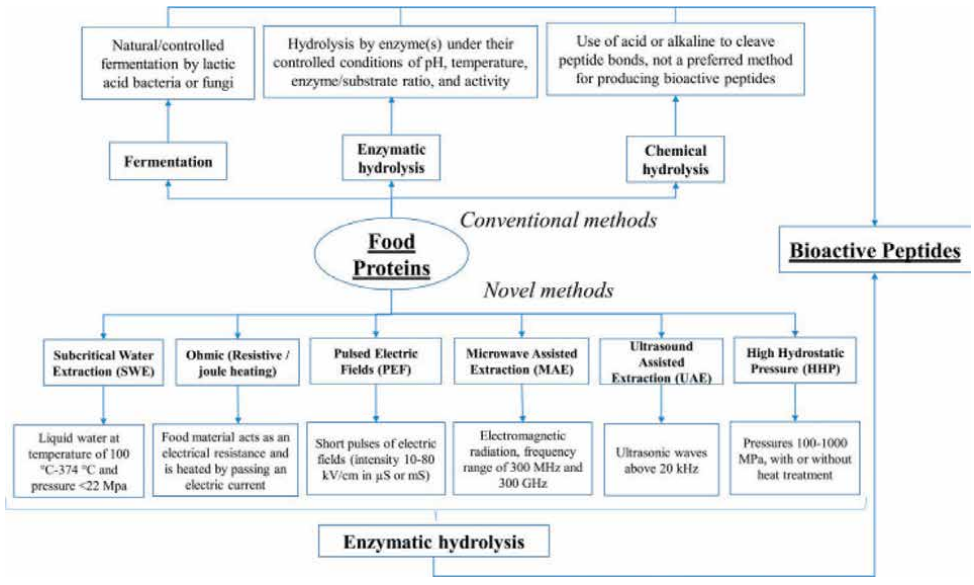


Figure 5.
Key features of conventional and novel methods used to produce bioactive peptides [107].

hydrolysis is high, and the enzyme type and reaction parameters need to be optimized according to the characteristics of the substrate [113]. This fact complicates experimental methods, so it is important to use computerized methods. Computer simulation analysis helps to select appropriate enzyme and protein sources, model hydrolysis, predict potential biological activity, anaphylaxis, and toxicity, and determine mechanisms of action [114]. In summary, enzymatic hydrolysis remains the cornerstone method for bioactive peptide production, valued for its specificity and mild conditions. While highly effective, optimization of enzyme cocktails, reaction parameters, and the integration of computational tools are essential to enhance yield, reduce bitterness, and control costs, addressing key limitations for scalability.

5.2 Fermentation technology

Microbial fermentation utilizes microorganisms (e.g., *Bacillus subtilis*, *lactic acid* bacteria, *Aspergillus oryzae*) to degrade proteins *via* their secreted proteases, yielding BPs. This process often benefits from the synergistic effects of microbial metabolites. Compared to enzymatic hydrolysis, fermentation is generally more cost-effective, as it avoids the need for expensive exogenous proteases [115]. It offers additional advantages, including improved peptide bioavailability, reduction of anti-nutritional factors (ANFs) in plant proteins (enhancing digestibility), shorter reaction times, and potentially higher peptide concentrations [73, 74, 116]. Fermentation can also mitigate issues like allergenicity, bitter taste, and inherent toxicity associated with some peptides or their sources [75]. Notably, *lactic acid* bacteria (LAB) fermentation of whey proteins targets α-lactalbumin and β-lactoglobulin, generating diverse peptides with antibacterial, antioxidant, antihypertensive, and antidiabetic properties [117]. Similarly, solid-state fermentation of soybean by-products (e.g., hulls, bran) by *Aspergillus niger* significantly increased antioxidant activity through the release of enzymes and peptides [118].

Despite its numerous advantages, fermentation remains challenged by the potential formation of undesirable by-products such as formaldehyde, methanol, nitrite, and biogenic amines [76, 119]. Technical hurdles involve maintaining consistent fermentation temperatures, controlling microbial contamination, ensuring adequate micronutrient supply, and achieving long-term storage stability. To overcome these limitations and enhance the effectiveness of fermentation for producing functional food ingredients, strategies such as optimizing process parameters (e.g., pH, temperature, time, inoculum), selecting or engineering improved microbial strains, and potentially integrating novel technologies are being actively pursued [77, 78]. These efforts aim to leverage plant and other proteins more effectively within fermentation systems, improving the sensory, nutritional, and functional qualities of the final products.

5.3 Other production technologies

In addition to enzymatic hydrolysis and fermentation, genetic engineering and chemical synthesis are emerging as key methods for producing BPs, especially the cloning and expression of genes through hosts such as *E. coli* to produce synthetic peptides with specific functions [115]. Genetic engineering allows for the production of peptides with controlled structures and high bioactivity, while chemical synthesis is particularly useful for producing short peptides [116]. Currently, many novel processing techniques such as high hydrostatic pressure (HHP), ultrasound, microwave-assisted

extraction, pulsed electric field, and subcritical hydrolysis have been developed to increase the degree of hydrolysis in the production of BPs [119]. Emerging technologies like genetic engineering and chemical synthesis provide precision and speed for producing specific, high-value peptides, particularly short sequences or those with complex modifications. Novel physical processing techniques (HHP, ultrasound, etc.) offer promising avenues to enhance the efficiency of traditional enzymatic and fermentation methods, potentially overcoming bottlenecks in yield and process time.

5.4 Purification and isolation

Purification is a critical step in the production of BPs, as it directly affects the product's purity, activity, and cost [117]. Achieving high purity and maintaining the bioactivity of the peptides while minimizing costs are key challenges in this process. Several traditional methods, such as ultrafiltration, ion exchange chromatography (IEC), and high-performance liquid chromatography (HPLC), are commonly used, but they have limitations in efficiency, scalability, and cost. These methods often require multiple steps, making the process time-consuming and expensive. As a result, emerging technologies have been developed to overcome these limitations and improve the overall process.

5.4.1 Traditional purification methods

Ultrafiltration is often used in the initial stages of peptide purification, especially for concentrating peptides and removing larger molecules. However, it is not precise enough for high-purity peptide isolation, and issues like membrane fouling reduce its efficiency over time [117]. Ion exchange chromatography (IEC) is widely used to separate peptides based on their charge but requires multiple steps to achieve optimal purity. This process is also prone to peptide degradation if not carefully controlled, and it requires large volumes of elution buffers, making it less efficient for large-scale production [120]. High-performance liquid chromatography (HPLC) offers precise separation based on size, hydrophobicity, and charge, but its high operational costs and time-consuming nature make it impractical for large-scale production [121].

5.4.2 Emerging technologies for peptide purification

Several emerging technologies offer promising alternatives to traditional methods. Molecular imprinting, for example, involves creating polymer matrices that can selectively bind to target peptides. This approach has the potential for high specificity and efficiency, though scalability remains a challenge [122]. Nanomaterials, such as nanoparticles and nanofibers, offer significant advantages due to their high surface area and customizable properties. They can be engineered to selectively capture peptides based on characteristics like size, charge, or hydrophobicity [123]. Although nanomaterials show great promise, challenges in reproducibility and scalability remain barriers to their widespread application. Supercritical fluid extraction (SFE) utilizes supercritical fluids like CO_2 to extract peptides from complex matrices, offering advantages like minimal solvent usage and mild operational conditions that help preserve peptide bioactivity [124]. However, the high cost of equipment and the need for specialized expertise are limitations that hinder its widespread use in large-scale production.

5.4.3 Challenges in peptide purification

Despite advances in purification technology, several challenges remain. The multistep nature of peptide purification often requires combining multiple methods, increasing both the time and cost involved in achieving high purity. Furthermore, peptides can degrade or lose bioactivity during the purification process due to factors like exposure to extreme conditions, pH shifts, or enzymatic activity [125]. The high operational costs of emerging technologies, such as HPLC and SFE, also pose significant barriers to large-scale, cost-effective peptide production [126]. Additionally, the complexity of maintaining the integrity of peptides while achieving high purity makes the process more difficult and expensive.

6. Commercial applications

The commercial production of BPs is the process of transforming laboratory research and development results into products with market value. Some successful commercial production cases demonstrate the great potential of the industrialization of BPs. For example, whey proteolytic peptides have been widely used as functional food ingredients in the market, especially in functional dairy products and sports nutrition products [127]. By utilizing a variety of BPs in whey proteins, manufacturers are able to develop products with multiple functions such as blood pressure lowering, antioxidant, and immune enhancement [128]. In addition, marine-derived collagen peptides have also achieved significant commercial success in the cosmetic industry, where they are widely used in antiaging and skin repair products [129, 130].

7. Conclusions and future perspectives

BPs are natural small molecules with diverse biological functions; they exert these effects through various mechanisms. Compared to synthetic drugs, BPs offer high specificity, favorable safety profiles, and low risk of inducing resistance, making them promising candidates for functional food ingredients, nutraceuticals, and therapeutic agents.

Despite significant progress, several challenges remain. The production of BPs often involves high cost, multistep purification, and low yield, especially in large-scale industrial applications. Traditional methods such as enzymatic hydrolysis and microbial fermentation continue to dominate, but they suffer from batch variability and scalability issues. Novel approaches, including recombinant expression systems, solid-phase synthesis, and in silico design, are emerging but require further validation and standardization.

Future research should prioritize the following key areas:

1. Identification of novel bioactive sequences from underexplored natural sources such as marine organisms, insect proteins, and plant by-products using omics technologies and bioinformatics tools;

2. Mechanistic studies at the molecular level, including receptor binding, signal transduction, and synergistic interactions, to deepen our understanding of structure-function relationships;

3. Development of efficient, cost-effective production platforms, such as microbial cell factories, continuous bioprocessing, and immobilized enzyme systems, to overcome industrial scalability issues;

4. Advanced purification and delivery systems, including nanocarriers, encapsulation, and molecular imprinting, to enhance peptide stability, bioavailability, and targeted delivery *in vivo*;

5. Clinical translation and regulatory validation remain a critical bottleneck. Few BPs have progressed to human trials due to limited pharmacokinetic data and unclear regulatory frameworks. Bridging this gap requires multidisciplinary collaboration and translational research strategies.

In conclusion, BPs hold substantial promise for future applications in functional foods, precision nutrition, and biopharmaceuticals. Addressing current knowledge gaps and technological barriers will be essential to fully realize their potential in improving human health.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter 2

Optimizing Enzymes for Dairy Processing: A Protein Engineering Perspective

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Abstract

The dairy industry relies heavily on enzymes for various processes, from milk coagulation to flavor development. Enzyme engineering has revolutionized these applications by creating more efficient, stable, and specific biocatalysts. Key advancements include recombinant production of chymosin replacing traditional calf rennet, development of cold-active and thermostable lactases for lactose-free products, creation of regioselective lipases for targeted flavor development, and modification of proteases for controlled cheese ripening. Modern techniques encompass rational protein design, directed evolution, computational modeling, and innovative immobilization technologies such as cross-linked enzyme aggregates and magnetic nanoparticle carriers. These engineered enzymes deliver significant industrial benefits, including higher yields, consistent product quality, reduced energy consumption, and novel dairy product development. Enzyme engineering also addresses growing consumer demands for clean-label products through improved specificity and reduced side reactions. The continuing evolution of these techniques promises further innovations in sustainability, functionality, and nutritional enhancement in dairy processing across global markets.

Keywords: dairy enzymes, directed evolution, rational enzyme design, enzyme engineering, dairy processing, biocatalyst optimization

1. Introduction

It is a vital component of the global economy, contributing significantly to the agricultural and food sectors. The industry in question encompasses a variety of activities, including, but not limited to, dairy farming, the production of milk, and its subsequent processing into various products, such as cheese, yogurt, and butter. The distribution of these products to consumers on a global scale is also an integral aspect of the industry [1]. Dairy products are classified as functional foods due to their inherent or possible enrichment with bioactive components, which confer health benefits that extend beyond basic nutritional requirements. The aforementioned benefits encompass the following: the prevention of disease, the enhancement of metabolism, and the optimization of physiological functions [2].

Technological advancements are significantly transforming the dairy sector. Furthermore, innovations in biotechnology and functional dairy product development are expanding the industry's product range. Enzymes, for instance, play a crucial and transformative role in the dairy industry, significantly enhancing production efficiency, product quality, and environmental sustainability. The utilization of these applications has been demonstrated to enhance the sensory quality and safety of products, while concurrently contributing to the industry's sustainability by minimizing waste and reducing energy consumption [3, 4].

Enzymes are proteins that act as pivotal biological catalysts, thereby accelerating essential chemical reactions in biological systems. It is evident that these substances fulfill a pivotal function in a multitude of physiological processes by accelerating reactions without being consumed in the process. This enables biological processes to proceed under milder conditions, such as lower temperatures and a neutral pH, than would otherwise be feasible [5, 6]. Beyond their role within biological systems, enzymes boast a plethora of industrial applications [7, 8]. Their utilization is pervasive within the food industry, encompassing processes such as starch processing, meat processing, dairy industry operations, wine industry practices, and the manufacture of predigested foods [9, 10]. For instance, they are employed in the degradation of oil spills and waste materials, the removal of fat stains, and the fermentation process for the production of alcoholic beverages.

Enzyme engineering customizes enzymes by altering their amino acid sequences to achieve improved properties. Enzyme engineering optimizes β-galactosidase activity to maximize lactose conversion while maintaining product quality and sensory characteristics, allowing the production of lactose-free or reduced-lactose dairy beverages. Additionally, enzymatic synthesis of prebiotic galactooligosaccharides (GOS) from lactose during fermentation creates functional symbiotic products that combine probiotic and prebiotic effects, further enhancing health benefits for consumers, including those with lactose intolerance.

2. Enzymes in the dairy industry

2.1 Historical overview of enzyme utilization in the dairy sector

In the dairy industry, enzymes are considered to be essential tools for the transformation of milk into a wide range of products, with specific characteristics that are in accordance with the desired product specifications. These enzymes are also known to extend the shelf life of the products and enhance their nutritional profiles [11–14]. The utilization of enzymes in the production of foodstuffs, including dairy products, constitutes an ancient practice with origins extending thousands of years. The earliest documented applications of biological agents in food processing date back to 6000 BCE, with practices such as bread baking, brewing beer, and the production of cheese and wine [7, 9, 15–17]. One of the most well-known and historically significant applications of enzymes in dairy is the use of rennet for milk coagulation in cheese production [13, 18, 19]. It is speculated that cheesemaking may have been discovered serendipitously 4000–5000 years ago when milk was stored in bags made from calf stomachs, where remnants of aspartic proteases from the stomach, along with natural milk flora, led to coagulation [19–21]. The first commercial standardized rennet preparation was produced and sold by Chr. Hansen A/S, Denmark, in 1874, making it arguably the first commercial enzyme of any type [22].

2.2 Applications of enzymes in the dairy industry

The global market for food enzymes, valued at USD 2058 million in 2020, is projected to reach USD 4194 million by 2032, with the dairy sector being a major demand area for enzymes [10, 12]. The dairy industry represents a significant domain for the implementation of enzymatic processes, with a diverse range of food products, including cheese, butter, and yogurt, being produced. The primary exogenous enzymes employed in dairy products are proteases (such as rennet and chymosin), lipases, and lactases, which are classified as hydrolases within the EC 3.x classification system [12].

Enzymes continue to play a pivotal and multifaceted role in modern dairy processing, offering innovative solutions for enhancing product quality, efficiency, and sustainability (curdling) of milk, a critical step in cheese production. The primary function of these enzymes is to hydrolyze κ-casein, a milk protein that stabilizes casein micelles, thereby allowing the micelles to aggregate and form a gel-like curd [16]. Enzymes are vital in the production of cheese, chiefly due to their ability to induce milk coagulation. Rennet, an enzyme complex that is traditionally derived from animal stomachs, is responsible for the breakdown of milk proteins, resulting in the formation of curds and whey. The utilization of sustainable sources of these enzymes, encompassing microbial and genetically engineered variants, has witnessed a marked increase in their application to meet the vegetarian and kosher demands within the industry [23].

Lactose intolerance, a condition characterized by the inability to digest lactose due to a lack of the necessary enzymes, affects a significant portion of the global population. This has resulted in a growing demand for lactose-free products, which are products that do not contain any lactose. Enzymes such as beta-galactosidase are pivotal in the hydrolysis of lactose into glucose and galactose, thus rendering dairy products more digestible for individuals who are lactose-intolerant. This process, catalyzed by enzymes, not only addresses consumers' health requirements but also enhances the sweetness of dairy products without the need for additional sugars [24].

Lactic acid bacteria, which are renowned for their ability to produce compounds that enhance flavor, possess enzymes capable of breaking down milk components into flavor-contributing peptides and amino acids. The fermentation process employs these enzymes to generate complex flavor profiles that are characteristic of products such as yogurt and cheese. Furthermore, proteolytic enzymes derived from these bacteria have been shown to enhance the maturation and flavor profiles of cheese [23].

Proteolytic enzymes have been identified as playing a pivotal role in the modification of milk proteins, thereby enhancing their nutritional and functional properties. Of particular interest are whey protein hydrolysates, which are obtained through enzymatic processes, yielding bioactive peptides that exhibit beneficial health effects such as antihypertensive and antioxidant properties. These modifications can be controlled to produce specific peptides for use in functional foods and nutraceuticals. Whey, a byproduct of cheese making, contains valuable proteins and lactose that can be processed using various enzymes to create high-value ingredients and products. Additionally, enzymes are used to modify whey proteins for improved functionality. Controlled proteolysis can enhance the solubility and emulsifying properties of whey proteins, making them more suitable for use in sports nutrition products and food applications [24, 25].

Enzymes also offer a sustainable alternative for cleaning and sanitation in dairy processing. Enzyme-based cleaning-in-place (CIP) systems are increasingly being

adopted as sustainable alternatives to traditional chemical detergents, offering significant reductions in water and energy consumption as well as environmental impact. Enzymes effectively break down milk residues on processing equipment, reducing biofilm formation and ensuring hygiene without the drawbacks of harsh chemicals [4].

Furthermore, enzymes are pivotal in the transformation of milk into functional foods through the production of bioactive components such as peptides, oligosaccharides, and other health-promoting molecules by means of targeted biochemical reactions.

2.3 Key enzymes in dairy processing

The dairy industry depends on a range of enzymes for the enhancement of product quality, the optimization of processing efficiency, and the development of specialized dairy products (**Table 1**). The following section provides a comprehensive overview of the key enzymes commonly employed in the field of dairy processing. These enzymes are accompanied by a detailed description of their sources, functions, and applications.

Enzymes in milk can be categorized into two main groups: first, indigenous enzymes are naturally present in milk and originate from the cow itself (e.g., blood plasma, mammary epithelial cells, milk fat globule membrane, somatic cells during infection) [14, 26, 27]. While the physiological role of certain indigenous enzymes in milk is not immediately apparent, others have been shown to have a significant impact on the quality and shelf life [26, 28]. Exogenous enzymes are added externally to milk or milk products during processing or are produced by bacteria, molds, or yeasts (microbial enzymes). It is imperative to acknowledge the significance of these components in the manufacturing process of numerous dairy products, which, in the absence of these elements, would remain unattainable [12, 26, 29, 30].

Application	Enzyme	Function/Benefit	Example Products
Cheese manufacturing	Chymosin (Rennet)	Coagulates milk by cleaving κ-casein, forming curds	Cheddar, Parmesan, soft cheeses
Lactose-free dairy products	Lactase (β-Galactosidase)	Hydrolyzes lactose into glucose and galactose, increases sweetness, enables lactose-free products	Lactose-free milk, yogurt, ice cream
Cheese ripening, flavor enhancement	Lipase	Hydrolyzes milk fats, develops characteristic flavors	Blue cheese, Parmesan, butter
Cheese maturation, protein hydrolysates	Protease	Breaks down milk proteins, enhances texture, reduces allergenicity	Yogurt, infant formula, protein supplements
Texture improvement, water retention	Transglutaminase	Cross-links proteins, improves texture and yield	Yogurt, processed cheese
Milk sterilization, shelf-life extension	Catalase	Decomposes hydrogen peroxide, prevents oxidation, preserves quality	Sterilized milk, cheese
Flavor development	Esterase	Hydrolyzes esters, contributes to flavor compound formation	Specialty cheeses

Table 1.
Applications of enzymes in the dairy industry.

2.3.1 Milk coagulants (rennets, chymosin, pepsin)

Due to their critical function in milk clotting, milk coagulants represent some of the most commercially important enzymes in cheese production. This enzymatic coagulation is a two-phase process [31]. In the primary, enzymatic phase, the coagulant, most notably chymosin, specifically cleaves the Phe105-Met106 peptide bond of kappa-casein (κ-casein) [3, 12, 19, 31]. Kappa-casein is located on the surface of casein micelles and typically stabilizes them through steric repulsion and a negative electrical charge [19, 32]. The cleavage of κ-casein yields a hydrophobic para-κ-casein, which remains in the curd, and a hydrophilic glycomacropeptide (also known as caseinomacropeptide), which is liberated into the milk serum (whey) [15, 31]. The secondary, nonenzymatic phase involves the aggregation of these destabilized casein micelles, which then precipitate in the presence of calcium ions (Ca^{2+}) to form a gel or curd [32, 33].

Traditionally, milk coagulants were sourced from animal rennet, primarily derived from the abomasa of young suckling calves, which are rich in chymosin [12, 31, 34, 35]. This type of rennet contains chymosin and pepsin, with their ratio influencing proteolysis during cheese ripening [35]. Calf chymosin is particularly valued for its specific proteolytic activity that coagulates milk without extensively damaging immunoglobulins, a characteristic favored in the evolution of young ruminants [36]. Rennet paste, derived from kid goats and lambs, contains lipase from the animals' glands, which is essential for producing piquant cheeses [32]. Besides calf rennet, other animal sources include lamb chymosin, which has similar coagulation properties but a slightly higher temperature dependency, and less-studied kid and buffalo rennet [35]. Recombinant camel chymosin is also gaining market share, known for its superior coagulation properties for both bovine and camel milk [20, 36].

However, due to limitations in the supply of animal rennet and other factors like increasing cheese production and dietary preferences, there has been a significant shift toward alternative milk coagulants [22, 31]. These alternatives include (I) microbial coagulants: These are produced from various microorganisms, including fungi (e.g., *Rhizomucor miehei*, *Mucor pusillus*, *Endothia parasitica*, *Aspergillus oryzae*, *Irpex lactis*, *Thermomucor indicae-seudaticae*) and certain bacteria (Bacillus subtilis) [3, 12, 15, 31, 32, 37]. While easily produced and inexhaustible, some early microbial coagulants had drawbacks such as heat stability, which could lead to clotting in rehydrated infant formulas, and high levels of nonspecific proteases, causing bitterness and poor yields in long-ripened cheeses [15, 32, 38]. Fermentation-produced chymosin (FPC), which involves cloning the calf chymosin gene into genetically modified food yeasts or molds, has largely overcome these issues and constitutes a significant portion of the market [22, 31, 36]. (II) Plant-derived coagulants: These include extracts from plants such as *Onopordum macracanthum*, kiwi fruit (*Actinidia deliciosa*), *Calotropis gigantea*, *Solanum incanum*, and lemon juice [3, 33, 39, 40]. Plant proteases show variability in their specificity, offering opportunities for diverse cheese characteristics. *Onopordum macracanthum* proteases, for example, have demonstrated promising technological properties [33].

The choice of coagulant significantly impacts cheese quality, affecting aspects such as texture, flavor, and whey drainage properties [14, 20, 28, 34]. Factors influencing milk-clotting activity (MCA) include temperature, pH, and calcium chloride concentration [33, 37]. For instance, increasing calcium chloride concentration can improve MCA by neutralizing negative charges on casein micelles and facilitating aggregation, although excessively high concentrations can reduce activity.

2.3.2 Lipases

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) are a crucial class of hydrolase enzymes widely utilized in the dairy industry [12, 28, 41, 42]. These enzymes are primarily responsible for catalyzing the hydrolysis of ester bonds in triglycerides, breaking them down into free fatty acids (FFAs), partial glycerides (mono- and diglycerides), and glycerol, predominantly at the water/oil interface [15, 28, 42–44]. Beyond hydrolysis, lipases are versatile biocatalysts capable of facilitating various other reactions, including esterification, interesterification, transesterification, acidolysis, aminolysis, and alcoholysis, which are vital for modifying lipid properties and creating value-added products [14, 18, 41, 45]. Their importance in the dairy sector stems from their ability to influence flavor, texture, ripening processes, and overall product quality [7, 11, 18, 21, 22, 46]. In addition to their role in flavor development, lipases contribute to the structural stability of dairy products.

Lipases found in dairy applications originate from both indigenous milk components and exogenous sources, namely animal, microbial, and, to a lesser extent, plant-derived materials [12, 20].

Indigenous Milk Lipases: The primary indigenous lipase in bovine milk is lipoprotein lipase (LPL; EC 3.1.1.34) [29, 42, 47]. LPL is a glycoprotein that exists as a homodimer with a molecular mass of approximately 90 kDa [41–43, 48]. It is thought to spill over from the mammary gland into milk, where it is primarily associated with casein micelles [48, 49]. While LPL plays a crucial role in lipid synthesis within the mammary gland, its presence in milk itself does not have an apparent biological purpose [29, 48]. LPL is unstable and can be significantly inactivated by heat treatments, such as High-Temperature Short-Time (HTST) pasteurization (72°C for 15 seconds) [48, 50]. Its optimal pH is around 8–9, and its optimal temperature is around 37°C [41]. Other less-studied indigenous esterases and colostral lipase also exist in milk [12].

Exogenous Lipases (Commercial Sources): Commercial lipases can be obtained from animals, plants, and microorganisms. *(I)* Historically, lipases were sourced from animal rennet, primarily from the abomasa of young suckling calves, kid goats, or lambs [7, 35, 51]. Pregastric esterase (PGE), secreted from the oral tissues of these animals, is particularly notable for its high specificity for short-chain fatty acids at the sn-3 position, contributing to a characteristic 'piccante' (pungent or peppery) flavor in cheeses like Provolone and Romano [12, 19]. *(II)* Due to advantages such as lower production costs, greater stability, and inexhaustible supply, microbial lipases have become the most commercially significant source [7, 15, 16, 46, 52]. These are produced by various microorganisms, including fungi (e.g., *Rhizomucor miehei*, *Aspergillus niger*, *Aspergillus oryzae, Candida* species, *Penicillium*, *Mucor pusillus*, *Mucor javanicus*, *Thermomyces*), yeasts (*Candida, Yarrowia*), and bacteria (*Bacillus subtilis*, *Pseudomonas* species, *Serratia marcescens*, *Staphylococcus aureus*) [3, 7, 15, 38]. Microbial lipases are increasingly important for dairy products, especially low-fat cheeses, where they enhance fatty acid flavor release [53]. While mentioned as a source, microbial lipases are favored for industrial-scale production.

2.3.2.1 Applications and functions of lipases in the dairy industry

Lipases are broadly applied across the dairy industry for a range of purposes, from enhancing flavor to improving product yield and even aiding in equipment cleaning.

Cheese production and ripening: This is arguably the most significant application of lipases in the dairy industry [9, 18, 35]. They contribute to the development of cheese

flavor, texture, and body characteristics by hydrolyzing milk fats [12, 14, 54]. This hydrolysis releases FFAs, which are key flavor components. Short-chain FFAs, such as butyric and caproic acids, impart pungent, tangy, peppery, or cheesy notes [18, 28, 47, 51, 55], while long-chain FFAs tend to develop a softer taste and odor [12, 55]. FFAs also serve as precursors for other volatile flavor compounds like esters, methyl ketones, and lactones, which contribute sweet, fruity, floral, buttery, or musty notes to cheese [38, 45]. Lipases also accelerate the cheese ripening process [9, 12, 15, 56]. They are specifically used in the production of enzyme-modified cheeses (EMCs) to achieve intense flavors [9, 29, 35].

Milk Fat modification and valorization: Lipases are used to enhance the flavor and structure of butter and dairy cream [28, 35]. They are also employed to produce modified milk fat products for other food applications in the confectionery, candy, chocolate, sauce, and snack food industries [22, 30]. Enzymatic interesterification of milk lipids can modify their rheological properties [30, 47], and lipases can be used to produce diglycerides (DGs) and monoglycerides (MGs) that function as emulsifiers in the food industry [47]. Furthermore, they enable the enrichment of milk fat with omega-3 fatty acids and the production of human milk fat substitutes [28, 38, 45].

Yield improvement: Fungal phospholipase A1, a type of lipase, can increase cheese yield, such as in mozzarella production, by improving the retention of fat and protein [12, 29, 35]. This occurs by enhancing the emulsifying property of milk, reducing fat losses in whey and cooking water [35, 38].

Addressing off-flavors (hydrolytic rancidity): While beneficial for specific cheese flavors, uncontrolled lipase activity, particularly from indigenous LPL and bacterial lipases, can lead to undesirable off-flavors in liquid milk and other dairy products, described as rancid, butyric, astringent, or soapy [26, 28, 41, 42, 57]. This "spontaneous lipolysis" can be triggered by factors related to the cow (e.g., stage of lactation, nutritional status) or induced by mechanical handling of raw milk (e.g., agitation, foaming, freezing, homogenization, pumping) that disrupts the milk fat globule membrane (MFGM) [42, 46, 47]. Lipolysis can also reduce the foaming capacity of milk due to the liberation of FFAs [26, 42, 47].

Cleaning-in-place (CIP) in the dairy industry: Lipases, often in combination with proteases and amylases, are employed in cleaning processes to hydrolyze lipid-based fouling deposits and biofilms that accumulate on dairy processing equipment. They represent the third most used enzyme class in detergents, highlighting their role in maintaining hygiene and operational efficiency [44].

2.3.3 Proteases

Proteases, or proteolytic enzymes, are crucial in the dairy industry, catalyzing the breakdown of proteins into smaller peptides or amino acids, thereby influencing the texture, taste, and aroma of dairy products and may also generate bioactive peptides with antihypertensive, antimicrobial, or immunomodulatory effects [3, 58, 59]. They are classified into serine, cysteine, aspartic, and metalloproteases based on their active site and catalytic mechanisms [60]. Proteolytic activity in milk comes from both indigenous enzymes, inherent to the cow, and exogenous enzymes, primarily from microbial contamination [59].

Indigenous milk proteases: Milk contains over 60 indigenous enzymes, many of which can survive heat treatments like High-Temperature Short-Time (HTST) pasteurization [12]. The most prominent indigenous proteinase is plasmin (EC 3.4.21.7), which exhibits trypsin-like activity [61]. Other indigenous proteases like cathepsin

D are also present in milk, originating from somatic cells, and can survive some heat treatments, contributing to post-processing proteolysis [41, 62].

Microbial proteases: Microbial enzymes are widely used in the dairy industry due to their efficiency and cost-effectiveness [58]. However, psychrotrophic bacteria, especially *Pseudomonas* species, are a major source of spoilage proteases in raw milk [41, 63, 64]. These bacteria produce significant amounts of heat-stable extracellular proteases, such as AprX (Protease) from *Pseudomonas fluorescens*, which are difficult to inactivate even with UHT treatment [59, 63, 64]. These heat-resistant proteases can cause undesirable changes like age gelation, off-flavor, and textural defects in processed dairy products, particularly ultra-high temperature processing (UHT) milk, thereby reducing shelf life and nutritional content [41, 63]. *Pseudomonas* proteases primarily degrade casein, with β-casein and κ-casein being particularly susceptible [63]. The thermal stability of AprX is mainly attributed to its flexible tertiary structure, which can refold quickly upon cooling [63]. Reducing the growth of psychrotrophic bacteria in raw milk by rapid processing and maintaining low temperatures (2–4°C) can suppress the production of these heat-stable enzymes [60].

2.3.3.1 Applications of proteases in dairy

Proteases are indispensable in various dairy applications, impacting product characteristics from coagulation to flavor development and shelf-life stability [8, 11, 15, 65].

Cheese production: Proteases are vital in cheese production, contributing to milk coagulation (see 2.3.1), accelerating ripening, and developing unique flavors and textures [9, 22, 50, 65, 66]. Proteases significantly enhance the aroma and flavor of cheese by breaking down casein proteins into various peptides and amino acids during the ripening process [9, 29, 49, 65]. The specific type of flavor generated depends on the amino acids released and the ability of cheese cultures to utilize them [20]. Proteases are also used to make enzyme-modified cheeses (EMCs), where cheese or curd is incubated with enzymes to achieve desired flavor intensity [29].

Shelf-life extension and spoilage prevention: Proteases are among the primary enzymes responsible for the deterioration of dairy products, particularly those with a long shelf life [14, 26, 41, 67]. Uncontrolled protease activity can lead to various defects such as casein coagulation, off-flavor development, textural changes, sedimentation, and creaming [26, 60]. For example, in UHT milk, proteolysis can lead to age gelation, a significant factor limiting shelf life [30, 41, 60]. By controlling these spoilage enzymes and their activities, raw milk quality can be maintained for extended shelf-life products [3, 7, 54].

Hypoallergenic products and protein hydrolysis: Proteases are crucial for the production of dairy protein hydrolysates used in infant, clinical, or sports nutrition, as they help reduce possible allergenic reactions and improve the digestibility of dairy proteins [12, 20, 29, 65, 66, 68]. They break down milk proteins (casein and whey proteins) into smaller peptides or amino acids, enhancing their nutritional value and digestibility [54, 62, 65, 69]. This enzymatic action also improves the viscosity, texture, and taste of fermented products like yogurt, contributing to a smooth and creamy consistency [54].

Cleaning-in-place (CIP) in the dairy industry: Proteases, along with lipases and amylases, are studied for their potential use in cleaning-in-place (CIP) systems in the dairy industry to remove fouling deposits, especially those that accumulate on heated surfaces during thermal processing [3, 44, 65]. These deposits, composed of protein, mineral, and fat, can obstruct flow, reduce heat transfer efficiency, and harbor microbial growth, including biofilms [44]. Proteases, particularly alkaline serine

proteases from *Bacillus* species, are effective in hydrolyzing proteinaceous substrates and disrupting biofilms [44].

2.3.4 Lactases (β-galactosidase)

Lactases, also known as β-galactosidases (EC 3.2.1.23), are a crucial class of glycoside hydrolase enzymes in the dairy industry, primarily catalyzing the breakdown of lactose. This enzymatic action plays a significant role in influencing the taste, texture, and aroma of dairy products and is central to various modern dairy applications [11, 51, 52, 70, 71]. Lactases perform two main types of reactions critical for dairy product development and consumer health.

2.3.4.1 Hydrolysis of lactose

This is the primary function: breaking down lactose into its constituent monosaccharides, d-glucose and d-galactose. This process is vital for producing lactose-free products [12, 32, 55, 70]. Lactose intolerance affects a significant portion of the global adult population, with rates as high as 70–90% in some Asian and American regions [52, 55, 72–74]. This condition stems from a deficiency or inefficiency of lactase in the human digestive system, leading to symptoms like diarrhea, abdominal cramping, and flatulence upon consuming lactose-containing dairy products [12, 20, 52]. Lactase enzymes are used to predigest lactose in milk, enabling the production of "low-lactose" or "lactose-free" dairy options such as milk, yogurt, ice cream, cheese, and dulce de leche [7, 11, 15, 18, 51, 75–77]. This not only facilitates lactose-free product development but also significantly enhances digestive health and comfort for lactose-intolerant individuals. The lactose content in these products can be reduced significantly, with thresholds varying by region (e.g., less than 10 mg/100 g for "lactose-free" in Denmark, 100 mg/100 g in Germany, and no FDA definition in the US beyond "truthful and not-misleading" labeling) [12, 20]. The hydrolysis products, glucose and galactose, are sweeter than lactose itself (approximately 70% of sucrose's sweetness compared to lactose's 20%) [7, 14, 18, 20, 29]. This allows for reduced sugar addition in dairy products like yogurts and desserts [11, 15], while also preventing lactose crystallization (often described as "sandiness") in products like ice cream and sweetened concentrated milk [15, 78]. Beyond lactose-free products, enzymatic hydrolysis improves the overall digestibility of dairy products and enhances fermentation efficiency and yield in products like yogurt and cottage cheese [14, 38].

2.3.4.2 Transgalactosylation for galactooligosaccharide (GOS) synthesis

Lactases can also catalyze transgalactosylation, synthesizing galactooligosaccharides (GOS) or lactulose from lactose [18, 70, 75, 79, 80]. GOS are nondigestible oligosaccharides that act as prebiotics, stimulating the growth and activity of beneficial microorganisms in the colon, such as *Lactobacillus* and *Bifidobacterium* species [12, 75, 80]. They contribute to various health benefits, including immune system support, protection against cancer, and prevention of cardiovascular and metabolic issues [80]. GOS are increasingly used as food ingredients in infant formulas, infant foods, livestock feed, and pet food industries [74, 80]. The successful synthesis of GOS from concentrated whey permeate utilizing a commercial β-galactosidase is reported. The yield of GOS was found to be up to 74% (g GOS/g lactose), with 63% lactose utilization after a mere 0.5 hours of reaction [81]. In a separate study, β-galactosidase from probiotic bacteria,

such as Lactobacillus delbrueckii subsp. bulgaricus CRL450, was employed to efficiently synthesize galactooligosaccharides (GOS) and lactulose oligosaccharides (OsLu) from lactose and lactulose *via* transgalactosylation. The resulting GOS was found to significantly enhance the growth of probiotic strains [82]. In another study, GOS were synthesized from whey permeate using commercial β-galactosidases from *Kluyveromyces lactis* and *Escherichia coli*, achieving yields up to 24%, which demonstrates a viable industrial process to turn a dairy byproduct into a value-added prebiotic [83]. A third approach combines specific enzymes like β-galactosidase with dietary fibers to generate tailored short-chain oligosaccharides that act as highly preferential food sources for probiotics, with the enzymatic process minimizing unwanted glucose production [84]. All these cases highlight that the enzymatic source and conditions can be controlled to create specific prebiotic structures that selectively stimulate beneficial gut bacteria.

2.3.4.3 Whey utilization

Lactase enzymes are used to hydrolyze lactose in whey, a byproduct of cheese production, converting it into more useful food ingredients and syrups for the bakery and confectionery industries [7, 29, 74, 78, 85]. This also provides better biodegradability to whey, making it suitable for ethanol production and addressing environmental concerns related to its high biological and chemical oxygen demand [7, 78].

2.3.4.4 Sources and types of lactases

Lactases can be derived from various sources, predominantly microbial, but also from plants and animals, and confer the desired properties [86–89].

β-galactosidases from yeasts are active at a rather neutral pH (between 6.0 and 7.0), making them highly suitable for producing lactose-free milk due to milk's pH of approximately 6.6 [22, 35, 73, 74]. *Kluyveromyces lactis* is the most commonly used yeast for industrial lactase production [74]. Other yeast sources include *Saccharomyces fragilis* and *Kluyveromyces fragilis* [22, 77]. These enzymes generally have low heat stability, becoming rapidly inactivated above 55°C [77].

Fungal β-galactosidases commonly work in an acidic pH range (from 2.5 to 5.0), making them most effective for lactose hydrolysis in acidic products like acid whey [20, 32, 65, 77]. They typically have a high optimum temperature (55–60°C) [77]. Notable fungal sources include *Aspergillus niger* and *Aspergillus oryzae* [22]. Fungal enzymes may contain other enzymes like amylase, lipase, and protease, limiting their application to high-acid products and pharmaceutical preparations [77].

Bacterial β-galactosidases generally exhibit high enzyme activity, good stability, and high production yield [70]. They are often suitable for milk and sweet whey hydrolysis, operating at a neutral pH (6.5–7.5) [15, 65]. Key bacterial sources include *Bacillus circulans* and *Bifidobacterium bifidum* [70, 75, 77]. Some *Bacillus* species lactases are noted for their thermostability and low product inhibition by galactose [15].

β-galactosidases are also found in plants (e.g., almonds, peaches, and apricots) and the intestinal tract of mammals, but these sources generally have less commercial value compared to microbial enzymes [74, 77, 90].

2.3.5 Transglutaminase (TGase)

Transglutaminase (EC 2.3.2.13) is an enzyme that catalyzes the cross-linking of proteins by forming covalent bonds between amino acid residues, particularly

between lysine and glutamine [8, 12, 15, 18]. TGases have been used to modify the rheological (flow and deformation) properties and improve the texture and stability of dairy products [16, 54, 69]. For instance, it can improve viscosity and texture in yogurt and contribute to firmness in cheese [54]. Furthermore, they have been shown to improve the yield of cheese [14]. Additionally, they are important for innovative dairy products and protein supplements [54].

2.3.6 Other specialized enzymes

Several other enzymes are utilized for specific functions within the dairy industry:

Catalase (EC 1.11.1.6): Breaks down peroxide compounds, preventing oxidation and prolonging the shelf life of dairy products [9, 51, 65, 67]. It acts as a natural preservative and helps control oxygen levels in milk solutions, which is particularly important in cheesemaking and packaging [16].

Lysozyme (EC 3.2.1.17): A hydrolase with antimicrobial properties that breaks down bacterial cell walls (peptidoglycan layer), extending shelf life and preventing spoilage [43, 65, 91]. It is used as a protective agent in cheese production, especially soft cheeses, and as an alternative to potassium nitrate for controlling "late blowing" caused by *Clostridium tyrobutyricum* in hard and semi-hard cheeses [16, 65]. It can also make cow's milk suitable as infant milk [65].

Lactoperoxidase (LPO; EC 1.11.1.7): An antimicrobial enzyme that catalyzes the oxidation of various substrates by hydrogen peroxide, playing a role in the lactoperoxidase-thiocyanate system for cold pasteurization of milk [30, 43, 91]. It is also used as an indicator of effective pasteurization [29, 30].

Xanthine oxidoreductase (XOR) / xanthine oxidase (XO): Can act as a pro-oxidant, is involved in oxidative flavor formation, and plays a role in cheese ripening [12, 30].

Sulfhydryl oxidase: Contributes to the amelioration of cooked flavor and milk preservation [30, 42].

Superoxide dismutase: An antioxidant enzyme involved in milk preservation [30, 42].

Alkaline phosphatase (ALP; EC 3.1.3.1): A phosphomonoesterase whose inactivation is a key indicator for ensuring adequate pasteurization of milk, thereby enhancing microbiological safety [12, 28, 29, 57].

Acid phosphatase (ACP): Involved in cheese ripening and can reduce the heat stability of milk. Its concentration increases during mastitis infection [12, 30].

N-acetyl-β-D-glucosaminidase (NAGase; EC 3.2.1.30): An enzyme whose elevated concentration indicates mastitis infection [29, 42, 73].

Glucose oxidase (EC 1.1.3.4): Used in conjunction with catalase for food preservation, removing harmful oxygen and stabilizing probiotic bacteria in yogurt [9, 65, 67].

The dairy industry's reliance on enzymes is continuously growing. Microbial enzymes offer significant advantages, including large-scale production, cost-effectiveness, environmental friendliness, and the ability to be genetically manipulated for superior properties like higher solubility, increased specificity, and improved product yield.

Advances in DNA technology and protein engineering allow for the production of tailored enzymes that optimize activity for specific applications. Furthermore, enzyme immobilization techniques (e.g., adsorption, covalent binding, entrapment, and encapsulation) are transforming milk processing by improving enzyme stability, activity, and reusability, leading to more efficient and sustainable production processes, reduced costs, and a lower environmental impact.

2.3.7 Enzymatic production of health-promoting molecules from milk

Milk proteins, predominantly casein and whey, comprise encrypted sequences that, upon cleavage by designated proteolytic enzymes (e.g., pepsin, trypsin, chymotrypsin, alcalase, thermolysin), yield bioactive peptides with diverse physiological effects [92–94]. The resultant peptides have been shown to display antihypertensive, antioxidant, immunomodulatory, antithrombotic, anticancer, and antimicrobial activities [92, 94]. The nature of the peptides formed is contingent on both the source substrate and the protease employed. For instance, chymotrypsin can target aromatic amino acids, while trypsin is able to target basic amino acids. This has a consequential effect on the final peptide's structure and function [93].

It is evident that human milk is a natural source of complex oligosaccharides (HMOs), which have been shown to possess significant prebiotic, immune-modulating, and anti-infective properties. However, cow's milk contains them at much lower levels. The employment of engineered enzymes (e.g., transglycosylases, fucosyltransferases, and sialidases) enables scientists to catalyze the formation of human-identical oligosaccharides such as 2'-fucosyllactose (2'-FL), lacto-N-tetraose, and sialyllactoses, either directly in bovine milk or within *in vitro* systems [95–97]. These oligosaccharides function as prebiotics, thereby promoting the proliferation of beneficial gut microbiota and contributing to immune system development and the inhibition of pathogens [96].

Lipolytic enzymes may release bioactive fatty acids and glycerol from milk triglycerides, further enhancing the nutritional profile and health benefits of fermented or ripened dairy products [98].

Enzymes enhance the bioavailability of nutrients in dairy products through various mechanisms. Phytases release minerals like phosphorus by breaking down phytate, making them more absorbable. Proteolytic enzymes hydrolyze proteins into easily digestible peptides and amino acids, improving the nutritional quality of milk proteins [45]. Enzymatic hydrolysis enhances mineral bioavailability by producing Casein Phosphopeptides (CPPs), which are bioactive peptides derived from caseins [99]. CPPs chelate essential minerals like calcium and iron, keeping them soluble in the gastrointestinal tract and preventing precipitation. This process increases the absorption of these minerals, leading to improved bone density and calcification from calcium and better iron uptake by vital organs [99]. In animal feed, fibrolytic enzymes boost nutrient absorption in dairy cows, indirectly increasing the nutrient density of milk [100]. Additionally, lactase breaks down lactose, improving carbohydrate digestibility and energy bioavailability for lactose-intolerant individuals.

3. Protein engineering for enzyme optimization

Enzyme engineering is an emerging field of research that focuses on the modification of enzymes to achieve specific properties for a range of applications [8, 101]. The process of altering the amino acid sequences of proteins through the genetic manipulation of their genes is a fundamental aspect of protein engineering [8, 102].

Natural enzymes often have properties that are not optimal for industrial or therapeutic applications [103–105]. Enzyme engineering aims to improve these limitations. Natural enzymes may not be efficient enough for large-scale industrial production or may be rate-limiting bottlenecks in metabolic pathways [89, 106, 107]. Enzyme engineering can significantly enhance the catalytic activity of the target

enzyme [108–110]. Enzymes obtained from natural sources often exhibit instability under nonphysiological conditions like elevated temperatures, extreme pH, or the presence of organic solvents [110–112]. Enzymes are highly specific to their natural substrates, limiting their application to a broader range of chemicals [113–115]. Many natural enzymes lack the desired stereoselectivity for producing optically pure compounds [8, 104, 116, 117]. Enzyme engineering can go beyond optimizing existing functions to design enzymes with entirely new capabilities not found in nature [118–121]. Enzyme engineering employs several key approaches to modify proteins for improved or novel functions, often used independently or in combination.

3.1 Directed evolution

Directed Evolution (DE) is a revolutionary protein engineering strategy that systematically mimics the process of natural evolution in a laboratory setting [122–126]. Its primary goal is to tailor the properties of proteins, particularly enzymes, to meet specific industrial demands or to design entirely new functions not found in nature [105, 120, 124]. This approach has become a cornerstone in various fields, including biocatalysis, metabolic engineering, and synthetic biology [127].

At its core, directed evolution operates through iterative cycles of introducing genetic diversity (mutagenesis) and then screening or selecting for improved enzyme variants [86, 122, 123, 128, 129]. This process is akin to guiding a "random walk" through the vast protein sequence space, aiming to climb "fitness peaks" on the fitness landscape (a plot of fitness versus sequence) [122, 126]. Unlike rational design, DE does not require detailed prior knowledge of the protein's structure, function, or the specific amino acid substitutions that would confer the desired properties [86–89]. Instead, it relies on the principle that evolution has already solved the complex mapping from amino acid sequence to function [130]. The method involves repeated local searches, taking advantage of the functional promiscuity and clustering of functional sequences in sequence space [129].

The general procedure of directed evolution encompasses two main steps: generating a mutant library to achieve genetic diversification and then screening or selecting beneficial mutants from this library [86, 125, 128].

3.1.1 Genetic diversification (mutagenesis)

This initial step is crucial for creating the raw material for evolution: a diverse library of genetic variants from a parental gene [86, 131]. The rate of natural mutation is typically too low for laboratory purposes, necessitating artificial enhancement of genetic diversification [86].

Random mutagenesis: This approach introduces mutations broadly across the gene sequence, without specific targeting [86, 132]. Error-prone PCR (epPCR) is a widely used technique that employs low-fidelity DNA polymerases to introduce random point mutations during DNA replication [126, 133–135]. EpPCR can be modified by adjusting experimental conditions (e.g., elevating magnesium levels, adding manganese, or using imbalanced dNTP concentrations) to significantly enhance mutation frequency [134]. It is particularly advantageous for changing global characteristics of enzymes, such as thermal and pH stability, and has been used to enhance enzyme activity [136].

Gene recombination/DNA shuffling: This technique reassembles gene fragments from different proteins, organisms, or variants of the same protein to create chimeric

proteins [87, 126, 137]. This can combine functions from parental enzymes or yield entirely new properties [137].

3.1.2 Screening and selection

Identifying beneficial mutants from the massive libraries generated is a critical and often rate-limiting step [111, 124, 138, 139]. Screening involves performing individual assays on each variant, often facilitated by robotics and high-throughput platforms [111, 134, 140]. Examples include colorimetric assays, fluorescent signals, or High-Performance Liquid Chromatography (HPLC) to detect the desired function [134, 140]. High-throughput screening methods, such as droplet-based microfluidics and Fluorescence-Activated Cell Sorting (FACS), enable the isolation and identification of enzymes with improved features [121, 134, 141, 142]. Selection identifies individual variants with a given property by separating them from those without the property, often by directly linking a fitness advantage to the desired activity [126, 140]. Techniques like Phage-Assisted Continuous Evolution (PACE), OrthoRep, and MutaT7 enable continuous mutation and selection *in vivo* [121, 138, 140, 143]. These methods significantly accelerate evolution cycles and can enhance gene mutation rates, overcoming the labor-intensive iterative steps of traditional DE [87, 138].

Directed evolution offers several compelling advantages that make it a powerful tool in protein engineering. A significant benefit is that it does not necessitate a detailed structural or mechanistic understanding of the protein, bypassing the challenges encountered when such knowledge is incomplete or unavailable [8, 86–88, 144]. This makes it particularly useful for enzymes that are not well-characterized [145]. DE can explore a much broader sequence space than rational design, allowing for the evolution of complex functionalities such as enhanced catalytic activity, improved stability (thermal, pH, solvent), altered substrate specificity, and tuned selectivity (enantio-, regio-, chemoselectivity) [86, 120, 125, 129, 133, 139, 146]. It also has the capability to generate novel functionalities or "new-to-nature" activities not found in nature, expanding the enzyme universe for nonnatural reactions [8, 87, 105, 124, 125]. Directed evolution effectively bypasses the limitations of purely rational design, especially when the sequence-structure-function relationship is difficult to predict or when structural information is lacking [126, 131, 147–149]. Additionally, it can identify beneficial mutations at sites distant from the active center, which are often challenging to predict computationally or with rational design approaches. These distal mutations can significantly influence enzyme function, including catalytic activity and allostery [87, 143, 148, 150–152].

Despite its power, directed evolution is not without its drawbacks. The process often requires significant resources, including time, labor, and financial investment, particularly for generating large libraries and implementing efficient screening methods [88, 124, 138, 144, 153–155]. Random mutagenesis techniques often produce libraries where a high percentage (60–70%) of the mutations are deleterious or inactive, while only a small fraction (less than 5%) provides functional gains [87, 88]. This necessitates robust screening methods to sift through many nonfunctional variants [88]. High-throughput screening remains a major challenge, especially for complex desired properties or when sensitive and high-throughput assays are not readily available [86, 138, 142, 144, 154]. Some random mutagenesis methods, such as epPCR using Taq polymerase, exhibit an inherent bias toward certain types of mutations, which can limit the diversity of the sequence space explored [132, 134].

3.2 Rational design

Rational design, also known as computational enzyme design, is a protein engineering strategy that focuses on creating or improving enzymes with desired functionalities by making precise, targeted changes to their amino acid sequences [8, 102, 110, 118, 126]. This approach is fundamentally based on a deep understanding of the relationship between an enzyme's structure and its function [106, 115, 117, 142, 156–158]. Unlike directed evolution, which relies on iterative cycles of random mutagenesis and high-throughput screening, rational design aims to significantly reduce the experimental workload by predicting beneficial mutations *in silico* [88, 89, 126, 159, 160].

The core principle is that an enzyme's three-dimensional (3D) structure dictates its function [160, 161]. Therefore, by rationally altering the protein structure, its properties can be modified [8]. This requires precise knowledge of the enzyme's structure, including substrate-binding pockets, catalytic sites, and sometimes even dynamic conformational changes [89, 110, 162, 163]. A detailed understanding of the enzyme's catalytic mechanism is crucial. This involves comprehending how the enzyme binds to substrates, stabilizes transition states (TS), and releases products, as well as the roles of specific amino acid residues in these processes [120, 157, 160, 163–165]. Rational design identifies "hotspots," or specific amino acid residues, for mutagenesis [162, 166–168]. These sites can be in the active site pocket, substrate-binding tunnels/channels [110, 148, 166, 169], surface regions [110, 115, 167], or even distal sites that influence enzyme function through allostery or dynamics [143, 148, 151, 165]. Highly conserved residues are generally important for protein function and are often non-variable. Highly variable regions, on the other hand, can be targeted for mutagenesis to alter surface properties [122] .

3.2.1 Computational tools and approaches

Rational design heavily relies on computational approaches to predict the impact of mutations and guide the design process, making it a "computer-aided" or "in silico" methodology (**Table 2**) [117, 126, 156, 160, 170].

When crystal structures are unavailable, homology modeling, often using tools like AlphaFold2 (AF2) or RosettaFold, can be used to predict the 3D structure of an enzyme based on known structures of related proteins [156, 158, 171–173].

Molecular docking predicts how a substrate or ligand binds to the enzyme's active site, revealing optimal binding modes and crucial interactions [174, 175]. Tools like AutoDock, Glide, and GOLD are frequently used [164, 167, 170, 175].

Molecular Dynamics (MD) simulations provide insights into the dynamic behavior of enzymes, including conformational changes during catalysis, substrate/product channels, and the flexibility of residues [110, 126, 160]. They can be used to screen for beneficial substitutions that repel/attract surface solvation or to understand mechanisms through which mutations enhance activity [176].

Quantum Mechanics (QM)/Molecular Mechanics (MM) calculations are advanced methods and are used to determine the catalytic mechanism of an enzyme and analyze the potential energy surface of reaction mechanisms, providing in-depth knowledge on the reaction [117, 157, 164, 177]. They are crucial for understanding high-energy transition states [157]. Monte Carlo (MC) simulations explore the conformational space of active sites and ligands to identify optimal positions for catalytic residues or to evaluate system energy [110, 178].

Software/Tool	The primary purpose in rational design	Key features/applications
AlphaFold2/AlphaFold3	Protein structure prediction	Predicts highly accurate 3D protein structures, including complexes with small molecules. Essential for design when experimental structures are unavailable.
RoseTTAFold/ESM-Fold	Protein structure prediction	Rapidly predicts single-sequence protein structures, comparable in accuracy to AlphaFold2. Used for de novo structure prediction.
YASARA	Homology modeling, molecular graphics	Simulates molecular mechanics, builds homology models, visualizes structures, and predicts molecular interactions (hydrogen bonds, salt bridges).
Swiss model	Homology modeling	Automates 3D protein structure homology modeling and assesses model quality.
Phyre2	Protein fold recognition/ homology modeling	An online tool for protein fold recognition, homology modeling, and active site studies.
MOE	Homology modeling and structure analysis	Used for all steps of homology modeling and calculating structural differences (RMSD).
MODELER	Comparative protein structure modeling	Performs comparative protein structure modeling. Performs comparative protein structure modeling.
AutoDock/AutoDock Vina	Molecular docking	Simulates how a ligand binds to an enzyme's active site to understand binding modes.
Discovery Studio (DS)	Homology modeling, docking, structure superimposition	Generates homology models, superimposes structures, and performs docking analysis.
PLIP	Protein-ligand interaction analysis	An automated tool for profiling protein-ligand interactions.
LigPlot+	Intermolecular interaction analysis	Analyzes interactions, hydrophobicity, and surface charge of enzymes and their complexes.
DOCK/GOLD/GLIDE	Molecular docking	Identifies binding sites, calculates distances between residues, and studies stereoselectivity.
PointSite	Binding pocket prediction	Predicts the binding pockets of an enzyme.
Rosetta (Suite)	De novo enzyme design, protein stabilization, activity/specificity enhancement	A versatile computational engine for de novo protein design, enzyme active site design, docking, and structure prediction.
FuncLib	Automated active site design and functional diversification	Designs multi-point mutations at active sites to enhance activity without sacrificing stability.

Software/Tool	The primary purpose in rational design	Key features/applications
SABER	Active site identification and redesign	Identifies active sites suitable for computational redesign and can be used for de novo design.
AsiteDesign	Semi-rational active site design	A Monte Carlo-based method for engineering active sites by introducing new catalytic residues.
PRODA	Computational enzyme design protocol (active site redesign)	Redesigns active sites and generates/ranks mutant sequences based on energy calculations.
OptZyme	Computational enzyme redesign (transition state analogues)	A tool for enzyme redesign based on transition state analogues.
CADEE	High-throughput screening of enzyme variants	A computational framework to screen thousands of enzyme variants for improved properties.
FoldX	Protein stability prediction	Calculates free energy changes upon mutations to predict protein stability.
FireProt/FireProtDB	Automated protein stabilization	An automated platform for designing thermostable proteins using energy- and evolution-based methods.
PROSS	Protein stabilization	Automates protein design for high bacterial expression and stability.
PoPMuSiC	Protein stability prediction	Predicts the effect of mutations on protein stability to aid in improving thermostability.
Disulfide by Design v2.0 (DbD2)	Disulfide bond prediction and optimization	Predicts the formation of disulfide bonds to improve protein stability.
LibGENiE	Mutant library design and deleterious mutation exclusion	Filters sequences to exclude deleterious mutations, creating more effective mutant libraries.
DynaMut / DynaMut2	Mutation impact prediction	Predicts how mutations impact a protein's conformation, flexibility, and stability.
HotSpot Wizard	Identification of hotspot residues and smart library design	Automates the design of targeted mutagenesis libraries by identifying "hotspot" residues critical for function.
IREDFisher	Structure-based computational screening workflow	Prioritizes protein sequences based on predicted activity, reducing lab screening efforts.
TurNup	Turnover number (kcat) prediction	Predicts the turnover number of enzyme variants to avoid those with poor activity.
COMPASS/CompassR	Organic-solvent tolerance / Activity enhancement	Yields enzymes with high tolerance to organic solvents by recombining compatible substitutions.
Pypef	Data-driven protein engineering framework	An integrated framework for data-driven protein engineering, used for screening and exploring combinatorial effects.

Software/Tool	The primary purpose in rational design	Key features/applications
EnzymeMiner	Soluble enzyme mining and alignment filtering	Generates function-specific alignments and filters them based on essential residues, predicting soluble expression.
A2CA	Sequence analysis and enzyme selection	A user-friendly tool to visualize sequence alignments and establish robust sequence-function relationships.
ConSurf	Evolutionary conservation analysis	Visualizes the evolutionary conservation of residues in proteins.
CoeViz	Coevolution analysis	A web tool for analyzing the coevolution of protein residues.
PELE	Ligand binding/unbinding simulations, substrate-induced fit	Predicts dynamic interactions and migration paths of ligands and proteins.
CAVER/CAVER Web	Tunnel and channel analysis	Identifies and characterizes transport pathways (tunnels and channels) within protein structures.
MOLEonline	Access tunnel detection and characterization	Detects and characterizes access tunnels in protein structures to guide substrate preference engineering.
EzMechanism	Automated catalytic mechanism proposal	Infers mechanistic paths for a given active site and reaction based on a database of catalytic rules.
GENZYME	Reaction-conditioned De novo enzyme design	A model that designs enzymes from a catalytic reaction as the input, generating the catalytic pocket and full enzyme structure.
Protein Language Models (e.g., ESM-2, RFdiffusionAA, ProteinMPNN, LigandMPNN, RIFGen, RIFDock)	De novo sequence generation, structure prediction, functional prediction	Models that efficiently generate new protein sequences and structures with desired functionalities.

Table 2.
Software used for rational enzyme design.

Rational design reduces the need for extensive experimental screening by using computational predictions to create "small but smart" mutant libraries. This approach allows for the precise customization of enzyme properties, such as enhancing catalytic activity, improving stability in harsh conditions, and fine-tuning selectivity. It can also create enzymes with entirely new functions, expanding the scope of biocatalysis. By relying on computational simulations, rational design significantly shortens the enzyme development cycle and deepens our understanding of protein function [8, 118, 126, 142, 156, 160, 165, 179].

Rational design has its challenges. It requires a deep understanding of the protein's structure and function, which can be difficult to obtain, especially for new or complex enzymes. Accurately predicting the effect of mutations is also a major challenge. Computational tools can be limited by biased training data and may not always accurately account for complex factors. Furthermore, some advanced methods are computationally expensive, requiring significant resources and expertise to use effectively [8, 88, 110, 117, 145, 180].

Despite these limitations, rational design, especially when combined with advanced computational tools and machine learning, is rapidly evolving and is considered a transformative approach in enzyme engineering, paving the way for more efficient and precise enzyme design [156, 165].

3.3 Semi-rational design

Semi-rational enzyme engineering is a sophisticated approach that strategically combines principles from both rational design and directed evolution to modify and enhance enzyme properties [88, 125, 160, 165, 181]. This hybrid methodology aims to generate "small but smart" mutant libraries, significantly reducing the extensive screening efforts typically required by purely random directed evolution methods, while offering more flexibility than solely rational approaches [88, 125, 173, 182].

Semi-rational design integrates the knowledge-based predictions of rational design with the diversity-generating power of directed evolution [126, 136, 160, 183]. This approach directly addresses the screening bottleneck of classical directed evolution and circumvents the need for an absolute understanding of the complex sequence-function relationship in enzymes, which can be computationally intensive [88]. It allows for a more efficient sampling of the vast sequence space compared to completely random methods, leading to a lower screening burden [88].

A core aspect of semi-rational design is the use of existing biochemical or structural information to guide mutagenesis [88, 117, 144, 148]. This often involves analyzing the enzyme's three-dimensional (3D) structure, particularly focusing on the catalytic pocket, substrate-binding sites, or access tunnels [110, 117, 184].

3.4 Artificial intelligence (AI) in enzyme engineering

Artificial Intelligence (AI), encompassing Machine Learning (ML) and Deep Learning (DL), plays a transformative role in enzyme engineering by enhancing the prediction of mutations, streamlining library design, and optimizing screening processes [147, 154, 185, 186].

In rational design, artificial intelligence (AI) is applied in two main ways. First, machine learning (ML) and deep learning (DL) models are used for predicting mutations. These models analyze huge datasets of protein sequences and structures to learn complex patterns and predict the effects of mutations on an enzyme's properties, like its stability, solubility, activity, and specificity [127, 144, 165, 177, 187]. They are especially useful for handling epistatic effects—nonadditive interactions between mutations that are very hard for traditional methods to predict accurately [106, 126, 188]. Specific tools and algorithms, such as protein language models (PLMs) and structure-based deep learning models, help identify beneficial mutations and predict stability changes [105, 106, 127, 189, 190].

Second, AI is used for designing libraries. By identifying promising "hot spots" for mutagenesis, ML/DL helps create "smart libraries" that have a much higher chance of producing a beneficial mutation. This significantly reduces the size and cost of screening compared to random mutagenesis [124, 142, 191]. ML also aids in combinatorial mutation design, allowing researchers to explore massive sequence spaces and find superior mutants with enhanced properties [103]. Generative models, such as Variational Autoencoders (VAEs) and Diffusion Models, can even create entirely novel protein sequences with functions not found in nature. By predicting variant

performance in a computer, AI greatly cuts down on the experimental work needed in traditional directed evolution [127, 147, 154].

ML approaches accelerate the screening and selection phase by predicting mutants with enhanced characteristics, allowing for multi-parameter optimization (e.g., catalytic activity, enantioselectivity, pH stability, thermostability, and binding affinity) [104, 131, 141, 147].

MLDE (Machine Learning-Guided Directed Evolution) reduces the burden of laboratory experiments by performing *in silico* population selection through model-based fitness prediction [128, 192–195].

These advancements enable rapid identification and engineering of enzymes, significantly reducing the complexity and cost of traditional screening processes.

3.5 Engineering of enzymes used in the dairy industry

Enzyme engineering in the dairy industry focuses on enhancing enzyme performance to improve product quality, process efficiency, and sustainability.

Improving the properties of lactase, particularly its thermostability and activity at low pH, is crucial for its widespread and efficient use in the dairy industry, enabling the production of lactose-free products, enhancing sweetness, and preventing lactose crystallization [12]. Enhancing lactase thermostability is a significant goal for industrial applications because higher temperatures can accelerate reaction rates, increase substrate solubility (especially for high lactose concentrations), and minimize microbial contamination risks [20, 79]. Several lactase enzymes have been successfully engineered. For instance, triple mutations (K166P, G307P, A833P) were introduced into a *Bacillus circulans* β-galactosidase, resulting in enhanced thermal stability at 60°C [7]. The half-life and catalytic efficiency of *Kluyveromyces lactis* lactase have been improved through rational mutation involving the addition of cysteine residues at subunit interfaces [7]. A thermoresistant variant of *Thermotoga maritima* beta-galactosidase was developed through rational design, specifically to improve its specificity for the biosynthesis of galactooligosaccharides (GOS). This engineered enzyme demonstrated optimal activity at 75°C, making it highly suitable for processes requiring elevated temperatures [79]. Rational design has also been successfully employed to overcome product inhibition in β-galactosidase by modifying the galactose-binding motif of *Aspergillus niger* β-galactosidase, thereby reducing the enzyme's affinity for galactose [7]. An evolved variant of β-galactosidase, achieved through directed evolution, showed high activity under industrial conditions relevant to milk processing (pH 6.75, 8°C) [7].

A protease (PB92) from *Bacillus alcalophilus* was engineered using site-directed mutagenesis, increasing its thermostability by up to 31 times at 65°C by identifying key residues like N18L-R143L-S97A and N18L-R143L-G100A [196]. Similarly, engineering the lactate dehydrogenase enzyme from *Bacillus stearothermophilus* altered its specificity from lactate to maleate by replacing glutamine at position 102 with arginine [8]. The thermostability of *Kluyveromyces lactis* β-galactosidase was improved by adding cysteine residues at subunit interfaces through rational mutation. The galactose-binding motif of *Aspergillus niger* β-galactosidase was modified to reduce product inhibition [7]. *Bacillus megaterium* M32 carboxypeptidase was modified through semi-rational design to enhance catalytic efficiency and thermal stability, improving food flavor and reducing bitterness in protein ingredients [17, 68].

Advanced enzyme engineering enables the scalable, controllable production of asymmetric and complex biantennary oligosaccharides that closely mimic those in

human milk, allowing for the enrichment of infant formula and functional foods with tailored health benefits.

4. Conclusions

The dairy industry, a vital component of the global economy, is being transformed by the strategic use of enzymes. Enzymes act as powerful biological catalysts, enabling crucial processes from milk coagulation to flavor development, all while enhancing product quality and sustainability. The global market for food enzymes is projected to grow significantly, with the dairy sector representing a major area of demand.

Enzyme optimization techniques are crucial in advancing the dairy industry, leading to the development of "next-generation" functional dairy products by enhancing their nutritional value, improving their sensory characteristics, extending their shelf life, and addressing specific dietary needs.

Enzyme engineering has emerged as a transformative field, allowing for the modification of enzymes to meet specific industrial needs. Through approaches like rational design, directed evolution, and semi-rational design, scientists can overcome the limitations of natural enzymes, such as poor stability or low catalytic efficiency. Rational design uses a deep understanding of protein structure to make precise, targeted changes, while directed evolution mimics natural selection to screen for improved variants without needing extensive prior knowledge. Semi-rational design combines the strengths of both, creating "small but smart" mutant libraries that reduce the experimental workload.

Modern computational tools and artificial intelligence (AI), including machine learning and deep learning, are increasingly integral to these processes. These technologies help predict beneficial mutations, handle complex interactions between mutations, and design efficient mutant libraries, significantly reducing the time and cost associated with enzyme development.

The continuous evolution of enzyme engineering techniques promises further innovations, offering solutions for higher yields, consistent product quality, and enhanced functionality. These advancements are not only crucial for meeting growing consumer demands for clean-label and functional foods but also for promoting sustainability through reduced energy consumption and more efficient processes. The integration of advanced computational methods ensures a future of increasingly precise and efficient biocatalyst optimization in the dairy sector.

5. Challenges and future perspectives

The dairy industry faces several challenges in enzyme application, as natural enzymes often have suboptimal properties like poor stability and low catalytic efficiency under processing conditions. For instance, heat-stable proteases from psychrotrophic bacteria cause undesirable age gelation and off-flavors in UHT milk. Furthermore, rational design is limited by the need for detailed structural knowledge, while directed evolution struggles with the costly and labor-intensive screening of large mutant libraries.

Future perspectives are highly promising due to the transformative role of AI and machine learning. These technologies will be crucial for predicting beneficial

mutations and designing "smart libraries," significantly reducing experimental effort and cost. This will enable the precise engineering of enzymes like cold-active and thermostable lactases for lactose-free products and regioselective lipases for targeted flavor development. Innovations in immobilization technologies will also improve enzyme stability and reusability. These advancements are critical for developing sustainable practices, such as enzyme-based cleaning systems, and for creating health-promoting products like infant formula enriched with human milk oligosaccharides (HMOs).

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Chapter 3

Enzymatic Biotransformations via the Recombinantly Produced Enzymes in the Production of Functional Foods

Fatma Ersöz

Abstract

Functional foods contain high nutrition and are defined as healthy foods. These foods positively affect metabolism through bioactive compounds. Our diets include many functional foods, such as yogurt, kefir, cheese, broccoli, chia, and meat products containing opioid and antioxidant peptides, as well as other foods like tempeh, kimchi, and kombucha. It is known that many functional foods are products of fermentations mediated by enzymes. Numerous enzymes play a critical role in the enzymatic bioprocessing of valuable functional foods. However, in a bioprocess, the characteristic properties of enzymes and environmental conditions can limit or completely block the bioconversion. In some situations, the amount of enzyme may be insufficient for the process. Therefore, to achieve the desired properties of enzymes, recombinant production using metabolic engineering methods has gained significant popularity in recent years. It is known that the recombinantly produced enzyme has several advantages, including substrate selectivity and specificity. Another benefit of using recombinant enzymes is that they can be designed through genetic manipulation according to the process, such as making them resistant to cold or hot temperatures and high or low pH conditions. In the production of functional foods, the food enzymes are mainly used. This chapter presents the literature data on the roles, types, applications, and sources of bioconversion enzymes. In particular, the recombinantly produced enzymes used in functional food products are thoroughly presented.

Keywords: biotransformation, functional foods, microbial process, metabolism, recombinant enzyme

1. Introduction

Functional foods are foods that contain high levels of nutrients. Due to their high content of bioactive ingredients, they are recognized as healthy foods and are frequently included in daily diets. It appears that using various nutrients, either alone or in combination, can protect health and prevent or treat specific diseases. These nutrients have antioxidant, antitoxic, antiallergic, anticarcinogenic, and antihypertensive

effects [1]. Following the emergence of the positive impact of these foods, people's nutritional preferences and eating habits underwent a shift, resulting in a corresponding increase in the tendency toward functional foods [2]. Functional foods are obtained by incorporating an active ingredient found in its natural form or by enriching it with bioactive elements or metabolites through technological processes. Recently, a new term, "superfood," has emerged, which contains a high concentration of nutrients and possesses a perfect bioactive ingredient profile [3]. Due to these properties, superfoods in their natural form, without any technological process, are categorized as natural. Health-conscious consumers often prefer these foods to enhance their welfare and prevent disease in their daily diet [4]. There are many plant-derived superfoods that have different pharmacological effects on the body's health. For example, the consumption of *Ficus carica* regularly in the diet has a positive impact on memory by promoting learning capacities [5]. In another study, it was reported that quercetin, found in *F. carica*, has neuroprotective effects and enhances memory [6]. The other superfood is *Phoenix dactylifera*, which has a positive impact against memory loss [7]. The pharmacological properties of *P. dactylifera* can be given as antioxidant, anti-hyperglycemic, anticancer, and antihypertensive [8]. The other superfood is Saffron (*Crocus sativus*), which has a healing effect on ocular and cardiovascular diseases [9]. Beetroot (*Beta vulgaris*) is another food that has antitumor activity, and it improves heart health and

Fruits	**Vegetables**	**Grains/cereals**	**Nuts and seeds**
Goji berry	Kale	Wheat	Chia seed
Pomegranate	Reishi (fungi)	Amaranth	Flaxseed
Choke berry	Cabbage	Rye	Hemp seed
Blackberry	Maca plant	Cocoa	Carob seed
Mangosteen	Broccoli	Oat	Almond
Passion fruit	Spinach	Buckwheat	Pistachio
Acai berries	Beet	Rice	Sunflower
Bilberry		Bean	Maize
Red raspberry	**Roots and tubers**	Barley	
Cape gooseberry	Ginger	Chickpea	**Others**
Jackfruit	Turmeric	Millet	Pollen
Acerola	Maca	Quinoa	Chlorella
Ficus carica	Chicory root	Sorghum	Spirulina
Camu	Ginseng	Maize	Honey
Avocado	Yam		Royal jelly
Lucuma	Burdock root		Waxes
Grape	Ashwagandha		Kefir
Cranberry	Ginger root	**Herbs and spices**	Donkey milk
Baobab		Matcha tea	Yogurt
Goji berries		Moringa	
Dragon fruit		Tea	
		Saffron	

Table 1.
The classification of superfoods.

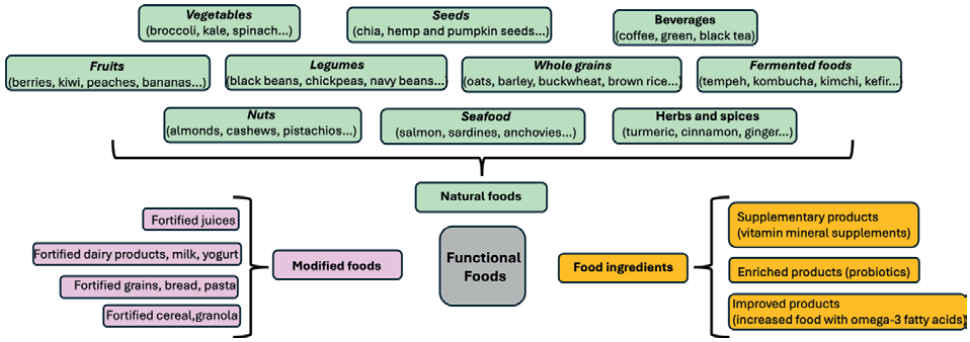


Figure 1.
Functional foods groups.

reduces blood pressure [10]. Numerous superfoods are incorporated into our diet, and their classification is presented in **Table 1**.

Superfoods offer numerous health benefits similar to those of functional foods, making it impossible to separate them with a sharp line; conversely, they can be grouped as intertwined foods. Functional foods, also known as nutraceuticals, are typically derived from plant or animal-based sources. It is known that they reduce the risk of cancer and metabolic disorders through the bioactive compounds they contain [11]. The functional foods are categorized (i) conventionally used food, (ii) modified food, and (iii) food ingredients [12] as given in **Figure 1**. While conventional foods are not modified and contain natural bioactive compounds, modified foods are enriched with specific nutrients such as calcium, omega-3, and folate [13–15]. Another group of functional foods is prebiotics, such as inulin, oligosaccharides, or non-digestible carbohydrates [12, 16].

The bioactive components found in functional foods are produced through chemically engineered extracts [17], microbial fermentations [18], or the use of commercial enzymes [19]. In these techniques, the chemical-mediated modifications can cause undesirable reactions, resulting in the formation of nonspecific transformation products [20]. The other method is microbial fermentation, which is widely used to produce valuable food products or ingredients, such as yogurt, vinegar, wine, beer, vitamin supplements, organic acids, and alcohol [21]. However, in some situations, unwanted or unexpected byproducts can form in microbial fermentations, such as alcohol and gases [22, 23]. It is well-known that processing methods such as hydrostatic pressure, pulse electric field, and ultrasonication are commonly used to produce functional foods [24]. However, these methods take heaps of time, and a significant amount of chemical waste is produced during the process. To overcome these limitations, enzyme-mediated modifications, which can catalyze reactions specifically, have gained popularity. Food enzymes are extensively used to produce modified functional foods or food ingredients. Many functional foods are the products of enzymatic bioprocess reactions. In this chapter, we will explain the enzymatic biotransformation techniques, the utilization of enzymes in these processes, the sources of enzymes, and recombinant enzymes associated with these processes.

2. Usage of enzymes in bioprocessing

Enzymes, which are protein-based biological catalysts, are utilized in various areas of technological processes. Specific enzymes are used to confer the desired function

to a food product, while others are employed to prevent the formation of undesirable byproducts. For example, a-amylase is used to increase bread quality [25], protease is used for the meat tenderization process [26], pectinase is used for the clarification of juice [27], and laccase is used for eliminating the polyphenol in wine [28].

It is known that, in fermentations using the same substrate as glucose or pyruvic acid, different fermentation products are produced by various microorganisms. These byproducts can be listed as CO_2 and propionic acid in cheese production, lactic acid in yogurt, CO_2 and ethanol in wine, acetic acid in vinegar, by using *Propionibacterium*, *Lactobacillus*, *Saccharomyces*, and *Acetobacter* microorganisms, respectively [29]. As is known, the food bioprocessing is a combination of fermentation and some enzymatic reactions. Fermentation is a metabolic process that enables the formation of different products from the same substrate, made possible by the enzymes present in microorganisms and the environmental conditions. In enzymatic biotransformation, microbial enzymes are primarily used in dairy products, bakery products, animal feed, food packaging, beverages, and the fruit and vegetable industries [30].

3. Enzymatic biotransformation in the functional food production

Enzymes selectively act on the substrates, and the region-selective properties of the enzymes are an advantage in the production of functional foods or bioactive molecules [31]. In certain conditions, the enzymes used in functional food production create a new product, while in other instances, they enhance the functional properties of the food through biotransformation. The enzymatic bioprocesses for producing functional foods, along with the enzymes involved, are schematized in **Figure 2**. The combination of enzyme and product should be optimal, and it is the most crucial factor in an enzyme-catalyzed bioprocess. In catalytic bioprocesses, enzymes isolated from different plant, animal tissues, or microorganisms are used; however, the

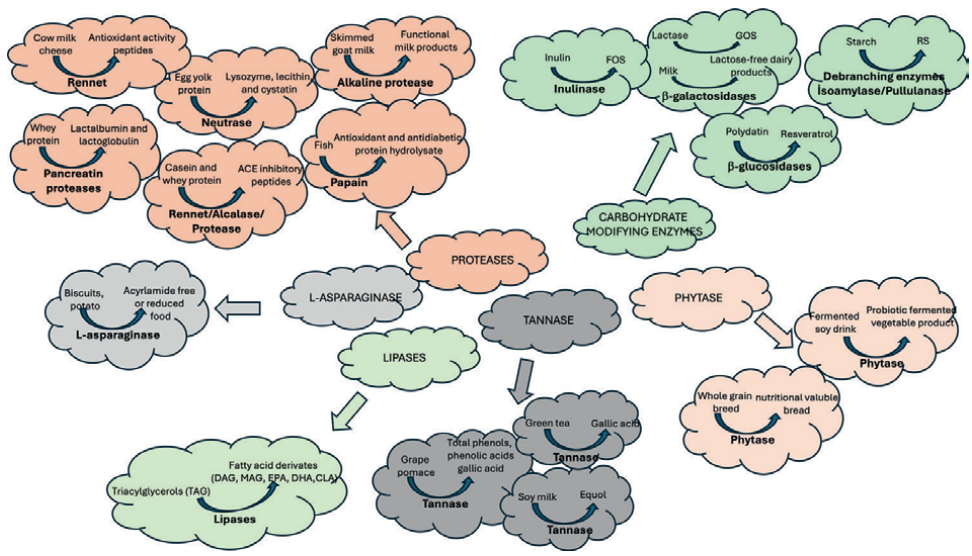


Figure 2.
Schematic representation of enzymes that are involved in the functional food production process (DAG: diacylglycerol, MAG: monoacylglycerol, EPA: eicosapentaenoic acid, and DHA: docosahexaenoic acid).

isolated enzymes cannot always be appropriately suited for the process. The source of enzymes affects the characteristic properties, such as optimum pH, temperature, and oxygen demand ratio. They are essential in extracting high-value food or food products, and food-derived proteins or peptides are obtained through enzymatic reactions. Numerous enzymes are involved in the production of functional foods or their derivatives, and their roles are explained in the following sections.

4. Recombinant production of the enzymes

Food processing enzymes are mainly obtained through extraction methods from plant, animal, and microbial origins. However, conventional extraction methods are time-consuming and inefficient for removing impurities, and the isolated enzyme can be denatured due to the high temperature and pH conditions [32]. The other disadvantages of naturally isolated enzymes are that they show low yields and cannot consistently achieve optimal performance in the processes [33]. The utilization of protein engineering techniques enables the elimination of disadvantages and the construction of enzymes with desired properties.

The isolated enzymes are obtained from plant or animal tissue in the form of a mixture with other intracellular enzymes, for example, rennet, which is isolated from the stomach of calves. Rennet is used for milk clotting due to its proteolytic activity, and it is a complex mixture of rennin (also known as chymosin) and pepsin [34]. During cheese production, cheese containing antioxidant peptides and exhibiting functional properties is produced by using rennet for clotting milk [35]. However, it is known that the rennet contains pepsin, at a ratio of up to 20%, which causes nonspecific proteolytic degradation [34]. These nonspecific degradations, which cause a bitter taste, also reduce the product's bioavailability. For these reasons, the use of another component of rennet, rennin, is preferred for producing cheese with functional properties. It is possible to produce the enzyme in its pure form rather than in a mixture by using the recombinant DNA technique.

In a recent study on the production of xylooligosaccharides (XOS), which has a prebiotic effect and decreases the risk of colon cancer, a recombinantly produced cold-adapted xylanase enzyme was utilized [36]. It was reported that the cold-adapted enzymes have broader substrate specificity than their thermophilic counterpart [37]. Therefore, the use of cold-adapted enzymes is an advantage for the XOS production process. In recombinant enzyme production techniques, the production of modified enzymes is possible. To achieve the goal of developing high-quality/quantity functional foods, genetically modified enzymes have been utilized. The production of enzymes with specific properties is possible using recombinant DNA techniques within protein engineering.

Several parameters must be considered in recombinant protein production, including the selection of host cell, promoter, and secretion signal, to achieve the optimal configuration. These factors directly affect the characteristics and yield of the produced enzyme. In a study on the recombinant expression of the nitrilase enzyme in *Pichia pastoris*, the enzyme production potency of two promoters, the constitutive glyceraldehyde-3-phosphate dehydrogenase (*GAP*) promoter and the methanol-inducible alcohol oxidase 1 (*AOX1*) promoter, was compared. It was reported that the inducible promoter produced the nitrilase enzyme at approximately a fourfold higher ratio [38]. In another study, the xylanase enzyme was produced under the control of the *GAP*, *AOX1*, and *ADH2* (alcohol dehydrogenase) promoters,

and the *ADH2* promoter exhibited the highest enzyme production capacity under the same conditions [39]. The other crucial factor is the secretion pathway, which is essential for producing proteins within or outside cells. It was reported that the recombinant secretion of recombinant proteins by using different secretion signals in gram-positive bacteria [40]. In recombinant protein production, various host systems are utilized, including bacteria, yeast, mold, and mammalian cells, and the expression platform type is selected based on the characteristics of the protein being produced. For example, while insulin is produced in the bacterial or yeast host system, many therapeutic agents are produced in mammalian cells [41]. It is also known that many food enzymes are produced in yeast expression systems.

In the production of recombinant proteins and enzymes, the primary goal is to design an optimal structure that achieves the desired properties. These properties can be specific to the reaction, such as the endurance of high or low temperature or pH conditions required for the product's formation. Transglutaminase is an enzyme used for cross-linking protein bonds in many foods. This enzyme is used as a reconstruction agent and is commonly referred to as meat glue in the meat industry. Although it varies depending on the gene source, the optimum temperature value is generally between 40°C and 50°C [42]. In a recent study, the biochemical properties of the transglutaminase enzyme were improved by producing a cold-adapted recombinant enzyme, which displays cold-adapted features under 8–16°C conditions [43]. Thus, the enzyme has been engineered to function in cold conditions, a desirable feature for the meat industry. Therefore, genetic engineering techniques have been used frequently to produce recombinant enzymes in recent years. It is known that commercial enzyme varieties produced by the recombinant production method used in functional food production, such as Phytex (recombinant phytase; LLC Sheridan, IN), Renmax-L (recombinant chymosin; Mayasan biotech), and Acyrlaway L (Novozymes). Numerous studies have shown that recombinant enzymes are used to produce functional foods. We summarized the studies that performed the recombinant production of enzymes responsible for producing functional foods, as given in **Table 2**.

4.1 Proteases

The peptide bonds in the protein structure are cleaved by proteases to obtain minor forms of peptides, and proteases are categorized as hydrolases with the Enzyme Commission (EC) number 3.4 [56]. Based on the catalysis mechanisms, proteases can be grouped as aspartic, serine, threonine, cysteine, glutamic, and metalloproteases [57]. The hydrolysis of protein, facilitated by the different characteristics of proteases, enhances the functional or nutritional properties of food. According to the hydrolysis type of protease, they have been used in various areas such as baking [58], beverage [59], detergent industry [60], leather industry [61], contact lens cleaning [62], meat tenderizing [63], protein modifications [64], and animal feeds [65].

Proteases are used to obtain different functional foods. The hydrolysis of soybean protein by protease imparts functional properties to foods, including antioxidant [66], antimicrobial [67], and antihypertensive [68] effects. The other bioactive peptide is an angiotensin I-converting enzyme (ACE) inhibitory peptide, which is used to reduce hypertension. It is known that ACE inhibitory peptides were produced using proteinase on α-casein substrate [69], rennet on casein substrate [70], and alcalase on whey protein [71]. The other protease is rennet, and its use in the cheese production process yields peptides with high antioxidant activity [72]. Using another protease,

Enzyme	EC number	Gene source	Host system	Production yield	Application/product	Reference
Alkaline protease	3.4.21-24	*B. clausii*	*B. licheniformis BL10*	15,435.1 U/mL	Functional milk products	[44]
Rennin (chymosin)	3.4.23.4	*B. taurus*	*P. pastoris*	39 IMCU/mL	Antioxidant activity peptides	[45]
Papain	3.4.22.2	*C. papaya*	*E. coli*	349 mg/L (titer)	Antioxidant and antidiabetic protein hydrolysate	[46]
β-Galactosidase	3.2.1.23	*Kluyveromyces* sp.	*E. coli BL21*	42,367 U/L	Lactose-free products and GOS	[47]
β-Glucosidase	3.2.1.21	*P. chrysosporium*	*K. pastoris (P. pastoris)*	2.79 U/mL	GOS and resveratrol production	[48]
Isoamylase	3.2.1.68	*T. fusca*	*E. coli MDS42*	22,983 U/mL	RS production	[49]
Pullulanase	3.2.1.41	*B. naganoensis.*	*B. subtilis*	102.75 U/mL	RS production	[50]
L-Asparaginase	3.5.1.1	*S. cerevisiae*	*E. coli K-12 BW25113*	93.52 μg/mL (titer)	Acrylamide-free food production	[51]
Lipase	3.1.1.3	*C. antarctica*	*P. pastoris*	7108.6 U/L	Fatty acid derivatives	[52]
Tannase	3.1.1.20	*P. albidiflava*	*E. coli*	—	Gallic acid	[53]
Phytase	3.1.3.8	*M. smegmatis*	*E. coli DH5*	118.91 U/mL	Nutritionally valuable product	[54]
Inulinase	3.2.1.7	*A. kawachii*	*P. pastoris*	840 U/mL	FOS production	[55]

Table 2.
The enzymes in the production of functional foods.

papain, bioactive peptides with antioxidant and antidiabetic properties have been extracted from fish and fisheries residues [73].

In the recombinant production of alkaline protease enzyme, *Bacillus licheniformis* was used as the host organism, and the enzyme was produced with a concentration of 15,435 U/mL. This enzyme activity was increased approximately 3-fold by optimizing the fermentation medium [44]. In another study on improving the production of alkaline protease, the same host was used, and genetically modified enzyme expression was achieved through genome-editing techniques [74]. It was reported that, by genetically modified techniques, the enzyme activity was improved by 62% compared to the unmodified producer clone. Another recombinant protein is the chymosin enzyme, and it has been reported that the enzyme level was increased twofold *via* the overexpression of the chaperone protein [45]. The other protease, papain, is used for fish products. In a study, the papain enzyme was recombinantly produced at a concentration of 350 mg/L in an *Escherichia coli* host [46], whereas the yield of the produced enzyme was 400 mg/L in a different study [75]. These situations demonstrate that the enzyme production level can be increased through genetic modifications and fermentation optimization.

4.2 Carbohydrate-modifying enzymes

Carbohydrases are the enzymes that catalyze the breakdown of carbohydrates. These enzymes are utilized in the production of various functional foods and nutraceuticals. Galactooligosaccharides (GOS), which are growth factors of bifidobacteria, are the prebiotics that are produced from lactose by β-galactosidases [76, 77]. Another known prebiotic is fructooligosaccharides (FOS), which is produced by the inulinase enzyme from inulin [78]. Resistance starch (RS) is another functional food that has an improving effect on human health by improving carbohydrate metabolism [79]. RS is a prebiotic, and isoamylase [80] or pullulanase [81] enzymes are responsible for producing resistant starch. The other functional foods are lactose-free products, produced by β-galactosidases [82], and resveratrol, which is used for its anti-inflammatory effect, is produced by β-glucosidases [83].

β-Galactosidase, the enzyme responsible for breaking down lactose into glucose and galactose, is utilized in the production of products for individuals with lactose intolerance who struggle to digest lactose. The production of β-galactosidase enzyme was performed in *E. coli*, resulting in an enzyme activity of 17,745 U/L [47]. In extracellular protein expression, the signal sequences are used to secrete the produced protein out of the cell. In a study, the β-galactosidase enzyme was produced in *Komagataella phaffii*, and various signal sequences were tested to increase the extracellular secretion of the enzyme [84]. However, contrary to expectation, the enzymes could not be expressed extracellularly, while the other enzyme, β-glucosidase, was expressed extracellularly by the optimized media components [48]. It was observed that the enzyme was produced 2.7-fold more in optimized media compared to standard media. In the production of resistant starch, debranching enzymes are used, and isoamylase is one of them. The isoamylase enzyme was produced in *E. coli* with an activity of 22,983 U/mL [49]. The other enzyme responsible for RS production is pullulanase, which is recombinantly produced in *Bacillus subtilis* at a concentration of 102.75 U/mL [50]. To enhance the produced pullulanase, the auto-inducer system was tested in a study [85]. It was observed that the combination of auto-induction and temperature-controlled production increased the enzyme's production levels.

4.3 L-Asparaginase

Fried and baked foods, such as biscuits, French fries, crackers, and chips, are frequently included in people's daily diets. During the preparation of these foods, the Maillard reaction, which leads to the formation of acrylamide, occurs between asparagine and reducing sugars [86]. To prevent the formation of highly carcinogenic acrylamide, the L-asparaginase enzyme (EC number 3.5.1.1) is utilized, which catalyzes the deamination of L-asparagine. It was reported that acrylamide-free or reduced biscuits were produced by using the L-asparaginase enzyme in the production process [87, 88]. In our daily diet, it is possible to consume foods that contain carcinogenic acrylamide. However, there should be a decrease in the amount of food. In a study, the L-asparaginase enzyme was recombinantly expressed in *E. coli* with a concentration of 93.52 mg/mL [51]. In another study on the production of this enzyme, waste materials were used as a substrate, and the microbial production of the enzyme was performed [89].

4.4 Lipases

Lipases are known as the digestive enzymes that catalyze the lipid triacylglycerol (TAG) into free fatty acids (FFA), diacylglycerol (DAG), and monoacylglycerol (MAG) [90]. It is known that DAG and MAG-enriched oils have anti-obesity properties, so lipase is an essential enzyme in the production of functional foods. Another catalytic product of the lipase enzyme is polyunsaturated fatty acids (PUFAs). Omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are the most well-known of the PUFAs. These functional foods have a protective effect against cardiovascular disease [91]. In a study on the production of recombinant lipase enzyme, crude glycerol, a byproduct of biodiesel, was utilized, and 7108.6 U/L of enzyme activity was detected in the *P. pastoris* host [52]. In another study, the lipase enzyme was produced in *E. coli*. The increase in activity was observed by using the disulfide bond formation catalyzing system [92].

4.5 Tannase

Numerous phenolic compounds are present in our diet. It is known that these phenolic compounds have a positive effect on the human body. These functional foods exhibit antioxidant, anti-inflammatory, anticarcinogenic, and antimicrobial properties, and are commonly found in plants, fruits, nuts, and teas [87, 93]. There are studies on the applications of tannase enzymes for the formation of gallic acid by using grape pomace [94] and green tea [95]. In another study on the application of tannase to citrus residues, an increase in aglycones, including hesperidin and naringenin, which are protective against breast cancer, was observed [96]. Another area of using tannase enzyme is in soymilk, which increases the equol content, a substance with a nutraceutical effect [97]. *Pseudoduganella albidiflava* tannase enzyme was recombinantly produced in *E. coli* with a high affinity for substrates [53].

4.6 Phytase

In foods, phytic acids are known to be antinutritional due to their complex formation with calcium, iron, magnesium, potassium, and proteins [98]. The complex formed with phytic acid and minerals reduces mineral absorption. The use of phytase

enzymes eliminates these adverse effects of phytic acid by reducing phosphorus pollution. The phytase enzyme, which facilitates the absorption of minerals, is found in plants, animals, and microorganisms [99]. In a study, the application of phytase in fermented soy drinks increases the bioavailability of Ca, Fe, and Zn, resulting in the production of a probiotic fermented vegetable food [100]. In bread, the use of bacterial phytase increases the bioavailability of minerals [101]. Phytase, which produces nutritionally valuable products, was produced in *E. coli* at a concentration of 118.91 U/mL [54]. In another study, the phytase enzyme, which has a wide pH stability, was produced in the *P. pastoris* host with a titer of 6413.5 U/g [102].

5. Challenges and future perspectives

Functional foods play a crucial role in human nutrition due to their health benefits. Although they are generally foods obtained directly from plant extracts, some functional food products are produced through enzymatic biotransformation, which is carried out by fermentation. The enzymes involved in biotransformation are primarily internal enzymes of a microorganism, and their characteristic features vary depending on the source. Additionally, the quality and quantity of the isolated enzyme are often insufficient. So, the recombinant production comes to the fore to produce the enzyme in high quantities and with the desired properties. In this study, the recombinant production of enzymes used in functional foods, produced through enzymatic biotransformation, is described. However, there is a need to improve the properties of the produced enzymes. Future studies should focus on providing enzymes with the required new, applicable, and sustainable properties through genetic modifications, such as those enabled by bioinformatics.

6. Conclusions

Functional foods contain high nutrition and are defined as healthy foods. Natural superfoods and functional foods, which are considered beneficial for our health, are often incorporated into our diet. The health benefits of functional foods can be listed as antioxidant, antihypertensive, antimicrobial, anti-inflammatory, antitoxic, antiallergic, and anticarcinogenic. While a food can be categorized as a functional food without any processing, it can be transformed from its native form to another form with high bioavailability through biotransformation. In the production of functional foods, chemical modification, microbial fermentation, and commercial enzymes are utilized. There are several disadvantages to producing functional food through chemical modification and microbial fermentation. Due to the extreme conditions, such as in the chemical modification method, the enzyme can be partially denatured. In the microbial fermentation process, the formation of byproducts is a serious problem. Therefore, the enzymatic biotransformation techniques are preferred. It is known that commercial enzymes used in enzymatic biotransformation are mainly produced from plant and animal tissue or as microbial isolates. However, the isolated enzymes cannot be in a pure form; they are typically found in a mixture. The mixture of isolated enzymes can cause purification problems or lead to the formation of undesirable products. To eliminate these problems, recombinant enzyme production techniques have been employed in recent years. In recombinant protein production, it is possible to gain the desired properties (such as resistance to high or low temperatures and

extreme pH conditions) with genetic modification techniques. Due to their superior properties, such as substrate specificity, stable behavior, and high enzyme activity, recombinant enzymes have been utilized in various food applications. Additionally, it may be possible to develop new functional foods by introducing novel properties to the enzymes used in their production through genetic modification. Future studies should focus on developing new recombinant enzymes for use in the production of functional foods.

Conflict of interest

The authors declare that they have no conflict of interest.

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Chapter 4

How Cold Plasma Processing Shapes the Volatile Compound Profile of Fruit Juices

Emine Ozen

Abstract

This review examines the impacts of cold plasma processing on the volatile compound profiles of fruit juices, with a focus on aroma preservation and enhancement. As an emerging nonthermal technology, cold plasma offers significant advantages over conventional thermal treatments in maintaining microbial safety while better preserving juice quality attributes. This chapter provides a brief overview of cold plasma fundamentals, including plasma physics, generation methods, and microbial inactivation mechanisms. Also, numerous studies have demonstrated that cold plasma treatment significantly alters volatile compositions in apple, cantaloupe, cashew apple, red dragon fruit, pineapple, and orange juices through selective chemical transformations of esters, aldehydes, alcohols, and other aroma-active compounds. Processing parameters—including gas composition, plasma type, frequency, and treatment duration—play crucial roles in determining these changes. It has been shown that cold plasma generally preserves more desirable aroma compounds and produces fewer off-flavors than thermal pasteurization, with some treatments even enhancing juice sensory profiles through the generation of new, favorable volatiles. This review synthesizes current knowledge and identifies research gaps regarding cold plasma's potential to optimize flavor quality in fruit juice processing, contributing to the development of high-quality, minimally processed, functional juice products with enhanced consumer acceptability.

Keywords: cold plasma processing, volatile compounds, fruit juice, nonthermal technology, flavor enhancement

1. Introduction

Growing interest in the idea that certain foods could enhance health has led to the creation of the broad term functional food. According to the Food and Nutrition Board (FNB) of the National Academy of Sciences, a functional food refers to "any modified food or food ingredient that may provide a health benefit beyond that of the traditional nutrients it contains" [1].

Global fruit juice consumption is on the rise, particularly within the "refrigerated single strength and not from concentrate" categories [2]. Consumers are increasingly willing to pay premium prices for high-quality juices, such as cold-pressed varieties,

favoring them over thermally processed options because of their fresh taste and perceived health benefits [2, 3]. In response to this demand, many countries—including the United States [4], Poland [5], and New Zealand [6, 7]—have developed functional fruit juices and beverages, as fruits are rich in essential nutrients and bioactive compounds such as polyphenols, vitamins A and C, minerals, and dietary fiber [8–14]. Research has demonstrated that fruit consumption contributes positively to human health and well-being [15, 16]; consequently, the intake of fruit juices has been associated with the prevention of chronic illnesses, including cancer and cardiovascular diseases [17].

Cold plasma processing is an emerging nonthermal technology that offers substantial advantages over traditional thermal methods in juice production. It effectively addresses key challenges, such as microbial safety, enzyme inactivation, and quality preservation. Traditional thermal processing, while widely used, often results in the degradation of juice quality, including undesirable changes in flavor, color, and nutrient content, which can lower consumer acceptability [18]. Moreover, it may not fully inactivate spoilage-related enzymes, such as polyphenol oxidase, leading to a reduced shelf life and compromised product stability [19].

In contrast, cold plasma has demonstrated a high capacity for microbial inactivation, effectively eliminating a broad range of pathogens and spoilage organisms [20–22]. It also preserves and can even enhance the nutritional profile of juices by retaining or increasing antioxidant activity and protecting sensitive compounds, such as anthocyanins and vitamin C [21, 23, 24]. Additionally, cold plasma is effective in inactivating enzymes that negatively affect juice quality, thereby extending shelf life and maintaining product integrity [19, 25]. Another key benefit is its ability to maintain the physicochemical properties of juices, with minimal alterations to color and flavor [22, 26]. Furthermore, several studies have shown that the antioxidant activity of fruit juices is often preserved or even enhanced after cold plasma treatment, which further supports their classification as functional foods [23, 27, 28].

Beyond quality benefits, the technology is cost-efficient, and highly adaptable for industrial use, offering scalable and flexible processing solutions [20, 21]. Hence, ongoing research is essential to adapt cold plasma treatments for different juice types and to fully realize their functional potential in a commercial setting.

In summary, cold plasma processing presents a promising and innovative approach for juice preservation, effectively overcoming many limitations of conventional methods while maintaining or improving product quality. However, the application of cold plasma requires careful optimization. The effectiveness of the treatment is highly dependent on parameters, such as exposure time, plasma type, and juice composition. Without proper optimization, there is a risk of degrading sensitive bioactive compounds instead of enhancing them [18, 27, 29]. Hence, advancing our understanding of cold plasma parameters across food matrices is crucial to achieve its full commercial potential.

Volatile compounds play an important role in determining the aroma and flavor profile of fruit juices, which are key quality attributes influencing consumer perception and acceptance. These compounds, comprising esters, aldehydes, alcohols, and terpenes, contribute to the characteristic sensory experience of freshness and fruit identity. As consumers increasingly prioritize natural flavor and sensory appeal in their food choices, preserving or enhancing these volatile profiles has become a central focus in food processing [30]. Understanding how emerging nonthermal technologies—such as cold plasma—affect these sensitive compounds is essential. This knowledge forms the basis for developing processing methods that ensure both safety and sensory quality in fruit juice products.

This chapter examines how cold plasma processing—an emerging nonthermal technology—affects the volatile compound profiles of fruit juices. Because these compounds are highly sensitive to thermal processing, there is increasing interest in alternative methods that can ensure microbial safety and shelf life while maintaining or enhancing sensory characteristics. To that end, the chapter explores the mechanisms through which cold plasma interacts with volatile compounds, the types and extent of compositional changes reported in recent studies, and how these effects vary among different juice matrices. It also evaluates the advantages and limitations of cold plasma technology. By synthesizing existing findings and highlighting areas requiring further investigation, this chapter aims to provide a comprehensive understanding of cold plasma's potential to preserve flavor quality in minimally processed fruit juice products.

2. Cold plasma technology

Plasma is the fourth state of matter, consisting of a quasi-neutral ionized gas. It contains ions, free electrons, atoms, and molecules in ground or excited states, but still maintains an overall neutral electric charge [31]. Plasma can be categorized based on the thermal equilibrium into two types: Thermal plasma and low-temperature plasma. In thermal plasma, all particles have the same temperature in thermodynamic equilibrium, typically around 10,000 K, as observed in arc plasmas. Low-temperature plasma is further divided into two subcategories: Quasi-equilibrium plasma (thermal low-temperature plasma), where particles maintain local thermal equilibrium, and nonequilibrium plasma (nonthermal plasma), where significant temperature differences exist between particles. In nonthermal plasma, such as glow discharges, electrons can reach 10,000–100,000 K, while heavier particles stay at lower temperatures (300–1000 K) [32]. In this chapter, the nonthermal plasma, where electrons and heavier particles maintain different temperatures, is referred to as "cold plasma." Plasma can also be categorized based on pressure conditions like atmospheric pressure and low pressure (vacuum) plasmas. Additionally, plasma can be created using various energy sources capable of ionizing gases, including electrical, thermal, optical (UV light), radioactive (gamma radiation), and X-ray electromagnetic radiation. Among these, electric or electromagnetic fields are the most commonly used methods for cold plasma generation [33].

Cold plasma generation techniques offer diverse designs that can be integrated with existing food industry equipment. Several atmospheric pressure plasma generation technologies are employed for food processing applications. These include microwave plasma (MW) [34], corona discharge plasma (CD) [35], dielectric barrier discharge plasma (DBD) [36], glow discharge plasma (GDP) [37], resistive barrier discharge plasma (RBD), and atmospheric pressure plasma jet (APPJ) [38]. Each of these plasma types generates and maintains electron collisions under specific conditions, such as radio frequency excitation, gas discharge, or photo ionization, to produce plasma [39, 40].

Among the available plasma systems, APPJ and DBD configurations have been most widely studied for juice treatment. This is because they are easy to build, commercially available, compatible with food equipment, and operate at atmospheric pressure (**Figure 1**). DBD devices utilize two metal electrodes with at least one covered by a dielectric material. This dielectric barrier serves two key functions—it prevents arc formation and promotes the creation of numerous micro-discharges, resulting

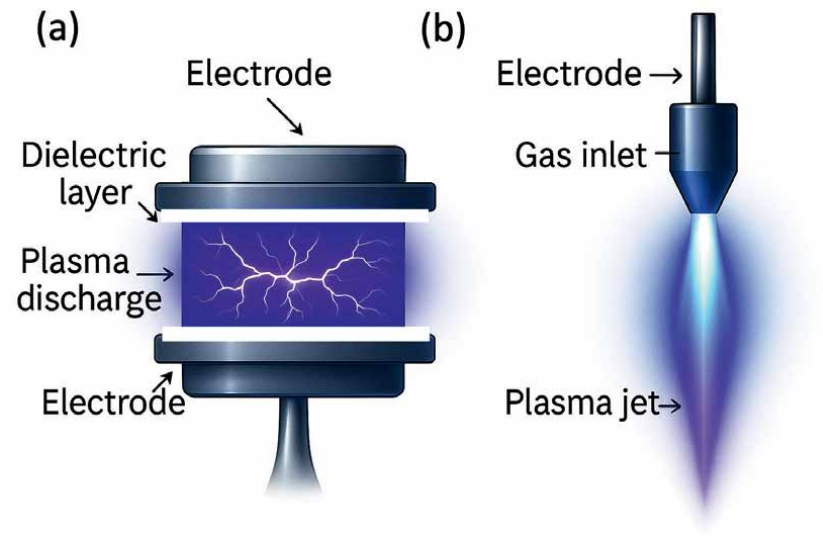


Figure 1.
Schematic diagrams of different plasma generation configurations: (a) Dielectric barrier discharge (DBD), (b) atmospheric pressure plasma jet (APPJ).

in more uniform treatment. Plasma jet systems are constructed with two concentric electrodes. The inner electrode connects to a high-frequency radio frequency power source that ionizes the working gas. As this ionized gas exits the nozzle, it creates a characteristic "jet-like" appearance. Additional technical details about these plasma generation systems can be found in other literature sources in detail [41–43].

Before discussing the effects of cold plasma processing on juice volatiles, it is important to briefly explain how cold plasma inactivates microorganisms, as this is a key factor in its application for food safety. The effectiveness of microbial inactivation through plasma treatment varies based on several key factors: The surface characteristics of the treated produce, the specific plasma device used, the composition of the gas employed, and the method of exposure [44, 45]. Plasma treatment has been shown to effectively deactivate a broad spectrum of microorganisms, including resilient bacterial spores and viruses [46–48]. The understanding of cold plasma's microbial inactivation mechanisms is critical for applying this novel technology to food products. Although several studies have successfully demonstrated these mechanisms in recent years, the findings remain somewhat elusive and inconsistent due to the complexity of both plasma and microbial systems [49]. Current findings demonstrate that cold plasma inactivation can be divided into biological and physical pathways. From a biological perspective, bacterial cells can be damaged through UV radiation, lipid peroxidation, protein modulation, and apoptosis induction. Plasma-generated UV radiation can induce dimerization of thymine bases in bacterial DNA, which blocks the replication ability of cells [50]. However, UV radiation plays a minor role compared to other plasma-derived components [51]. Membrane lipids are among the most vulnerable cell compounds to plasma components, especially reactive oxygen species (ROS). This vulnerability is due to their location near the cell membrane and their inherent sensitivity [37, 49]. These lipids are oxidized by reactive plasma species, which alters the cell's ability to transport materials in and out [52]. The plasma interaction with amino acids is less clear [49], but Dobrynin et al. proposed that plasma alters the three-dimensional structure of proteins

by cleaving peptide bonds and directly decimating their two-dimensional structure [53]. Recent years have seen discoveries regarding the effect of ROS on intracellular oxidative stress and programmed cell death (PCD) in bacteria, similar to processes in mammalian cells [54, 55]. Studies have proposed that intracellular ROS generated from nonthermal plasma induces bacterial apoptosis, which involves ordered cellular disassembly in living cells [56, 57]. However, there is insufficient research to fully prove this claim, indicating a need for more effort in this area.

Microorganisms are also decontaminated through physical mechanisms, such as electrostatic disruption and electroporation. Electrostatic disruption, one of the most studied physical mechanisms, is caused by the accumulation of charged particles on the cell surface [50]. These particles create an electrostatic force through charge accumulation on the outer cell membrane, resulting in membrane rupture [58]. When cells are subjected to pulsed electric fields, instability in the cell membrane is triggered, and the electric field causes perforations in the membranes of microorganisms [59]. It is proposed that electric pulses from cold plasma can lead to electroporation in the cell membrane [60], further compromising cellular integrity.

3. Volatile compounds in fruit juices

Volatile compounds are chemical substances that easily vaporize at ambient temperatures and play a critical role in defining the aroma and flavor of foods, oils, and beverages. These compounds are essential in sensory evaluation, as they contribute significantly to how products are perceived and categorized. In the realm of food and oil analysis, volatiles are frequently employed as indicators of quality and are integral to classification systems used in both research and industry practices [30].

Although thousands of volatiles have been identified in foods [61], only a small subset meaningfully affects human sensory perception because of their concentrations relative to odor thresholds. These volatiles fall into three major biochemical groups based on their precursor compounds. The first group consists of volatiles derived from essential fatty acids, such as linoleic and linolenic acid, producing compounds like hexanal, cis-3-hexenal, and trans-2-hexenal, which contribute to "green" and "grassy" notes in foods like tomato, apple, and tea [62–65]. The second group includes volatiles derived from essential amino acids, such as leucine, isoleucine, and phenylalanine, yielding compounds like 2-methylbutanal, 3-methylbutanol, and 2-phenylethanol that are key to the aroma of strawberries, apples, and breads [63]. The third group, apocarotenoids, is formed when carotenoids, such as β-carotene and lycopene, are broken down by oxidation. This process produces compounds such as β-ionone and 6-methyl-5-hepten-2-one, which help signal fruit ripeness and contribute antioxidant properties [65–68]. These volatiles serve not only as indicators of flavor but also of nutritional and health value. They help link sensory perception to essential nutrients. Thus, plant volatiles are both critical sensory cues and indicators of nutritional content, shaped by evolutionary pressures and plant-animal interactions.

To isolate, identify, and quantify volatile compounds, several sophisticated analytical tools are utilized. Headspace Solid-Phase Microextraction (HS-SPME) is a fast and solvent-free technique widely used for analyzing volatile content in complex matrices like food and oil samples. Gas Chromatography–Mass Spectrometry (GC-MS) remains the most widely adopted method due to its high sensitivity, specificity, and ability to detect trace compounds. Another option, Gas Chromatography-Flame

Ionization Detection (GC-FID), offers good selectivity and a broader detection range, although it is generally less sensitive than GC-MS [69, 70]. Simultaneous Distillation and Extraction (SDE) is another approach for isolating volatiles from complex food matrices and is often followed by GC-MS for identification [71]. In recent years, sensor arrays and electronic noses—including technologies based on functionalized carbon nanotubes and biologically modified field-effect transistors (bio-FETs)—have shown promise for real-time, low-cost detection of volatile organic compounds such as alcohols and aldehydes [72, 73].

4. Impact of cold plasma on volatiles of fruit juices

Volatile compounds are key contributors to juice flavor and are susceptible to alterations during processing. In a study by Ozen et al., atmospheric cold plasma (ACP) treatment using simulated air (SA; 80% N_2, 20% O_2) and combined gas (CG; 90% N_2, 10% O_2) with 90 s led to notable changes in the volatile compound profiles of cloudy apple juice (AJ) and cantaloupe juice (CJ), compared to untreated and thermally pasteurized counterparts [74]. In AJ, untreated samples consisted of 74% aldehydes and ketones, 13% alcohols, and 13% esters. SA-treated samples showed a decrease in aldehydes and ketones to 65%, while alcohols and esters increased to 20 and 15%, respectively. The effect was more striking with CG, where aldehydes and ketones dropped sharply to 11%, and alcohols and esters rose to 48 and 41%. In contrast, thermal pasteurization resulted in a volatile profile that was heavily dominated by aldehydes and ketones, accounting for 91% of the total. Among these, nonanal—a compound associated with an orange-rose aroma [75]—made up 79%, likely due to the thermal degradation of precursor compounds. Several flavor-active compounds in AJ were especially affected by ACP. Hexanal, associated with green apple aroma [64], decreased following plasma treatment, though the change was not statistically significant with SA. The metallic off-flavor compound 1-octen-3-one [76] was found in untreated, pasteurized, and SA-treated AJ, but was completely absent in CG-treated samples, highlighting CG's potential to eliminate undesirable notes. Another off-flavor, 3-octanone, was detected only in ACP-treated juices, but in negligible amounts. Desirable volatiles, such as 1-hexanol, which imparts an herbaceous aroma [77], increased significantly following SA treatment. Likewise, 1-butanol, 2-methyl-, acetate—a compound with fruity notes [78]—also increased after ACP, though not significantly. In CJ, untreated samples contained 33% aldehydes and ketones, 16% alcohols, and 23% esters. SA treatment increased aldehydes and ketones to 58% and alcohols to 33%, while reducing esters to 8%. CG treatment resulted in a more balanced composition: 41% aldehydes/ketones, 35% alcohols, and 19% esters. Notably, aldehydes, such as hexanal, heptanal, nonanal, and decanal—which were absent in untreated and minimally present in pasteurized samples—were detected in ACP-treated CJ. These compounds contribute to green apple (hexanal), pungent (heptanal), orange-rose (nonanal), and citrusy (decanal) aromas, with the highest levels observed in SA-treated samples. The alcohol fraction of CJ increased markedly following ACP. For example, 1-hexanol rose from 3.09 ± 0.07 μg/L in untreated CJ to 12.02 ± 2.53 μg/L (SA) and 17.15 ± 0.85 μg/L (CG). 1-heptanol (green aroma) and 1-nonanol (watermelon aroma) were detected exclusively in ACP-treated samples, further enriching the juice's flavor profile. Additionally, esters, such as methyl nonanoate and methyl decanoate—known for imparting fruity-sweet notes [79]—were primarily found in ACP-treated CJ and either absent or detected at much lower levels

in untreated and pasteurized samples. Ozen et al. summarized that ACP—especially when applied with CG—favorably altered the volatile profile of fruit juices by increasing flavor-enhancing compounds such as alcohols and esters while reducing or eliminating off-flavor compounds like 1-octen-3-one. In contrast, thermal pasteurization led to an accumulation of aldehydes (notably nonanal) [74].

Wang et al. evaluated the effect of a continuous cold plasma (CCP) reactor on the volatile composition of fresh apple juice (AJ) [80]. The reactor utilized a cold plasma jet generator (PG-1000Z) with argon (99.99%) and compressed air at a 25% air mixing ratio, operated under a gas flow rate of 470 L/h and high voltage of 65 kV. Juice was delivered at a feed flow rate of 12.7 mL/min, and treatment was conducted in a liquid film-forming chamber designed to maximize plasma-liquid contact. The CCP-treated juice was passed through the system three times to achieve microbial safety targets. GC-MS analysis identified 46 volatile compounds, including 15 esters, 8 alcohols, 11 aldehydes, 5 acids, and 4 ketones. CCP treatment resulted in a 14.8% reduction in total volatile content, significantly lower than the 35.1% reduction observed in pasteurized (PT) juices. Among compound classes, esters and alcohols were most affected, but CCP better preserved their concentrations compared to PT. Interestingly, CCP-treated juice exhibited 22.3% more aldehydes than fresh AJ, attributed to oxidation of alcohols and fatty acid cleavage *via* cold plasma-generated free radicals. During storage (30 days), total volatile content decreased in both treatment groups. At 4°C, CCP-treated samples lost ~33% of their initial volatile content, while PT samples lost ~52%. At 25°C, losses were greater, but CCP still outperformed PT in retaining volatiles. The relative odor activity value (rOVA) was used to assess aroma relevance. Six volatiles—isoamyl acetate, hexyl acetate, hexanal, 2-hexen-1-al, ethyl hexanoate, and octanal—had high rOVA values, identifying them as key aroma compounds. CCP processes juices retained significantly higher rOVA values of hexyl acetate, isoamyl acetate, and ethyl hexanoate, preserving fruity notes better than PT. Moreover, 2-hexen-1-al and nonanal levels were higher in CCP samples, contributing to green and floral notes, respectively. In contrast, octanal, associated with aldehydic off-flavors, was reduced in CCP samples. Principal component analysis (PCA) based on rOVA values showed that CCP-treated and AJ samples clustered closely, reflecting similar volatile profiles, whereas PT samples, particularly after 30 days at 25°C, showed significant deviation. This confirmed that CCP treatment better maintained the original aroma profile of AJ over time [80].

Maia et al. explored the impact of dielectric barrier discharge plasma (DBD) treatment on the volatile compounds and aroma profile of cashew apple juice (CAJ) [81]. The researchers applied plasma treatment with varying frequencies (50–1000 Hz) and processing times (5–20 minutes) while keeping the voltage constant at 18 kV. The primary objective was to modify the volatile profile of juice to improve its flavor by altering undesirable compounds-particularly styrene, which is responsible for the main off-flavor in the fruit and imparts an unpleasant aldehydic note for some consumers. Results showed that cold plasma significantly altered the volatile composition of the juice. Notably, there was a marked reduction in organic acids (73%) and an increase in aldehydes (52%), alcohols (67%), and short-chain esters (11%). Plasma treatment induced scission reactions in fatty acids and esters, which generated new compounds like short-chain esters and aldehydes. Further oxidation of aldehydes led to the formation of alcohols and isomerized short-chain esters, contributing to changes in aroma. However, despite these changes, DBD treatment did not significantly reduce styrene levels, which continued to influence the off-flavor profile. The plasma processing selectively targeted organic and free fatty acids, converting them

into alcohols and esters, with the formation of "green leaf volatiles," such as alcohols, aldehydes, and organic acids. The oxidation of aldehydes, particularly at longer treatment times, resulted in an increase in alcohols, such as octanol and nonanol, from the oxidation of aldehydes like octanal and nonanal. Additionally, the formation of branched esters was more pronounced in the DBD system compared to other plasma treatments, suggesting that DBD induces ester isomerization. The study concludes that while cold plasma can enhance certain volatile compounds to improve the aroma of CAJ, it does not fully address the off-flavor caused by styrene, which remains a challenge [81].

Chen et al. investigated the impact of different processing methods—namely pasteurization (PT), atmospheric cold plasma jet (CP), and a control (untreated)—on the physicochemical properties and volatile metabolite profiles of red dragon fruit juice (RDFJ) [82]. Using HS-SPME-GC-MS, 104 volatile organic compounds (VOCs) were identified across treatments, with a notable overall reduction in total VOC content. Pasteurization caused the greatest loss (43.14%), whereas CP treatment showed a more moderate decrease (27.73%). Cold plasma demonstrated a superior ability to preserve key flavor compounds compared to pasteurization. While esters, ketones, and acids were reduced across treatments, CP-treated samples retained higher levels of desirable esters (e.g., ethyl caprate, ethyl acetate) and ketones, such as acetoin—a creamy aroma compound—than their pasteurized counterparts. Additionally, CP treatment resulted in increased levels of palmitoleic and palmitic acids, which contribute to health-promoting properties and antioxidant activity. The study also highlighted the formation of furan compounds, known for their potential toxicity. Pasteurization led to the highest furan content, while CP treatment had the lowest, suggesting a significant advantage of CP in preserving juice safety and quality. Principal component analysis (PLS-DA) identified 17 key differential VOCs (VIP > 1) responsible for flavor differentiation among treatments. Notably, CP-treated juices exhibited the emergence of new aroma compounds, such as heptyl acetate and costol, suggesting that cold plasma not only preserves but may also enhance the aroma complexity of RDFJ. Overall, the findings support CP as a promising nonthermal method for retaining flavor and reducing the formation of undesirable compounds in fruit juice processing [82].

Maia et al. investigated the effects of glow discharge plasma (GDP) treatment on the volatile compound profile of cashew apple juice (CAJ) [83]. Their experimental setup utilized a plasma etch model PE-50 system operating at 0.4 bar pressure with synthetic air flow rates of 10–30 mL/min for durations of 10–20 minutes. The study revealed significant modifications in the volatile composition following plasma treatment. Most notably, plasma processing substantially reduced fatty acid and fatty acid ester concentrations (by 92%), particularly palmitic acid, palmitelaidic acid, myristic acid, methyl stearate, and stearic acid. Conversely, plasma treatment increased concentrations of aldehydes (50%), esters (21%), linear hydrocarbons (37%), and sesquiterpenes (44%). The researchers observed that fatty acids and their esters underwent internal scission reactions induced by plasma-generated free radicals. These reactions produced "green leaf volatiles"—primarily short-chain aldehydes, alcohols, and organic acids. For instance, the reduction in palmitic acid, methyl palmitate, and ethyl palmitate corresponded with increased hexadecanal formation through dehydration and methyl group abstraction processes. Additionally, the researchers detected N_2+, Hα, Hβ, and atomic oxygen as primary plasma reactive species through optical emission spectroscopy. Furthermore, the study demonstrated that increases in specific aldehydes (2-decenal, nonanal, octanal, hexanal) resulted

from the scission of various fatty acids and esters. Corresponding alcohols (hexanol, octanol, and nonanol) increased through hydrogenation of these newly formed aldehydes. The researchers also noted that plasma-induced scission of ethyl and methyl esters generated aldehydes and short-chain esters, with reactions typically occurring at carbon double bonds through oxygen-free radical mechanisms. Extended plasma treatment times (20 minutes) favored the production of shorter esters, likely due to methyl group abstraction processes. Ethyl esters demonstrated greater stability during plasma application compared to methyl esters and fatty acids. This study provides valuable insights into how cold plasma treatment chemically modifies volatile compounds in CAJ, with implications for aroma profile engineering and juice processing technologies [83].

Porto et al. investigated how atmospheric cold plasma (ACP) treatment affects the volatile compounds in pineapple juice [84]. Using a DBD plasma system (two 8 cm aluminum electrodes with acrylic dielectric barriers), they treated 20 mL samples at 20 kV for 10–20 minutes across different frequencies (50, 500, and 1000 Hz). The volatile profile of untreated pineapple juice consisted predominantly of esters (71.4%), thioesters (14.8%), and minor compounds including acids, aldehydes, furans, and hydrocarbons (13.8%). The primary compounds were methyl hexanoate (53.2%), methyl 3-(methylthio) propanoate (12.5%), ethyl hexanoate (10.0%), and methyl 2-methyl butanoate (7.8%). Cold plasma processing significantly altered the ester composition, particularly reducing higher carbon-number esters through methyl unit abstraction. This transformation pathway preferentially affected esters with more than six carbons, while shorter-chain esters remained relatively stable. Consequently, the relative abundance of ethyl acetate, methyl butanoate, and methyl 2-methyl butanoate increased in the treated juice. While the absolute abundance of thioesters remained relatively unchanged during plasma treatment, their mass fraction increased due to the reduction in ester content. The researchers observed that methyl radicals abstracted from esters reacted with methyl thioesters to form ethyl thioesters, particularly at higher plasma frequencies (1000 Hz). The researchers also measured reactive oxygen species formed during plasma treatment. Treatment at 500 Hz generated the highest overall reactive oxygen content, with elevated levels of hydrogen peroxide, superoxide anion, and hydroxyl radicals. This treatment condition also produced the most significant changes to the volatile profile of the juice. The 50 Hz treatment produced the highest hydroxyl radical content but low superoxide anion levels, while the 1000 Hz treatment yielded high hydrogen peroxide content but lower levels of hydroxyl radical and superoxide anion. The study demonstrates that cold plasma treatment can significantly modify the volatile compound profile of pineapple juice through selective chemical transformations, specifically through methyl group abstraction and grafting reactions that are influenced by plasma frequency and treatment duration [84].

A study by Rodrigues and Fernandes investigated how dielectric barrier discharge plasma (DBD) treatment affects the volatile composition and aroma of pasteurized orange juice [85]. The researchers employed a plasma generator with varying excitation frequencies (50, 200, 400, and 600 Hz), applying 20 kV across a 15-mm gap for 15 minutes. Their findings revealed that all plasma treatments significantly altered the volatile profile of juice compared to untreated samples. While limonene constitutes over 90% of orange juice volatiles, even small changes in its content significantly impacted aroma perception due to its conversion into compounds with lower odor thresholds. The research identified distinct chemical modification pathways based on frequency ranges. At lower frequencies (50–400 Hz), limonene and ethyl butanoate

slightly increased, while α-pinene, camphene, β-pinene, 3-carene, p-cymene, and oxidation products decreased. These reactions likely involved hydrogen radicals interacting with limonene to form terpinyl radicals, which underwent isomerization and dehydrogenation to produce various compounds. At a higher frequency (600 Hz), a different reaction pathway emerged, with increased limonene concentration and higher levels of alcohols and aldehydes. The chemical equilibrium shifted toward terpinyl radical formation and direct oxidation products, while compounds derived from pinenyl radicals decreased. The study also observed increases in sesquiterpenoids (germacrene D and eremophila-1(10),11-diene) at 600 Hz, though these compounds do not contribute significantly to orange juice aroma. Additionally, aldehydes and alcohols (octanal, 1-octanol, nonanal, decanal, dodecanal) increased during plasma application, particularly at 600 Hz, likely due to fatty acid oxidation through the lipoxygenase enzymatic route or direct oxidation by reactive oxygen species. These findings demonstrate that excitation frequency is a crucial processing variable in DBD processing, as it significantly influences the volatile composition and resulting aroma profile of orange juice.

The effect of cold plasma treatment on juice aroma is highly dependent on processing conditions, such as gas composition, plasma type, frequency, and treatment duration. Studies have shown that atmospheric cold plasma (ACP) treatments using different gas mixtures can selectively increase desirable volatiles like alcohols and esters while reducing off-flavor compounds. For example, using nitrogen-rich gases or adjusting plasma parameters (e.g., frequency or exposure time) significantly influences the breakdown and formation of aroma compounds. Continuous and dielectric barrier discharge plasma (DBD) systems, depending on their configurations, also vary in their ability to preserve or enhance fruity, floral, and herbaceous notes by modifying esters, aldehydes, and alcohols. In general, nonthermal plasma methods tend to better retain the complex aroma profiles of fresh juice compared to thermal pasteurization, with some treatments even generating new, desirable volatile compounds.

5. Challenges and future perspectives

Cold plasma treatment of fruit juices is a promising technology in that it is rapid, and the temperature rise is at an acceptable level [86]. This nonthermal technique offers several advantages over conventional thermal processing methods, particularly in the preservation of heat-sensitive nutrients, aroma compounds, and color. Cold plasma can inactivate a wide range of spoilage and pathogenic microorganisms without significantly altering the nutritional quality of the juice [18]. Furthermore, the technology is environmentally friendly and requires relatively low energy input. Among the available gases, the use of air in the system is much more cost-effective than other types of gases [87]. Air as a processing gas was used in most of the juice studies because it is a cheap and effective way to inactivate microorganisms while protecting the product quality. The versatility of cold plasma, in terms of customizable treatment parameters such as voltage, exposure time, and gas composition, allows it to be optimized for specific applications in juice processing.

Despite these promising advantages, cold plasma treatment also presents some limitations that need to be addressed. One of the primary concerns is the inconsistency of results across different studies. The effect of cold plasma on volatile compounds varies significantly depending on several factors, including the juice type,

the plasma generation device used, the treatment duration, the type and flow rate of gas, and the volume of juice treated. These variabilities make it difficult to generalize findings or develop standardized protocols. Even though cold plasma treatment demonstrates a notable inactivation efficacy, most studies have been conducted using small sample volumes. Larger-scale studies are required to accurately predict industrially relevant process parameters, such as processing time and gas flow rates for juice treatment.

Moreover, another challenge is the lack of clearly established regulatory frameworks. Approvals require extensive data on efficacy, safety, and the nature of any treatment-induced by-products, leading to significant delays in market adoption. Regulatory agencies also demand rigorous, product-specific validation to demonstrate that cold plasma is both safe and effective for each unique application. Additionally, concerns remain about the reactive species generated during plasma treatments and their potential long-term effects on consumer health, food quality, and the environment [88].

6. Conclusion

Cold plasma processing represents a notable advancement in fruit juice preservation, effectively addressing key limitations of conventional thermal methods, such as nutrient degradation, loss of sensory quality, high energy intensity, limited effectiveness against certain microbes, and the formation of undesirable chemical changes. This review has demonstrated that cold plasma technology significantly impacts volatile compound profiles in various fruit juices, with effects that are largely beneficial to sensory quality when processing parameters are optimized.

The evidence from multiple studies indicates that cold plasma treatments generally preserve key aroma-active compounds compared to thermal pasteurization. While both processing methods reduce total volatile content, cold plasma typically causes less severe losses (approximately 25–30% reduction versus 35–45% for thermal treatments). Moreover, cold plasma selectively affects compound classes in ways that often enhance the overall aroma profile. For instance, the technology tends to preserve fruity and floral notes by maintaining higher levels of key esters while sometimes generating new desirable compounds through controlled oxidation reactions. Plasma-generated reactive species induce various chemical transformations, including oxidation of alcohols to aldehydes, scission of fatty acids and esters to form "green leaf volatiles," and isomerization reactions that alter ester structures. The specificity of these reactions can be manipulated through careful control of processing parameters, particularly gas composition, plasma type, frequency, and treatment duration. Importantly, cold plasma processing not only preserves desirable compounds but, in some cases, reduces off-flavors. Several studies reported decreased levels of compounds associated with metallic or unpleasant notes, such as 1-octen-3-one in apple juice. Additionally, cold plasma produces lower levels of potentially harmful compounds, such as furans compared to thermal processing, further supporting its potential as a cleaner and safer alternative. By preserving bioactive compounds and enhancing sensory attributes, cold plasma processing supports the production of functional fruit juices that align with consumer preferences for health-promoting, minimally processed foods.

Despite these promising results, several challenges remain. The effectiveness of cold plasma is highly dependent on juice composition and treatment parameters, requiring careful optimization for each product. Some undesirable compounds, such

as styrene in cashew apple juice, have proven resistant to plasma-induced modifications. Furthermore, the long-term stability of plasma-altered volatile profiles during storage requires additional investigation.

Future research should focus on developing standardized approaches for optimizing plasma treatments based on specific juice types and desired sensory outcomes. In-depth sensory evaluation studies are also needed to correlate instrumental volatile analysis with consumer perception and acceptance. Additionally, exploration of combination technologies that leverage the strengths of cold plasma with other nonthermal methods could yield further improvements in juice quality and safety.

In conclusion, cold plasma technology offers a viable alternative to conventional thermal processing for fruit juices, with significant potential to enhance functional and sensory qualities. By preserving and improving volatile profiles, cold plasma processing can contribute to the development of premium juice products that meet growing consumer demand for minimally processed foods with fresh-like characteristics and enhanced health benefits.

Conflict of interest

The authors declare no conflict of interest.

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Chapter 5

Valorization of Grape Waste for Sustainable Mushroom Cultivation

Somali Ghosh, Nongmaithem Babita Devi, Arivazhagan Suganya and Chinnappan A. Kalpna

Abstract

The global wine industry generates substantial volumes of grape pomace (GP), a lignocellulosic by-product rich in fiber, phenolic compounds, and unsaturated fatty acids. Improper disposal of GP contributes to environmental pollution and underutilizes a resource abundant in bioactive molecules. This chapter explores the valorization of grape waste through its integration into sustainable mushroom cultivation, particularly with *Pleurotus* species (oyster mushrooms). Given their strong enzymatic capacity to degrade lignocellulose, oyster mushrooms can efficiently convert GP into high-value edible biomass. Incorporation of this waste material into mushroom substrates not only promotes mycelial growth and fruiting but also enriches the nutritional and functional profiles of the harvested mushrooms, enhancing levels of ergosterol, β-glucans, resveratrol, and antioxidant activity. Comparative analyses with traditional substrates (e.g., paddy straw, wheat straw, sawdust) demonstrate that GP offers competitive or superior yield performance when optimized with co-substrates. The health-promoting properties of mushrooms cultivated on GP, including antidiabetic, anticancer, and immunomodulatory effects, further highlight their functional food potential. This valorization approach exemplifies sustainable bioconversion, offering economic, environmental, and nutritional benefits through synergistic integration of winery and mushroom production sectors.

Keywords: antioxidant, food technologies, functional ingredients, health benefits, mushroom cultivation

1. Introduction

The exponential growth of agro-industrial activities, particularly winemaking, has resulted in the generation of significant volumes of organic waste, with grape pomace (GP) representing one of the largest by-products, globally in millions of tons annually. Improper disposal of this lignocellulosic residue not only poses serious environmental challenges, including soil and water pollution, but also represents a loss of valuable bioactive compounds, such as phenolics, flavonoids, and dietary fibers, which possess antioxidant, antimicrobial, and anti-inflammatory properties [1].

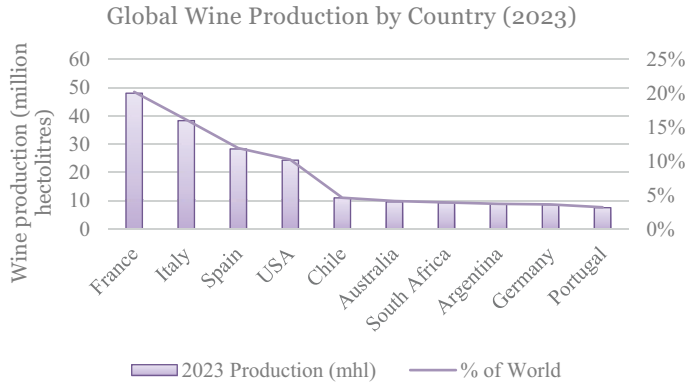


Figure 1.
Global wine production by country in 2023, measured in million hectolitres (mhl) [2].

France leads, followed by Italy and Spain, with the USA also contributing significantly [2].

According to State of the World Vine and Wine Sector in 2023 report, about 99 million hectolitres of wine were exported worldwide with an export value of ~ €36 billion and major export countries are European based but Asia-Pacific region also growing significantly (**Figure 1**) [3]. Per liter of wine produced, approximately 0.16–0.17 kg of GP is generated, corresponding to about 1 kg of pomace for every 6 liters of wine. For every 1,000 kg of grapes processed, around 130 kg of GP is typically obtained, representing approximately 13% of the grape input mass. Overall, pomace accounts for 20%–30% of the weight of grapes used in vinification, with variations depending on grape variety, vinification method, and pressing intensity [4]. In 2022, global grape production reached 77.3 million tonnes, and wine production was estimated at 258 million hectoliters, according to data from the International Organisation of Vine and Wine [2]. Applying standard yield coefficients, the total global production of GP is estimated to fall within the range of 8 to 13 million tonnes per year (fresh weight) [5].

In recent years, the valorization of grape waste has emerged as a promising strategy to support sustainable development within the circular bioeconomy framework. Among the innovative approaches explored, the bioconversion of GP through mushroom cultivation offers a dual advantage: effective waste management and the production of nutritionally enriched edible mushrooms. *Pleurotus* species, commonly known as oyster mushrooms, are particularly suitable for this purpose due to their strong lignocellulolytic enzyme systems, which enable them to utilize a wide range of agricultural and agro-industrial residues as growth substrates [6].

Research has demonstrated that incorporating GP into mushroom cultivation substrates not only supports satisfactory mycelial growth and fruiting but also enhances the nutritional and functional quality of the harvested mushrooms [7, 8]. Mushrooms grown on GP-based substrates have shown increased levels of bioactive compounds, including phenolic acids, resveratrol, triterpenic acids, and ergosterol, which are linked to higher antioxidant activity and improved health-promoting properties [8]. Additionally, the spent mushroom substrate, enriched through fungal bioconversion, can be repurposed as a high-value animal feed ingredient, further expanding its environmental and economic benefits [9].

Integrating GP into mushroom cultivation aligns with the principles of sustainable agriculture by reducing reliance on traditional substrates such as wheat

straw and minimizing the ecological footprint of the wine industry. It also contributes to the diversification of mushroom cultivation practices, promoting the production of functional foods and nutraceuticals that cater to contemporary consumer demands for healthier and more eco-friendly products [1, 8].

Given these multifaceted advantages, the valorization of grape waste for sustainable mushroom cultivation represents an innovative and practical solution to address both environmental waste management and food system sustainability. This approach not only exemplifies circular economy practices but also offers opportunities to develop value-added products with potential health benefits.

This chapter delves into the comprehensive aspects of integrating grape waste into mushroom production systems, exploring its chemical characteristics, influence on mushroom growth and nutritional composition, and evaluating its broader environmental and economic impacts as a sustainable resource for a greener agri-food sector.

2. Health benefits of isolated bioactive compounds from mushroom

Mushrooms contain greater in dietary fibre, vitamins, minerals, proteins and amino acids, PUFA, which function as a nutraceutical studied by Rahi and Malik (2016). The combined effects of mushrooms have many beneficial properties to health, including the maintenance of blood pressure, management of diabetes and cancer, antibacterial and antiviral potential and aids in wound healing and boosts immunity. According to the Global Mushroom Market (GMM) Report 2015–2021 predicted that the value of mushrooms will reach 60 billion USD by 2021 and acceptance also increase due to their medicinal and nutraceutical properties. The bioactive chemical compounds of mushrooms were identified as one of the food sources and also used as the pharmaceutical industry. GMM created a greater number of secondary metabolites over 20-year study, was conducted that the bioavailability of mushrooms has increased (**Figure 2**) [10].

Mushrooms cultivated using grape marc contains stilbenoid – resveratrol in a significant level. *P. ostreatus* mushrooms grown from grape marc has the resveratrol content increased up to 15 times higher. Mushrooms cultivated by grape marc substrate, including *P. eryngii* and *Pleurotus* species exhibit bioactive compounds like resveratrol, β-glucans, and phenolics from the growth media. The mushrooms from grape marc substrate were 60 times higher in resveratrol, triterpenes, total phenolic content (TPC), antioxidant activity, and ergosterol [12]

In the past 20 years, many studies have been carried out, in-depth investigation on the bioactive components of mushrooms, which demonstrated that mushrooms are known to make the variety of bioactive components that acts as a immunomodulatory, antibacterial, antidiabetic, antioxidant, and anticancer properties. These therapeutic properties get minimised by many factors including mushroom family, environmental condition when fruiting, age and developmental stage of mushroom, condition of storage, methods of cooking and the effectiveness of bioactive components. One or more hydroxyl groups joined to a benzene ring constitute the primary characteristic of the broad class of chemicals known as phenols. Flavonoids, quinones, hydroxybenzoic acids, phenolic acids (including curcuminoids), lignans, lignins, stilbenes, tocopherols, oxidized polyphenols, and salicylic acid are some of these. Mushroom contains plenty of phenolic compounds, which carries their presence in food and pharmaceutical industries.

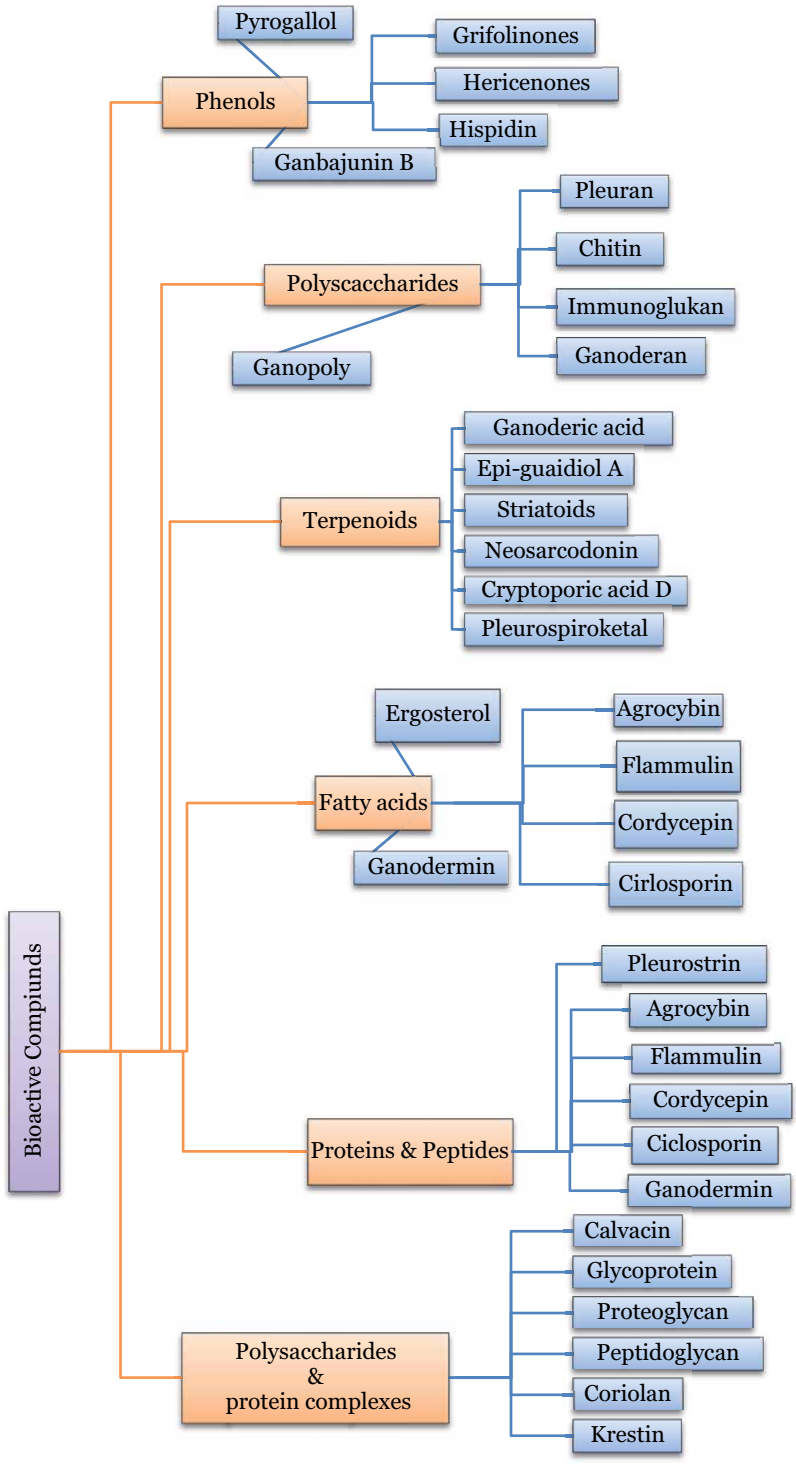


Figure 2.
Bioactive compounds of mushroom [11].

Natural and traditional medicine from plant source is reserved a great deal of interest in the development of new medications as well as maintenance of health from illness. The use of mushroom as a natural treatment for antibacterial, anti-inflammatory and anti-cancer has been raised [13]. Dietary intake of mushroom may aid in increasing innate immunity that act against cancer by boosting natural killer cell activity [14]. Most of the cancer patients prefer herbal medicines or natural products from mushrooms [15].

According to data published by the International Diabetes Federation in the eighth edition of Global Diabetes Map in 2017, the total number of patients aged 20–79 years afflicted with diabetes worldwide reached 415 million, with approximately 73 million of those patients coming from India. Diabetes mellitus is a dreadful metabolic disorder represented by hyperglycemia due to absolute or relative deficiency of insulin. It is the third most common non-infectious disease that is becoming more and more widespread and uncontrollable worldwide. Mushroom reflects the better food choice for diabetic people due to its dietary fibre, high protein, low fat and cholesterol level. Furthermore, antioxidants of mushroom such as Vitamin C, vitamin D and B_{12}, folate, ergothioneine may contain hypo-cholesterolemic and hypoglycemic effects [16].

GP enhances the nutritional value of mushrooms, particularly by increasing their polyunsaturated fatty acid (PUFA) content-especially linoleic acid. Linoleic acid is beneficial to human health as it serves as a precursor to arachidonic acid and eicosanoids (like prostaglandins and leukotrienes), which are essential for inflammation control, vascular function, immune response, and reproductive health [17]. Additionally, GP high antioxidant content, mainly from vitamin E and polyphenols, helps protect PUFAs from oxidative degradation, preserving their nutritional value. Polyphenols scavenge free radicals, while phytosterols in GP may contribute to cholesterol-lowering effects [18].

Mushrooms contain essential and non-essential amino acid contents, which are known to affect prostaglandin metabolism, are associated with their anti-inflammatory properties. The inclusion of amino acids including leucine, isoleucine, tyrosine, and phenylalanine in *P. ostreatus* (oyster mushroom) has been largely attributed to its anti-inflammatory qualities [19]. The mushroom, *Basidiomycota* species offers β-glucans, which are known as biological response modifiers because of their broad range of immune system action. Moreover, β-glucans have antioxidant activity which protects against DNA damage, and lower the amount of metabolites that have carcinogenic qualities. Additionally, it was shown that β-glucans affected the synthesis of cytokines that promote and inhibit inflammation [20]. Research revealed *Pleurotus* (oyster mushrooms) holding dietary fibre, vitamins, β-glucans and antioxidants which contribute anticancer, anti- inflammatory, hypoglycemic and antibacterial properties [8]. Study demonstrated that adding forest and agri-food waste GP to the growth substrate of *G. lucidum* a medicinal mushroom may enhance its antibacterial, antioxidant, and anti-inflammatory activities. As a result, *G. lucidum* can be produced effectively using this agricultural waste as an excellent supplement [21].

3. Grape pomace

Waste material from the wine industry, a semi-solid residue reported as “grape pomace” by the United States Department of Agriculture. From the European perspective, it is termed “grape marc”. Pomace, which includes skin, seeds, and

other solids, accounts for 20%–25% of grape weight. It also has a complicated substrate comprising 30% neutral polysaccharides, 20% pectic material, 15% insoluble proanthocyanidins, structural proteins, and phenolic chemicals.

This solid by-product is obtained following the pressing of whole grapes during wine production. It typically constitutes approximately 20%–25% of the total mass of processed grapes, although this proportion may vary depending on factors such as grape variety, vineyard terroir, ripeness at harvest, and the pressing technique employed[1]. It is estimated that approximately 1 kg of GP is produced for every 6 liters of wine. On average, one metric ton of GP comprises approximately 425 kg of grape skins, 225 kg of grape seeds, and 249 kg of grape stalks, along with minor constituents including residual moisture and soluble solids [22].

The diverse chemical constituents of wine GP collectively confer its notable nutritional and functional attributes, as detailed in **Table 1**. Dietary fiber is the predominant component, typically accounting for 50% to 75% of the dry weight, and is mainly composed of cellulose, hemicellulose, and lignin. This makes GP a valuable source of insoluble fiber for food and feed applications. The protein content ranges from 8% to 12%, and includes biologically important proteins such as globulins and albumins, which supply essential amino acids[22]. Fibre is well known to improve gut health, promote satiety, and reduce cholesterol. It helps normalize bowel movements, supports beneficial gut microbiota, and blunts postprandial glucose spikes. Epidemiological and intervention studies consistently show that higher fiber intake is linked to lower cardiovascular risk and mortality. A recent umbrella review found that high fiber intake significantly improves glycemic control, lowers total and LDL cholesterol, and modestly reduces blood pressure. Every additional 7 g/day of fiber has been estimated to reduce CVD risk by ~ 9%. Current guidelines (USDA/DGA) recommend ~ 25–30 g/day (14 g per 1,000 kcal) for adults. GP can be milled into fiber-rich flour or added to foods to boost fiber content. Industrially, GP fiber is used in baked goods, snacks and dietary supplements (fiber powders, bars). It can also serve as a prebiotic substrate for fermentative foods [23–25].

The lipid content, found mostly in grape seeds, constitutes approximately 7% to 12% of the dry matter. These lipids are predominantly unsaturated fatty acids, particularly linoleic and oleic acids, which have recognized health benefits. GP is also rich in phenolic compounds, ranging between 4% and 5% of the dry weight. These include various flavan-3-ols (such as catechins and proanthocyanidins), anthocyanins (in red pomace), and phenolic acids, which are responsible for much of the antioxidant activity associated with grape waste [17–26].

In GP, catechins occur at ~ 1.97 mg/g (197 mg/100 g)[27]. Clinical trials of pure catechin supplements have shown modest weight and metabolic benefits (e.g., slight weight loss or fat oxidation), though results are heterogeneous. Industrially, catechins are marketed as part of grape seed or green tea extracts for heart health and weight management. They are also used in skincare (antioxidant serums) and as natural preservatives in functional beverages. Notably, catechin-related applications overlap heavily with grape seed extract products [28–30].

About ~ 128.7 μg/g Quercetin has been found in GP, and the industrial uses include dietary supplements (often combined with vitamin C for immune support) and cosmetic formulations (as an anti-inflammatory, skin-protective antioxidant) [31]. The incorporation of quercetin and catechin from grape by-products into skincare creams has been proposed to provide antioxidative UV protection [32, 33].

Compound	Dry matter content
Physico-chemical parameters	
Ash	4.65 ± 0.05 g/100 g
Moisture content	3.33 ± 0.04 g/100 g
Fiber	46.17 ± 0.80 g/100 g
Lipids	8.16 ± 0.01 g/100 g
Proteins	8.49 ± 0.02 g/100 g
Carbohydrates	29.20 g/100 g
Fructose	8.91 ± 0.08 g/100 g
Glucose	7.95 ± 0.07 g/100 g
Energy value	224.00 kcal/100 g
Mineral substances	
Calcium	9.90 g/kg
Phosphrous	2.70 g/kg
Magnesium	0.80 g/kg
Potassium	13.90 g/kg
Sodium	0.22 g/kg
Sulfur	1.50 g/kg
Manganese	13.00 mg/kg
Zinc	25.00 mg/kg
Copper	49.00 mg/kg
Iron	361.00 mg/kg
Selenium	0.20 mg/kg
Cobalt	0.40 mg/kg
Bioactive compounds	
Vitamin E	5.00 mg/kg
Vitamin C	26.25 ± 0.01 mg AAE/g
Soluble fiber	9.76 ± 0.03 g/100 g
Insoluble fiber	36.40 ± 0.84 g/100 g
Total anthocyanin content	131.00 ± 0.40 mg/100 g
Total phenolic content	60.10 ± 0.10 mg GAE/g
Catehic tannins	13.10 ± 0.80 mg CE/g
Hydrolysable tannins	3.70 ± 0.10 mg TAE/g
Quercitin	128.70 ± 5.90 μg/g
Gallic acid	607.00 ± 9.00 μg/g
Catechin	1973.40 ± 17.60 μg/g
Procyanidin B2	1071.00 ± 17.70 μg/g

AAE: ascorbic acid equivalent; GAE: gallic acid equivalent; CE: catechin equivalent; TAE: tannic acid equivalent [22].

Table 1.
*Physico-chemical composition of GP (*Vitis vinifera l.*).*

Experimentally, grape seed proanthocyanidins have shown potential benefits for cardiovascular health (improving endothelial function and lowering blood pressure), anti-inflammatory and anti-microbial actions, and modulation of glucose metabolism, though human data are limited. One umbrella review noted improved CVD risk markers with fiber, but specific RCTs on isolated grape tannins are scarce. Industrially, condensed tannins from grape seeds are extracted as grape seed extract supplements (marketed for antioxidant and vascular support) and as natural antioxidants in foods and cosmetics. They are also used in traditional industries: leather tanning, adhesive manufacture, and wine fining, leveraging their protein-binding properties [34–36].

Anthocyanins are pigmented flavonoids (red-blue pigments) abundant in grape skins. They are potent antioxidants and are being studied for cardiometabolic and anti-inflammatory effects. In GP (red varieties), total anthocyanins can exceed 100 mg per 100 g dry weight (e.g., 131 mg/100 g). Anthocyanins also support eye health (e.g., slow macular degeneration) and may improve vascular endothelial function, though evidence is mixed. Industrially, anthocyanin-rich grape extracts are used as natural colorants and antioxidants in foods and beverages, and in cosmetics as UV-protective antioxidants. For example, one review notes that anthocyanins inhibit LDL oxidation and can be incorporated into functional foods and supplements for their health-promoting color and antioxidant properties [37–40].

One report measured about ~ 607 μg/g (0.607 mg/g) of gallic acid present in GP seed and skin. After ingestion, gallic acid is rapidly absorbed and metabolized, leading to limited bioavailability. Industrially, gallic acid is used as a food additive (antioxidant preservative, E314), in ink and dye manufacture, and as a building block for pharmaceuticals. Nutraceutical products and cosmetics sometimes include grape-derived gallates for their protective effects. A 2024 review notes its emerging use in functional foods and supplements aimed at improving [41–44].

Meta-analytical evidence highlights the potential health benefits of key bioactive compounds found in GP, notably Vitamin E, Vitamin C, and catechins. Although Vitamin E (primarily α-tocopherol) is present in GP at relatively low concentrations (~5 mg/kg dry weight), higher levels are found in grape seed oil (1–53 mg/100 g). Despite its established role as a lipid-soluble antioxidant, a comprehensive meta-analysis by Miller et al. (2005) involving 135,967 participants across 19 trials found that high-dose Vitamin E supplementation (≥400 IU/day) was associated with a statistically significant increase in all-cause mortality (risk difference = 0.03%, $p < 0.001$), indicating potential harm at pharmacologic doses, with no consistent benefit for cardiovascular or cancer outcomes [45].

For Vitamin C, while its presence in GP is minimal or undetectable, its physiological relevance as a water-soluble antioxidant remains well-supported. A meta-analysis by Hemilä and Chalker (2013) showed that regular vitamin C supplementation (≥200 mg/day) did not reduce the incidence of the common cold in the general population (RR = 0.97; 95% confidence interval [CI]: 0.94–1.00), though it reduced cold duration by 8% in adults. Other large-scale analyses have failed to demonstrate cardiovascular or cancer risk reduction with vitamin C supplementation [46–48].

Regarding catechins, particularly those derived from grape seeds and skins, robust meta-analytic data support their role in improving cardiometabolic health. Xu et al. (2020), in a meta-analysis of 31 randomized controlled trials (n = 3,321), reported that green tea catechin supplementation significantly reduced total cholesterol (weighted

mean difference [WMD]: – 4.66 mg/dL; 95% CI: – 8.65 to – 0.68) and LDL cholesterol (WMD: – 4.55 mg/dL; 95% CI: – 8.73 to – 0.37), with additional modest reductions in fasting glucose and HbA1c [49]. These findings reinforce the therapeutic potential of catechin-rich extracts – like those found in GP – as natural agents for lipid regulation and glycemic control [50, 51].

Additionally, GP contains residual soluble sugars, primarily glucose and fructose, which remain from the unfermented pulp and typically constitute about 4% to 17% of the dry mass. Ash or mineral content accounts for roughly 1.3% to 3.8%, and includes essential minerals such as potassium (K), calcium (Ca), magnesium (Mg), and trace elements. These components highlight the potential of GP as a sustainable source of dietary nutrients and bioactive compounds [22, 52–54].

4. Grape pomace composting

Studies show GP composts best with an initial C: N ~ 25–30. For example, one trial mixed GP to C/N ≈ 28 (dry basis); during 30–60 days of aerated composting, the C: N fell to < 20, a sign of maturity. Key interventions include particle size reduction and moisture control. Grinding the pomace accelerated microbial activity: smaller particles gave higher peak temperatures and faster stabilization, and yielded richer humic fractions. Moisture ~ 60% and turning or forced aeration ensure oxygen supply. Reported composts reached ≥ 45% organic matter and neutral pH. Nutrients (N, P, K) are largely retained: nitrogen losses are modest if C: N is balanced. In practice, adding N-rich amendments (manure or legume residues) to reach C: N ≈ 25–30 is recommended. Under optimized conditions (moisture 65%–75%, moderate C: N), GP composting can be complete in 1%–2% and C: N ≈ 15–20 [55, 56].

4.1 Anaerobic Digestion (AD) of GP

GP alone is lignocellulosic (C:N often > 25) and yields limited biogas unless co-digested. Recent studies find that co-digestion with nitrogen-rich wastes markedly boosts performance. For example, co-digesting GP (grape marc) with cheese whey (3:1 ratio) at thermophilic conditions (55°C) produced ~ 85% CH_4 in the biogas and ~ 24.4 L CH_4 per kg volatile solids. In contrast, GP mono-digestion gave only ~ 13.7 L biogas/kg VS (much lower methane). This improvement is attributed to a balanced C: N and extra easily degradable substrates from whey [57]. Methane production ramps up after approximately one- to two-week lag, and reaches steady output (~64 days). Carbon conversion of organics to methane was ~ 23.7% in the mixed feed. The digestate retains most P/K and substantial N, providing nutrient recycling (though caution: high phenolics in GP can inhibit microbes unless diluted) [58, 59].

Pre-treatment also enhance anaerobic digestion. Mechanical grinding or thermal hydrolysis improves digestibility: one report found steam/explosion at 180°C (severity 3.8) increased GP methane potential significantly. Similarly, hydrothermal (subcritical water) processing can solubilize cellulose/hemicellulose into sugars. (Optimal AD C: N is ~ 20–30; co-substrates like manure or whey typically adjust GP's ratio into this range.) Biodegradability thus improves with such pretreatments, raising cumulative CH_4 yield. Overall, optimized AD of GP yields on the order of 0.02–0.03 m^3 CH_4 per kg VS (8–15 MJ/kg VS), with higher

yields under co-digestion. Environmental performance is enhanced by converting GP to energy and stabilizing waste; however, process stability requires monitoring pH and potential inhibitors (acids, salts) [60–63].

4.2 Biofuel and bioenergy production

4.2.1 Fermentative biofuels

GP can be hydrolysed and fermented to ethanol or chemicals, but lignin hinders this. Green pre-treatments (thermal/alkaline) improve sugar yields. Subcritical water hydrolysis of freeze-dried GP at 150°C/150 bar released ~296 mg reducing sugars per g pomace – a high sugar recovery while minimizing degradation products. Such sugar-rich hydrolysates are suitable for yeast fermentation to ethanol. (Acid or alkaline hydrolysis is also used, sometimes followed by enzymatic saccharification.) Reported ethanol yields (after pretreatment) can approach theoretical maxima (~80%–90% of sugar conversion) under optimized conditions [64–66].

4.2.2 Thermochemical biofuels

GP can be processed via pyrolysis or gasification. Fast pyrolysis yields a bio-oil, char, and syngas: for example, in one pilot study, pyrolyzing exhausted grape marc at 450°C –550°C gave ~ 37%–41% char, ~ 19%–24% bio-oil, and ~ 36%–42% permanent gases (by weight). Higher temperature (550°C) shifts products: char yield fell (~38%), gas rose (~42%). The biochar has high fixed carbon (68%–73%), low volatile matter (17%–23%), and a heating value of ~ 21–22 MJ/kg, making it a solid biofuel or soil amendment. Bio-oil from GP (rich in phenolics) can be upgraded to fuels or chemicals. Gasification (esp. at > 800°C) produces H_2-rich syngas; modeling predicts maximal hydrogen yield above 800°C (though practical trials are limited) [67–70].

4.2.3 Solid biofuels

GP and its residues can be pelletized or briquetted. Pellets blend GP with woody material to lower ash; for example, wine byproducts pellets (with mixed feedstocks) showed acceptable hardness and calorific value (~16 MJ/kg). Raw GP pellets have high ash (~4%–6%) and sulfur, which must be managed (via blending or cleaning). Thermochemical routes from GP yield energy products (gas, oil, char) with net energy content depending on process (e.g., combined pyrolysis products at optimum ~ 550°C maximize energy). Lignin and moisture are challenges; pretreatments (drying, torrefaction, steam explosion) can improve yield and product quality [67–71].

4.3 Soil amendment uses

GP can amend soils either fresh/composted or as biochar. Field trials over three years and more, reported that GP composted or vermi-composted into amendments had mixed effects. In one vineyard study, annual application of GP vermicompost (C:N ≈ 15.8) reduced soil organic C and N stocks, likely by a priming effect: the low C:N and labile carbon stimulated microbes that mineralized native SOM. By contrast, GP compost (C:N ≈ 23.9) did not significantly raise soil C or N;

it neither depleted nor increased SOM over three years. Thus, using highly processed (vermicompost) GP can accelerate nutrient turnover (potentially leading to N leaching), whereas higher-C compost is more inert. Reported C:N: vermicompost ~ 16, compost ~ 24. Both forms add K and micronutrients, but effects on soil C storage are modest [55, 72, 73].

Grape marc pyrolysis char (e.g., at 450°C) greatly increases fixed carbon (68–73%) and pH (~10.5). In pot trials, GP biochar at 450°C (EGM_{450}) boosted plant growth by ~ 16% (ryegrass dry mass 1.87 g vs. 1.61 g in control), likely due to liming and nutrient effects. Higher-temperature char (550°C) was less beneficial (1.14 g yield) [74–76].

GP derived biochars improve soil carbon stability and can enhance crop growth if produced at moderate temperature. Compost/vermicompost do add organic matter and nutrients, but managers should note the "priming" risk from low C: N vermicomposts. Properly matured GP compost (C: N ≈ 20–30) is the safest amendment for sustained soil health [24, 77–83].

5. Antioxidant properties

5.1 *In vitro* antioxidant activity

GP extracts exhibit strong antioxidant activity in standard chemical assays. Common tests (DPPH, $ABTS^{+}$, FRAP, ORAC, etc.) all show high radical-scavenging and reducing capacity. For example, one study of a Syrah pomace extract reported ORAC ≈ 1913 μmol Trolox equivalents (TE)/g, ABTS ≈ 2615 μmol TE/g, FRAP ≈ 1.52 mmol Fe^{2+}/g, and DPPH ≈ 1392 μmol TE/g. Other red pomace extracts have ORAC values in the 1000–2000 μmol TE/g range and ABTS values > 1000 μmol TE/g. In general, antioxidant capacity parallels phenolic content: red pomace > white pomace, seeds rich in procyanidins are especially potent, and micronization or solvent extraction methods can further enhance measured activity [81, 82].

5.2 *In vivo* antioxidant effects

A few animal studies suggest biological antioxidant effects of GP. In cell and rat models of oxidative stress, grape seed/pomace extracts reduced reactive oxygen species (ROS) and lipid peroxidation. For instance, pre-treatment of human skin cells with grape seed extract markedly lowered UV-induced ROS. In mice, GP polysaccharides significantly protected the liver from CCl_4-induced oxidative injury, as evidenced by reduced malondialdehyde (MDA, a lipid-peroxidation marker) and inflammatory cytokines (TNF-α, IL-6) and by restoration of antioxidant enzymes (SOD, CAT, GSH). Similarly, in hypertensive rat models, chronic pomace extract intake showed modest attenuation of blood-pressure elevation, presumably via antioxidant mechanisms. However, well-controlled human clinical data remain scarce [83].

5.3 Functional food applications

The high antioxidant content of pomace makes it attractive as a functional food ingredient. Enrichment of foods with GP (powders or extracts) consistently boosts

phenolic levels and antioxidant activity. For example, adding 5%–10% GP to white bread increased total polyphenols and radical-scavenging capacity (measured by FRAP and ABTS) while also raising dietary fiber content. Yoghurt and salad dressings formulated with GP fibers showed higher radical-scavenging activity and lower peroxide values during storage. In meat products, incorporating grape-seed extracts or pomace powder markedly retarded lipid oxidation: chicken and sausage products with pomace had significantly lower TBARS (malondialdehyde) levels than controls, and warmed-over flavor in pork was suppressed by grape-seed polyphenols. Overall, these applications harness GP's antioxidants to extend shelf life and add healthful phenolics [24].

As illustrated in **Table 2**, these values demonstrate the very high radical-scavenging power of red GP extracts, with variations dependent on variety, extraction, and assay method.

6. Nutritional potential of oyster mushroom

Despite its richness in phenolic compounds, bioactive compounds, and minerals, the utilization of GP from grape juice and wine-making industries in ruminant fodder is limited due to the indigestible nature of grape skin [9]. In response to these challenges, current techniques of mushroom cultivation are given importance to reutilize organic waste products. Since some mushroom species, especially white-rot fungi (oyster mushroom), can produce lignolytic enzymes in plant biomass (like GP, straw, and sawdust), they can convert lignocellulose materials into highly nutritious food rich in protein, vitamins, dietary fibre, and minerals [84]. Usage of organic waste as a substrate in mushroom cultivation is one of the innovative strategies to reduce environmental pollution as well as production of healthy and nutritious products [9, 85]. Zuffi et al., (2025) reported that the yield and quality of mushrooms grown on GP substrate were high, especially when incorporated with anaerobic digestate [85].

6.1 Enzymatic activity

Researchers also demonstrated that GP could be sustainable and an alternative to the conventional way of cultivation. Studies reported that Trametes versicolor (white-rot-fungi) is well known for the production of lignolytic enzyme called Laccase (multi-copper oxidase enzyme found in fungi which catalyses the oxidation of phenolic and non-phenolic compounds), which is useful for the enzymatic

Assay	Result (Syrah GP extract)	Units
ORAC*	1,912.6 μmol Trolox equivalents/g [82]	μmol TE/g DW
ABTS**	2,614.5 μmol Trolox equivalents/g [82]	μmol TE/g DW
FRAP*	1.52 mmol Fe^{2+}/g [82]	mmol Fe^{2+}/g DW
DPPH (EC_{50})*	~1.32 g sample/g DPPH (EC_{50}) [81]	g DW/g DPPH

**The extracts were prepared using 70% ethanol.*

Table 2.
Antioxidant capacities of syrah GP.

polymerization and oligomerization of polyphenols and formation of phenoxy radicals and quinones by catalysation of phenolic compounds. Other than laccase, *T. versicolor* also induces other enzymes (oxidases, peroxidases, reductases, hydrolases, and pectinases) which are involved in the metabolism of lignocellulosic compounds. Laccase enables the lignin degradation and produces cellulose and hemicellulose, whereas xylanase contributes degradation of hemicellulose, which improves the yield of fermentable sugars that can be used as source of carbon and energy by the microorganisms. The most importance of cellulose degradation, β-glucosidase converts oligosaccharides into glucose by while hydrolysis of sucrose into glucose and fructose by the catalysis of invertase [86]. The yielding and biological efficacy of mushrooms is influenced by the fermentation media used pre or during treatment. GP was utilized as Solid-state (SSF), semi-liquid (SLF), and submerged (SmF) fermentations substrate for the cultivation of *P. ostreatus* and *P. pulmonarius*. Additionally, the production and activity of Laccase enzyme is also influenced by the type of fungal strains and fermentation media. Study found that highest Laccase enzyme activity was found in SSF and lowest Laccase enzyme activity was found in SLF for both the mushroom species. Researchers also highlighted that both *P. ostreatus* and *P. pulmonarius* could degrade till 79% of the total phenolic compound (TPC) from the GP as substrate regardless mode of the culture [87] Therefore, lignolytic and hydrolytic enzyme plays an important role on the SSF process in fermentation and degradation of lignocellulosic and phenolic compounds present in GP which would influence the nutritional and bioactive compounds of the fruiting bodies of mushrooms.

Traditionally, mushrooms have been used as culinary medicine since immemorial time because of their unique umami taste and flavour [88]. Consumption and preferences of mushrooms are not only for the taste, but mushrooms also possess high protein, dietary fibre, vitamins and minerals, low calorie, low fat and cholesterol, presence of glucans, lectin, chitinous substance, high molecular weight polysaccharides and secondary metabolites (alkaloids, terpenoids, phenols, antibiotics) [85–89]. Mushrooms are well recognized as a rich source of bioactive compounds, which have the potential role of antibiotics, antioxidants, and anti-inflammatory properties. They are also the only vitamin D-rich vegan source; ergosterol (a precursor of vitamin D2) present in mushrooms will convert to vitamin D2 in the presence of ultraviolet light [89]. Mushrooms also possess nutraceutical compounds (terpenes, bioactive proteins and antioxidants) that can fight against different degenerative diseases [90]. The squalene and phenolic compounds present in the substrate are closely related to the ergosterol level and antioxidant activity in the fruiting body of mushrooms. Koutrotsios et al. (2018) demonstrated that mushrooms (*Pleurotus ostreatus*, *P. eryngii*, and *P. nebrodensis*), which were cultivated in GP and olive mill byproducts (OMP), exhibited a significant increase of phenolic compounds, resveratrol, ergosterol, and triterpenic compounds in fruiting bodies of the mushroom [8]. A 27% increase in β-glucans of the mushroom was observed when compared to the control (WS), and a significant increase of 2 to 8 fold of antioxidant activity was also noticed in mushrooms, mainly in *P. eryngii*. A study exhibited that the IR region of glucan was found to be broader in mushrooms grown on GP substrate when compared to corn digestate (CD) and wheat straw (WS); the band of Lignin was also obtained in both GP and CD. Researchers also demonstrated that there was a significant increase in the yield of mushrooms grown in GP (100%) substrate alone as well as a mixture with either GP and CD (50:50) or

GP and WS (50:50). Since the high-level chemical compounds such as lingo-cellulose and phenols mainly in GP which urged the mycelium colonization of the substrates. Study evidenced that GP & CD (50:50) was found to be more favorable substrate than GP; WS for the cultivation of Pleurotus species because of equal combination of pH and required nutrients like carbohydrates, proteins, lipids and polyphenols [85].

Antioxidant properties of mushrooms are influenced by the content of phenolic compounds, flavonoids, and alkaloids [91]. Total phenolic compounds (TPC) were found to be high in mushroom extracts which was grown on 80% GP & 20% WS (27.09 ± 0.56 mg/g) rather than 100% GP (19.83 ± 0.32 mg/g) on 7th and more higher on 14th days (37.39 ± 2.42 mg/g and 25.11 ± 0.28 mg/g respectively) of growth with an extensive shelf-life period. Mushroom growth on 100% GP had the highest scavenging activity against DPPH and ABTS assays on both 7th (2.60 ± 0.14 mg/mL and < 0.65 mg/mL, respectively) and 14th days (8.08 ± 0.13 mg/mL and 3.91 ± 0.11 mg/mL, respectively) of growth. The TPC and antioxidant content in the mushroom may be related to the polyphenols obtained from the GP substrate [15].

7. Other agricultural waste on mushroom production

Unlike plants, mushrooms grow naturally in organic matter (as substrate) and damp places like decaying wood, straw, and sawdust, which are rich in nutrients. Commercially, mushrooms are cultivated indoors on different substrates like wheat straw, sawdust, and other agricultural waste in a well-maintained environment [92]. Cultivation of mushrooms on agro-waste or industrial waste is one strategy to control pollution and environmental hazards [93]. Agro-wastes are a rich source of lignocellulosic components, which are low-cost, renewable, and have abundant natural resources, difficult to decompose; however, they can be useful in mushroom production [94, 95].

7.1 Organic fertiliser in mushroom production

Rice straw, wheat straw, paddy straw, coffee straw, molasses, cotton straw, banana leaves, sawdust, tea leaves, and rice bran are most commonly used substrates for the cultivation of mushrooms. Wheat straw, rice straw, and cotton straw are commonly used substrates to cultivate oyster mushroom (*Pleurotus*), while wheat straw is mainly used to cultivate button mushroom (*Agaricus*). For the cultivation of shitake mushroom (*Lentinula edodes*), coffee pulp and rice bran, the substrates that are most commonly used. Banana leaves and tea leaves are mainly used as substrate for the cultivation of paddy straw mushroom (*Volvariella*) and oyster mushroom (*Pleurotus spp*.). Sawdust is a non-nutritious substrate that needs to be enriched with nitrogen sources (bran, urea, sunflower seed & horse manure). By using sawdust, Reishi (*Ganoderma*) can be cultivated [94]. Some common edible and medicinal mushrooms and their suitable substrates are illustrated in **Table 3**. Tavarwisa et al., (2021) reported that baobab fruit shells and corn cob could be utilized as substrate for the cultivation of oyster mushroom which had an average yielding of 482 and 518 g respectively per 1 kg of dry substrate but low yield was observed while compared to saw dust and wheat straw which had an average yielding of 682 and 594 g per 1 kg of dry substrate [95].

S.No.	Type of mushroom	Substrate
1.	Oyster mushroom (*Pleurotus spp.*)	Rice straw, wheat straw, tea leaves, banana leaves, sugarcane bagasse, saw dust, water hyacinth,
2.	Button mushroom (*Agaricus bisporus*)	Wheat straw
3.	Shitake mushroom (*Lentinula edodes*)	Rice bran, coffee pulp
4.	Paddy straw mushroom (*Volvariella volvacea*)	Tea leaves
5.	Reishi (*Ganoderma lingzhi*)	Saw dust

Source: Ref. [94].

Table 3.
Some common edible and medicinal mushrooms and their suitable substrates.

A combination of different substrates of agro-waste or industry waste has been used to promote mushroom production and reduce wastage of nutrient-rich organic waste, as well as to control environmental pollution. Kumar et al. (2022) demonstrated that growing shitake mushrooms on substrate containing (80% rice straw + 20% sugar cane bagasse), sterilized with secondarily treated dairy waste and sugar mill wastewaters, enhanced the nutrient contents of the developed substrates, which increases the production of mushrooms when harvested [96]. The researchers also added that this concept could be implemented for the production of high-quality mushrooms. A study done by Ellatar et al. (2019) reported that the yield of oyster mushroom was observed to be highest in the substrate mixing of rice straw & wheat straw, followed by rice straw alone (7,600 and 6,650 g, respectively) [97]. *Clitocybe maxima*, *Ganoderma lucidum*, and *Lentinula edodes* were found to be highest yielding and mineral content on substrate, which was based on a mixture of alder and beech sawdust, whereas *Agrocybe cylindracea* and *Flammulina velutipes* were found to be higher yielding on the substrate, which was based on a mixture of oak sawdust and flax shives. However, *P. eryngii* was found to yield the same yield for both substrates. The yielding and nutritional level of the mushroom might be correlated with the chemical composition of the substrates [98].

8. Conclusion

The integration of GP into mushroom cultivation systems offers a promising avenue for establishing practical synergies between the wine industry and the edible fungi sector. By redirecting GP – traditionally considered a waste product – toward mushroom farms, it becomes possible to generate high-value functional foods while simultaneously mitigating environmental burden. For mushroom producers, GP presents a viable alternative to conventional substrates such as straw, particularly when it is used alone or in combination with other agricultural residues and appropriately optimized, for instance by adjusting the carbon-to-nitrogen ratio or applying pre-treatment methods to enhance mycelial development and yield. Furthermore, the spent substrate derived from GP-based mushroom cultivation is

enriched in protein and lower in lignin, making it suitable for secondary applications such as animal feed or organic fertilizer. Strategically, vineyards and wineries are encouraged to collaborate with nearby mushroom growers to channel GP into substrate production. Testing of co-substrate formulations, such as GP mixed with straw or digestate, is recommended to identify optimal combinations for maximizing both quantity and quality of mushroom yields. Such circular approaches not only reduce disposal costs but also generate additional revenue streams for agricultural producers. Despite encouraging preliminary data, current evidence is primarily limited to laboratory and small-scale pilot studies. Therefore, comprehensive field trials are essential to assess economic viability, logistical feasibility, and large-scale applicability. In parallel, post-harvest aspects, including the shelf life, sensory attributes and storage conditions of mushrooms cultivated on GP substrates, warrant further research due to the perishable nature of fresh mushrooms. Although oyster mushrooms grown on GP have shown considerable antioxidant activity and potential health-promoting effects *in vitro* and in animal studies, robust human clinical trials are still lacking. To support broader implementation, it is imperative to develop regulatory frameworks and incentive mechanisms, such as funding for waste-to-mushroom conversion initiatives and technical guidelines for the reuse of agro-industrial by-products. These measures would facilitate the alignment of mushroom and wine production with circular economy and sustainability objectives. In contrast to traditional GP disposal methods, which may lead to soil contamination due to phytotoxic phenolic compounds, its bioconversion via fungal cultivation offers an environmentally sound and economically advantageous solution.

Conflict of Interest

There is no conflict of interest.

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Chapter 6

The Utilization of Cassava Leaves as a Functional Food Based on Local Wisdom

Syamsul Rahman

Abstract

The global functional food market was valued at approximately USD 280.7 billion in 2021, with a projected growth rate of 8.5% from 2022 to 2030. Functional foods are attractive to a diverse global population due to their potential to improve and maintain human health. Plant-based diets contain numerous secondary metabolites that may influence health and disease prevention, with a primary focus on the potential bioactivity and nutritional relevance of several classes of phytochemicals, such as polyphenols, flavonoids, saponins, tannins, carotenoids, phytoestrogens, and fructooligosaccharides, many of which are found in cassava leaves (*Manihot esculenta* Crantz). The purpose of this chapter is to provide an in-depth overview of the potential use of cassava leaves as functional foods, with an emphasis on traditional knowledge and practices. Evidence from various regions in Indonesia indicates that cassava leaves have long been utilized, both as food and as conventional medicine. Its widespread use is due to its rich composition of nutrients, minerals, vitamins, and phytochemicals, including carbohydrates, essential amino acids, dietary fiber, β-carotene, phenolics, anthocyanins, flavonoids, tannins, and saponins, which are widely associated with its various beneficial properties for health such as antidiabetic, anti-inflammatory, antibacterial, and anti-obesity effects.

Keywords: cassava leaves, functional food, local wisdom, supporting public, health in Indonesia

1. Introduction

Cassava (*Manihot esculenta* Crantz.) is an agricultural commodity that has been cultivated for generations and is widely known in Indonesian society. In addition, this commodity has several benefits and uses, including food ingredients, industrial raw materials, and animal feed [1]. In addition to fulfilling basic human nutritional needs, food ingredients also play an essential role in improving the quality of life and health. This is evidenced by the increasing interest of global consumers in recent years towards functional foods containing bioactive compounds that can provide health benefits beyond their basic nutritional content [2]. Bioactive compounds are a diverse group of molecules with potential health benefits that exhibit various properties such

as antioxidant, anti-inflammatory, antimicrobial, antiatherogenic, antithrombotic, cardioprotective, and vasodilatory effects [3].

Functional foods are appealing to a wide range of global populations because they can improve and maintain human health and well-being [4]. Increasing health awareness and the prevalence of lifestyle-related diseases have led consumers to choose foods that provide additional health benefits, such as probiotic-enriched vitamins [3].

In addition to meeting nutritional needs, functional foods provide health benefits, especially in the prevention and treatment of diseases, which have been widely recognized by various international health organizations, including the World Health Organization (WHO). WHO emphasizes that a healthy and balanced diet, which includes the consumption of foods rich in fiber and antioxidants, is a key strategy to prevent noncommunicable diseases [5, 6]. Food, as one of the sources of vital needs in human life, is needed because it is a source of energy and also a builder of body cells [7]. According to reference [8] a plant-based diet contains many secondary metabolites that can affect health and disease prevention, which are primarily focused on the potential bioactivity and nutritional relevance of several classes of phytochemicals, such as polyphenols, flavonoids, carotenoids, phyto-estrogens, and frucro-oligosaccharides, and of these compounds are found in many fruits, spices, and vegetables. One of them is cassava leaves (*Manihot esculenta* Crantz).

Likewise, reference [9] reported that the health benefits of cassava leaves are due to their nutritional, mineral, and phytochemical content, such as carbohydrates, essential amino acids, dietary fiber, β-carotene, vitamins, phenolics, anthocyanins, and flavonoids. In addition, reference [10] explained that edible cassava leaves contain a large number of phenolic compounds, including flavonoids and tannins, which exhibit antidiabetic and anti-obesity properties. These compounds exhibit high inhibitory activity against enzymes such as α-amylase and lipase, making them beneficial for metabolic health.

2. Nutritional composition, bioactive constituents, and functional properties of cassava leaves

Cassava is a shrub, growing up to 5 m in height, and is part of the Euphorbiaceae family, considered one of the most complex and diverse families of Angiosperms, with about 300 genera and 8000 species [11]. Stem cuttings generally propagate Cassava. The leaves consist of palmate, heart-shaped leaflets with 3–9 lobes. The color of the leaves can change with age, such as purple, dark green, or light green in mature leaves, while young leaves have purple buds, which eventually turn green. The leaves also consist of petioles that vary from green to purple [12].

Besides tubers, other parts that are beneficial for human health are the leaves, because they contain crude protein ranging from 29.3 to 32% based on dry weight (DW), fat, carbohydrates, vitamin A, vitamin B, and vitamin C [13], and the crude protein content can reach 38.1% DW [11], as well as minerals (Ca, Mg, P, Zn, Mn) in high amounts [12, 14]. Cassava leaves are also an important source of essential amino acids, including valine and leucine [15], as well as methionine and phenylalanine [10]. **Table 1** presents data reported by the Ministry of Health of the Republic of Indonesia and the FAO Database [16] for comparison with the above values.

Cassava leaves, especially the buds, contain vitamins A and C, making them a very effective antioxidant against free radicals. The inhibitory activity was 88.09% and the IC50 was 45.16 ppm [13]. Due to its protein, vitamin, and mineral content,

Component	Content per 100 g (fresh leaves)
Energy	64 kcal
Protein	6.8 g
Fiber	1.9 g
Carbohydrate	7.1 g
Vitamin A (β-carotene)	1310 mcg
Vitamin C	275 mg
Calcium	165 mg
Iron	1.4 mg

Table 1.
Presenting data reported by the Ministry of Health of the Republic of Indonesia and FAO Database [16].

cassava leaves are a valuable source of additional nutrition for humans [17]. They can contribute to increasing mineral intake from food, especially essential elements such as calcium and iron [18].

Vitamin A deficiency is a public health problem, that can cause impaired vision and immunity, growth and development, epithelial cell integrity, and reproductive function [15]. According to traditional medicine in Nigeria, cassava leaves are used to reduce fever, headache, rheumatism, hemorrhoids, ringworm infections, conjunctivitis, wounds, and abscesses [9, 19], as well as inflammation and gastritis. Cassava leaves have been used for centuries for their antioxidant properties [19]. Furthermore, [19] explains that phenolic compounds, found in cassava leaves and tubers, contribute to the antidiabetic effect by inhibiting starch digestive enzymes such asα-amylase and α-glucosidase, and according to [9], the presence of flavonoids such as rutin and kaempferol in cassava leaves can increase antioxidant properties and support collagen synthesis.

Cassava has significant medicinal value, as its stems and leaves contain various phytochemical compounds (**Table 2**) that possess antioxidant, anthelmintic, and antidiabetic properties [12]. In particular, the compound isoginkgetin, which is the main component identified in cassava leaf extract, is associated with various biological activities such as antitumor and anti-inflammatory effects [17]. Furthermore, the potential of Cassava leaves as a cosmetic ingredient is related to its chemical composition, which contains rutin, apigenin, and kaempferol, and several high levels of beneficial bioactive components, such as antimelanogenesis, increased collagen synthesis, antiadipogenesis, antioxidation, and ultraviolet filtering capabilities [9].

Natural phenolic compounds are secondary metabolites [5, 20, 21], which are found in various plants that function as plant protectors from ultraviolet radiation, predators, pathogens, and so on [5]. Phenolic compounds are widely distributed in plants, mainly synthesized by lactic acid, pentose phosphate, and phenylpropanoid pathways [21]. Phenolic compounds (C6H5OH) have one or more hydroxyl groups directly bound to an aromatic ring [5]. According to reference [22], the importance of phenolic compounds is related to their function as defense or prevention of various diseases, such as antioxidant, anti-inflammatory, antidiabetic, antiproliferative, antimicrobial, anticancer, antihypertensive, anticardiovascular, and antiaging activities.

Flavonoids are secondary metabolites consisting mainly of benzopyrones with phenolic or polyphenolic groups at various positions [19], and have attracted

Functional components	Impact on human health
Phenolic	It has anticancer and anti-inflammatory properties, protecting against various diseases such as diabetes mellitus, arthritis, neurodegenerative disorders, and headaches.
Flavonoid	Flavonoid-derived compounds can reduce oxidative stress and inflammation, act as anti-inflammatories, help control cell growth, are anticancer agents, have antidiabetic effects, and show antiviral effects and antibacterial properties.
Carotenoids	Reduces the incidence of cataracts in the eyes and cardiovascular diseases, improves immune response, reduces the likelihood of diseases such as cancer, muscle, and degenerative diseases, contributes to the maintenance of heart cells, kidneys, and other organs, as well as reduces the risk of type 2 diabetes and metabolic syndrome.
Vitamin C	Inhibits the growth of cancer cells, reduces the oxidation of low and high-density lipoproteins, reduces oxidative stress, acts as an antihypertensive, improves the immune system, and is used to treat diseases such as scabies and the common cold, and is involved in collagen synthesis.
Dietary fiber	Improves the digestive process, prevents cancer, and has antidiabetic properties.

Table 2.
Several bioactive compounds in cassava leaves have properties functional and impact on human health [18].

considerable attention due to their potential as functional ingredients and natural food additives. Flavonoids are widely distributed in plant parts, including leaves, flowers, roots, stems, barks, and seeds [17, 18]. Approximately 10,000 flavonoids have been reported in the literature, placing flavonoids among the most abundant bioactive compounds in plants [15]. Flavonoids are common polyphenolic compounds that exhibit diverse biological and pharmacological potentials, such as anticancer, antiviral, antioxidant, anti-inflammatory, immunomodulatory, and antibacterial [20].

Carotenoids are isoprenoids widely distributed in food [21], which are the primary source of vitamin A in the diet of most of the world's population, and about 600 carotenoids are found in nature. Still, only three are essential precursors of vitamin A in humans, namely β-carotene, α-carotene, and β-cryptoxanthin [22]. Some carotenoids can be converted into retinoids that exhibit vitamin A activity, which are essential for humans and versatile, because they are not only relevant in food, but also as sources of vitamin A, natural pigments, antioxidants, and health-promoting compounds [21].

Carotenoids have different physiological and pathophysiological functions, ranging from fetal development to adult homeostasis [23]. Carotenoids also have a high potential to inhibit pancreatic lipase, which may indicate a potential anti-obesity effect [24]. In addition, carotenoids, which are natural antioxidants, can help neutralize free radicals produced by complex physiological reactions and, consequently, can protect the eye from oxidative stress, apoptosis, mitochondrial dysfunction, and warts [23]. Recently, carotenoids have also been studied in the context of nutricosmetics, because they have been shown to provide cosmetic benefits when consumed in appropriate amounts [21].

Vitamin C, also known as ascorbic acid, has attracted significant attention in both the medical and food sectors for many years [25]. Vitamin C is an essential nutritional antioxidant that protects the immune system from the detrimental effects of reactive oxygen species. Although it is one of the most essential nutrients, vitamin C cannot be

stored by the body, so it is necessary to maintain optimal levels of vitamin C through dietary intake [26]. It occurs naturally in various plants and fruits, thus playing an essential role in maintaining health [25].

The role of antioxidants is to prevent and slow down the oxidation process by interfering with the transfer of electrons between substances and reactive agents. This is important at the cellular level because oxidation reactions cause the formation of free radicals and contribute to various diseases [27]. A comprehensive analysis of the functional properties of vitamin C revealed that, in addition to its well-known antioxidant properties, vitamin C also exerts substantial effects on CD4+ cells [26], and other functional properties are primarily related to the oxidation-reduction properties of vitamin C [25].

The vital biological functions and health benefits of vitamin C are extensive, making it a subject of ongoing research and interest among health professionals and scientists [25]. As research in the health field advances, a deeper understanding of the role of vitamin C in shaping T cell-mediated adaptive immune responses may open new avenues for personalized approaches to immune modulation [26].

Dietary fiber is a food macromolecule that is resistant to digestion and breakdown in the human digestive tract up to the large intestine, where a number of microbial organisms can begin to break it down [28]. In the dynamic intestinal ecosystem, dietary fiber plays an important role in fermentation and serves as a significant nutrient source for gut bacteria. Several studies have shown that a high-fiber diet is closely associated with increased production of short-chain fatty acids [29]. Dietary fiber is classified as soluble and insoluble fiber, with each having a unique function in gut health. Unlike other nutrients, fiber cannot be digested by the intestine [30].

Dietary fiber found in plant foods functions as a prebiotic, meaning it plays a role in nourishing good bacteria in the gut and supporting microbial diversity and overall digestive function [30]. Dietary fiber also plays a vital role in the regulation of metabolism, inflammation, and microbiome composition [31]. In addition, several studies have shown that high fiber intake of foods, such as cereals and whole grains, is associated with a reduced risk of type 2 diabetes, cancer, and cardiovascular disease [32], reduced systemic inflammation, and altered microbial and metabolic profiles in experimental periodontitis [32]. Meanwhile, a persistent decrease in dietary fiber consumption over time can contribute to an imbalance in the gut microbiota, which can negatively impact human health and contribute to the global increase in diabetes, cancer, and other noncommunicable diseases [29].

3. Local wisdom in the use of cassava leaves

Local wisdom is a cultural heritage that has been acquired and passed down from generation to generation within a community or society. Specifically, it encompasses the knowledge, values, technology, practices, and expertise possessed by a community in utilizing the natural resources around them [20]. One form of integration between nature and culture is the local wisdom of communities in utilizing natural food ingredients, namely, cassava leaves. The use of cassava leaves is location-specific and important, considering their potential for development in various regions for future plant breeding to better utilize their use and consumption globally [15].

Cassava leaves in Indonesia are used as a vegetable, either boiled or mashed. For example, all Padang restaurants and local Indonesian restaurants generally serve cassava leaf vegetables [13]. One of the famous traditional dishes from

West Sumatra, Indonesia, is cassava leaf rendang. This type of dish is made from cassava leaves, which have their own uniqueness because they use many types of leaves, in addition to the leaves that are usually added to make rendang. This dish is often served at traditional events such as weddings, circumcisions, turun mandi events, batagak pangulu, and others [33]. **Table 3** presents a selection of conventional Indonesian vegetable dishes made from cassava leaves, as reported in several unpublished sources.

No	Type of cuisine	Region of origin	Characteristic
1.	Cassava leaf curry 	South Sulawesi & West Sumatra (Minang)	Cooked with thick coconut milk, with curry spices such as shallots, galangal, turmeric, and chilies.
2.	Mashed cassava leaves 	North Sumatra (Batak)	It uses finely ground young cassava leaves, then cooked with coconut milk, anchovies, and traditional Batak spices like andaliman. The pounding process gives it a unique texture and enhances the flavor.
3.	Cassava leaf salad 	Java and Bali	Cassava leaves are boiled and then mixed with grated coconut seasoned with onions, galangal, and chilies, so that the taste is fresh, savory, and slightly spicy.
4.	Cassava leaf salad 	East Java	Boiled cassava leaves are served with peanut sauce, so they taste sweet, spicy, and savory.

No	Type of cuisine	Region of origin	Characteristic
5.	Cassava leaf lodeh 	Central Java	Cassava leaves are cooked in coconut milk with pumpkin, tempeh, and lodeh spices, so they taste soft, savory, and slightly sweet.
6.	Sour vegetables with cassava leaves 	Javanese (Betawi)	Cassava leaves are added as a filling in a fresh sour sauce containing other vegetables such as melinjo, long beans, and others, resulting in a fresh, sour, and slightly sweet taste.
7.	Cassava leaf, coconut milk, and vegetable soup 	Common in various regions of Indonesia	Cassava leaves are boiled and then cooked in a light coconut milk sauce with simple spices. The vegetable has a soft, savory flavor, perfect as a side dish with fried tempeh or tofu.

Table 3.
Various types of typical Indonesian dishes based on cassava leaves.

Seven types of Indonesian cassava leaf vegetable dishes are prepared using various methods. One method involves separating the Cassava leaves from their stalks, cutting them into pieces approximately 2 cm wide and 4 cm long, washing them, and cooking them [34]. At the household level, the most common preparation method is washing, pounding, and boiling or cooking the cassava leaves [35]. Another technique involves slicing and pounding the cassava leaves, then cooking them for approximately 2 hours [36].

The seven types of cassava leaf preparations mentioned above are generally prepared by boiling them in water, a standard method in food preparation [37]. Traditional processing techniques include chopping, pounding, fermentation for one or 2 days, or longer boiling to reduce cyanide content [35]. Additional detoxification approaches include drying, soaking in water, and kinetic washing of the pounded cassava leaves [37, 38].

The cyanide content in Cassava leaves ranges from 141.64 mg/100 g dry weight [39], with an average of 52.9 mg/100 g for all varieties. Boiling can reduce the bound cyanide level to 5.9 mg/kg [36]. To enhance detoxification, it is recommended to boil cassava leaves in a pan without a lid to allow the hydrogen cyanide to evaporate [34].

Among the various processing methods, boiling is the most widely used and effective for cyanide removal [36]. However, heat treatment causes significant losses of protein, thiamine, riboflavin, nicotinic acid, vitamin C, and amino acids, especially methionine and cysteine [35]. Pre-cooking treatments, such as pounding or grinding, further reduce the content of ascorbic acid and thiamine, with slightly higher losses observed in grinding (78.61 and 38.95%, respectively), compared to mashing (76.87 and 35.14%, respectively). These nutrient losses are mainly due to the immersion in the cooking water and are accompanied by a 4.40% decrease in protein content [40].

Unboiled cassava leaves contain 20.73 ppm cyanide, while the safe range for humans is 0.5–3.4 mg/kg body weight; WHO (2019) recommends a maximum of 10 mg/kg in raw materials [41]. A study involving 20 volunteers who consumed 200 g of boiled cassava leaves twice daily for 12 consecutive days reported a significant decrease in thiodothyronine and thyroxine levels within 9 days, leading to a reduction in urinary iodine excretion and potential risk of goiter. Based on these findings, a daily intake of 50–100 g of boiled cassava leaves is recommended [42].

4. Innovation of functional food products based on cassava leaves

Given its nutritional composition and health benefits, cassava leaves have great potential for development into various functional food products. Converting cassava leaves into flour facilitates their incorporation into various formulations [13]. For example, partially replacing wheat flour with cassava leaf flour in cake making (**Table 4**) has been shown to increase protein and fiber content while reducing gluten levels, making this product suitable for individuals with gluten intolerance [43]. In addition, cassava leaves can be processed into jerky with a crunchy texture and flavor profile comparable to beef jerky. This process usually involves partially replacing the formula with tapioca flour to improve

No	Product name	Utility	Reference
1.	Cookie cake	The addition of cassava leaf flour in making cookies, to increase the protein and fiber content, while reducing the gluten content, for individuals who are intolerant to gluten.	[43]
2.	Cassava leaf jerky	Making cassava leaf flour jerky with tapioca flour substitution can improve the quality, making it a source of protein and fiber, which is a traditional food typical of the people of Bukittinggi City.	[44, 45]
3.	Tempeh	The addition of cassava leaves in the manufacture of tempeh is to increase nutritional value and to increase the diversity of alternative food products.	[46]
4.	Mushroom nuggets	The addition of cassava leaf flour in making mushroom nuggets is an alternative food choice that vegetarians can eat.	[47]
5.	Sago crackers	The addition of cassava leaf flour in making sago crackers increases the nutritional content and provides an attractive color to the resulting sago crackers.	[48]
6.	Cassava leaf granules	Instant granules are a practical and easy-to-consume pharmaceutical preparation. To be well-received by the public, they must have a pleasant taste.	[49]

Table 4.
Functional food innovation products based on cassava leaves.

product quality, resulting in jerky that serves as a valuable source of protein and dietary fiber and can be classified as a functional food [44, 45].

In addition, cassava leaves can be incorporated into tempeh products to increase the diversity of alternative food products [46]. Cassava leaf flour has also been used in the manufacture of straw mushroom nuggets [47] and sago crackers [48]. In the latter, its addition not only increases the nutritional value but also provides an attractive green color to the final product. Furthermore, considering the antibacterial properties of the bioactive compounds present in cassava leaves, a study formulated cassava leaf granules combined with mannitol, sucrose, and honey. Antibacterial testing of this combination showed that the optimum mixture exhibited potent inhibitory activity [49].

5. Cassava leaves as a strategic resource for resilience food and health promotion

Approximately 800 million people worldwide depend on Cassava as a staple food, with approximately 500 million of these people in sub-Saharan Africa [50]. In this region, Cassava accounts for 61 percent of annual production [19] and plays a crucial role in food security. Globally, Cassava ranks fifth in terms of production, after maize, rice, wheat, and potatoes. Cassava is a drought-tolerant crop that requires relatively few inputs, can grow on marginal land where other crops cannot survive, and is available for up to 10 months each year in East Africa [50].

Food security encompasses efforts to ensure adequate food supplies, which can be supported by production systems rooted in local wisdom and cultural practices while maintaining nutritional quality. Strategies include replacing commonly consumed foods with nutrient-rich alternatives and diversifying processed food products [7]. Raising awareness of the dietary benefits of native leafy vegetables such as cassava leaves can contribute significantly to food security, as these leaves provide essential nutrients [28].

In Cameroon, cassava tubers are a staple food, and the leaves are used to treat malnutrition because they are rich in protein, phenolics, carotenoids, vitamin B, and vitamin E [10]. Cassava leaves are a source of the minerals Ca, Mg, Fe, Mn, Zn, vitamin A, and vitamin B2 (riboflavin) [51], as well as carbohydrates, protein, and other vitamins and minerals [15]. According to [13], cassava leaves are low in calories and can increase satiety, improve digestion, and relieve constipation. Cassava leaves are also traditionally used to relieve headaches, treat the flu, expel worms, treat rheumatism, heal boils, treat diarrhea, and increase stamina.

6. Conclusion

Cassava leaves are known as a functional food that is gaining global popularity due to their ability to improve and maintain health. Beyond meeting basic needs, functional foods made from cassava leaves offer additional health benefits, particularly in disease prevention and management, through their antioxidant, anti-inflammatory, antidiabetic, antiproliferative, antimicrobial, anticancer, antihypertensive, cardioprotective, and antiaging activities.

A key example of the integration of nature and culture is reflected in the local wisdom of communities utilizing cassava leaves as a natural food source. In Indonesia, Cassava is commonly consumed as a vegetable, boiled or mashed, not only as a component of traditional dishes but also as a practical way to reduce cyanide levels.

Processing cassava leaves into flour facilitates their incorporation into various processed products, expanding their potential applications. A practical approach to increasing food availability is to raise public awareness of the diverse benefits of cassava leaves, particularly their role in preventing and managing degenerative diseases, while also providing essential nutrients. Such efforts can strengthen cassava leaves' position as a valuable functional food in both traditional and modern food systems.

Conflict of interest

The author declares that there is no conflict of interest, either moral or financial, arising from the writing of this chapter.

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Chapter 7

Engineered Microbial Factories: Next-Generation Food Production Systems for Emergency and Military Applications

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Abstract

The increasing fragility of global supply chains, starkly highlighted by recent geopolitical and public health crises, presents a critical vulnerability for military and humanitarian operations. This chapter explores the emergence of Engineered Microbial Factories (EMFs) as a transformative solution, enabling a paradigm shift from long, precarious logistical tails to resilient, on-demand, point-of-need production. Powered by advances in synthetic biology and metabolic engineering, EMFs are microorganisms designed to convert a range of feedstocks—from atmospheric gases and water to plastic waste—into essential supplies, including nutritionally complete and functionally enhanced food. This technological revolution is driven by the twin engines of strategic defense investment and a burgeoning commercial market. Flagship U.S. Department of Defense programs, such as Defense Advanced Research Projects Agency (DARPA's) Cornucopia and ReSource, are analyzed; they aim to create deployable systems for producing food from air and valorizing battlefield waste. Concurrently, the explosive growth of the commercial fermentation sector, exemplified by pioneers such as Solar Foods and Perfect Day, is examined; their success derisks the technology and creates a powerful dual-use ecosystem. The chapter details the profound implications for military logistical independence and humanitarian aid, where EMFs offer an empowerment model that fosters local self-reliance. However, significant challenges remain. The critical hurdles of technical scalability, advanced bioprocessing, the unique economic calculus of fully burdened cost versus commercial price parity, and the complex global regulatory and social gauntlet for these novel foods are addressed. Ultimately, EMFs represent a technology at a strategic inflection point, offering a credible path to enhanced operational endurance, superior nutrition, and global resilience.

Keywords: engineered microbial factories, synthetic biology, precision fermentation, food security, military logistics, waste valorization, supply chain resilience, functional foods, novel food regulation, consumer acceptance

1. Introduction

1.1 The fragility of global logistics: A shared military and humanitarian vulnerability

Modern military and humanitarian operations are precariously reliant on complex global supply chains, now widely recognized as a paramount strategic liability. For the U.S. Department of Defense (DoD), missions in contested or austere environments are severely hampered by what experts describe as "costly and brittle food supply chains" [1–3]. The extensive logistical "tail" required to transport and protect provisions, such as food, water, and fuel, consumes a vast portion of operational resources and, more critically, exposes personnel to significant risk, especially within vulnerable supply convoys [2]. The COVID-19 pandemic acted as a global stress test, starkly revealing the inherent fragility of these systems and their susceptibility to widespread disruption [4]. The impact resonated even within the disciplined confines of military food logistics, forcing immediate modifications to ensure food security and highlighting the urgent need for resilient alternatives.

This vulnerability is alarmingly magnified in the context of Humanitarian Assistance and Disaster Relief (HADR). In the wake of natural disasters or during protracted conflicts, established food systems are often the first infrastructure to collapse, leaving entire populations dependent on external aid. The current model of shipping bulk rations, while indispensable, is frequently slow, expensive, and can foster long-term dependency, undermining the goal of sustainable self-reliance in relief and recovery efforts [5]. The scale of this challenge is immense: In 2024, an estimated 37.2 million people across 47 countries faced emergency, or worse, levels of acute food insecurity, demanding immediate, life-saving assistance [6].

1.2 The new paradigm: From logistics-centric to production-centric operations

In response to this shared vulnerability, a transformative technological vision is emerging: To revolutionize the procurement of critical supplies by decoupling operations from fragile, long-distance supply lines. The core strategic objective is to enable the "on-demand production… on site" of essential goods, particularly food. Such a capability would fundamentally alter the operational calculus, extending mission endurance, bolstering force protection by minimizing reliance on vulnerable convoys, and dramatically expanding the scope of what is achievable in both military and humanitarian theaters [7–9].

This paradigm shift represents a move away from the linear, extractive model of logistics—growing, harvesting, processing, and shipping food over vast distances—toward a resilient, circular bioeconomy. In this new model, local resources and even waste streams are repurposed to create high-value products at the point-of-need. The very concept of "feedstock," the raw material for production, is being radically expanded. While commercial fermentation has historically depended on agricultural inputs like sugar, advanced research now explores inputs previously dismissed as waste or those that are ubiquitously available. DARPA's ReSource program [2], for instance, explicitly targets the conversion of battlefield waste—including notoriously difficult-to-degrade plastics—into valuable resources [10]. Concurrently, the DARPA Cornucopia [1] program and commercial pioneers like Solar Foods are taking this concept to its logical extreme, aiming to use the fundamental building blocks of life—water, air (specifically

CO_2 and nitrogen), and energy—as primary feedstocks. This evolution heralds a future of extreme feedstock flexibility, where a single deployed bioreactor could be configured to generate vital supplies from any available input, be it generator exhaust, plastic packaging from spent rations, or the very air itself [11, 12].

1.3 A spectrum of fermentation: From ancient foods to engineered bio-functional factories

At the heart of this new logistical paradigm are Engineered Microbial Factories (EMFs), also known as Microbial Cell Factories (MCFs). EMFs are microorganisms—such as bacteria, yeast, or algae—specifically designed and cultivated to function as microscopic biocatalysts, efficiently producing high-value biomolecules. This technology is a sophisticated evolution of fermentation, a process humans have utilized for millennia to preserve foods like kimchi, produce beverages like beer, and leaven bread. The modern food industry is now built upon three distinct pillars of fermentation technology [13].

Traditional Fermentation utilizes intact, often unmodified, live microorganisms to process and transform ingredients. This is the ancient practice behind products like yogurt, sauerkraut, and kefir, where microbes fundamentally alter the food's chemistry, preservation qualities, and nutritional profile. These foods are increasingly recognized as "functional foods" because the fermentation process generates bioactive compounds and beneficial microorganisms (probiotics) that can improve gut health and bolster the immune system.

Biomass Fermentation, in contrast, leverages the rapid growth of microorganisms to produce a large quantity of microbial mass, which itself becomes the primary food ingredient. The mycoprotein used in established consumer products from companies like Quorn and Meati Foods is a prime example, where the fungal biomass provides the bulk protein and texture [14, 15].

Precision Fermentation represents a third, more advanced pillar. Described as a "game-changing technique," it uses biotechnology to program microbial hosts to function as highly specialized "cell factories" [16, 17]. These factories can be instructed to produce specific, predetermined molecules—such as proteins identical to those in milk or eggs, essential fats, or vitamins—with remarkable purity and efficiency. This approach focuses on creating discrete, high-value ingredients rather than simply processing existing ones or using the entire microbe.

1.4 The scientific engine: Synthetic biology and metabolic engineering

The power to create these sophisticated microbial factories stems from the convergence of two powerful scientific disciplines: Metabolic engineering and synthetic biology [13, 18, 19].

Metabolic Engineering is the foundational practice of purposefully redesigning an organism's metabolism to optimize its cellular processes for the overproduction of a desired compound. Scientists employ a range of strategies to achieve this, including amplifying genes for key enzymes (gene overexpression), deleting genes for competing pathways (gene deletion), and even introducing "heterologous" pathways from other organisms to produce entirely non-native compounds. The ultimate goal of these interventions is to fine-tune the microbial strain's performance, maximizing its titer (the final product's concentration), yield (the amount of product per unit of input), and productivity (the rate of production) [20, 21].

Synthetic Biology provides the advanced toolkit that makes modern metabolic engineering possible. It is an engineering-driven discipline focused on designing and constructing new biological parts, devices, and systems [19]. It provides the methods for reading (DNA sequencing), writing (DNA synthesis), and editing (e.g., CRISPR/Cas9) an organism's genetic code with unprecedented precision [22–24]. This enables the rational design of novel biosynthetic pathways and the systematic engineering of robust, efficient production hosts.

The convergence of EMF technology with the science of functional foods means that deployed systems could produce not just basic nutrition, but *targeted* nutrition. The original military objective is to produce macronutrients—protein, carbohydrates, and fats—to replace standard rations. However, extensive research demonstrates that fermentation can be engineered to produce specific bioactive compounds with known health benefits, such as peptides that regulate blood pressure, γ-aminobutyric acid (GABA) for stress reduction, or conjugated linoleic acid (CLA) for reducing inflammation. Military and HADR environments are characterized by extreme physiological and psychological stress, which these compounds are known to mitigate. Therefore, the strategic value of an EMF is not just replacing a Meal, Ready-to-Eat (MRE)—a logistical benefit—but potentially creating a superior, mission-specific nutritional intervention. A bioreactor could be programmed to produce food enriched with immunomodulatory compounds for a unit operating in a high-disease-risk environment, or neuroprotective compounds for high-stress special operations. This elevates the technology from a "nice-to-have" logistical tool to a "must-have" force multiplier that enhances warfighter and survivor performance and resilience [25].

2. The military vision for logistical independence

2.1 The end of the supply chain: A biological revolution

The modern U.S. military's logistical capability, while a marvel of planning and execution, relies on a vast and extended supply chain that is a well-understood vulnerability. Recognizing this challenge, the Department of Defense has identified biotechnology as one of its 11 key technology modernization priorities [26]. Within this field, synthetic biology is a critical enabler for future capabilities, particularly for the on-demand, in-theater production of advanced materials, chemicals, energy, and, crucially, food. This strategic focus aims to reduce logistical dependencies, increase operational endurance, and enhance the resilience of the force. The overarching goal is not merely logistical efficiency but a fundamental enhancement of military power, with the stated aim to "harness energy and resources in place [to] maximize lethality, warfighter performance and dominance."

The Defense Advanced Research Projects Agency (DARPA), specifically its Biological Technologies Office (BTO), is the primary engine for this vision within the DoD. The BTO's mission is to leverage the unique properties of biology to revolutionize national security capabilities. A core thrust of this mission is "Logistics," which explicitly aims to develop solutions for "distributed forward manufacturing" and to "enable point-of-need production" of critical commodities. DARPA's investment in synthetic biology has been substantial, growing from almost no funding in 2010 to $100 million per year by 2014. This investment has led to foundational programs like "Living Foundries," which sought to dramatically increase the speed and decrease the cost of generating new production strains of organisms. It is from this strategic

imperative that DARPA has launched two of its most ambitious and potentially transformative programs: Cornucopia and ReSource [1–3, 10, 27].

2.2 The Cornucopia program: Food from air, water, and electricity

Announced in 2021, the Cornucopia program embodies the most forward-looking aspect of DARPA's vision for logistical independence. Its goal is to create a deployable, transportable system that can produce "appetizing, microbial-origin food" on-demand, using only the most fundamental and universally available inputs: Air, water, and electricity, with minimal supplementation. If successful, the program could eliminate the need for traditional food supply chains, allowing deployed units to sustain themselves indefinitely if power and water are available.

The technical objectives of Cornucopia are highly ambitious. The program aims to produce all four human dietary macronutrients—protein, carbohydrates, fats, and dietary fiber—in customizable ratios that meet the stringent Military Dietary Reference Intake (MDRI) standards for complete nutrition. A critical aspect of the program is ensuring the final products are not just nutritious but also palatable. The target outputs include familiar formats like shakes, bars, gels, and even jerky, all of which must meet military standards for taste and texture to ensure troop acceptance and morale. As of early 2023, DARPA had contracted several teams, each pursuing a unique technical approach to this challenge. This has created a diverse portfolio of 13 distinct approaches to carbon and nitrogen fixation, utilizing 12 different microbial and algal species and numerous food processing methods [27, 28]. The program's designated transition partner is the U.S. Army Combat Capabilities Development Command (DEVCOM) Soldier Center, the organization responsible for developing and fielding soldier sustenance solutions.

2.3 The ReSource program: Valorizing waste into critical materiel

Running in parallel with the forward-looking Cornucopia program is ReSource, a similarly ambitious yet more pragmatically grounded effort to solve the pressing battlefield problem of waste. Military forward operating bases generate tons of waste, primarily single-use plastics and packaging, which are often managed through environmentally hazardous burn pits. The ReSource program aims to turn this liability into a strategic asset [10]. The target feedstock for the ReSource system is the heterogeneous mix of trash generated in a field environment, with a particular focus on energy-dense but difficult-to-recycle plastics like polyethylene and polyethylene terephthalate (PET). The envisioned system, roughly the size of a pickup truck, would pre-treat and break down this mixed waste at a molecular level, reformulating the constituent parts into strategic materials. The desired outputs were diverse and strategically vital, including edible macronutrients, industrial lubricants (POLs), adhesives, tactical fibers, and potable water [29].

Performer teams from institutions like Battelle, Iowa State University, MIT, and Michigan Technological University were tasked with developing novel conversion processes. Battelle's approach, for example, focused on using banks of bioreactors housing genetically engineered microorganisms to degrade plastic waste and upgrade it into gun lubricant, a material critical to warfighters in the field [10].

Together, Cornucopia and ReSource represent two prongs of a unified strategy to unburden the warfighter and reduce the military's logistical tail (**Table 1**).

Feature	DARPA Cornucopia	DARPA ReSource
Core concept	Produce food from air, water, and electricity	Convert military waste (plastics) into food and materials
Primary inputs	Atmospheric CO_2 and N_2, water, electricity	Mixed plastic waste, packaging, and other organic waste
Target outputs	Nutritionally complete, palatable food (shakes, bars, jerky)	Edible macronutrients (protein powder), lubricants, adhesives, and tactical fibers
Military scenario	Feed a small combat unit for 45 days	On-site waste management and resource production at a forward operating base
HADR scenario	Feed 100 civilians for 21–30 days	Dual benefit: Post-disaster cleanup and emergency food production
System footprint	Humvee-transportable	Tricon/Humvee-transportable container
Program status	Ongoing, performer teams in development	Concluded, technologies being explored for transition

Table 1.
Key DARPA program specifications.

3. The commercial ecosystem: De-risking and accelerating the technology

3.1 The commercial fermentation market: A dual-use powerhouse

The strategic vision for military and humanitarian food production is being powerfully accelerated by a vibrant and rapidly growing commercial sector. Driven by consumer demand for sustainable and ethical alternatives to animal agriculture, the fermentation-based food industry has attracted billions of dollars in investment and is making rapid technical progress. Multiple industry analyses project extraordinary growth rates for the precision fermentation market, with forecasts calculating its size at approximately $3 billion in 2024 and projecting it to reach between $54 billion and $104 billion by 2032–2034, representing a staggering compound annual growth rate (CAGR) of over 40% [30, 31].

Investment patterns are also maturing. While early funding was dominated by venture capital, there is a noticeable shift toward strategic corporate partnerships and government funding, indicating market validation and a move toward industrial scale-up. Geographically, while North America has historically been a leader, the Asia-Pacific region has captured a dominant market share, driven by pressing food security concerns and supportive government industrial policies that parallel the strategic interests of the DoD.

3.2 Case study: Solar foods and the Solein® revolution

Solar Foods, a Finnish food-tech company, exemplifies the potential to create high-quality protein from the most basic of inputs, closely mirroring the ambitions of DARPA's Cornucopia program. The company's core technology is a proprietary form of gas fermentation using a single, naturally occurring, non-genetically modified hydrogen-oxidizing bacterium. This microbe is cultivated in a bioreactor and fed a simple diet of carbon dioxide and nitrogen from the air, along with hydrogen produced *via* water electrolysis powered by electricity. The result is a nutritionally

rich powder called Solein®, which is approximately 78% protein, 10% dietary fiber, 6% fat, and 4% minerals [32]. Crucially, it provides a complete amino acid profile and contains essential micronutrients like iron (1.1 g/kg) and vitamin B12 (50 μg/kg), which are often lacking in purely plant-based diets [33, 34].

This method is completely independent of agriculture, allowing for consistent production in any location with access to electricity and water. The company achieved a critical milestone in April 2024 with the launch of "Factory 01" in Vantaa, Finland, a commercial facility capable of producing up to 160 tons of Solein® annually [32, 34]. Functionally, Solein® is highly versatile, with excellent emulsification and fibrillation properties that allow it to be incorporated into products ranging from dairy alternatives to meat analogues, helping to solve the critical challenge of palatability. Solar Foods has pursued a shrewd regulatory strategy, first achieving novel food approval in Singapore in 2022, followed by self-affirmed Generally Recognized as Safe (GRAS) status in the U.S. in 2024, providing a clear roadmap for other companies in the sector. The company has also built a strong intellectual property portfolio, with over 160 patent applications, including a key patent on the production organism itself [35].

3.3 Case study: Perfect day and precision-fermented whey

Perfect Day has pioneered the production of animal-free whey protein using precision fermentation. Their technology employs a genetically modified strain of the fungus *Trichoderma reesei* to produce beta-lactoglobulin, a protein that is molecularly identical to the primary whey protein found in cow's milk. The company makes strong functional claims, stating that its protein delivers the same taste, texture, and culinary functionality (e.g., solubility, gelation, foaming) as conventional whey, but is free of lactose, cholesterol, and hormones [36].

A key part of Perfect Day's value proposition is its environmental benefit. A commissioned Life Cycle Assessment (LCA) concluded that their animal-free whey produces up to 97% fewer greenhouse gas emissions, consumes up to 99% less blue water, and uses up to 60% less nonrenewable energy compared to conventional bovine dairy protein [37]. The company has secured a "no questions" letter from the Food and Drug Administration (FDA) regarding the GRAS status of its protein and has built an extensive patent portfolio, with numerous granted patents and applications focusing heavily on compositions containing casein, another major milk protein. However, the company has faced legal and scientific challenges. A 2024 lawsuit alleged "false and deceptive" marketing, citing a study that claimed to find 93 unknown fungal compounds and fungicide residue in a consumer product made with Perfect Day's protein [38]. Perfect Day has refuted these claims, stating that all microflora are filtered out of the final product and that no fungicides are used in their process.

3.4 The industrial landscape: Ingredient producers and platform enablers

The case studies of Solar Foods and Perfect Day reveal two fundamentally different strategic philosophies for bringing novel microbial proteins to market, each with distinct risks and rewards. Solar Foods pursues a "novel organism, natural process" approach. They emphasize that their microbe is a "non-modified, single-cell organism found in nature," with their core intellectual property being the organism itself. This simplifies the public narrative but requires building a safety and efficacy case from scratch for a completely new food. In contrast, Perfect Day uses an "engineered organism, known product" approach. They employ a well-established industrial fungus,

Trichoderma reesei, but genetically engineer it to produce a familiar protein, beta-lactoglobulin [39]. This strategy leverages the known functionality and consumer familiarity of whey but inherits the public relations and regulatory baggage of genetic modification.

These divergent paths create different vulnerabilities. Solar Foods' risk is primarily technical and regulatory, proving the safety of a new organism. Perfect Day's risk is primarily social and legal—managing public perception of GMOs and defending against legal challenges to their "nature-identical" marketing claims. For a strategic user like the DoD, this implies that there is no single "best" commercial model to emulate. A portfolio approach is necessary. A Cornucopia system might leverage a robust but novel extremophile (akin to Solar Foods' model), while a ReSource system might require a highly engineered specialist microbe to break down plastics (akin to Perfect Day's model). Understanding these divergent commercial strategies is critical for shaping military R&D and acquisition strategy. The broader commercial landscape can be categorized into key segments, each contributing to a powerful dual-use technology base (**Table 2**).

Company	Primary technology	Key products/ services	Regulatory status/ milestones	Relevance to military/ HADR Apps
Solar foods	Biomass fermentation (Hydrogen-oxidizing bacteria)	Solein™ (complete protein powder from CO_2)	Singapore novel food (2022); US GRAS (2024); Factory 01 operational	Direct model for Cornucopia. Validates the food from air concept and offers a robust chassis for extreme environments.
Perfect day	Precision fermentation	Animal-free whey and casein proteins	US GRAS ("no questions" letter); B2B pivot; multiple commercial products	Source for high-quality protein. Demonstrates scalable production of a key macronutrient for nutritionally complete rations.
Meati foods	Biomass fermentation (Mycelium)	Mycelium-based whole-cut meat alternatives	Expanded retail presence; $100 M Series C funding	Solves the palatability/ texture problem. Provides a high-protein, textured food base for high-morale meal formats.
The EVERY Co.	Precision fermentation	Animal-free egg proteins (e.g., ovalbumin)	Commercial product partnerships	Source of functional, high-quality protein; useful as a binder and nutritional supplement in ration formulation.
Ginkgo bioworks	Platform technology	Custom microbial strain engineering ("foundries")	Leader in organism design; partnerships with major corporations	Potential partner for custom microbe development for specific waste conversion (ReSource) or extremotolerance.
Impossible foods	Precision fermentation	Soy leghemoglobin ("heme") for flavor	Established brand; wide market penetration	Proof-of-concept for sensory attributes. Demonstrates fermentation can create key flavor compounds, crucial for troop acceptance.

Table 2.
Leading commercial entities in the fermentation-for-food sector.

4. Humanitarian and disaster relief (HADR): An empowerment model

The same logistical vulnerabilities that challenge military operations are magnified in the context of humanitarian crises. Events such as pandemics, armed conflicts, and natural disasters can shatter local and national food systems, severely impacting the availability, affordability, and safety of food for affected populations. The standard response, while vital, is often slow, logistically intensive, and can inadvertently create a cycle of dependency that hinders long-term recovery [40–43].

Engineered Microbial Factories (EMFs) present a transformative alternative, offering a "people-centric approach" to build resilience directly within affected communities. This technology represents a paradigm shift from a dependency model, which involves the continuous shipment of food, to an empowerment model. Deploying a bioreactor is a one-time capital investment that provides a community with the means of production, fostering local resilience, creating technical skills, and allowing aid organizations to transition from a role of perpetual provider to one of trainer and technical support [5, 44].

The models envisioned in DARPA's programs, designed to fit within standard shipping containers, provide a clear blueprint for a deployable HADR system. Such a system could be transported to a disaster area and quickly brought online to produce nutritionally complete food. Furthermore, by producing foods fortified with specific bioactive compounds, these systems could address common post-disaster health issues like immune suppression or stress. The waste valorization concept from the ReSource program is particularly powerful, offering the dual benefit of contributing to environmental cleanup while simultaneously addressing urgent human needs. This suggests a potential tiered deployment strategy: A ReSource-type system in the first wave to tackle waste and provide initial food security, followed by a more efficient Cornucopia-type system for high-volume, continuous production once the situation stabilizes.

5. Engineering the factories: From microbial chassis to final product

5.1 Selecting the microbial chassis: A comparative analysis

The success of any engineered microbial factory depends on its biological foundation: The host organism, or "chassis," that is engineered for production. There is no single "best" microbe; the selection is a trade-off between robustness, productivity, input requirements, and final product characteristics. While the bacterium *Escherichia coli* and the yeast *Saccharomyces cerevisiae* are the most established platforms due to their well-characterized genetics, the field is actively expanding to include a more diverse set of chassis, each with unique, innate characteristics suitable for specific applications (**Table 3**) [45, 46].

5.2 Metabolic engineering for enhanced functionality

Metabolic engineering provides the tools to transform a basic microbial chassis into a high-performance factory for specific, high-value functional ingredients. The core strategies involve the precise manipulation of an organism's genetic and regulatory processes. One common approach is to overexpress the gene encoding the rate-limiting enzyme in a biosynthetic pathway, thereby increasing the overall rate of production. Another is to block competing metabolic pathways by deleting key genes, which redirects cellular resources toward the desired product. A

Chassis type	Key advantages	Key disadvantages	Typical products	Example programs/ companies
Bacteria (*E. coli*)	Very fast growth; extensive genetic toolkit; high-density cultivation possible.	Endotoxin production in some strains raises safety concerns; protein secretion can be difficult.	Amino acids, vitamins, some proteins, biofuels.	Zymergen, Genomatica, numerous academic programs.
Yeast (*S. cerevisiae*)	Generally Recognized as Safe (GRAS) status; robust and tolerant of industrial conditions.	Slower growth than bacteria; can have lower product yields for some compounds.	Proteins (whey, egg), enzymes, flavors, vitamins.	Perfect Day, The EVERY Co., DARPA Cornucopia performers.
Filamentous fungi (*Aspergillus*)	Excellent protein secretion capacity; biomass itself is a high-protein food source (mycoprotein).	More complex genetics; morphology can create viscosity challenges in bioreactors.	Mycoprotein (whole-food meat alternatives), enzymes, lipids.	Meati, quorn, infinite roots.
Microalgae (*Chlorella*)	Photosynthetic (can use CO_2 and light); can grow in non-arable land/water.	Slower growth rates; cultivation requires large surface area for light exposure; harvesting can be costly.	Proteins, lipids (omega-3 s), carbohydrates, pigments.	SRI international (in DARPA Cornucopia), Corbion.
Extremophiles (*D. radiodurans*)	Innately tolerant to extreme conditions (radiation, drought), reducing need for complex containment.	Less characterized than model organisms; limited genetic tools; typically slower growth and lower yields.	Robust enzymes, biopolymers; potential for any product in a hardened system.	Primarily academic and early-stage defense research.

Table 3.
Comparative analysis of microbial chassis for food production.

third, powerful strategy is heterologous gene expression, where genes from other organisms are introduced to create entirely new production capabilities in the host microbe [20, 21].

These strategies have been successfully applied to produce a range of functional food ingredients:

Vitamins: Researchers have engineered *S. cerevisiae* for high-titer Vitamin B5 (pantothenic acid) production. This was achieved by screening key biosynthetic genes from diverse species (bacteria, fungi, plants), assembling an efficient heterologous pathway, and then fine-tuning gene copy numbers and knocking out competing pathways to maximize yield [47].

Omega-3 fatty acids: The oleaginous yeast *Yarrowia lipolytica* has been engineered to produce eicosapentaenoic acid (EPA), a crucial omega-3 fatty acid. When using waste cooking oil as a feedstock, a key challenge was the accumulation of toxic free fatty acids (FFAs). Engineers solved this by deleting the *TGL4* gene, which encodes a lipase responsible for degrading stored triglycerides back into FFAs. This single deletion prevented FFA accumulation and boosted EPA production by 300% compared to the parent strain under the same conditions [48].

Bioactive peptides: To produce bioactive peptides, which are short chains of amino acids, the precursor amino acids must first be accumulated. In *E. coli*, engineers have increased the production of L-homoserine (a precursor) by knocking out the *metA*, *thrB*, and *lysA* genes. This blocks the pathways that would normally consume L-homoserine to produce other amino acids like methionine, threonine, and lysine, thereby forcing it to accumulate for peptide synthesis [49].

5.3 Bioprocess engineering: From upstream fermentation to downstream processing

The engineering challenges of moving from a controlled laboratory environment to a robust, field-deployable system are immense. The bioprocess involves two main stages: Upstream processing (fermentation) and downstream processing (purification and formulation). While fermentation requires precise control of environmental parameters, downstream processing is often the primary bottleneck, potentially accounting for over 60% of total production costs. For deployable systems, these steps must be radically simplified, highly efficient, and automated for "push-button" operation by nonspecialist users [50].

5.3.1 Downstream I: Cell disruption and protein recovery

For many EMFs, the valuable protein product is intracellular, trapped behind a tough cell wall. Therefore, a critical downstream step is cell lysis, or disruption, to

Method	Principle	Advantages	Disadvantages	Suitability for deployable systems
Bead milling	Mechanical shear and impact from grinding beads.	High disruption efficiency, scalable, good temperature control.	High energy demand; can generate fine cell debris, complicating purification.	Moderate. Requires a robust, low-power milling device. Best for hardened, stationary systems.
High-Pressure Homogenization (HPH)	High-velocity fluid shear, cavitation, and impact forces.	Highly efficient; scalable; continuous process.	Very high energy consumption; can denature proteins due to heat/shear; expensive equipment.	Low. High power and maintenance requirements make it unsuitable for most field applications.
Sonication	Cavitation and acoustic shockwaves from high-frequency sound.	Scalable; low operational costs compared to HPH.	It can generate significant heat, risking protein denaturation; efficiency varies by species.	Moderate to High. Solid-state, low-maintenance transducers make it a promising option for compact, automated systems.
Enzymatic lysis	Use of enzymes (e.g., lysozyme, zymolyase) to digest cell wall components.	Gentle, preserving protein activity; highly specific.	Can be slow; enzymes are costly; requires removal of enzymes/ reagents downstream.	High. Ideal for low-power systems. Enzymes could be stored and added automatically, simplifying hardware.

Table 4.
Comparison of cell disruption techniques for microbial protein recovery.

release the contents for recovery. This is a crucial consideration for system design, as different methods have varying impacts on energy consumption, equipment footprint, and the integrity of the final product (**Table 4**) [51, 52].

5.3.2 Downstream II: Enhancing nutritional value and safety

Raw microbial biomass is not always inherently optimal for human consumption. It can contain "anti-nutritional factors" (ANFs)—compounds like phytates, lectins, and cyanogenic glycosides that are part of the microbe's natural defenses but can interfere with human digestion and nutrient absorption. Ensuring the final product is free of these compounds is a critical quality control challenge.

A powerful solution is to incorporate an additional downstream processing step using enzymatic hydrolysis or a secondary microbial fermentation. This process uses specific enzymes (e.g., phytases, β-glucosidases) to break down and neutralize ANFs, significantly improving the bioavailability of minerals and the overall nutritional quality and safety of the final product [53]. This same principle can be used not just to remove unwanted compounds but to create valuable new ones. Enzymatic hydrolysis of the bulk protein can release smaller, encrypted bioactive peptides, which have demonstrated health benefits such as antioxidant, antihypertensive, and antimicrobial properties [54]. This turns a simple purification step into a value-adding functionalization step.

6. Critical challenges to deployment

6.1 Technical hurdles: Scalability, robustness, and palatability

Despite the immense promise of EMFs, the path from laboratory proof-of-concept to widespread field deployment is fraught with significant technical challenges. The transition from small-scale lab experiments to robust, reliable production at a field-deployable scale is a notorious bottleneck in biotechnology [55]. A microbial strain that performs well in the pristine conditions of a petri dish may fail under the stresses of a large-scale bioreactor. Strains must be engineered not only for high productivity but also for robustness to withstand process conditions like shear stress, temperature fluctuations, and pH changes. For deployable systems, the microbes must remain viable and productive after long-term storage and transport, often in suboptimal conditions [56].

6.2 The economic calculus: Fully burdened cost vs. commercial price parity

Ultimately, the deployment of EMFs will be governed by their economic feasibility. The primary economic barrier is the high capital investment for bioreactors and downstream processing equipment, with current production costs for microbial proteins remaining significantly higher than conventional sources. However, the economic calculation for military and humanitarian applications is fundamentally different from that of a commercial food company. For a commercial entity, viability is determined by a direct comparison of its production cost to the market price of a commodity like whey protein [57, 58].

For the DoD, the calculation is not so simple. The cost of a deployable EMF system must be weighed against the *fully burdened cost* of the alternative: Delivering MREs to a forward operating base. This fully burdened cost includes not only the price of

the MRE itself but also the enormous expense of fuel for transport, the cost of the personnel required for the logistical operation, and, most critically, the intangible but immense cost in operational risk and human lives associated with protecting vulnerable supply convoys. From this strategic perspective, an EMF system that produces food at a cost that would be commercially nonviable could be considered exceptionally cost-effective if it eliminates the need for a single dangerous supply mission. This suggests that military and HADR applications will likely serve as a crucial "beachhead market," providing the demand needed to drive the technology down the cost curve long before it achieves mass-market price parity [59, 60].

6.3 The regulatory gauntlet: Navigating global novel food frameworks

Perhaps the most complex set of challenges lies in the regulatory domain. The products of EMFs, particularly those using genetically modified organisms (GMOs) or novel feedstocks, must navigate a labyrinthine global regulatory framework. The two largest and most influential regulatory systems are those of the United States and the European Union, which take fundamentally different approaches (**Table 5**) [61, 62].

In the U.S., there is no single law governing biotechnology products; they fall under statutes managed by the FDA, USDA, and EPA. For food ingredients, companies typically pursue a Generally Recognized as Safe (GRAS) determination from the FDA. This can be achieved through a formal notification process or *via* "self-affirmation," a path successfully used by Solar Foods to gain market access. In contrast, the European Union has a highly stringent, centralized pre-market authorization system under Regulation (EU) 2015/2283, the "Novel Food Regulation" [63]. The European Food Safety Authority (EFSA) conducts a rigorous scientific safety assessment that

Feature	United States (GRAS)	European Union (Novel Food)
Key regulation	Federal food, drug, and cosmetic act (FD&C Act)	Regulation (EU) 2015/2283
Competent authority	Food and Drug Administration (FDA)	European Commission (EC) and European Food Safety Authority (EFSA)
Definition of "Novel"	An ingredient not widely consumed before 1958, or produced by a novel process.	Food not consumed to a significant degree within the EU before May 15, 1997.
Assessment process	Company-led assessment. Can be "self-affirmed" or submitted to the FDA for a "no questions" letter.	Centralized pre-market authorization. Mandatory application to the EC, followed by a scientific risk assessment by EFSA.
Data requirements	Evidence of safety based on scientific procedures or history of use. Less prescriptive than the EU.	Highly prescriptive: composition, production process, toxicology, allergenicity, whole genome sequence of the microbe, etc.
Timeline	Can be relatively fast (months) for self-affirmed GRAS. FDA review takes longer.	Minimum of 9 months for EFSA assessment, often extended. The total process can take several years.
Market authorization	No formal pre-market approval is required for GRAS. Market access upon determination of safety.	Formal authorization by the EC is required. The novel food is added to a "Union list" before it can be sold.

Table 5.
Comparison of US (GRAS) and EU (Novel Food) regulatory pathways.

can take 9 months or longer, with demanding data requirements, including whole genome sequencing of production microbes and testing for antimicrobial resistance genes [64].

The most profound challenge, however, is not navigating these existing frameworks but confronting their limitations. For a program like ReSource, which aims to create food from plastic waste, the current regulatory paradigm breaks down. Global food safety regulations are built on comparing a new food to an existing, conventional counterpart—a principle known as "substantial equivalence" [65, 66]. A protein powder derived from microbes that have consumed pyrolyzed polyethylene has no conventional counterpart. The concept is meaningless. This implies that the existing regulatory *process* is insufficient; a new *scientific methodology* for risk assessment must be invented. What tests can definitively prove that complex, novel breakdown products from plastic are not present or are harmless? How does one conduct toxicology studies for a substance never consumed by humans? The "regulatory challenge" is therefore not a bureaucratic problem to be solved after the technology is perfected. It is a fundamental scientific problem that must be solved *in parallel* with the engineering. The DoD and its partners must fund the development of this new regulatory science, or they risk creating a technically brilliant system that can never be legally used.

6.4 The social gauntlet: Overcoming psychological barriers to acceptance

Beyond technical and regulatory hurdles, the final gatekeeper for EMF-derived foods is the end-user. Public and consumer acceptance is shaped by a complex interplay of psychological factors, and overcoming innate skepticism is a critical mission [67, 68]. General consumer attitudes toward novel food technologies are often driven by heuristics rather than deep technical knowledge. Familiarity with the technology and trust in the institutions developing and regulating it are key predictors of acceptance [69].

Several specific psychological barriers are particularly relevant:

Food neophobia: An innate and powerful reluctance to try unfamiliar foods. This trait, which has evolutionary roots as a defense mechanism, is a major hurdle for products from entirely new sources like microbes grown on air or waste [70].

The "Ick Factor" and disgust: Disgust is a strong emotional response that can override rational assessments of benefit. It is particularly potent for foods derived from sources perceived as unclean, such as waste (as in the ReSource program), or processes seen as tampering with nature [71].

Perceptions of unnaturalness: This is a dominant concern for consumers regarding both cultured meat and precision fermentation products. The perception that a food is "unnatural" is a powerful negative heuristic that can be difficult to overcome with factual information alone [72].

Effective communication strategies are essential to navigate this social gauntlet. The approach must move beyond a "deficit model"—the assumption that simply providing more facts will change minds—to a more sophisticated, dialogue-based engagement. This involves acknowledging concerns with empathy and building trust. A powerful tool is values-based messaging, which connects the technology to widely held values like sustainability, health, environmental protection, and animal welfare, creating a foundation of shared interest before introducing the technical details [73]. Ultimately, communication must be tailored to the audience, transparently addressing perceived risks while clearly articulating the tangible benefits that matter most to them, whether that is taste, nutrition, or mission success [74].

7. Strategic outlook and future frontiers

7.1 The next horizon: Deep space exploration

The ultimate application and most stringent testing ground for EMF technology is long-duration human space exploration. The immense logistical challenge of sustaining a human presence on the Moon or Mars, where resupply from Earth is impractical, makes the development of self-sufficient, closed-loop systems an absolute necessity [75, 76]. Bioregenerative Life Support Systems (BLSS), which can reliably produce food, recycle water, and regenerate air, are an enabling technology for humanity's future in space. NASA's BioNutrients Program, which tests on-demand vitamin production on the ISS, and ESA's MELiSSA project, a closed-loop ecosystem based on microbial bioreactors, are at the forefront of this research [77]. The extreme environment of space serves as the ultimate technology accelerator; systems proven for a Mars mission will be exceptionally robust, efficient, and automated, making this research the high-performance testbed for the very systems needed on Earth [78, 79].

7.2 The hybrid food system: EMFs vs. adjacent technologies

Engineered Microbial Factories do not exist in a technological vacuum. They are part of a broader landscape of emerging food technologies [80, 81]. Cellular agriculture, which cultivates animal cells to create meat *in vitro*, is a promising field but is generally considered less mature and scalable than microbial fermentation due to the use of more delicate, slow-growing animal cells [82, 83]. Vertical farming, which grows produce like leafy greens indoors, is not a competitor but a highly complementary technology. An optimal, resilient food system is a hybrid one: An EMF bioreactor efficiently provides dense macronutrients (proteins, fats), while a co-located vertical farm supplements this with fresh produce, supplying essential micronutrients and a critical boost to morale [84, 85].

7.3 Environmental impact and sustainability

The environmental benefits of EMFs are profound. As demonstrated by lifecycle analyses of products like Solein, the technology offers a dramatic reduction in environmental impact compared to both animal and plant-based agriculture, with some estimates suggesting it is about 1% of the impact of meat production. Key benefits include a massive reduction in land and water usage, the elimination of fertilizers and pesticides, and, in the case of systems that use.

CO_2 as a feedstock, the potential for a negative carbon footprint [86]. The technology's inherent climate resilience and potential for circular economy integration position it as a key strategic tool in an era of increasing environmental instability [87, 88].

7.4 International cooperation and strategic competition

The field of synthetic biology and biomanufacturing is a critical arena for geopolitical competition. China has made massive, state-directed investments in synthetic biology, highlighting the strategic importance of maintaining a technological lead [89]. This reality necessitates strong international cooperation among allied nations to pool resources, share research, and establish common standards. Frameworks within

NATO for resilience, partnerships with international development organizations, and shared research through space agencies are critical mechanisms for maintaining a technological edge and ensuring the responsible deployment of this transformative technology [90, 91].

8. Conclusion

Engineered Microbial Factories represent a fundamental alteration of the strategic calculus for national security and crisis response, offering a credible path to shift from vulnerable, extended supply chains to resilient, on-demand production. This technology has moved beyond theoretical promise to a strategic inflection point, powered by converging advances in biotechnology and a robust commercial ecosystem. However, realizing this potential requires a decisive pivot from foundational research to applied development and deployment.

The critical path forward runs through three coequal, parallel lines of effort. First is Applied Engineering, focusing investment on integrated, containerized, and field-ready prototypes. This work must prioritize the development of low-power systems, closed-loop water recycling, full automation for operation by nonspecialists, and rigorous testing in simulated austere conditions. Second, and perhaps most critical for the most ambitious applications, is regulatory science. Proactive policy and funding are needed to create a streamlined regulatory framework. This requires a joint working group of the DoD, FDA, and USDA, not just to navigate existing rules, but to invent the new scientific methodologies needed to assess the safety of foods derived from unprecedented feedstocks like plastic waste. Third is Strategic Communication. The most advanced system is useless if soldiers or aid recipients refuse to consume its products. A concerted effort, grounded in social science, must be made to develop values-based communication strategies that build trust, address psychological barriers like neophobia, and ensure end-user acceptance.

By pursuing a deliberate strategy of focused investment in engineering, pioneering new regulatory science, and engaging in sophisticated communication, the promise of EMFs can be accelerated from prototype to deployed reality. This will secure a significant and enduring strategic advantage in operational endurance and global resilience.

Conflict of interest

The authors declare no conflict of interest.

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Advances in Functional Foods – New Perspectives and Sustainable Practices offers a comprehensive and forward-looking overview of current research in the rapidly evolving field of functional foods. Within the context of global challenges such as public health, food security, and environmental sustainability, the book examines how bioactive compounds, advanced processing technologies, enzyme-based innovations, and biotechnological approaches can be applied to develop health-supporting food systems. In doing so, the volume presents an integrated perspective that considers the contribution of functional foods to human health alongside principles of resource efficiency and responsible production. By bringing together diverse perspectives from food science, nutrition, biotechnology, and sustainable processing, this book aims to highlight emerging trends, practical solutions, and future directions in functional food research and development. As such, it serves as a valuable reference for researchers, graduate students, food technologists, and professionals working in related fields.

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