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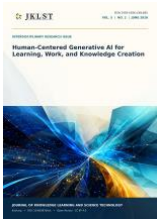

SYNERGISTIC EFFECTS OF TARGETED MULTI-ENZYME SYSTEMS IN BIORECOVERY OF SECONDARY METABOLITES: FROM MECHANISTIC INSIGHTS TO OPTIMIZATION

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Abstract

The efficient recovery of plant-derived secondary metabolites has become a major research priority in response to the growing demand for sustainable production of high-value functional ingredients within the circular bioeconomy. Enzyme-assisted extraction (EAE) has emerged as a promising green extraction technology because of its high substrate specificity, mild operating conditions, and ability to preserve thermolabile bioactive compounds. However, the heterogeneous architecture of plant cell walls often limits the effectiveness of individual enzymes, emphasizing the need for rationally designed targeted multi-enzyme systems. This review provides a comprehensive and mechanism-oriented overview of recent advances in targeted multi-enzyme-assisted extraction for the recovery of plant secondary metabolites. Particular attention is given to plant cell wall architecture, molecular mechanisms of enzyme synergism, sequential deconstruction of pectin, hemicellulose, and cellulose, and the release mechanisms of matrix-bound bioactive compounds. The review further discusses rational strategies for designing biomass-specific enzyme cocktails based on substrate composition, enzyme complementarity, catalytic compatibility, and hydrolysis strategies. Recent developments in response surface methodology, kinetic modeling, artificial intelligence, and machine learning are critically evaluated as advanced tools for predictive process optimization. Emerging technologies, including multi-enzyme co-immobilization, protein engineering, synthetic biology, continuous bioprocessing, and integrated biorefinery concepts, are also reviewed with respect to their potential for improving catalyst utilization, process scalability, and industrial sustainability. Overall, this review demonstrates the transition of enzyme-assisted extraction from empirical optimization toward knowledge-driven, precision-designed bioprocesses integrating molecular enzymology, process engineering, and computational intelligence, providing a scientific framework for sustainable biomass valorization and next-generation circular biorefineries.

Keywords: *Enzyme-assisted extraction; Targeted multi-enzyme systems; Enzyme synergism; Secondary metabolites; Process optimization*

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1. Introduction

The transition toward a circular bioeconomy has fundamentally transformed the perception of agricultural biomass from low-value waste into a strategic renewable resource for the sustainable production of high-value bioactive compounds. Growing environmental concerns, increasingly stringent regulations on green manufacturing, and the rising demand for naturally derived functional ingredients have accelerated the development of environmentally friendly extraction technologies capable of maximizing resource utilization while minimizing environmental impacts [1], [2], [3], [4], [5]. Within this context, agricultural residues and plant by-products are increasingly recognized as promising feedstocks for integrated biorefinery systems, providing abundant sources of polyphenols, flavonoids, carotenoids, alkaloids, terpenoids, and bioactive polysaccharides for pharmaceutical, nutraceutical, cosmetic, and functional food applications [6], [7].

Despite the enormous commercial potential of these secondary metabolites, their efficient recovery remains a significant technological challenge. Conventional extraction methods based on organic solvents generally require long extraction times, high solvent consumption, elevated energy input, and complex downstream purification, resulting in considerable environmental burdens and increased production costs [1], [3]. Although emerging physical techniques, including ultrasound-assisted extraction and microwave-assisted extraction, have substantially improved extraction efficiency, they may still induce thermal degradation, oxidation, or structural modification of thermolabile phytochemicals, particularly during industrial-scale processing [2], [8]. Consequently, the development of sustainable extraction strategies capable of preserving molecular integrity while improving extraction efficiency has become one of the major research priorities in modern food biotechnology.

Among currently available green extraction technologies, enzyme-assisted extraction (EAE) has emerged as one of the most promising approaches because it utilizes highly selective biological catalysts to disrupt structural barriers within plant tissues under relatively mild operating conditions. Compared with conventional extraction technologies, EAE offers numerous advantages, including high substrate specificity, reduced solvent consumption, lower energy requirements, improved extraction selectivity, and superior preservation of bioactive compound stability [4], [7], [9], [10]. These advantages have positioned EAE as an essential enabling technology for sustainable biomass valorization and green biorefinery development.

The extraction performance of EAE is primarily determined by the complex structural organization of plant cell walls. Plant tissues consist of highly heterogeneous composite matrices in which cellulose microfibrils are embedded within interconnected networks of hemicellulose, pectin, lignin, and structural proteins. This sophisticated three-dimensional architecture functions as a mechanical barrier that restricts solvent penetration and limits the release of intracellular metabolites [11], [12]. Because the chemical composition and ultrastructure of cell walls vary considerably among plant species, tissues, and developmental stages, extraction efficiency largely depends on the compatibility between enzyme specificity and substrate architecture.

For many years, enzyme-assisted extraction research mainly focused on empirical optimization of operational parameters, including enzyme concentration, extraction temperature, pH, liquid-

to-solid ratio, and extraction time [8], [9]. Although these studies demonstrated significant improvements in extraction yield, they generally employed individual commercial enzymes and treated enzymatic hydrolysis as an isolated catalytic event rather than a coordinated biological process. Consequently, many optimized extraction protocols remain substrate-specific and often exhibit limited scalability and reproducibility during industrial implementation [4], [13].

Recent advances in plant cell wall biology and enzymology have shifted the research paradigm toward the rational design of targeted multi-enzyme systems. Rather than relying on individual enzymes, modern enzyme cocktails are engineered according to the hierarchical organization of cell wall polymers to maximize catalytic cooperation. Within these systems, pectinases initially degrade the middle lamella and increase cell wall porosity, thereby facilitating the penetration of cellulases and hemicellulases into deeper structural layers. The sequential and cooperative degradation of different polysaccharide components substantially accelerates biomass deconstruction, enhances mass transfer, and increases metabolite release beyond the additive effects of individual enzymes [12], [14]. Such synergistic hydrolysis has become the fundamental principle underlying next-generation enzyme cocktail engineering.

In parallel with advances in enzymology, mathematical optimization and digital technologies have significantly expanded the capability of EAE process design. Response surface methodology (RSM), artificial neural networks, machine learning algorithms, and kinetic modeling have been increasingly employed to predict extraction performance, optimize enzyme formulations, and minimize experimental costs [15], [16]. Furthermore, recent developments in enzyme immobilization, enzyme recycling, and integrated continuous bioprocesses have greatly improved the economic feasibility and industrial applicability of enzyme-assisted extraction, supporting the transition from laboratory-scale research toward commercial biorefinery production [5], [17].

Despite these remarkable advances, current review articles generally address molecular enzymatic mechanisms, statistical optimization, kinetic modeling, enzyme immobilization, and industrial applications as separate research topics. Comprehensive integration of these disciplines into a unified framework that links plant cell wall architecture, enzyme synergism, process optimization, kinetic prediction, and industrial scalability remains limited [4], [5]. In particular, few reviews systematically explain how molecular-level understanding can guide the rational design of enzyme cocktails while simultaneously supporting predictive process engineering and techno-economic optimization.

Therefore, this review provides a comprehensive and mechanism-oriented overview of targeted multi-enzyme systems for the recovery of plant-derived secondary metabolites. Particular emphasis is placed on molecular mechanisms of enzyme synergism, structure-guided enzyme cocktail engineering, advanced statistical optimization, kinetic modeling, enzyme immobilization, and industrial biorefinery integration. By bridging molecular enzymology with process engineering and industrial implementation, this review proposes a comprehensive technological roadmap for developing highly efficient, economically sustainable, and environmentally responsible enzyme-assisted biorecovery systems suitable for next-generation circular bioeconomy applications.

2. Advanced Extraction and Bio-valorization Strategies

To objectively evaluate the technical prospects and strategic positioning of enzyme-assisted extraction (EAE) within downstream bioprocess engineering, this technology should be considered within a comprehensive framework that compares conventional extraction methods with emerging green and advanced extraction technologies. The evolution of extraction engineering has been driven by three fundamental objectives: maximizing extraction yield, preserving the structural integrity and biological activity of target metabolites, and minimizing environmental impacts through sustainable processing technologies [1], [3], [4], [5].

2.1. Conventional extraction methodologies and architectural bottlenecks

Conventional extraction techniques, including Soxhlet extraction, heat reflux extraction, and solid–liquid maceration, rely primarily on passive diffusion and concentration gradients between plant tissues and extraction solvents. Mass transfer is generally enhanced through prolonged heating and solvent recirculation, allowing intracellular compounds to gradually diffuse into the surrounding medium [1], [2]. Although these methods remain widely employed because of their operational simplicity and relatively low equipment costs, they present several intrinsic limitations that hinder their application in modern sustainable bioprocesses.

First, prolonged exposure to elevated temperatures frequently induces oxidation, hydrolysis, isomerization, and thermal degradation of thermolabile bioactive compounds, leading to substantial losses in antioxidant activity and biological functionality, particularly for polyphenols and other oxidation-sensitive phytochemicals [3], [6], [7]. Second, conventional extraction generally requires large volumes of organic solvents, such as methanol, ethanol, acetone, hexane, or chloroform, thereby increasing solvent recovery costs, environmental burdens, and the potential risk of residual solvent contamination in final products [1], [3]. Consequently, conventional solvent extraction increasingly conflicts with the principles of green chemistry and sustainable industrial production.

2.2. Advanced physical modalities and energy-intensive barriers

To overcome the diffusion limitations associated with conventional solvent extraction, numerous advanced physical extraction technologies have been developed, including ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), and supercritical fluid extraction (SFE). These technologies significantly shorten extraction time while improving extraction efficiency through enhanced disruption of plant cellular structures [2], [4], [8].

Ultrasound-assisted extraction enhances mass transfer primarily through acoustic cavitation, where the formation and collapse of microscopic bubbles generate localized shear forces capable of disrupting plant tissues and increasing solvent penetration. Microwave-assisted extraction accelerates extraction by rapidly heating intracellular water molecules, producing internal pressure that promotes cell rupture and metabolite release [1], [3]. Despite these advantages, both technologies may generate localized hot spots and reactive oxygen species that promote oxidation or structural degradation of highly sensitive bioactive compounds, thereby reducing product quality under inappropriate processing conditions [4], [6].

Supercritical fluid extraction, particularly using supercritical carbon dioxide ($scCO_2$), has attracted considerable attention because it produces solvent-free extracts with excellent purity

while operating under relatively mild temperatures. However, its industrial application remains constrained by the high capital investment required for high-pressure equipment and its relatively poor extraction efficiency toward polar phytochemicals unless suitable co-solvents are incorporated into the extraction system [1], [3], [4].

2.3. Enzyme-assisted extraction (EAE) – A green, target-specific, and mild deconstruction pathway

In contrast to the non-specific mechanical disruption employed by physical extraction technologies, enzyme-assisted extraction (EAE) utilizes highly selective biocatalytic reactions to achieve controlled and targeted deconstruction of plant biomass. Rather than indiscriminately disrupting cellular structures through external physical energy, EAE selectively hydrolyzes structural polysaccharides and the molecular linkages that entrap intracellular bioactive compounds, thereby facilitating metabolite release while preserving their structural integrity [4], [9], [12].

The scientific and technological advantages of EAE arise from several distinctive characteristics. First, enzymatic hydrolysis is typically performed under relatively mild operating conditions, generally at temperatures of 40–55 °C and slightly acidic pH values, thereby minimizing thermal degradation, oxidation, and structural modification of thermolabile phytochemicals. Consequently, the biological activity, stereochemical configuration, and antioxidant capacity of released metabolites are better preserved than those obtained using conventional thermal extraction methods [4], [7], [9].

Second, owing to the remarkable substrate specificity of carbohydrate-active enzymes, the hydrolytic process selectively targets structural components of the plant cell wall, including cellulose, hemicellulose, and pectin, while minimizing undesirable degradation of the target bioactive compounds. This selective degradation substantially reduces the co-extraction of non-functional macromolecules, facilitating downstream purification and improving overall process efficiency [11], [12].

Third, EAE predominantly employs aqueous media as the extraction solvent, significantly reducing or even eliminating the requirement for hazardous organic solvents. Together with lower energy consumption and reduced environmental emissions, these characteristics position EAE as one of the most sustainable extraction technologies currently available for biomass valorization and green biorefinery development [3], [5], [7], [17].

Beyond its environmental benefits, EAE is increasingly recognized as a key enabling technology for integrated biorefinery systems because it can be readily combined with complementary green extraction techniques, statistical optimization approaches, enzyme immobilization strategies, and continuous processing technologies. Such integration substantially improves extraction efficiency, process economics, and industrial scalability while supporting the transition toward sustainable circular bioeconomy models based on comprehensive biomass utilization [4], [5], [15], [16].

3. Molecular Mechanisms Governing Targeted Multi-Enzyme-Assisted Extraction

The extraction efficiency of enzyme-assisted extraction (EAE) is fundamentally determined by the complex interactions between plant cell wall architecture, enzyme specificity, substrate

accessibility, and mass transfer phenomena. Unlike conventional solvent extraction or physical disruption techniques, EAE employs highly selective carbohydrate-active enzymes (CAZymes) to progressively dismantle the structural polysaccharide network surrounding intracellular metabolites. This targeted enzymatic deconstruction enhances solvent penetration, reduces diffusion resistance, and facilitates the release of both free and matrix-bound bioactive compounds while preserving their structural integrity under mild processing conditions [4], [9], [12].

3.1. Structural organization of plant cell walls before and after enzymatic hydrolysis

3.1.1. Native architecture of plant cell walls

Plant cell walls are sophisticated composite materials that provide both mechanical support and protection while simultaneously regulating the transport of water, nutrients, and metabolites. Their hierarchical organization creates one of the principal barriers limiting the efficient extraction of intracellular phytochemicals. Primary cell walls consist predominantly of crystalline cellulose microfibrils embedded within amorphous matrices of hemicellulose and pectin, whereas secondary cell walls additionally contain substantial amounts of lignin that further increase mechanical rigidity and resistance to enzymatic degradation [11], [12].

Cellulose represents the primary structural scaffold of the cell wall. Individual cellulose chains are linked through extensive intra- and intermolecular hydrogen bonds, assembling into highly ordered crystalline microfibrils with exceptional tensile strength. These microfibrils are surrounded by branched hemicellulosic polysaccharides, primarily xylans, mannans, and arabinoxylans, which act as molecular cross-links between adjacent cellulose fibrils. The entire network is further embedded within a hydrated pectin matrix that occupies the middle lamella and functions as a biological adhesive maintaining cell-to-cell cohesion [4], [11], [12].

This highly cross-linked three-dimensional architecture severely restricts solvent penetration and creates substantial diffusion resistance during extraction. Consequently, numerous intracellular secondary metabolites—including polyphenols, flavonoids, carotenoids, anthocyanins, alkaloids, and bioactive polysaccharides—remain physically entrapped within intact cellular compartments or chemically associated with structural polysaccharides through hydrogen bonding, hydrophobic interactions, and ester linkages [3], [6], [7].

3.1.2. Sequential structural disassembly during multi-enzyme hydrolysis

Unlike mechanical extraction techniques that disrupt tissues indiscriminately, multi-enzyme-assisted extraction proceeds through a coordinated sequence of catalytic reactions in which different carbohydrate-active enzymes act synergistically to dismantle specific structural components of the plant cell wall. The progressive degradation of pectin, hemicellulose, and cellulose substantially increases wall porosity, weakens mechanical integrity, and improves accessibility of intracellular metabolites [12].

Pectin degradation and initiation of cell wall loosening: The enzymatic process generally begins with pectin degradation. Pectinases—including polygalacturonases, pectin lyases, and pectin methylesterases—hydrolyze α-1,4-glycosidic linkages within homogalacturonan and other pectic domains. Depolymerization of the middle lamella weakens intercellular adhesion, decreases matrix viscosity, and markedly increases cell wall porosity. As a result, steric

hindrance surrounding cellulose and hemicellulose is substantially reduced, allowing subsequent enzymes to penetrate deeper into the lignocellulosic network [12].

Hemicellulose degradation and exposure of cellulose microfibrils: Following pectin degradation, hemicellulases—particularly xylanases, mannanases, and arabinofuranosidases—hydrolyze branched hemicellulosic polysaccharides that tether cellulose microfibrils together. The removal of hemicellulose disrupts intermolecular cross-linking, exposes previously inaccessible cellulose surfaces, and significantly increases enzyme accessibility to the structural scaffold of the cell wall [4], [12].

Cellulose degradation and breakdown of the structural framework: Cellulose hydrolysis is subsequently performed by a coordinated cellulase system comprising endoglucanases, cellobiohydrolases, and β-glucosidases. Endoglucanases randomly cleave internal β-1,4-glucosidic bonds within amorphous cellulose regions, whereas cellobiohydrolases progressively hydrolyze chain ends to release cellobiose. Finally, β-glucosidases convert soluble oligosaccharides into glucose, preventing product inhibition and sustaining continuous hydrolysis. The combined action of these enzymes progressively dismantles both amorphous and partially crystalline cellulose domains, resulting in collapse of the structural framework supporting the plant cell wall [12].

Morphological consequences and enhancement of mass transfer: Sequential degradation of pectin, hemicellulose, and cellulose produces profound structural alterations that can be observed using scanning electron microscopy (SEM), confocal microscopy, and atomic force microscopy. Typical morphological changes include surface erosion, formation of numerous micro-pores, enlargement of existing pores, fragmentation of cell wall fragments, and eventual collapse of cellular architecture. These modifications substantially shorten diffusion pathways, increase solvent accessibility, reduce internal mass transfer resistance, and facilitate rapid release of intracellular phytochemicals into the extraction medium [4], [15], [17].

3.2. Molecular synergism among carbohydrate-active enzymes

The superior performance of enzyme-assisted extraction does not arise solely from the catalytic activity of individual enzymes but rather from the synergistic interactions established among multiple carbohydrate-active enzymes. Such cooperation enables sequential removal of structural barriers, improves substrate accessibility, and enhances catalytic efficiency beyond the additive effects of individual enzymes [12].

Pectinase–cellulase synergism: Pectinases initiate cell wall loosening by degrading the middle lamella, thereby increasing wall permeability and exposing cellulose microfibrils. This preliminary modification significantly enhances cellulase adsorption and catalytic efficiency, resulting in improved cellulose hydrolysis and metabolite release [12].

Cellulase–hemicellulase cooperation: Hemicellulose forms a physical barrier surrounding cellulose microfibrils. Xylanases remove this barrier, enabling cellulases to access cellulose more efficiently. Simultaneously, cellulase activity further exposes residual hemicellulose, creating a positive feedback mechanism that accelerates biomass deconstruction [4].

Accessory enzymes: Accessory enzymes—including feruloyl esterases, acetyl xylan esterases, and arabinofuranosidases—hydrolyze ester bonds and side-chain substitutions that limit

accessibility of the primary hydrolytic enzymes. Although present in relatively small proportions, these enzymes substantially improve overall hydrolysis efficiency by removing steric constraints within the cell wall matrix [12].

Rational design of enzyme cocktails: Recent advances emphasize the rational engineering of enzyme cocktails according to biomass composition rather than relying on individual commercial enzymes. Tailoring enzyme combinations to the structural characteristics of specific plant materials has emerged as a promising strategy for maximizing extraction efficiency while reducing enzyme dosage and processing costs [5], [15], [16].

3.3. Mechanisms of bioactive compound release

The progressive dismantling of plant cell wall architecture ultimately facilitates the release of intracellular bioactive compounds through several complementary mechanisms. Free metabolites are rapidly liberated following membrane disruption, whereas cell wall-bound compounds require enzymatic cleavage of hydrogen bonds, ester linkages, or polysaccharide associations before becoming extractable. Consequently, EAE substantially increases the recovery of polyphenols, flavonoids, carotenoids, anthocyanins, and other phytochemicals that are otherwise inaccessible using conventional solvent extraction [4], [6], [7].

4. Molecular Mechanisms of Bioactive Compound Release During Enzyme-Assisted Extraction

Unlike the structural deconstruction mechanisms discussed in the previous section, the ultimate objective of enzyme-assisted extraction is the efficient liberation of intracellular bioactive compounds from the plant matrix. Following enzymatic degradation of structural polysaccharides, a series of molecular events govern the transformation of bound phytochemicals into extractable soluble forms. These events involve disruption of intermolecular interactions, cleavage of covalent linkages, enhanced solvent accessibility, and accelerated mass transfer across the partially degraded cell wall [4], [12].

Molecular release of matrix-bound phytochemicals: Many phytochemicals exist in plant tissues not as freely soluble molecules but as structural complexes associated with polysaccharides, proteins, or lignin. Polyphenols, tannins, flavonoids, and phenolic acids frequently interact with cell wall polymers through hydrogen bonding, hydrophobic interactions, ionic interactions, and ester or ether linkages, which substantially reduce their extractability under conventional solvent extraction [6], [7].

During enzyme-assisted extraction, progressive degradation of surrounding polysaccharides destabilizes these molecular associations. Simultaneously, accessory enzymes—including esterases and proteases—hydrolyze ester bonds and protein–phenolic complexes, facilitating conversion of bound metabolites into soluble forms that readily diffuse into the extraction medium [12].

Phase transition from bound to free bioactive forms: A major advantage of enzyme-assisted extraction lies in its ability to convert matrix-bound bioactive compounds into freely soluble molecules. Enzymatic cleavage of structural linkages disrupts the physicochemical interactions responsible for metabolite immobilization, allowing previously inaccessible phytochemicals to become available for solvent extraction. This transformation is particularly important for

insoluble-bound phenolics, condensed tannins, and esterified phenolic acids, which typically exhibit poor recovery using conventional extraction methods [6], [7]. Rather than generating new bioactive compounds, EAE increases the proportion of extractable metabolites by enhancing molecular accessibility while preserving their native chemical structures under mild processing conditions [3], [4].

Enhancement of solubilization and mass transfer: Following molecular liberation, extraction efficiency becomes controlled primarily by diffusion and solubilization processes. Enzymatic degradation increases cell wall porosity, shortens diffusion pathways, and facilitates solvent penetration into intracellular compartments. These structural modifications increase the effective mass transfer coefficient and accelerate transport of liberated phytochemicals toward the bulk extraction medium [4], [15]. Because enzymatic extraction is generally performed under relatively mild temperatures and aqueous conditions, oxidation, isomerization, and thermal degradation of thermolabile compounds are substantially reduced. Consequently, higher recovery yields are achieved while maintaining the structural integrity and biological activity of sensitive metabolites [7], [9].

Integrated molecular model of bioactive release: The molecular release of plant bioactive compounds during enzyme-assisted extraction may be summarized as a sequential process involving: (i) enzymatic degradation of structural polysaccharides; (ii) increased cell wall porosity and solvent accessibility; (iii) disruption of intermolecular interactions retaining phytochemicals; (iv) conversion of bound metabolites into soluble free forms; and (v) accelerated diffusion of liberated compounds into the extraction medium. The coordinated progression of these events explains the superior extraction efficiency achieved by targeted multi-enzyme systems compared with conventional solvent extraction and single-enzyme treatments [5], [15], [16].

5. Rational design and optimization of targeted multi-enzyme systems

5.1. Rational selection of enzyme cocktails based on biomass characteristics

The successful implementation of enzyme-assisted extraction (EAE) relies not only on the catalytic efficiency of individual enzymes but also on the rational selection of enzyme combinations according to the structural characteristics of the target biomass. Plant-derived biomass exhibits remarkable diversity in cell wall composition, polymer architecture, lignification degree, and metabolite localization. Consequently, no universal enzyme cocktail is capable of efficiently processing all biomass types. Instead, the selection of enzyme systems should be guided by detailed characterization of substrate composition, structural organization, and the physicochemical interactions governing metabolite retention within the cell wall matrix [11], [12].

The first step in enzyme cocktail design involves comprehensive characterization of biomass composition. Cellulose, hemicellulose, pectin, lignin, structural proteins, and other accessory polymers contribute differently to the mechanical stability of plant tissues depending on botanical origin, developmental stage, and processing history. Fruit peels generally possess pectin-rich primary cell walls, cereal residues are dominated by cellulose and arabinoxylan, whereas woody biomass contains substantial amounts of lignin that markedly restrict enzyme accessibility. Marine biomass, in contrast, exhibits completely different polysaccharide

compositions, including alginate, laminarin, fucoidan, and carrageenan, requiring specialized hydrolytic enzymes rather than conventional cellulases or pectinases [4], [17], [5].

To accurately determine biomass composition, a combination of compositional analysis and structural characterization techniques is commonly employed. Quantitative fiber analysis based on detergent fiber fractionation provides estimates of cellulose, hemicellulose, and lignin contents, whereas Fourier-transform infrared spectroscopy (FTIR) enables rapid identification of characteristic functional groups associated with structural polysaccharides and phenolic cross-links. More advanced analytical tools—including X-ray diffraction (XRD), Raman spectroscopy, nuclear magnetic resonance (NMR), scanning electron microscopy (SEM), confocal laser scanning microscopy (CLSM), and atomic force microscopy (AFM)—provide complementary information regarding cellulose crystallinity, polymer organization, surface morphology, and microstructural integrity. Together, these analytical approaches establish the structural foundation required for rational enzyme selection and process optimization [11], [15], [16].

Beyond overall polymer composition, understanding the localization of target bioactive compounds is equally important. Polyphenols may occur as free molecules within vacuoles, as soluble conjugates, or as insoluble-bound forms associated with cellulose, hemicellulose, lignin, or structural proteins through hydrogen bonding, hydrophobic interactions, and ester linkages. Similarly, carotenoids are primarily localized within chromoplasts, whereas many bioactive polysaccharides remain embedded within the cell wall matrix itself. These differences strongly influence enzyme selection because each metabolite class requires distinct mechanisms for efficient release. Consequently, enzyme cocktails should be designed not only to degrade structural polysaccharides but also to disrupt the specific molecular interactions responsible for metabolite immobilization [6], [7].

The relationship between biomass characteristics and enzyme selection has become increasingly substrate-specific (Table 1). Biomass rich in pectin, such as citrus peel, mango peel, passion fruit peel, apple pomace, and sugar beet pulp, generally requires pectinase-dominant enzyme systems supplemented with cellulases to enhance tissue disintegration. Conversely, cereal by-products, spent coffee grounds, corn stover, and wheat bran contain higher proportions of cellulose and arabinoxylan, making cellulase–xylanase combinations more appropriate. Marine biomass, including brown seaweeds, requires entirely different enzyme systems such as alginate lyases and laminarinases because conventional plant cell wall-degrading enzymes exhibit limited activity toward algal polysaccharides [4], [17], [5].

Recent advances in enzyme cocktail engineering have shifted the design philosophy from empirical enzyme selection toward knowledge-driven formulation based on substrate architecture. Instead of relying on individual commercial enzymes, researchers increasingly integrate compositional analysis, structural characterization, enzyme specificity, and statistical optimization to construct targeted enzyme systems tailored to particular biomass types. This strategy not only improves extraction efficiency but also reduces enzyme consumption, minimizes processing costs, and enhances scalability for industrial biorefinery applications [15], [16].

Table 1. Structural characteristics of representative biomass and corresponding enzyme systems recommended for enzyme-assisted extraction.

Biomass	Major structural barrier	Major bioactive compounds	Recommended enzymes	Design rationale
Citrus peel	Pectin-rich cell wall	Flavanones, phenolic acids	Pectinase + Cellulase	Degradation of middle lamella enhances solvent penetration
Apple pomace	Pectin + Cellulose	Polyphenols	Pectinase + Cellulase	Increased porosity and release of bound phenolics
Mango peel	Pectin + Cellulose + Protein	β-Carotene, mangiferin	Pectinase + Cellulase + Protease	Cell wall disruption and protein complex hydrolysis
Passion fruit peel	Highly esterified pectin	Pectin, flavonoids	Pectinase + Cellulase	Pectin depolymerization
Coffee husk	Cellulose + Arabinoxylan	Chlorogenic acids	Cellulase + Xylanase	Hemicellulose degradation
Spent coffee grounds	Cellulose + Galactomannan	Polyphenols	Cellulase + Mannanase	Cell wall loosening
Wheat bran	Arabinoxylan	Ferulic acid	Xylanase + Feruloyl esterase	Cleavage of ester linkages
Corn stover	Lignocellulose	Phenolic compounds	Cellulase + Xylanase	Structural deconstruction
Tomato pomace	Pectin + Cutin	Lycopene	Pectinase + Cellulase	Improved chromoplast accessibility
Grape pomace	Cellulose + Tannin complexes	Anthocyanins	Cellulase + Pectinase + Tannase	Release of bound polyphenols
Brown seaweed	Alginate	Phlorotannins	Alginate lyase	Alginate depolymerization
Red seaweed	Carrageenan	Sulfated polysaccharides	Carrageenase	Cell wall degradation

5.2. Design principles for targeted multi-enzyme cocktails

The superior performance of targeted multi-enzyme systems arises not merely from combining multiple hydrolytic enzymes but from the rational engineering of enzyme cocktails that maximize catalytic cooperation while minimizing operational incompatibilities. The effectiveness of an enzyme cocktail is determined by several interrelated factors, including enzyme specificity, catalytic complementarity, dosage ratio, mode of enzyme addition, substrate accessibility, and compatibility of reaction conditions. Optimizing these parameters is essential for achieving efficient biomass deconstruction, enhanced bioactive compound recovery, and economically viable industrial processes [12], [16].

Selection of complementary enzyme classes: The first principle in enzyme cocktail engineering is the selection of complementary enzymes that target different structural components of the biomass simultaneously. Because plant cell walls comprise multiple interconnected polymers,

efficient hydrolysis requires coordinated degradation of pectin, hemicellulose, cellulose, and, in some cases, protein or ester linkages. Enzyme combinations should therefore be selected according to substrate composition rather than relying on a single dominant enzyme. For pectin-rich biomass such as citrus peels or mango peels, pectinases serve as the primary hydrolytic enzymes by degrading the middle lamella and increasing tissue permeability. Cellulases subsequently hydrolyze exposed cellulose microfibrils, whereas accessory enzymes such as proteases or feruloyl esterases facilitate the release of protein-bound or esterified phenolic compounds. Conversely, lignocellulosic residues containing abundant cellulose and arabinoxylan generally require cellulase–xylanase combinations, while marine biomass benefits from alginate lyases, agarases, or carrageenases specifically adapted to algal polysaccharides [4], [5], [17]. This complementary enzyme selection ensures that each catalyst performs a distinct yet cooperative function, thereby maximizing substrate accessibility and minimizing redundant catalytic activity.

Optimization of enzyme ratios: Beyond enzyme selection, determining the appropriate proportion of each enzyme within the cocktail is critical for maximizing synergistic hydrolysis. Excessive loading of one enzyme rarely improves extraction efficiency if another structural barrier remains intact. Instead, enzyme ratios should reflect the relative abundance of structural polymers within the target biomass. For example, biomass rich in pectin generally benefits from pectinase-dominant formulations, whereas cellulose-rich agricultural residues require higher cellulase activities supplemented by hemicellulases. Statistical optimization methods such as Response Surface Methodology (RSM), Box–Behnken Design (BBD), Central Composite Design (CCD), and mixture design experiments have become widely adopted for identifying optimal enzyme ratios while minimizing experimental effort and enzyme consumption [15], [16]. Rather than maximizing individual enzyme activity, these optimization strategies aim to maximize overall system performance by identifying synergistic interactions among multiple catalytic components.

Simultaneous versus sequential enzymatic hydrolysis: The mode of enzyme application also significantly influences extraction performance. In simultaneous hydrolysis, all enzymes are added at the beginning of the process, allowing concurrent degradation of multiple structural polymers. This approach generally reduces processing time, simplifies operation, and is well suited for industrial-scale production when the selected enzymes exhibit compatible operating conditions.

Alternatively, sequential hydrolysis applies enzymes in a predefined order according to substrate accessibility. Pectinases are often introduced first to disrupt the middle lamella and increase tissue permeability, followed by hemicellulases and cellulases that progressively degrade the exposed structural network. Sequential processing can improve catalytic efficiency for highly recalcitrant biomass by minimizing steric hindrance before cellulase action, although it typically increases operational complexity and processing time [12]. The choice between simultaneous and sequential hydrolysis therefore depends on biomass composition, enzyme compatibility, production scale, and economic considerations rather than a universally superior strategy.

Operational compatibility of multi-enzyme systems: Efficient multi-enzyme hydrolysis requires that all enzymes operate within sufficiently overlapping reaction conditions. Parameters including pH, temperature, ionic strength, reaction time, and substrate concentration influence not only the catalytic activity of individual enzymes but also the overall stability of the enzyme cocktail. Commercial fungal cellulases, hemicellulases, and pectinases commonly exhibit optimal activity under mildly acidic conditions (approximately pH 4.5–5.5) and moderate temperatures ranging from 40 to 55 °C. Maintaining reaction conditions within this shared operational window enables simultaneous enzyme activity while minimizing thermal denaturation and preserving the stability of thermolabile bioactive compounds [3], [4]. Another important consideration is the prevention of product inhibition. During cellulose hydrolysis, accumulation of cellobiose inhibits cellulase activity, whereas β-glucosidase rapidly converts cellobiose into glucose, thereby sustaining continuous hydrolysis. Similar complementary relationships exist among other enzyme classes, emphasizing that operational compatibility extends beyond environmental conditions to include coordinated catalytic turnover and efficient intermediate utilization [12].

Integration of statistical and artificial intelligence-based optimization: Recent developments have shifted enzyme cocktail engineering from empirical trial-and-error approaches toward predictive optimization supported by computational tools. Classical statistical techniques, including RSM, BBD, and CCD, remain widely used for optimizing enzyme dosage, reaction time, pH, and temperature. More recently, machine learning algorithms, artificial neural networks (ANNs), and hybrid optimization models have demonstrated increasing potential for predicting enzyme interactions, identifying optimal reaction conditions, and reducing the number of required experiments. These data-driven approaches facilitate rapid optimization of complex multi-enzyme systems while improving process robustness and scalability, making them increasingly attractive for industrial biorefinery applications [5], [15], [16]. Representative examples illustrating the relationship between biomass characteristics, enzyme cocktails, catalytic mechanisms, and extraction performance are summarized in Table 2.

Table 2. Rational selection of enzyme cocktails according to biomass structural characteristics in enzyme-assisted extraction

Biomass	Major structural barrier	Dominant bioactive compounds	Recommended enzyme cocktail	Primary mechanism of action	Expected extraction improvement	Key references
Citrus peel	Pectin-rich middle lamella	Flavanones, phenolic acids	Pectinase + Cellulase	Pectin depolymerization and cellulose hydrolysis	Increased permeability and flavonoid recovery	4
Mango peel	Pectin + cellulose + protein complexes	β-Carotene, mangiferin	Pectinase + Cellulase + Protease	Cell wall disruption and protein complex degradation	Enhanced carotenoid release	17
Coffee grounds	Cellulose + galactomannan	Chlorogenic acids	Cellulase + Mannanase + Xylanase	Hemicellulose degradation	Improved chlorogenic	15

					acid extraction	
Apple pomace	Pectin-rich primary wall	Polyphenols	Pectinase + Cellulase	Increased porosity	Enhanced release of bound phenolics	12
Brown seaweed	Alginate matrix	Phlorotannins	Alginate lyase + Cellulase	Alginate depolymerization	Improved phlorotannin recovery	5

5.3. Emerging strategies in multi-enzyme cocktail engineering

The development of targeted multi-enzyme systems has evolved substantially over the past decade, shifting from empirical enzyme combinations toward knowledge-driven engineering strategies supported by advanced analytical techniques, computational optimization, and integrated biorefinery concepts. Rather than focusing solely on maximizing extraction yield, current research emphasizes improving process robustness, enzyme utilization efficiency, sustainability, and industrial scalability. These emerging approaches are expected to play a pivotal role in expanding the application of enzyme-assisted extraction across food, pharmaceutical, cosmetic, and nutraceutical industries [5], [15], [16].

Immobilized multi-enzyme systems: One of the major limitations of conventional soluble enzymes is their limited operational stability and inability to be economically recovered after extraction. Enzyme immobilization has therefore emerged as an effective strategy for improving catalyst stability, facilitating enzyme recycling, and reducing production costs. Immobilization techniques—including adsorption, covalent attachment, entrapment, encapsulation, and carrier-free cross-linked enzyme aggregates (CLEAs)—allow enzymes to retain catalytic activity over multiple reaction cycles while improving resistance to thermal denaturation and pH fluctuations. Multi-enzyme co-immobilization further enables spatial proximity between complementary enzymes, facilitating rapid transfer of intermediate products and improving overall catalytic efficiency. Such systems are particularly attractive for continuous processing and industrial-scale extraction where catalyst reuse significantly influences economic feasibility [12], [15]. Nevertheless, immobilization may also introduce diffusional limitations or reduce enzyme flexibility, making careful selection of support materials and immobilization chemistry essential for maintaining catalytic performance.

Artificial intelligence and data-driven optimization: Optimization of multi-enzyme systems traditionally relies on response surface methodology (RSM), central composite design (CCD), and Box–Behnken design (BBD). Although these statistical approaches remain highly effective, they become increasingly challenging when numerous enzymes and interacting variables are simultaneously optimized. Recent advances in artificial intelligence (AI) and machine learning provide powerful alternatives for modeling highly nonlinear relationships between biomass characteristics, enzyme composition, reaction parameters, and extraction performance. Artificial neural networks (ANNs), random forest algorithms, support vector machines, Bayesian optimization, and genetic algorithms have demonstrated considerable potential for predicting optimal enzyme combinations while substantially reducing experimental workload. Integration of AI with high-throughput screening technologies further enables rapid identification of enzyme formulations tailored to specific biomass compositions,

accelerating process development and improving reproducibility across different raw materials [5], [15], [16].

Continuous bioprocessing and process intensification: Industrial implementation of enzyme-assisted extraction increasingly favors continuous rather than batch operation. Continuous bioprocessing provides improved productivity, more consistent product quality, and reduced energy consumption through better control of reaction conditions and mass transfer. Integration of immobilized enzymes into packed-bed, membrane, or continuous-flow reactors allows prolonged catalyst utilization while minimizing enzyme loss. Moreover, coupling enzyme-assisted extraction with membrane separation, ultrasound-assisted extraction, microwave-assisted extraction, pulsed electric fields, or supercritical fluid extraction has created hybrid extraction platforms capable of combining the selectivity of enzymatic hydrolysis with the enhanced mass transfer provided by physical technologies. Such process intensification strategies improve extraction efficiency while maintaining mild operating conditions that preserve sensitive bioactive compounds [4], [17].

Integration into sustainable biorefinery systems: Perhaps the most significant trend in enzyme-assisted extraction is its integration into circular biorefinery platforms. Rather than targeting a single product, modern biorefineries seek to sequentially recover multiple high-value compounds from the same biomass, thereby maximizing resource utilization and minimizing waste generation.

Within this framework, enzyme-assisted extraction often serves as the initial valorization step, enabling selective recovery of polyphenols, carotenoids, polysaccharides, proteins, or functional oligosaccharides before the remaining biomass undergoes subsequent conversion into biofuels, biofertilizers, biodegradable materials, or biochar. Such cascading utilization strategies are fully aligned with the principles of the circular bioeconomy and contribute substantially to improving both environmental sustainability and economic profitability [9], [15], [16]. The compatibility of enzyme-assisted extraction with aqueous processing, renewable catalysts, and low-energy operation further reinforces its importance as a core technology within next-generation sustainable biorefineries.

Future perspectives for precision enzyme engineering: Future advances in enzyme-assisted extraction are expected to focus on precision enzyme engineering through integration of enzyme discovery, protein engineering, systems biology, and computational modeling. Metagenomics and synthetic biology are expanding the diversity of available carbohydrate-active enzymes, while protein engineering enables the development of enzymes with enhanced thermal stability, broader substrate specificity, and improved catalytic efficiency. Simultaneously, molecular docking, molecular dynamics simulations, and digital twin technologies are beginning to facilitate predictive design of enzyme cocktails by modeling enzyme–substrate interactions before experimental validation. These approaches are expected to substantially shorten process development cycles while enabling customized enzyme formulations for increasingly diverse biomass resources. As computational tools continue to evolve, enzyme cocktail engineering is likely to transition from empirical optimization toward predictive, data-driven design capable of supporting highly efficient and sustainable industrial

bioprocesses [5], [16]. The major emerging engineering strategies that are expected to shape the next generation of enzyme-assisted extraction systems are summarized in Table 3.

Table 3. Emerging engineering strategies for next-generation targeted multi-enzyme-assisted extraction systems

Strategy	Scientific principle	Major advantages	Current limitations	Industrial readiness	Future prospects
Multi-enzyme co-immobilization	Immobilization of complementary enzymes on a common support to enable substrate channeling	Improved stability, enzyme reuse, lower operating cost	Diffusion limitations, support cost	Pilot–industrial	Continuous bioprocessing
Artificial intelligence (AI) & Machine learning	Predict nonlinear relationships between biomass, enzyme formulation, and extraction yield	Reduced experimental workload, rapid optimization	Requires large, high-quality datasets	Pilot	Intelligent process design
High-throughput screening (HTS)	Automated parallel screening of enzyme combinations	Fast identification of optimal cocktails	Specialized equipment	Laboratory–pilot	Rapid enzyme discovery
Protein engineering & Directed evolution	Modification of enzyme structure to improve activity and stability	Enhanced catalytic efficiency, broader substrate specificity	High development cost	Laboratory	Customized enzymes
Synthetic biology & Metagenomics	Discovery and production of novel CAZymes from microbial communities	Access to new enzymes with unique functions	Complex validation	Early stage	Tailor-made enzyme libraries
Digital twins	Virtual simulation of enzymatic extraction processes using real-time data	Predictive optimization, process control	High computational demand	Emerging	Smart manufacturing
Continuous-flow bioprocessing	Integration of immobilized enzymes into continuous reactors	High productivity, reduced downtime	Reactor design complexity	Industrial	Large-scale biorefineries

Integrated biorefinery	Sequential recovery of multiple products from one biomass	Maximum biomass utilization, circular economy	Process integration challenges	Industrial	Zero-waste production

6. Advanced system optimization tools in enzyme-assisted extraction

The successful industrial implementation of enzyme-assisted extraction requires not only appropriate enzyme selection but also systematic optimization of process variables governing catalytic efficiency, mass transfer, and product stability. Because EAE involves multiple interacting parameters—including enzyme dosage, substrate characteristics, pH, temperature, extraction time, and solvent composition—its optimization cannot rely solely on empirical trial-and-error approaches. Instead, advanced statistical, mathematical, and computational tools have become indispensable for maximizing extraction efficiency while minimizing enzyme consumption, processing time, and operational costs [5], [15], [16]. Conventional one-factor-at-a-time (OFAT) experimentation remains useful for preliminary investigations but fails to identify interaction effects among variables and generally requires a large number of experimental trials. Modern optimization strategies therefore employ Design of Experiments (DoE), response surface methodology (RSM), kinetic modeling, artificial intelligence (AI), and computational simulation to provide predictive models capable of supporting both laboratory-scale optimization and industrial process scale-up [12], [15].

6.1. Statistical design of experiments and response surface methodology

Response Surface Methodology (RSM) has become one of the most widely applied optimization tools in enzyme-assisted extraction because it simultaneously evaluates the individual and interactive effects of multiple process variables. Rather than examining each parameter independently, RSM constructs empirical mathematical models that describe the relationship between independent variables—such as enzyme concentration, pH, temperature, extraction time, and liquid-to-solid ratio—and process responses including extraction yield, total phenolic content, antioxidant activity, or polysaccharide recovery [15], [16]. Optimization typically follows two sequential stages. The first stage consists of experimental screening, during which fractional factorial or Plackett–Burman designs identify statistically significant variables while eliminating factors with negligible influence. Once the critical variables have been identified, optimization proceeds using second-order experimental designs such as Central Composite Design (CCD) or Box–Behnken Design (BBD). These approaches generate quadratic regression models that quantify not only the individual effects of process variables but also their interaction terms and quadratic responses. The resulting mathematical models are commonly evaluated using analysis of variance (ANOVA), regression coefficients, coefficients of determination (R^2), lack-of-fit tests, and response surface visualization. Such analyses enable precise identification of optimal operating conditions while substantially reducing the number of experiments compared with conventional optimization approaches [15], [16].

6.2. Kinetic modeling of enzyme-assisted extraction

Whereas statistical optimization identifies optimal operating conditions, kinetic modeling provides mechanistic insight into the temporal evolution of enzymatic hydrolysis and bioactive

compound release. These models describe reaction rates, substrate conversion, enzyme deactivation, and mass transfer phenomena, thereby facilitating prediction of extraction performance under different operating conditions and reactor configurations [12]. For enzymatic hydrolysis, modified Michaelis–Menten equations remain the most widely adopted framework for describing heterogeneous enzyme–substrate interactions. However, because EAE involves insoluble biomass particles rather than homogeneous substrates, kinetic models frequently incorporate diffusion resistance, substrate accessibility, and structural changes occurring during hydrolysis. Additional empirical models—including Peleg, Weibull, first-order, and diffusion-controlled kinetic models—have also been successfully applied to describe the extraction behavior of different classes of bioactive compounds [4], [17]. Another important aspect of kinetic modeling concerns enzyme deactivation. Catalytic activity gradually decreases during extraction owing to thermal denaturation, product inhibition, irreversible adsorption onto biomass surfaces, or interactions with naturally occurring inhibitors. Incorporating enzyme deactivation into kinetic models substantially improves predictive accuracy and supports optimization of reaction duration and enzyme dosage for industrial applications [12].

6.3. Artificial intelligence and digital process optimization

Recent advances in artificial intelligence (AI) have significantly expanded the capabilities of process optimization beyond conventional statistical approaches. Machine learning algorithms—including artificial neural networks (ANNs), random forest models, support vector machines (SVMs), and Bayesian optimization—are increasingly employed to predict extraction performance from complex experimental datasets involving multiple interacting variables. Compared with classical regression-based methods, AI models often exhibit superior predictive performance when nonlinear interactions dominate system behavior. Moreover, integration of machine learning with high-throughput experimentation enables rapid identification of optimal enzyme formulations while substantially reducing the number of required experiments [15], [16]. Emerging concepts such as digital twins and hybrid mechanistic–data-driven models further allow real-time simulation and optimization of industrial extraction systems, supporting predictive process control, fault detection, and adaptive operation under continuously changing production conditions [5].

6.4. Future perspectives in process optimization

Future optimization strategies are expected to integrate experimental design, mechanistic kinetic modeling, artificial intelligence, and real-time sensor technologies into unified decision-support platforms. Such integrated frameworks will enable predictive optimization throughout the entire production chain, from biomass characterization and enzyme cocktail formulation to industrial process control and product quality assurance. As enzyme-assisted extraction continues to evolve toward precision bioprocessing, optimization tools will play an increasingly important role in improving catalyst utilization, reducing energy consumption, enhancing reproducibility, and facilitating large-scale implementation within sustainable biorefinery systems [5], [15], [16].

2. Conclusion

Targeted multi-enzyme-assisted extraction (EAE) has evolved from a conventional extraction technique into a knowledge-driven bioprocess that integrates plant cell wall biology,

enzymology, process engineering, and computational optimization. This review demonstrates that the superior performance of modern EAE is not determined solely by the catalytic activity of individual enzymes but rather by the rational design of synergistic multi-enzyme systems tailored to the structural organization and biochemical characteristics of specific biomass. A comprehensive understanding of plant cell wall architecture, enzyme specificity, catalytic cooperation, and substrate accessibility provides the scientific foundation for improving extraction efficiency while preserving the structural integrity and biological activity of target metabolites.

Recent advances in analytical characterization, statistical experimental design, kinetic modeling, artificial intelligence, and digital process optimization have substantially expanded the capability to develop predictive and highly efficient enzyme-assisted extraction systems. At the same time, emerging technologies—including multi-enzyme co-immobilization, protein engineering, synthetic biology, high-throughput enzyme discovery, continuous bioprocessing, and integrated biorefinery concepts—are accelerating the transition from empirical optimization toward precision-designed and economically sustainable extraction platforms.

Despite these remarkable advances, several challenges remain before the full industrial potential of targeted multi-enzyme systems can be realized. Future research should focus on improving the robustness and reusability of enzyme cocktails, developing standardized methodologies for biomass characterization and enzyme selection, integrating mechanistic models with artificial intelligence for predictive process control, and establishing scalable continuous processing strategies capable of handling the inherent variability of agricultural biomass. Addressing these challenges will be essential for enhancing process reproducibility, reducing production costs, and facilitating industrial implementation.

Overall, targeted multi-enzyme systems represent a key enabling technology for sustainable biomass valorization and next-generation biorefinery development. By bridging molecular enzymology, systems biology, advanced process engineering, and data-driven optimization, future enzyme-assisted extraction technologies are expected to evolve toward intelligent, precision-designed, and fully integrated bioprocesses that maximize resource utilization while supporting the transition to a circular and sustainable bioeconomy.

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The authors declare no conflict of interest.

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Informed Consent Statement

Not applicable.

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No new data were generated or analyzed in this study. All information presented in this review was obtained from previously published literature cited in the reference list.

Declaration of Generative AI and AI-Assisted Technologies

During the preparation of this work, the authors used ChatGPT (OpenAI) solely to improve the language and readability of the manuscript. After using this tool, the authors carefully reviewed and edited the content and take full responsibility for the content of the publication.

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