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Review article

Enzymatic Delignification Technology for Bioethanol Production from *Ulva reticulata* Macroalgae: A Review

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Abstract

Bioethanol is a promising renewable fuel that can reduce dependence on fossil fuels and mitigate greenhouse gas emissions. *Ulva reticulata* has attracted attention as a third-generation bioethanol feedstock because of its carbohydrate-rich biomass, containing cellulose, hemicellulose-like fractions, and ulvan-rich sulfated polysaccharides that can be converted into fermentable sugars. Although a lignin-like or acid-insoluble fraction has been reported in *U. reticulata*, this macroalga is generally considered a low-lignin biomass, and its recalcitrance is primarily associated with the ulvan-rich cell-wall matrix rather than a dominant terrestrial-type lignin network. Consequently, the role of delignification in *U. reticulata* should be viewed as the modification of phenolic or lignin-like residues and the enhancement of cell-wall accessibility rather than the removal of structural lignin. This review critically evaluates the potential of enzymatic delignification technologies to improve bioethanol production from *U. reticulata* through a PRISMA-guided literature analysis. The findings indicate that ligninolytic enzymes, including laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP), may enhance polysaccharide accessibility and downstream saccharification. Among these enzymes, fungal laccase, particularly from *Pleurotus* spp., appears promising because it uses molecular oxygen as the electron acceptor and produces water as the primary by-product, offering a potentially more environmentally friendly alternative to peroxide-dependent systems. However, most available evidence is derived from terrestrial lignocellulosic biomass, and direct studies on *U. reticulata* remain limited. Overall, enzymatic delignification is a promising yet experimental strategy for improving *U. reticulata* bioconversion. Future studies should validate the effects of ligninolytic enzymes on the modification of phenolic or lignin-like residues, ulvan-matrix disruption, sugar release, inhibitor formation, and ethanol production to establish their practical role in macroalgae-based bioethanol processes.

Keywords: bioethanol; delignification; fungal laccase (EC.1.10.3.2); *Ulva reticulata*

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1. Introduction

Fuel oil remains a major energy source for transportation and daily economic activities in Indonesia (Bria & Kolo, 2023). In 2024, fuel production in Indonesia was estimated at 39.68 million kL, while consumption is projected at 84.65 million kL, indicating a production-consumption mismatch. To address this issue, the Indonesian government has been importing fuel oil from other countries, accounting for 37.74% of total imports over the past five years (Kementerian ESDM, 2025). The high volume of fuel oil imports has led to rising fuel prices (Nggai et al., 2022). From 2022 to 2025, fuel prices in Indonesia had been risen, particularly subsidized fuel, which rose from Rp. 6,450/L in 2022 to Rp. 10,000/L in 2025 (Lestari & Asyiqin, 2022). To address this issue, the development of renewable alternative energy sources, such as bioethanol, is essential. Bioethanol is a renewable alternative energy source that can be used as a substitute for petroleum fuels due to its abundant supply, renewable nature, and environmental friendliness (Sari et al., 2018). Furthermore, bioethanol has the potential to reduce CO_2 emissions by up to 18% (Bria & Kolo, 2024). Bioethanol also contains oxygen and has a high research octane number, which may improve combustion efficiency and reduce exhaust emissions when blended with gasoline (Permatasari et al., 2020). Bioethanol can typically be produced from carbohydrate-rich sources such as cassava, sweet potatoes, corn, and sugarcane. However, the development of these sources still faces challenges in meeting human food needs (Kolo et al., 2022). For this reason, non-food marine biomass such as seaweed is increasingly relevant as a third-generation bioethanol feedstock.

Seaweed is one of the main ingredients used in bioethanol production, given its very high production rate. Seaweed holds significant potential as a biofuel source because it is largely underutilized and grows rapidly in saline environments, including less-than-ideal conditions and saltwater. The carbohydrate and lipid content of seaweed is considered a potential third-generation renewable energy source (Kolo et al., 2023). Among marine macroalgae, *Ulva reticulata* is particularly relevant because it is abundant in several Indonesian coastal areas, including East Nusa Tenggara, where its reported density reaches 50.42% (Supit et al., 2021). Chemically, *Ulva reticulata* contains 22.29% hemicellulose, 14.38% cellulose, and a reported 10.03% lignin-like or acid-insoluble fraction (Kolo et al., 2024). However, this fraction should not be automatically interpreted as true phenylpropanoid lignin, as Ulva is a green macroalga with a cell-wall structure that differs from that of terrestrial lignocellulosic biomass. Its cell wall is mainly composed of ulvan-rich sulfated polysaccharides, cellulose-containing regions, hemicellulose-like fractions, minerals, and salts. At the same time, phenolic or lignin-like residues are expected to occur only as minor components. Therefore, the term lignin in *U. reticulata* requires careful interpretation and further chemical verification before it can be treated as equivalent to structural lignin in terrestrial plants. This distinction is important because the conversion of *U. reticulata* into bioethanol depends not only on reducing lignin-like residues but also on improving access to the ulvan-associated carbohydrate matrix that can release mixed sugars during hydrolysis.

Bioethanol production from *Ulva reticulata* involves converting its carbohydrate content, such as cellulose and hemicellulose, through hydrolysis, either acidic or enzymatic. In *Ulva* biomass, hydrolysis may release mixed sugars derived from cellulose, hemicellulose-like fractions, and ulvan, including glucose, xylose, rhamnose, and uronic acid derivatives. This sugar heterogeneity can influence fermentation efficiency because not all sugars are equally fermentable by conventional ethanol-producing microorganisms. Therefore, the conversion of *Ulva* biomass into bioethanol should be understood as an

integrated process that involves pretreatment, hydrolysis, microorganism selection, and a fermentation strategy. However, the barrier to hydrolysis in *U. reticulata* should not be explained only through a terrestrial lignin model. The ulvan-rich matrix is likely to be a major structural barrier, while phenolic or lignin-like residues may still contribute to biomass recalcitrance by limiting enzyme access to polysaccharides. Enzyme-based delignification catalyzes the oxidation or modification of phenolic and lignin-like structures, promoting oxidation, degradation, or cross-linking, thus significantly increasing separation efficiency (Gan et al., 2025). Enzymatic delignification methods are widely used to degrade lignin due to their environmental friendliness, low energy consumption, and mild reaction conditions (Deng et al., 2019). The extracellular lignolytic enzymes used in delignification, such as laccase, manganese peroxidase, and lignin peroxidase, can be produced by microorganisms, including white-rot fungi (Suryadi et al., 2022). For *U. reticulata*, however, the rationale for using ligninolytic enzymes should not be based on the assumption that lignin is the dominant structural barrier. Instead, their scientific value lies in testing whether minor phenolic or lignin-like residues contribute to cell-wall recalcitrance, restrict enzyme access to carbohydrate polymers, and reduce saccharification efficiency. This focus is meaningful because the ulvan-rich matrix is the primary barrier, while lignin-like residues may act as secondary constraints that further limit polysaccharide accessibility. Therefore, ligninolytic enzymes should be evaluated as part of an integrated pretreatment strategy that also considers ulvan disruption, mixed-sugar release, inhibitor formation, and fermentation performance. This review, therefore, aims to critically evaluate the potential role of ligninolytic enzymes, particularly laccase and peroxidase systems, in modifying phenolic or lignin-like residues in *U. reticulata*, while acknowledging that direct experimental validation on macroalgal biomass remains limited.

2. Biomass Characteristics of *Ulva reticulata*

Ulva reticulata is classified in the class Ulvophyceae, order Ulvales, family Ulvaceae, and genus Ulva, species *Ulva reticulata*. *Ulva reticulata* is one of the most common and abundant macrogreen algae (Tran et al., 2017). In Indonesia, this species has been reported in several coastal regions, including Maluku, Bali, Java, and East Nusa Tenggara, but its utilisation as a bioenergy feedstock remains limited (Meiyasa et al., 2025). This underutilisation provides an opportunity to explore *U. reticulata* as a non-food third-generation bioethanol feedstock.

Chemically, *U. reticulata* contains 33.82% carbohydrates, 10.68% protein, 0.33% lipid, and 23.1% ash content (Tarigan et al., 2023). The carbohydrate content of *Ulva reticulata* is 55.8% (Hofmann et al., 2025). The difference between these carbohydrate values may arise from variations in sampling location, season, biomass maturity, analytical method, moisture basis, and compositional reporting. Therefore, these values should be interpreted as literature-based ranges rather than directly comparable fixed values. According to research conducted by Kolo et al. (2024), *U. reticulata* contains 14.38% cellulose, 22.29% hemicellulose, a reported 10.03% lignin-like or acid-insoluble fraction, and 53.15% water. However, the 10.03% fraction should be interpreted with caution, as standard fiber analysis methods developed for terrestrial lignocellulosic biomass may overestimate acid-insoluble residues in seaweed, rather than the true phenylpropanoid lignin. This distinction is critical because *U. reticulata* is a green macroalga whose cell wall is dominated by ulvan-rich sulfated polysaccharides, cellulose-containing regions, hemicellulose-like fractions, minerals, ash, and associated salts, rather than a dominant lignin network (Duong et al. 2025). In addition, Rodríguez-iglesias et al. (2024) reported

that the main constituents of *Ulva reticulata* include ulvans such as rhamnose (6.5-24.9%), xylose (2.1-12.0%), and sulfate (3.8-32%). According to research by Meiyasa et al. (2025), *U. reticulata* also contains minerals such as calcium (806.92 ppm), followed by magnesium (692.95 ppm), sodium (650.17 ppm), potassium (470.93 ppm), iron (2.21 ppm), manganese (1.27 ppm), and zinc (0.04 ppm). *U. reticulata* is a biomass that is considered ideal to be used as raw material in bioethanol production because of its low lignin content when compared with terrestrial lignocellulosic biomass such as eucalyptus 31.9%, pine sawdust 27.7%, bamboo 28.4%, bagasse 20.0%, switchgrass 12.9%, coconut fiber 19.4%, empty oil palm fruit bunches 20.4%, wheat straw 17.7%, cotton stalks 15.4%, corn straw 17.0%, and corn cobs 15.0% (Niu et al., 2024). Although the reported lignin-like fraction in *U. reticulata* is lower than the lignin content in terrestrial lignocellulosic biomass, it should not be described as a dominant lignin matrix that covers the polysaccharide wall. A more appropriate interpretation is that the recalcitrance of *U. reticulata* is mainly associated with its ulvan-rich sulfated polysaccharide matrix, cellulose-containing regions, hemicellulose-like fractions, minerals, salts, and minor phenolic or lignin-like residues that may limit enzyme access to fermentable polysaccharides.

Strategies for improving the conversion of *U. reticulata* into bioethanol should therefore focus not only on removing lignin-like residues, but also on modifying the ulvan-rich cell-wall matrix to improve polysaccharide accessibility. Research conducted by Kolo et al. (2024) used a chemical treatment with NaOH solution to remove lignin from *U. reticulata*, yielding promising results, lowering the lignin content from 10.03% to 3.86%. However, this decrease should be discussed as a reduction in the measured lignin-like or acid-insoluble fraction unless additional chemical analysis confirms that the removed fraction is true lignin. Although research shows promise for the use of chemicals in renewable energy production, very low concentrations are recommended to minimize environmental pollution from the resulting waste. Therefore, developing mild and selective pretreatment technologies for *U. reticulata* is needed to improve access to cellulose, hemicellulose-like polymers, and ulvan-derived sugars, while avoiding overstating lignin as the only structural barrier.

Because *U. reticulata* is a green macroalga, its cell-wall architecture should not be interpreted as identical to that of terrestrial lignocellulosic biomass. Instead, its recalcitrance is more appropriately related to the arrangement of ulvan-rich sulfated polysaccharides, cellulose-containing regions, hemicellulose-like fractions, proteins, ash, minerals, and associated salts. Accordingly, Figure 1 presents a schematic model of macroalgal cell-wall of *U. reticulata*, rather than a representation of a generic terrestrial plant cell-wall structure. As illustrated in Figure 1, the cell wall of *U. reticulata* consists mainly of cellulose microfibrils embedded within a complex matrix of ulvan-rich sulfated polysaccharides and hemicellulose-like components. Cellulose is a linear polymer composed of β-D-glucose units linked by β-1,4-glycosidic bonds, forming rigid and highly ordered microfibrils that contribute mechanical strength to the cell wall. Hemicellulose-like polysaccharides, including the xylan-, mannan-, and galactan-type fractions, act as interstitial materials that connect cellulose microfibrils and contribute to wall cohesion and flexibility. The ulvan fraction, which is unique to many green macroalgae, consists primarily of sulfated rhamnose-containing polysaccharides and represents a major structural component influencing cell-wall integrity, hydration properties, and resistance to enzymatic degradation. In addition, proteins, sulfate groups, minerals, and associated salts contribute to the structural complexity of algal biomass. Although phenolic or lignin-like compounds have occasionally been detected in some macroalgal species, these components are generally present at relatively low concentrations and do not play the same dominant structural role as lignin in terrestrial lignocellulosic biomass (Al-Battashi et al., 2019; Hasanov et al., 2020).

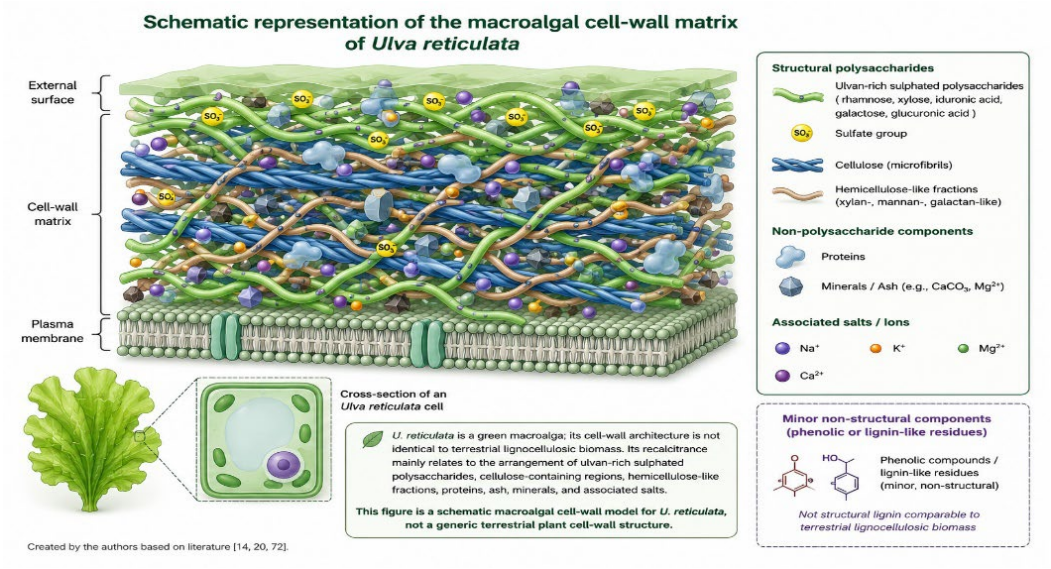


Figure 1. Schematic representation of the macroalgal cell-wall matrix of *Ulva reticulata*, highlighting ulvan-rich sulfated polysaccharides, cellulose, hemicellulose-like fractions, proteins, minerals/ash, sulfate groups, and associated salts. Phenolic or lignin-like residues, if detected, are shown as minor non-structural components and should not be interpreted as dominant structural lignin comparable to terrestrial lignocellulosic biomass. Created by the authors based on the literature (Rodríguez-iglesias et al., 2024)

3. The Concept of Delignification in Bioethanol Conversion

Delignification is generally defined as the reduction or removal of lignin from lignocellulosic biomass to improve the accessibility of cellulose and hemicellulose for enzymatic hydrolysis. In terrestrial biomass, lignin forms a major structural barrier that restricts enzyme penetration and limits the release of fermentable sugars (Farheen et al., 2026). However, this concept should be applied with caution to *Ulva reticulata*, as a dense lignin network does not primarily govern its biomass recalcitrance. Instead, the cell wall is composed mainly of an ulvan-rich sulfated polysaccharide matrix, cellulose-containing regions, hemicellulose-like fractions, proteins, minerals, salts, and minor phenolic or lignin-like residues.

Therefore, in the context of *U. reticulata*, delignification should be regarded as the selective modification of phenolic or lignin-like residues and the enhancement of cell-wall accessibility rather than the removal of dominant structural lignin. The primary objective is to improve access to carbohydrate-rich fractions for subsequent hydrolysis and fermentation. Following pretreatment, cellulolytic and other carbohydrate-active enzymes hydrolyze polysaccharides into fermentable sugars, which are then converted into bioethanol by microorganisms (Dandash et al., 2025; Farheen et al., 2026). Because *U. reticulata* hydrolysates may contain glucose, xylose, rhamnose, and uronic acid derivatives, fermentation efficiency depends not only on sugar release but also on the ability of microorganisms to utilize mixed sugar streams effectively.

The effectiveness of biomass pretreatment is influenced by several structural factors, including cellulose crystallinity, degree of polymerization, porosity, lignin composition, and carbohydrate accessibility (Weidener et al., 2020). In macroalgal biomass, these considerations should be expanded to include disruption of the ulvan-rich matrix and improved accessibility to mixed carbohydrate fractions. Consequently, the goals of pretreatment in *U. reticulata* include increasing cell-wall porosity, modifying lignin-like or phenolic residues, altering structural polysaccharide organization, and enhancing enzymatic accessibility (Suryadi et al., 2022).

Various pretreatment approaches, including physical, chemical, physicochemical, and biological methods, have been applied to improve biomass conversion efficiency (Baruah et al., 2018). Their effectiveness for *U. reticulata* should be evaluated not only by the reduction of lignin-like residues but also by carbohydrate preservation, sugar recovery, inhibitor formation, compatibility with ulvan degradation, and subsequent fermentation performance. Among these approaches, biological pretreatment, particularly enzyme-based systems, is attractive because it operates under mild conditions and can be tailored to target specific biomass components while minimizing inhibitor formation (Tsegaye et al., 2019; Kumar & Chandra, 2020). For *U. reticulata*, the most promising strategy is likely an integrated approach combining ligninolytic enzymes with carbohydrate-active enzymes, such as cellulases, hemicellulases, and ulvan-degrading enzymes, to improve overall bioethanol conversion efficiency.

4. Enzymatic Pretreatment: Principles and Mechanisms

Enzymatic pretreatment utilizes enzymes to break down lignin in lignocellulosic biomass, making cellulose more accessible to cellulases during hydrolysis. In *Ulva reticulata*, however, enzymatic pretreatment should not be described as a conventional lignin-removal process because this macroalga has a low-lignin cell-wall system. Instead, it should be understood as a selective cell-wall modification strategy aimed at improving access to cellulose, hemicellulose-like fractions, and ulvan-associated polysaccharides. The main recalcitrant barrier in *U. reticulata* is more closely related to the ulvan-rich sulfated polysaccharide matrix, minerals, salts, and minor phenolic or lignin-like residues than to a dominant terrestrial-type lignin network. Ligninolytic enzymes such as laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) (Figure 2) play a crucial role in the decomposition and neutralization of lignocellulosic waste, the decomposition of litter, and the formation of organic matter in the soil. During this process, cellulose and hemicellulose fibers become exposed, allowing cellulolytic and xylanolytic enzymes to access them (Vilar et al., 2021). For *U. reticulata*, its expected role should be limited to modifying minor phenolic or lignin-like residues that may restrict polysaccharide accessibility. Therefore, these enzymes should be evaluated alongside carbohydrate-active enzymes targeting cellulose, hemicellulose-like polymers, and ulvan. This combined approach is more consistent with the macroalgal cell-wall structure and avoids overstating lignin as the primary barrier. Enzymatic pretreatment is considered environmentally favourable because it can operate under mild conditions and reduce the use of harsh acids, alkalis, and other reactive chemicals (Ponnusamy et al., 2019). It may also reduce inhibitor formation, preserve fermentable carbohydrates, and support downstream hydrolysis and fermentation when pretreatment severity is properly controlled (Rezania et al., 2020). These advantages are relevant for macroalgal bioethanol production because excessive pretreatment severity may reduce sugar recovery, generate fermentation inhibitors, and lower the overall efficiency of hydrolysis and ethanol conversion.

The strategy of utilizing enzymes to decompose lignocellulosic biomass is considered more effective because it can overcome the challenges posed by the direct use of fungi. These challenges include difficulty meeting large-scale growth requirements, long incubation times, the mycelium's ability to penetrate the substrate, excessive polysaccharide utilization by fungi, which can lead to unnecessary carbohydrate degradation, and contamination (Masran et al., 2016). The use of lignolytic enzymes in the direct processing of lignocellulose can reduce problems associated with fungal pretreatment and offers a simpler, more efficient method. Moreover, the enzymatic approach is attractive for further investigation due to its ability to operate within a relatively short reaction time (a few hours) and it requires less nutrient input for the enzyme reaction (Suryadi et al., 2022). However, enzyme selection for *U. reticulata* should not be based only on general lignocellulosic biomass literature. The selected enzymes should be assessed according to their ability to improve polysaccharide accessibility, preserve fermentable sugars, reduce inhibitor formation, tolerate marine biomass conditions, and support downstream hydrolysis and fermentation.

The effect of enzymes on lignocellulosic biomass depends on the type of enzyme used and the composition of the biomass. This is because enzyme specificity is related to the type of reaction catalyzed (Moreno et al., 2019). This principle is particularly important for *U. reticulata* because its cell-wall composition differs from terrestrial biomass.

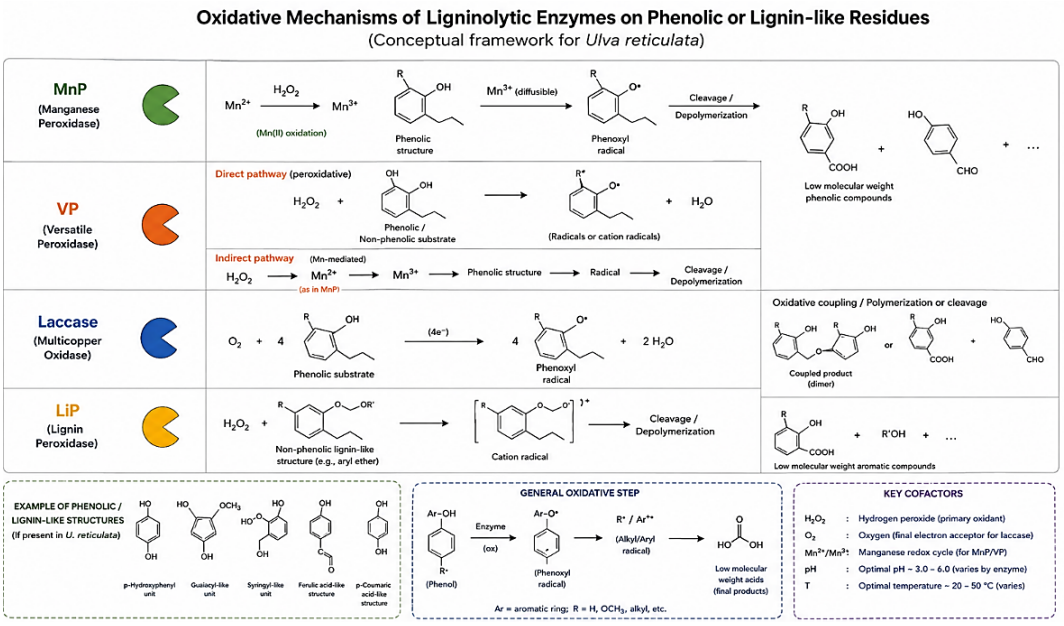


Figure 2. Illustrates the general oxidative mechanisms of ligninolytic enzymes, including laccase, LiP, MnP, and VP. However, this mechanism is primarily derived from studies of terrestrial lignocellulosic biomass. Therefore, its relevance to *U. reticulata* should be interpreted as a conceptual basis for testing the modification of minor phenolic or lignin-like residues, rather than as direct evidence that these enzymes have already been validated for macroalgal delignification (Janusz et al., 2017).

Laccase, LiP, MnP, and VP differ in electron acceptor requirements, substrate preferences, redox mechanisms, mediator dependence, and sensitivity to process conditions. These differences may influence delignification efficiency, saccharification performance, enzyme cost, and process scalability. However, there are also disadvantages associated with the use of enzymes. When compared directly with the use of fungal biomass, enzymes are more expensive to produce at scale. This problem can be overcome through advances in enzyme immobilization technology that increase the effectiveness of enzyme reuse, both in terms of quantity and activity, potentially exceeding that of fungal biomass (Rodriguez-Couto, 2017). Thus, future studies on *U. reticulata* should evaluate ligninolytic enzymes through measurable process indicators, including reduction of phenolic or lignin-like residues, sugar release, saccharification yield, inhibitor formation, enzyme loading, enzyme reuse, and compatibility with fermentation microorganisms.

5. Enzyme Types for Delignification and Deconstruction of Algal Biomass

5.1 Lignin oxidative enzymes

Lignin-based enzymes are a diverse group of enzymes from the oxidoreductase class that play a crucial role in the degradation of lignin, a complex and difficult-to-degrade polymer found in plant cell walls. These enzymes are primarily produced by white-rot fungi, some members of other fungal groups, and some bacteria, and function in the depolymerisation of lignin into smaller molecules, making it a key component of the natural carbon cycle. Some of the most crucial lignolytic enzymes include laccase (Lac), lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), and supporting enzymes such as aryl alcohol oxidase (AAO), glucose oxidase (GOX), and dye-decolorising peroxidase (DyP) (Asemoloye et al., 2021). In the context of *Ulva reticulata*, these enzymes should not be discussed as tools for degrading a dominant plant-type lignin network. Instead, they should be evaluated as candidate enzymes for modifying minor phenolic or lignin-like residues that may restrict access to cellulose, hemicellulose-like fractions, and the ulvan-rich matrix. Therefore, their relevance should be assessed using practical process indicators, including reductions in phenolic or lignin-like residues, polysaccharide accessibility, saccharification yield, sugar profile, inhibitor formation, enzyme loading, operational cost, and compatibility with downstream fermentation.

5.1.1 Laccase

Laccase (ρ-diphenol: dioxygen oxidoreductases; EC 1.10.3.2) is a multicopper oxidase containing four copper ions that are organized into three copper sites: one Type 1 (T1) copper site and a trinuclear cluster consisting of one Type 2 (T2) and two Type 3 (T3) copper ions (Janusz et al., 2020). The laccase structure consists of three cupredoxin-like domains, each containing four copper ions. These ions are categorised by their spectral properties into Type 1 (T1), Type 2 (T2), and Type 3 (T3). The T1 site plays a crucial role in substrate oxidation and contains a hydrophobic pocket rich in histidine residues that adjusts the redox potential and substrate affinity (Galazka et al., 2025). For *U. reticulata*, laccase is relevant because it may selectively modify phenolic or lignin-like residues without requiring hydrogen peroxide, thereby reducing the risk of oxidative enzyme inactivation and excessive inhibitor formation. Laccase activity is influenced by pH, temperature, and the presence of small-molecule mediators that enhance the enzyme's

oxidative capacity toward substrates that it cannot oxidize directly. Furthermore, laccase exhibits varying levels of tolerance to various environmental compounds (Galazka et al., 2025; Wozniak et al., 2025). Protein engineering has also been used to modify laccase properties, with engineered high-redox-potential laccases providing an example of how structural and biochemical engineering can be applied to improve laccase performance (Salas et al., 2019). However, its effectiveness against *U. reticulata* remains a testable hypothesis because direct comparative studies using macroalgal biomass remain limited. Future evaluation should focus on whether laccase improves access to ulvan-associated polysaccharides, increases the release of fermentable sugars, reduces inhibitory compounds, and improves ethanol yield after hydrolysis and fermentation (Kaczmarek et al., 2017).

5.1.2 Lignin peroxidase

Lignin peroxidase (LiP) is a heme-containing oxidoreductase that can oxidise high-redox-potential aromatic compounds and non-phenolic lignin model substrates. It has been reported in several white-rot fungi, including *Phanerochaete chrysosporium*, *Trametes versicolor*, *Bjerkandera* sp., and *Phlebia tremellosa* (Janusz et al., 2017; Galazka et al., 2025). The practical advantage of LiP is its strong oxidative capacity, but its dependence on hydrogen peroxide can increase process sensitivity. Excessive H_2O_2 may reduce enzyme stability, increase operational complexity, and create challenges for process control (Falade et al., 2017; Singh et al., 2021b). Studies on terrestrial lignocellulosic biomass have demonstrated that LiP activity can be enhanced through synergistic interactions with other enzymes and small molecules, although such findings cannot be directly extrapolated to *U. reticulata* (Chen et al., 2022). For *U. reticulata*, LiP should not be assumed superior because the target fraction is likely to consist of minor phenolic or acid-insoluble residues rather than abundant structural lignin. Its suitability should therefore be tested through measurable outcomes such as reduction of the measured lignin-like or acid-insoluble fraction, sugar recovery, inhibitor formation, enzyme loading, and fermentation compatibility.

5.1.3 Manganese peroxidase

Manganese peroxidase (MnP, EC 1.11.1.13) is a heme-containing enzyme that oxidises Mn^{2+} to Mn^{3+} in the presence of hydrogen peroxide. The generated Mn^{3+} species can diffuse and oxidize phenolic substrates, making MnP useful in lignin-related oxidative modification (Shi et al., 2020; Galazka et al., 2025). For *U. reticulata*, MnP should not be discussed primarily in terms of detailed structural features, amino acid residues, or catalytic-cycle intermediates, but rather in terms of its practical relevance for modifying minor phenolic or lignin-like residues that may restrict polysaccharide accessibility. Compared with laccase, MnP may provide broader oxidative action through diffusible manganese complexes; however, its dependence on hydrogen peroxide, manganese ions, and chelating organic acids can increase operational complexity, process sensitivity, and cost. Therefore, MnP should be evaluated not only by its ability to reduce measured phenolic or lignin-like residues, but also by its effects on ulvan-rich matrix accessibility, fermentable sugar recovery, inhibitor formation, enzyme loading, and downstream fermentation performance. Direct validation on *U. reticulata* remains necessary before MnP can be recommended as a suitable enzyme for macroalgal pretreatment.

5.1.4 Versatile peroxidase

Versatile peroxidase (VP) (EC 1.11.1.16) combines the catalytic features of LiP and MnP and can oxidise both phenolic and non-phenolic substrates. This broad substrate range makes VP theoretically attractive for oxidative biomass modification (Ravichandran & Sridhar, 2017; Galazka et al., 2025). However, VP depends on hydrogen peroxide and may experience oxidative inactivation when peroxide concentration is not properly controlled. This limitation may reduce its industrial practicality unless reaction conditions are carefully optimized. In the case of *U. reticulata*, VP should be treated as a candidate enzyme for future testing rather than an established delignification enzyme. Its relevance should be measured using macroalgal-specific indicators, including changes in phenolic or lignin-like residues, improvements in saccharification yield, sugar profile, inhibitor formation, and ethanol fermentation efficiency.

Overall, laccase, LiP, MnP, and VP differ in electron-acceptor requirements, substrate range, mediator- or cofactor-dependence, operational stability, process cost, and scalability. For *U. reticulata*, these differences are more important than detailed structural mechanisms because enzyme selection must be linked to practical conversion performance. Laccase appears promising because it uses oxygen and produces water as the main by-product. Still, this advantage should be regarded as a testable hypothesis rather than an established conclusion until direct comparative studies on *U. reticulata* are available.

5.2 Supporting hydrolytic enzymes

Because *U. reticulata* contains cellulose, hemicellulose-like fractions, and ulvan-rich sulfated polysaccharides, ligninolytic enzymes alone are unlikely to achieve efficient bioethanol conversion. Supporting hydrolytic and polysaccharide-degrading enzymes are needed to release fermentable and partially fermentable sugars from the algal cell-wall matrix. These enzymes should be discussed as part of an integrated enzyme system that connects pretreatment, hydrolysis, and fermentation.

5.2.1 Cellulase

Cellulase is a widely used enzyme in industry and has been commercially available for over three decades. This enzyme can be produced by various microorganisms, such as bacteria and fungi, provided they grow on cellulose-containing materials. Cellulose is converted into a simple sugar, namely glucose, which can be fermented into cellulose-based biofuels (Ejaz et al., 2021). Cellulase consists of three types of enzymes: β-glucosidase, endo-1,4-β-D-glucanase (endoglucanase), and exo-1,4-β-D-glucanase (exoglucanase). These three enzymes play a role in cellulose hydrolysis by working synergistically to ensure efficient hydrolysis (Patel et al., 2019). Specifically, cellulase degrades cellulose by hydrolysing β-1,4-glycosidic bonds. Endoglucanase acts on the internal structure of oligosaccharides present in carboxymethyl cellulose, cellooligosaccharides, or amorphous cellulose. Exoglucanase cleaves the non-reducing sites of crystalline cellulose, producing cellobiose or glucose as the primary end product. β-glucosidase acts on the non-reducing sites of cellobiose and cellodextrin (Figure 3) (Ejaz et al., 2021). Cellulase can be produced through both dry and wet fermentation processes by microorganisms such as bacteria and fungi. As an activatable enzyme, its production rate is influenced by activation and stress. Several studies have shown that cellulase is more stable at high temperatures than other enzymes that break down plant cell walls (Chang et al., 2016).

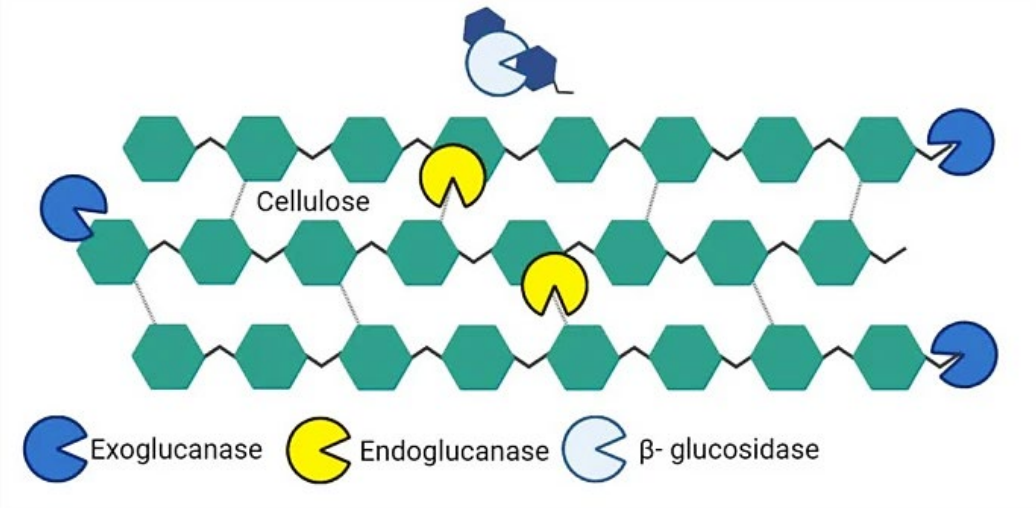


Figure 3. Shows the synergistic action of cellulase components in cellulose hydrolysis. In *U. reticulata*, cellulase activity may improve glucose release from cellulose-containing regions, but the surrounding ulvan-rich matrix and hemicellulose-like components can limit its efficiency. Therefore, cellulase should be applied together with enzymes that target hemicellulose-like polymers and ulvan to improve overall saccharification (Basera et al., 2024).

5.2.2 Hemicellulose-activated oxidative enzymes

Organisms that degrade lignocellulose also use oxidative enzymes such as lytic polysaccharide monooxygenases (LPMOs), to depolymerize hemicellulose. These enzymes play a crucial role in lignocellulose degradation because much like endo-acting GHs, LPMOs generate new chain ends, facilitating the action of exo-acting enzymes (Østby & Varnai, 2023). Hemicellulose degradation is influenced by various enzymes, most of which function hydrolytically and act in a similar manner to cellulolytic enzymes. The main xylan chain is attacked by xylanase endoenzymes, which reduce the substrate's ability to polymerise by hydrolyzing the β-d-xylopyranosyl bond, releasing xylobiose, xylooligosaccharides, and xylose (Sharma et al., 2020).

The β-xylosidase exoenzyme initiates the stepwise degradation process after the xylanase action is complete, starting from the intact xylooligosaccharide ends and producing xylose as the final product (Najjarzadeh et al., 2020). Xylan- and glucomannan-specific enzymes have been the primary focus among hemicellulose-active enzymes. In general, hemicellulolytic enzymes include those that cleave the main chain, namely endo-β-1,4-xylanase (xylanase), endo-β-1,4-mannanase (mannanase), and debranching enzymes that remove substitutions from the polysaccharide backbone, such as deacetylase, arabinosidase, and galactosidase (Østby & Varnai, 2023).

Endoxylanase cleaves the β-1,4 glycosidic bonds present in the xylan chain of hemicellulose. Endo-1,4-beta-xylanase (EC 3.2.1.8) breaks down xylan to form xylooligosaccharides, while endo-1,3-beta-xylanase (EC 3.2.1.32) produces xylobiose, xylotriose, and xylotetrose by hydrolyzing the glycosidic bonds at carbon positions 1 and 3. The non-reducing terminal residue of α-D-xylose is hydrolyzed by α-D-xyloside xylohydrolase (EC 3.2.1.177), which releases α-D-xylose (Saldarriaga-Hernández et al.,

2020). β-D-xylosidase (EC 3.2.1.37) acts on the non-reducing end of xylooligosaccharides, including xylose units. After the stepwise hydrolysis of xylan by xylanase, β-xylosidase plays a crucial role in converting smaller oligomers into monomers. The ideal substrate for this enzyme is xylobiose, and its affinity for oligosaccharides is inversely proportional to the degree of polymerization (Alokika & Singh, 2019).

Arabinofuranosidase, also known as arabinase, releases arabinose from arabinose-containing oligosaccharides and polysaccharides through exo- and endo-acting mechanisms. In these substrates, α-L-arabinofuranosidase (EC 3.2.1.55) cleaves α-1,2-, α-1,3-, and α-1,5-L-arabinofuranosyl residues from the non-reducing end. This enzyme is also known to remove 4-O-methylglucuronic acid substituents from xylan, thereby supporting the debranching of hemicellulose-like polymers and improving access for other hydrolytic enzymes (Contesini et al., 2017; Karnaouri et al., 2019). For *U. reticulata*, hemicellulose-active enzymes may support the release of xylose and other sugars from hemicellulose-like fractions. However, the resulting sugar mixture may not be fermented as efficiently by conventional ethanol-producing microorganisms. Therefore, the role of these enzymes should be linked to both saccharification performance and the selection of downstream microorganisms.

5.2.3 Ulvan lyase

Ulvan lyase is an enzyme that breaks down ulvan, converting ulvan polysaccharides into ulvan oligosaccharides through a beta-elimination process. Therefore, this enzyme belongs to the polysaccharide lyase (PL) family and has been divided into five PL groups based on amino acid sequence. All β-1,4-glycosidases are endolytic enzymes capable of hydrolyzing β-1,4-glycosidic bonds and producing various oligosaccharides. Many marine bacteria can produce ulvan lyase to utilize ulvan, a polysaccharide crucial to the marine carbon cycle. To date, every ulvan lyase enzyme discovered is capable of breaking down ulvan into ulvan oligosaccharides endolytically. For ulvan lyases from the PL24 family, the primary products are disaccharides and tetrasaccharides (Li et al., 2020).

The first ulvan lyase isolated was from a flavobacterium, *Persicivirga ulvanivorans*. The gene encoding the catalytic module of this enzyme has no sequence similarity to any other in the GenBank sequence database. It is categorized as a new group of polysaccharide lyases. This enzyme is recognized as an endo-type enzyme that cleaves the glycosidic bond between glucuronic acid and iduronic acid. Currently, species known to produce ulvan lyase generally include *Alteromonas*, *Pseudoalteromonas*, *Formosa agariphila*, and *Nonlabens ulvanivorans* (Huang et al., 2024). Temperature and pH are two crucial factors that influence ulvan lyase activity. Ulvan lyase exhibits the highest activity in the pH range of 7.5 to 9.0. This occurs because most ulvan lyases identified are derived from marine bacteria and the digestive environments of marine organisms, indicating potential evolutionary adaptation of these bacteria to weakly alkaline conditions (Qin et al., 2019).

Ulvan lyase activity is influenced by surfactants, metal ions, and chelating compounds, and these effects can be categorized into two, namely enhancing effects and reducing effects. Enzymes from the PL24 family derived from *Pseudoalteromonas* sp. PLSV_3875 showed increased activity when exposed to Ca^{2+} and CO^{2+}, with increases of 45% and 60%, respectively. However, this activity was inhibited by Mn^{2+} and Zn^{2+}, and the presence of ethylenediaminetetraacetic acid (EDTA) also decreased the enzyme's performance (Huang et al., 2024). Biochemical characterization of ulvan lyase revealed its function as an endonuclease that cleaves the glycosidic bond between Rha3S and GlcA or IdoA via a β-elimination mechanism. This β-elimination mechanism was proposed by

Gacesa and has been observed in various polysaccharide lyases (Li et al., 2021). This mechanism involves three stages: (1) neutralizing the negative charge on the carboxyl group at the +1 position of the substrate, resulting in a lower pKa of the C5 proton; (2) uptake of the C5 proton by the base; and (3) electron transfer within the carboxyl group, which triggers the cleavage of the C-O4 glycosidic bond, forming an unsaturated C4-C5 double bond at the non-reducing end (Huang et al., 2024). Ulvan lyase is particularly relevant for *U. reticulata* because ulvan is a major structural polysaccharide in its cell wall and may restrict cellulase access. By degrading ulvan into oligosaccharides, this enzyme can help open the algal cell wall matrix and improve the accessibility of cellulose- and hemicellulose-like fractions. However, ulvan-derived sugars such as rhamnose and uronic acid derivatives may be poorly fermented by conventional *Saccharomyces cerevisiae*. Therefore, ulvan lyase should be evaluated not only for biomass deconstruction but also for its effects on sugar composition and fermentation strategy.

5.2.4 Pectinase

Pectinase is a group of enzymes involved in the breakdown of pectin. Complete pectin breakdown requires the cooperation of various types of pectinases, each with its own substrate specificity. Several criteria exist for classifying pectinases. Based on their optimal pH, pectinases can be divided into two categories: acidic and alkaline pectinases. Based on the substrate used and their mode of action, pectinases are usually divided into pectin esterases, pectin hydrolases, propectinases, pectin lyases, and pectate lyases (Zheng et al., 2021). Pectinase plays a role in cell wall metabolism, cell growth, aging, fruit ripening, disease development, and abscission. Since the 1970s, pectinase has been commercially produced from microorganisms, primarily fungi (Haile & Ayele, 2022).

Pectin lyase (PMGL) and pectate lyase (PGL) are capable of directly cleaving the α-1,4-glycosidic bonds in the pectin molecule through a β-elimination process without producing harmful methanol, which has attracted increasing attention. Both PGL and PMGL belong to the pectinolytic lyase family, also known as pectin transeliminases. However, PGL and PMGL exhibit significant differences in their substrate specificity. Specifically, PGL acts on low-methylated pectin acids to produce oligogalacturonic acid or digalacturonic acid, while PMGL catalyzes the cleavage of highly methylated pectin to produce methylated oligogalacturonic acid (Lima et al., 2017).

Although pectinase is not the primary enzyme for *U. reticulata* deconstruction, it may support the removal of pectin-like or acidic polysaccharide components when present in algal biomass. Its use should therefore be considered part of an auxiliary enzyme cocktail rather than a primary delignification enzyme. The practical relevance of pectinase should be assessed by whether it improves sugar release, reduces viscosity, or enhances the activities of cellulase, hemicellulase, and ulvan lyase.

5.2.5 Xylanase

Xylanase is a type of glycosidase that hydrolytically breaks down the β-1,4-glycosidic bonds in xylan. This enzyme is also known as pentosanase. In scientific terminology, xylanase is referred to as endo-1,4-β-xylanase (EC 3.2.1.8) and is also known as β-xylanase, xylanase, β-1,4-xylanase, endoxylanase, endo-1,4-β-d-xylanase, and β-1,4-d-xylan-xylanohydrolase (Kumar et al., 2018). The enzymatic degradation of xylan involves complex steps and requires the collaboration of several hydrolytic enzymes (Malgas et al., 2019). Endo-1,4-β-d-xylanase works by randomly cleaving linear xylan chains, while β-d-xylosidase (EC 3.2.1.37) functions to release xylose monomer units from the non-reducing

ends of xylobiose and xylo-oligosaccharide chains. Other enzymes, such as acetyl xylanesterase (EC 3.1.1.72), α-L-arabinofuranosidase (EC 3.2.1.55), and α-d-glucuronidase (EC 3.2.1.139), remove side-chain groups. These polymers are crucial for fiber stability and cell wall integrity in plants (Dhiman & Mukherjee, 2018).

Various types of microorganisms, including filamentous fungi, bacteria, yeast, and actinomycetes, are known to produce xylanase. Xylanases are used in various industrial applications, such as pulping and bleaching in the paper and pulp sector (using xylanase formulations that do not contain cellulase); textile processing; the saccharification stage in the biofuel industry; organic waste management; and the food and animal feed industry. Most of these industrial activities take place at high temperatures and extreme pH values, so heat-resistant, alkaline- or acidic-stable enzymes are more advantageous (Kumar et al., 2018).

For *U. reticulata*, xylanase may contribute to the hydrolysis of hemicellulose-like xylan fractions and improve the release of xylose or xylooligosaccharides. However, its value in bioethanol production depends on whether selected microorganisms can effectively ferment the released sugars. Therefore, xylanase should be discussed in relation to mixed-sugar release, saccharification yield, and fermentation compatibility rather than as an isolated hydrolytic enzyme.

In summary, enzymatic deconstruction of *U. reticulata* should be designed as a coordinated enzyme system. Lignin oxidative enzymes may modify minor phenolic or lignin-like residues, while cellulase, xylanase, hemicellulose-active enzymes, pectinase, and ulvan lyase act on carbohydrate fractions within the algal cell wall. This integrated interpretation is more appropriate than treating *U. reticulata* as a conventional terrestrial lignocellulosic substrate. Future studies should compare these enzymes using quantitative indicators, including delignification or lignin-like residue reduction, total reducing sugar, glucose release, mixed-sugar profile, ethanol yield, inhibitor formation, enzyme loading, enzyme reuse, and process scalability.

6. Enzyme Sources and Biocatalyst Production

Enzymes used as biocatalysts for biomass deconstruction can be obtained from several biological sources, including fungi, marine bacteria, and genetically engineered hosts. However, because *U. reticulata* is a green macroalga with a low lignin or lignin-like fraction, enzyme sources should be discussed not only in relation to lignin degradation, but also in relation to their ability to modify minor phenolic or lignin-like residues, improve access to ulvan-rich polysaccharides, support saccharification, and remain feasible for downstream fermentation. Therefore, fungi, marine bacteria, and genetically engineered hosts should be evaluated based on enzyme activity, stability, production cost, enzyme-loading requirements, potential for immobilization, reusability, and scalability.

6.1 Enzymes from Fungi

Fungi produce enzymes that break down lignocellulose. With their extraordinary adaptability, demonstrated through their wide phylogenetic and phenotypic variation, they can degrade and absorb even the most resistant organic polymers. Fungi utilize a variety of extracellular enzymes to reduce the physical and chemical stability of lignocellulosic biomass (Kracher & Ludwig, 2016). The fungal system consists of two distinct types of decomposition processes: extracellular, which play a key role in the breakdown of polysaccharides, and intracellular, which are associated with the outer protective layer of

cells. In addition, the extracellular enzyme system comprises two distinct types of enzymes: hydrolytic enzymes, which break down polysaccharides, and oxidative enzymes, which degrade lignin and open aromatic rings. Three distinct categories of fungi have been described: brown-rot fungi, white-rot fungi, and soft-rot fungi, each with distinct effects and procedures on lignocellulose decomposition (Andlar et al., 2018).

Soft-rot fungi belong to the ascomycetes and deuteromycetes and typically colonize wood before the onset of the brown- and white-rot phases. These fungi function as slow decomposers and are capable of hydrolyzing polysaccharides on the surface of plant biomass and of breaking down wood under extreme environmental conditions (Saldarriaga-Hernández et al., 2020). Brown rot fungi belong to the group of basidiomycetes, and several species of ascomycetes can rapidly degrade the polysaccharides in lignocellulosic biomass, namely cellulose and hemicellulose, but not lignin. The use and application of these fungi do not rely on specific enzymes or low-molecular-weight compounds such as oxalic acid; instead, they employ a unique reduction mechanism to hydrolyze complex carbohydrates in biomass. Chelator-mediated Fenton system (CMF) for non-enzymatic fractionation of lignocellulosic biomass (Nargotra et al., 2023). White rot fungi break down three types of polymers in lignocellulosic biomass: cellulose, hemicellulose, and lignin. Because of their ability to completely break down lignin into CO_2, they can degrade lignin faster than other organisms, such as soft-rot and brown-rot fungi, and use lignin as the sole source of carbon and energy. White rot fungi produce various enzymes, such as lignin peroxidase, laccase, manganese peroxidase, and multipurpose peroxidase, which play a role in the effective decomposition of lignin (Zabed et al., 2019).

For *U. reticulata*, fungal enzymes are relevant mainly because white-rot fungi can produce oxidative enzymes, especially laccase, LiP, MnP, and VP, that may modify phenolic or lignin-like residues. However, fungal sources should not be interpreted as direct evidence of effective delignification in *U. reticulata*, because most available data still come from terrestrial lignocellulosic substrates. Their application to macroalgal biomass must be validated using *Ulva*-specific indicators, including reductions in the measured lignin-like or acid-insoluble fraction, improvements in polysaccharide accessibility, reducing sugar yield, glucose release, mixed-sugar profile, inhibitor formation, and ethanol fermentation performance. From a production perspective, fungi offer a strong advantage because they naturally secrete extracellular oxidative and hydrolytic enzymes. This secretion can simplify enzyme recovery compared with intracellular enzyme systems. Nevertheless, fungal enzyme production may require strict control of the culture medium, pH, aeration, incubation time, nutrient supply, and the risk of contamination. These factors can increase production cost and limit industrial scalability if the enzyme preparation is not stable or reusable. Therefore, fungal enzymes for *U. reticulata* should be assessed not only on catalytic potential but also on enzyme yield, production cost, operational stability, and compatibility with immobilization or recycling strategies.

6.2 Enzymes from marine bacteria

Bacterial enzymes can also degrade lignin found in lignocellulosic biomass. Bacteria can degrade lignin, though not as effectively as fungi. It is generally believed that bacteria produce a group of enzymes called lignolytic enzymes. These enzymes can break down lignin molecules into smaller aromatic compounds. These compounds can then enter the cell and undergo subsequent aromatic hydrolysis (Chauhan, 2020). Various bacterial types have been identified to accelerate lignin degradation, specifically α-proteobacteria,

actinobacteria, and γ-proteobacteria. Actinomycetes are filamentous bacteria that produce various enzymes essential for the decomposition of organic matter. The breakdown of organic matter by these bacteria occurs because their body shape allows them to penetrate and thrive within it. They are known to produce extracellular enzymes that hydrolyze lignin, cellulose, and hemicellulose (Limaye et al., 2017).

Currently, marine bacteria are being studied for their roles in degrading lignin and its derivatives and in contributing to the marine carbon cycle. Research conducted by Ley et al. (2023) identified two bacterial consortia capable of degrading alkaline lignin (a model lignin compound), named LIG-B and LIG-S, enriched from sediments near the coasts of the East and South China Seas. The LIG-B and LIG-S consortia mainly consisted of the phylum Proteobacteria with *Nitratireductor* sp. (71.6%) and *Halomonas* sp. (91.6%), respectively. Lignin degradation was found to be more favourable in the LIG-B consortium (max. 57%) than in the LIG-S consortium (max. 18%). The ligninolytic enzymes laccase (Lac), manganese peroxidase (MnP), and lignin peroxidase (LiP), which are capable of decomposing lignin into smaller fragments, were all active in both consortia. The newly formed low-molecular-weight aromatic compounds, organic acids, and other lignin-derived compounds in the biologically treated alkali lignin also clearly indicated that both consortia depolymerized lignin. The reconstructed lignin degradation pathway of the LIG-S consortium was found to be more comprehensive compared to that of the LIG-B consortium. It was further revealed that catabolic genes, involved in the degradation of lignin and its derivatives through various pathways via protocatechuate and catechol, were present not only in the lignin-degrading consortia LIG-B and LIG-S but also in 783 publicly available metagenomic genomes from nine coastal regions (Ley et al., 2023).

Marine bacteria are particularly relevant to *U. reticulata* because they originate from environments closer to macroalgal habitats than terrestrial fungi do. In addition to potential lignin-like residue modification, marine bacteria may produce carbohydrate-active enzymes, including ulvan lyases, cellulases, xylanases, and other polysaccharide-degrading enzymes that are more compatible with saline conditions and sulfated algal polysaccharides. This feature makes marine bacteria attractive for developing enzyme cocktails that target both minor phenolic or lignin-like residues and the ulvan-rich matrix of *U. reticulata*.

However, evidence from marine lignin-degrading consortia should be interpreted carefully. Studies using alkaline lignin or plant-derived lignin models do not automatically confirm activity on *U. reticulata* biomass. Therefore, bacterial enzyme sources should be validated using direct macroalgal substrates and process indicators, including enzyme loading, sugar yield, ulvan degradation, inhibitor formation, and ethanol yield. In terms of industrial feasibility, bacterial systems may offer faster growth and easier genetic manipulation than many fungi. Still, enzyme secretion, purification, stability, salt tolerance, and large-scale production cost remain key issues that need further evaluation.

6.3 Genetic engineering

In addition to enhancing the natural growth of microorganisms, genetic engineering is another strategy frequently used to improve and enable the production and application of lignocellulolytic enzymes on an industrial scale. Several techniques can be applied in this area, such as directed evolution, gene editing, and heterologous expression. Furthermore, many aspects can be improved, such as catalytic activity, stability, and resistance to inhibitors, as well as the microorganism's regulatory system, metabolism, and physical form, and improving the cooperation and efficiency of enzyme mixtures (Phillips, 2019).

For example, replacing the native cbhI gene of *T. reesei* with its counterpart from *Chaetomium thermophilum* increased the catalytic efficiency of *T. reesei* cellulase, resulting in a 2.2-fold increase in cellulase activity. It is also possible to construct chimeric complexes with enzymes from different microorganisms through gene editing (Jiang et al., 2020). Improving the stability of lignocellulolytic enzymes under adverse environmental conditions is also highly sought after, as it allows for high-temperature hydrolysis, which is often required for industrial biomass conversion processes (Ajeje et al., 2021).

Another focus of genetic engineering of lignocellulolytic microorganisms is the modification of their metabolic networks. Some examples of what has been done in this regard include removing specific proteases to reduce lignocellulolytic enzyme degradation, increasing strain growth and protein production rates, and improving metabolic control to balance the synthesis of other enzymes competing for precursors and energy, and altering the morphology of fungal mycelia to reduce medium viscosity, allowing for greater mass transfer and oxygen supply (Kracher & Ludwig, 2016; Andlar et al., 2018; Nargotra et al 2023).

Regarding heterologous expression of lignocellulolytic enzymes, efforts are underway to engineer functional lignocellulolytic systems that enable non-lignocellulolytic microorganisms to hydrolyze and transform lignocellulosic biomass. The most commonly used non-lignocellulolytic microorganisms for this purpose are *Zymomonas mobilis*, *Escherichia coli*, *Pichia pastoris*, and *Saccharomyces cerevisiae*. For example, active manganese peroxidase has been produced in *Escherichia coli* through co-expression of molecular chaperones followed by in vitro maturation, demonstrating the potential of heterologous expression systems for the production of functional ligninolytic enzymes (Al et al., 2019).The production of recombinant lignocellulases could address the limitations of high substrate costs and the need to maintain conditions for enzyme production and enable the development of more resistant and stable strains and higher enzyme yields. However, these modified enzymes and microorganisms still lack widespread industrial applications, so efforts should be made to optimize these aspects (Benatti & Polizeli, 2023).

For *U. reticulata*, genetic engineering has two potential roles. First, it can improve enzyme production by increasing the yield, secretion, thermostability, salt tolerance, pH tolerance, and inhibitor resistance of ligninolytic and carbohydrate-active enzymes. Second, it can support the design of integrated enzyme systems that combine laccase or peroxidase with cellulase, xylanase, and ulvan lyase to target the specific cell-wall architecture of *U. reticulata*. This approach is more appropriate than relying on a single lignin-degrading enzyme because *U. reticulata* contains an ulvan-rich matrix and only a limited lignin-like or acid-insoluble fraction.

Genetic engineering may also improve downstream fermentation by developing microorganisms that can use mixed sugars derived from *U. reticulata*, including glucose, xylose, rhamnose, and uronic acid derivatives. For example, engineered yeast has been investigated for the enzymatic hydrolysis of laminarin from brown macroalgae, demonstrating the potential of engineered microorganisms to improve the utilization of macroalgal polysaccharides (Rocher et al., 2021).This is important because conventional *S. cerevisiae* may ferment glucose efficiently but has limited ability to ferment several algal sugars. Therefore, engineered microorganisms or microbial consortia may be needed to connect pretreatment, hydrolysis, and fermentation into a more efficient bioethanol production system.

Despite these advantages, genetically engineered enzymes and host strains still require careful techno-economic assessment before industrial application. Key parameters include enzyme production yield, purification cost, enzyme loading, operational stability, immobilization potential, reusability, regulatory acceptance, biosafety, and scalability. For

this reason, genetic engineering should be presented as a future development strategy for *U. reticulata* bioconversion rather than as an already established industrial solution.

Overall, enzyme sources for *U. reticulata* should be selected based on both biological function and process feasibility. Fungal enzymes provide strong oxidative capacity; marine bacteria may offer better compatibility with algal polysaccharides and saline conditions; and genetic engineering can improve enzyme performance and mixed-sugar fermentation. However, direct validation on *U. reticulata* remains limited. Future studies should compare these biocatalyst sources using quantitative indicators such as enzyme activity, enzyme loading, lignin-like residue reduction, ulvan disruption, sugar yield, ethanol yield, inhibitor formation, enzyme recycling, and process cost.

6.4 Fermentation-based enzyme production

The development of enzymatic systems for efficient hydrolysis of lignocellulosic biomass on an industrial scale has been studied since the 1950s. Currently, two methods are used to produce lignocellulase, both in the laboratory and in industry: submerged fermentation (SmF) and solid-state fermentation (SSF) (Benatti & Polizeli, 2023). SmF is a fermentation process involving excess water, in which the substrate is immersed in a liquid. This process requires a free-floating liquid. The fermentation process is carried out on a solid, insoluble substrate without the use of free liquid. In this type of fermentation, in addition to providing physical support, the substrate also acts as a food source for the growth of microorganisms (Singh et al., 2021a).

Submerged fermentation is more commonly used in industrial production than solid-state fermentation because it allows better control of parameters such as pH, temperature, and agitation, and facilitates recovery and improves reproducibility. However, this method also has disadvantages, such as high electricity requirements, higher investment, and lower production and yield due to longer fermentation times (Benatti & Polizeli, 2023). On the other hand, although this technique requires a medium that maintains moisture for microbial growth, solid-state fermentation allows microorganisms to take up the substrate readily, reduces the risk of contamination, and saves water and energy, while producing higher yields than submerged fermentation. Solid-state fermentation is considered an important alternative for the hydrolysis of lignocellulosic biomass and the subsequent production of by-products (Maurice, 2019).

Various types of bioreactors can be used in both situations. In this way, the bioreactor functions as a mechanical device that creates in vitro conditions for the growth of microorganisms, thereby producing the desired product from the raw material. For submerged fermentation, there are three types of bioreactors used. The most commonly used is the stirred-tank bioreactor, which enables more effective temperature control and oxygen transfer during the reaction (Benatti & Polizeli, 2023). Additionally, there are air-transport bioreactors that enable efficient mixing by continuously flowing liquid through channels in a closed circuit, and bubble-column bioreactors that are more suitable for microorganisms susceptible to disturbances from the carrier. In solid-state fermentation processes, bioreactors can be divided into four categories. In a tray bioreactor, the bed is nearly static, and air circulates freely around it. In a dense-bed bioreactor, the bed remains stationary, but air remains within it. In a fluidized bioreactor, the solid substrate particles are continuously suspended and mixed by an upward flow of air, which promotes aeration and mass transfer within the bed (Leite et al., 2021).

The selection of the appropriate method for each case depends on various factors, including the type of microorganism and the substrate used. In general, the main criteria

for selecting an option include: adequate mass transfer, low shear stress, sterility, aeration, pH and temperature control, low energy consumption, appropriate material sizing, and an efficient mixing system (Pino et al., 2018).

7. Enzymatic Delignification Process Parameters

The effectiveness of enzymatic delignification in *U. reticulata* depends on several process parameters, including pH, temperature, incubation time, enzyme type, enzyme loading, and substrate characteristics. Unlike terrestrial lignocellulosic biomass, *U. reticulata* contains an ulvan-rich sulfated polysaccharide matrix, cellulose-containing regions, hemicellulose-like fractions, minerals, salts, and minor phenolic or lignin-like residues. Consequently, pretreatment should aim not only to modify lignin-like components but also to improve accessibility to carbohydrate-rich fractions for subsequent hydrolysis and fermentation (Fillat et al., 2017).

The choice of ligninolytic enzyme is a critical factor because laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) differ in catalytic mechanisms, substrate specificity, cofactor requirements, and operating conditions. Laccase uses molecular oxygen as an electron acceptor and may require mediators to oxidize recalcitrant substrates, whereas LiP, MnP, and VP depend on hydrogen peroxide, making process control more complex. LiP has been reported to exhibit optimal activity at pH 5.5 and 55°C, with an incubation period of 120 h, when veratryl alcohol is used as the substrate (Suryadi et al., 2022). Experimental and theoretical studies have also demonstrated that pH influences the catalytic bond-cleavage activity of LiP from *Phanerochaete chrysosporium*, highlighting the importance of pH optimization for LiP-mediated reactions (Pham et al., 2021). However, enzyme production conditions and catalytic activity conditions are not necessarily identical and should be optimized separately. For the optimization of *U. reticulata*, salinity, sulfate content, ash levels, pH fluctuations, and metal ions should also be considered as all of these factors may influence enzyme stability and activity.

Enzyme performance can be further enhanced through appropriate cofactor supplementation. Copper and other metal ions are known to stimulate laccase production and activity, although excessive concentration may increase process costs and wastewater treatment requirements. Because no single enzyme can fully deconstruct the macroalgal cell-wall matrix, enzyme cocktails combining ligninolytic enzymes with cellulases, hemicellulose-active enzymes, xylanases, and ulvan lyases are likely to be more effective than individual enzymes. Nevertheless, the optimal enzyme combination and ratio for *U. reticulata* remain largely unknown and require experimental validation.

Process optimization should be evaluated using multiple performance indicators rather than lignin-like residue reduction alone. Relevant parameters include reducing sugar production, glucose and xylose release, rhamnose and uronic acid profiles, saccharification efficiency, inhibitor formation, ethanol yield, and fermentation performance. This broader assessment is important because macroalgal hydrolysates contain a mixture of sugars that are not equally fermentable by conventional microorganisms such as Saccharomyces cerevisiae. For industrial applications, enzyme loading, immobilization, recycling, and operational stability are important economic considerations. Immobilized enzymes may improve stability and reusability, but they may also reduce catalytic accessibility if contact between the enzyme and biomass is restricted. Therefore, comparisons between free and immobilized enzyme systems should include residual activity, reuse cycles, saccharification efficiency, ethanol yield, and process cost.

Overall, the key process parameters for enzymatic delignification of *U. reticulata* include pH, temperature, incubation time, enzyme type, enzyme loading, enzyme ratio, cofactor requirements, biomass particle size, solid loading, salinity tolerance, immobilization strategy, and compatibility with downstream hydrolysis and fermentation. Because direct experimental studies on *U. reticulata* remain limited, current parameter recommendations should be regarded as preliminary guidelines requiring further validation.

8. Integration of Enzymatic Delignification with Bioethanol Production

8.1 Process scheme

The main steps in converting lignocellulose to bioethanol include pretreatment (delignification), enzymatic hydrolysis, and fermentation (Pino et al., 2018). For *Ulva reticulata*, this process scheme should be adjusted to the specific structure of macroalgal biomass. The pretreatment stage should not be interpreted only as conventional lignin removal, but also as modification of the ulvan-rich cell-wall matrix, hemicellulose-like fractions, minerals, salts, and minor phenolic or lignin-like residues that may restrict enzyme access to polysaccharides. In an integrated enzymatic delignification process, the preparatory phase is designed to remove or modify lignin and hemicellulose, allowing hydrolytic enzymes to more easily access structural carbohydrates before fermentation. In *U. reticulata*, this preparatory phase should be linked to the release of mixed sugars from cellulose, hemicellulose-like fractions, and ulvan. These sugars may include glucose, xylose, rhamnose, and uronic acid derivatives, which differ in fermentability and therefore influence microorganism selection and ethanol yield.

In laccase-dependent systems, delignification and/or detoxification can be performed after the preparatory phase and before the hydrolysis-fermentation process, either in the solid state or in the process stream entering an SSF or SSCF setup. Functionally, this integration aims to reduce lignin-induced inhibition of hydrolysis, reduce the adsorption of unproductive enzymes, and reduce inhibitory compounds produced in the previous preparatory phase. Therefore, the most descriptive integration scheme can be expressed as enzymatic delignification, enzymatic hydrolysis, and ethanol fermentation (Fillat et al., 2017). For *U. reticulata*, this scheme should be expanded as follows: enzymatic modification of phenolic or lignin-like residues and the ulvan-rich matrix; enzymatic hydrolysis of cellulose- and hemicellulose-like fractions; ulvan depolymerization; mixed-sugar release; and fermentation using microorganisms capable of utilizing the resulting sugar profile. This integrated scheme avoids treating *U. reticulata* as a conventional terrestrial lignocellulosic feedstock and provides a clearer link between pretreatment performance and ethanol production.

In a SHF (separate hydrolysis and fermentation) system, the enzymatic hydrolysis and fermentation processes are carried out in two separate stages. On the other hand, in the SSF (simultaneous saccharification and fermentation) system, hydrolysis and fermentation are carried out simultaneously in a single vessel. In terms of initial enzymatic treatment, SSF is often considered more advantageous from a process perspective because this combination can reduce equipment and operational costs and mitigate cellulase feedback inhibition, since the sugars from hydrolysis are immediately converted to ethanol (Pino et al., 2018). In processes involving high solids concentrations, pre-saccharification before adding the inoculum is also considered important because it can soften the medium and facilitate the implementation of pre-saccharification followed by

simultaneous saccharification and fermentation (PSSF) and pre-saccharification followed by simultaneous saccharification and co-fermentation (PSSCF) after the initial enzymatic treatment (Fillat et al., 2017).

In a PSSF configuration, biomass is first partially hydrolyzed before fermentation begins, thereby reducing viscosity and improving sugar availability. In a PSSCF configuration, the initial pre-saccharification phase is followed by simultaneous saccharification and co-fermentation, enabling selected microorganisms or microbial consortia to convert multiple sugar types in a single integrated process. These configurations are relevant for *U. reticulata* because macroalgal hydrolysates contain mixed sugars that may require co-fermentation strategies rather than glucose-only fermentation.

8.2 Impact of delignification on bioethanol production

Enzymatic delignification generally improves bioethanol production by increasing the accessibility of polysaccharides to hydrolytic enzymes and reducing non-productive enzyme binding to lignin or lignin-like components (Fillat et al., 2017). However, most quantitative evidence originates from terrestrial lignocellulosic biomass, including wheat straw, eucalyptus, spruce, date palm waste, bagasse, rice straw, and cotton waste. Therefore, these findings should be regarded as indirect evidence supporting the potential of enzymatic delignification rather than direct proof of its effectiveness in *Ulva reticulata*, for which experimental validation remains limited.

Studies on terrestrial biomass have consistently shown that laccase-based pretreatment or detoxification can enhance saccharification efficiency. Reported improvements include a 26% increase in sugar release from wheat straw, 21-30% increases in glucose and xylose yields following laccase-assisted pretreatment, a 30% increase in glucose yield from eucalyptus wood, up to an eightfold increase in sugar production from date palm waste, and 36-54% improvements in cellulose hydrolysis in spruce wood (Fillat et al., 2017; Deng et al., 2019). These benefits are attributed not only to lignin removal but also to modifications in lignin properties, such as hydrophobicity, surface charge, and enzyme-binding behavior. Nevertheless, the effects are not always positive, as some laccase-mediated reactions can generate phenolic oligomers that inhibit cellulase and β-glucosidase activity.

The positive effects of enzymatic delignification frequently extend to fermentation performance. Laccase-based detoxification has been reported to improve yeast growth, glucose consumption, fermentation efficiency, ethanol productivity, and ethanol yield in several lignocellulosic systems (Fillat et al., 2017). For example, ethanol concentrations ranging from 16.7 to 58.6 g/L have been reported in different wheat straw fermentation processes following laccase-assisted detoxification. Similarly, integrated pretreatment strategies applied to cotton waste increased glucose yields by 23-41% and ethanol yields by 31-64% (Fillat et al., 2017).

Another important advantage of enzymatic delignification is the reduction of fermentation inhibitors. Laccase-based detoxification has been shown to remove approximately 80-95% of phenolic compounds from pretreated lignocellulosic hydrolysates, thereby improving fermentation performance and ethanol production (Fillat et al., 2017). Likewise, laccase treatment of rice straw reduced both phenolic content and lignin levels, highlighting its potential as a cleaner pretreatment approach (Farheen et al., 2026). Because enzymatic processes operate under milder conditions than many physicochemical methods, they generally produce fewer inhibitory by-products and support more stable fermentation (Pino et al., 2018).

Overall, available evidence indicates that enzymatic delignification can improve saccharification, reduce inhibitor levels, and enhance ethanol production. However, the magnitude of improvement varies considerably among biomass types, pretreatment conditions, and fermentation systems. Importantly, increased sugar release does not always translate into higher ethanol yields because fermentation performance depends on sugar composition, inhibitor concentration, substrate loading, and microorganism selection. This consideration is particularly relevant for *U. reticulata*, whose hydrolysates may contain glucose, xylose, rhamnose, and uronic acid derivatives that are not equally fermentable by conventional *Saccharomyces cerevisiae*. Therefore, future studies should evaluate not only sugar release but also sugar composition, inhibitor formation, ethanol concentration, ethanol yield, ethanol productivity, and microorganism performance to accurately assess the benefits of enzymatic delignification in *U. reticulata*.

9. Comparison with Non-Enzymatic Pretreatment Methods

Pretreatment technologies for biomass conversion can be broadly classified into enzymatic and non-enzymatic approaches. Enzymatic pretreatment operates under mild conditions and selectively modifies target biomass components, thereby reducing the risk of carbohydrate degradation and inhibitor formation (Fillat et al., 2017). In contrast, non-enzymatic methods, including acid, alkali, hydrothermal, steam-explosion, and microwave-assisted pretreatments, are technologically mature and often provide more extensive biomass disruption (Moreno et al., 2019). However, for *Ulva reticulata*, pretreatment performance should not be evaluated solely by lignin removal because this macroalga contains relatively low levels of lignin-like material and is primarily composed of ulvan-rich sulfated polysaccharides, cellulose-containing regions, hemicellulose-like fractions, minerals, and salts. Therefore, key evaluation criteria should include ulvan disruption, sugar release, inhibitor formation, enzyme accessibility, fermentation compatibility, and process cost. Comparison of enzymatic and non-enzymatic pretreatment methods to produce bioethanol from *Ulva reticulata* macroalgae is concluded in Table 1.

Acid pretreatment effectively hydrolyzes hemicellulose and disrupts lignin–carbohydrate linkages, thereby improving biomass accessibility. However, its low selectivity may promote sugar degradation and the formation of inhibitory compounds, including furfural, 5-hydroxymethylfurfural (HMF), organic acids, and phenolic derivatives. In U. reticulata, acid pretreatment may enhance ulvan depolymerization and carbohydrate release, but its effectiveness depends on balancing sugar recovery with inhibitor generation.

Alkaline pretreatment is generally more effective for lignin removal because it cleaves aryl-ether and ester linkages within lignin–carbohydrate complexes (Gan et al., 2025). Nevertheless, chemical consumption, reagent recovery, and potential carbohydrate losses remain important limitations (Moreno et al., 2019). For *U. reticulata*, the practical value of alkali pretreatment should be assessed through carbohydrate preservation, ulvan modification, sugar yield, and fermentation performance rather than lignin-like residue reduction alone.

Hydrothermal pretreatment offers the advantage of operating without added chemicals and can effectively disrupt polysaccharide structures while reducing environmental impacts (Gan et al., 2025). Similarly, steam explosion can improve biomass fractionation and hydrolysis efficiency but may cause sugar degradation and inhibitor formation under severe operating conditions (Moreno et al., 2019; Gan et al., 2025). Because the primary structural barrier in *U. reticulata* is not a dense lignin network, the benefits of these high-severity pretreatments require further validation on macroalgal biomass.

Table 1. Comparison of enzymatic and non-enzymatic pretreatment methods to produce bioethanol from *Ulva reticulata* macroalgae.

Pretreatment Methods	Main Mechanism or Role	Cost	Environmental Effect	Potential for Inhibitor Formation	Suitability for *Ulva reticulata*	Resources
Enzymatic	Selective conversion or separation of the desired lignin/polysaccharide can be achieved under simpler conditions, thereby improving separation effectiveness and yielding higher-quality results.	The cost of enzymes remains quite high at the industrial level, but the required reaction conditions are simpler, and enzyme reactions typically last only a few hours.	This approach is very environmentally friendly as it does not use harmful acids, bases, or chemicals, and it also helps reduce waste and costs associated with further processing.	Low; pretreatment with biological or enzymatic methods indicates that only small amounts of inhibitor are formed and that no by-products hinder hydrolysis.	It is very suitable because ulvan is a water-soluble polysaccharide in the cell wall of Ulva, and the bio-enzymatic hydrolysis process is known to operate under gentle conditions, with high efficiency and specificity towards the substrate.	(Gan et al., 2025; Suryadi et al.,2022; Huang et al., 2024)
Acid	It is highly efficient at separating cross-links in biomass by breaking glycosidic bonds and lignin-carbohydrate linkages; it also achieves good cellulose retention and removes hemicellulose.	Costs tend to be high due to equipment damage, demand for anti-acid materials, and the processing of acidic wastewater, all of which increase operational expenses.	Relatively less, because it causes environmental pressure due to acidic liquid waste.	High, especially in extreme situations, because it can produce furfural, HMF, phenolic derivatives, and aldehydes that inhibit the fermentation process.	Medium, because acid dissolution is regareded as an ulvan degradation methods, its nature is less selective than enzymatic hydrolysis.	(Gan et al., 2025; Farheen & Moorthy, 2026; Huang et al., 2024).
Alkali	Excellent for delignification because it breaks aryl-ether bonds, saponifies ester bonds, and dissociates the lignin-hemicellulose network; it also increases enzyme access to cellulose.	Medium, because the operating temperature is generally milder than acid/hydrothermal, but still burdened by the use of chemicals and the need for reagent handling/recovery.	Medium: milder than acid in some aspects, but still carries the chemical burden of the process and the potential for hazardous waste.	Moderate to high; under certain conditions, inhibitory compounds such as phenols, furans, and organic acids can form, reducing the efficiency of further hydrolysis.	Low–moderate, because Ulva does have a minor component in the form of alkali-soluble linear xyloglucan, but Ulva's main target is ulvan so the alkali is less specific.	(Gan et al., 2025; Farheen & Moorthy, 2026; Suryadi et al.,2022; Huang et al., 2024).

Table 1. Comparison of enzymatic and non-enzymatic pretreatment methods to produce bioethanol from *Ulva reticulata* macroalgae. (continued)

Pretreatment Methods	Main Mechanism or Role	Cost	Environmental Effect	Potential for Inhibitor Formation	Suitability for *Ulva reticulata*	Resources
Hydrothermal / Liquid Hot Water	Effective for hemicellulose dissolution and maintaining high cellulose retention, but its lignin removal ability is limited.	Relatively low–medium because it does not require additional chemicals and reduces waste processing costs, although it still requires high temperatures.	Good because it does not use chemical agents, reduces liquid-waste pollution, and is considered economical and operationally flexible.	Relatively low; at neutral pH, liquid hot water is reported to produce the least inhibitor.	Low–medium, because this method is more suitable for terrestrial lignocellulose and is not specifically designed for ulvan depolymerization in Ulva.	(Gan et al., 2025; Farheen & Moorthy, 2026; Huang et al., 2024).
Physics: Steam explosion	Effective for biomass fractionation, increasing hydrolyzability, and supporting sugar recovery and fermentability.	Medium–high because it requires control of pressure, particle size, and water content, and increases the equipment's design and maintenance costs.	Medium; does not necessarily require harsh reagents, but its thermomechanical intensity is high and can increase the process load.	High, because harsh conditions can cause sugar degradation and the formation of furfural and HMF; in addition, lignin can melt and redistribute on the fiber surface.	Low, because the advantage of steam explosion is mainly for recalcitrant lignocellulosic biomass, while Ulva is dominated by ulvan and not a lignin matrix like terrestrial biomass.	(Moreno et al., 2019; Gan et al., 2025; Farheen & Moorthy, 2026; Huang et al., 2024).
Physics: Microwave	Especially effective when combined with alkali or other catalysts; in one study, microwave-alkali reduced lignin by about 60.8%.	Costs vary, but for high efficiency, a pressurised reactor and good process control are required.	Medium; its advantage is rapid, uniform heating, but its environmental profile depends heavily on the reagents used.	The explicit data in this collection of articles are less robust than those for acid or steam explosions, so the inhibitory potential is more accurately assessed on a process-by-process basis.	Medium, because microwave degradation is indeed considered to be an ulvan degradation methods, it is still less selective than ulvan lyase.	(Farheen et al., 2026; Huang et al., 2024).

Microwave-assisted pretreatment is commonly combined with chemical catalysts to enhance heating efficiency and biomass disruption (Pino et al., 2018). Although substantial lignin removal has been reported for terrestrial feedstocks such as wheat straw (Farheen et al., 2026), evidence for *U. reticulata* remains limited. Its potential application should therefore be evaluated based on ulvan disruption, sugar recovery, and inhibitor formation rather than extrapolated from terrestrial biomass studies.

Overall, enzymatic pretreatment appears particularly attractive for *U. reticulata* because it can be tailored to modify specific biomass fractions, including phenolic or lignin-like residues, cellulose-containing regions, hemicellulose-like polysaccharides, and ulvan, and can operate under mild conditions. However, challenges related to enzyme cost, stability, loading, and reusability remain significant. Non-enzymatic methods generally provide faster and more extensive biomass disruption but often require higher energy inputs, chemical consumption, and additional inhibitor management. Consequently, a combined strategy involving mild non-enzymatic pretreatment followed by enzymatic hydrolysis or enzyme-assisted matrix modification may offer the most promising route for improving sugar release and bioethanol production from *U. reticulata*.

10. Technological Challenges

The conversion of *Ulva reticulata* into bioethanol faces several technological challenges related to its unique cell-wall composition and the complexity of downstream bioconversion processes. Unlike terrestrial lignocellulosic biomass, *U. reticulata* contains an ulvan-rich sulfated polysaccharide matrix, cellulose-containing regions, hemicellulose-like fractions, minerals, salts, and minor phenolic or lignin-like residues. Consequently, efficient biomass deconstruction requires not only modification of lignin-like components but also disruption of the ulvan-rich matrix to improve carbohydrate accessibility. A major limitation is the incomplete degradation of ulvan, as ulvan lyase initiates depolymerisation but additional glycosidases are required to convert the resulting oligosaccharides into fermentable monosaccharides (Li et al., 2020).

Enzyme performance is another important challenge. Salinity, pH, temperature, sulfate content, ash content, and metal ions can influence catalytic activity and stability during macroalgal processing. Although some enzymes remain active under moderate salt concentrations, excessive salinity may reduce catalytic efficiency (Huang et al., 2024). Similarly, only a limited number of enzymes exhibit sufficient stability under marine biomass processing conditions (Qin et al., 2019). Therefore, lignolytic enzymes such as laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), and versatile peroxidase (VP) should be evaluated alongside cellulases, hemicellulose-active enzymes, and ulvan lyases as integrated enzyme systems rather than as individual catalysts.

The high cost of enzymes remains a major barrier to commercialization. Although enzyme cocktails can improve biomass conversion efficiency, they substantially increase operational costs (Suryadi et al., 2022). Enzyme recycling and immobilization may reduce enzyme consumption, but repeated use can decrease hydrolysis performance, while immobilization may limit enzyme accessibility to wet macroalgal particles (Zabed et al., 2019). Consequently, enzyme loading, enzyme ratio, operational stability, immobilization efficiency, and reusability are critical techno-economic considerations. Another challenge is the efficient fermentation of mixed sugars. Hydrolysis of *U. reticulata* may generate glucose, xylose, rhamnose, and uronic acid derivatives. While conventional Saccharomyces cerevisiae efficiently ferments glucose, its ability to utilize several algal-derived sugars is limited. As a result, high saccharification efficiency does not necessarily

translate into high ethanol yields. This limitation highlights the need for engineered microorganisms, adapted marine strains, or microbial consortia capable of fermenting diverse sugar mixtures.

Pretreatment optimization also remains critical. Acid, alkali, hydrothermal, steam-explosion, and microwave-assisted pretreatments can improve biomass accessibility. Still, it may also promote sugar degradation and the formation of inhibitory compounds, including furfural, 5-hydroxymethylfurfural, organic acids, and phenolic derivatives. These compounds can negatively affect both enzymatic hydrolysis and fermentation. Therefore, pretreatment performance should be assessed not only by lignin-like residue reduction but also by sugar preservation, inhibitor formation, and ethanol productivity.

On the industrial scale, process efficiency, substrate variability, and operational costs remain significant constraints (Niu et al., 2024). Seasonal and geographical variation in *U. reticulata* composition can influence carbohydrate content, ash levels, sulfate concentration, moisture content, and the measured lignin-like or acid-insoluble fraction, thereby affecting pretreatment response, enzyme requirements, and fermentation performance (Benatti & Polizeli, 2023). Biomass standardization, compositional screening, moisture control, and particle-size optimization are therefore essential for process scale-up.

Overall, the major challenges include biomass recalcitrance, enzyme limitations, mixed-sugar fermentation, inhibitor formation, substrate variability, and high process costs. Addressing these constraints will require integrated optimization of pretreatment, enzymatic hydrolysis, fermentation, and process economics (Wozniak et al., 2025). Because direct evidence for enzymatic delignification in U. reticulata remains limited, current strategies should be regarded as promising yet experimental and require systematic laboratory and pilot-scale validation.

11. Innovation and Future Research Directions

Future advances in bioethanol production from *U. reticulata* should focus on enzyme engineering, process integration, and biorefinery development. Directed evolution, rational design, and semi-rational design offer opportunities to improve the catalytic efficiency, stability, and operational robustness of lignolytic and carbohydrate-active enzymes under macroalgal processing conditions, including high salinity, sulfate-rich polysaccharides, elevated ash content, and variable pH (Asemoloye et al., 2021). Rather than targeting lignin degradation alone, future studies should develop integrated enzyme systems combining laccase, lignin peroxidase (LiP), manganese peroxidase (MnP), versatile peroxidase (VP), cellulase, xylanase, and ulvan lyase to maximize polysaccharide accessibility and sugar release. The design of enzyme cascades for ulvan conversion represents another important research direction. Because ulvan lyase only initiates ulvan depolymerization, additional glycosidases are required to generate fermentable monosaccharides (Li et al., 2020). Enzymes derived from marine and extremophilic microorganisms are particularly promising because they can retain activity under the salinity and temperature conditions commonly encountered during marine biomass processing (Qin et al., 2019). Future research should also clarify whether the reported lignin-like fraction in *U. reticulata* represents true phenylpropanoid lignin or primarily acid-insoluble residues and phenolic compounds, as this distinction will determine the scientific basis for enzymatic delignification strategies.

Enzyme immobilization and recycling technologies should be further explored to improve enzyme stability, reusability, and process economics (Singh et al., 2021a). Their

effectiveness should be evaluated using macroalgae-specific performance indicators, including residual enzyme activity, reuse cycles, lignin-like residue reduction, ulvan disruption, sugar release, inhibitor formation, and ethanol yield. At the process level, integrated bioconversion systems linking pretreatment, enzymatic hydrolysis, mixed-sugar fermentation, and residue valorization offer significant potential to enhance overall resource efficiency (Wozniak et al., 2025). Because *U. reticulata* hydrolysates contain diverse sugars, including glucose, xylose, rhamnose, and uronic acid derivatives, future studies should investigate engineered yeasts, marine microorganisms, and microbial consortia that efficiently ferment mixed sugars. Integration of bioethanol production with broader algal biorefinery concepts may also enable the generation of value-added products from ulvan and residual biomass, improving economic feasibility while contributing to the management of green-tide biomass (Huang et al., 2024).

A major priority is the direct comparison of laccase, LiP, MnP, and VP using standardized experimental conditions and the same *U. reticulata* substrate. Such studies should evaluate lignin-like residue reduction, saccharification efficiency, sugar profiles, inhibitor formation, ethanol yield, productivity, enzyme reusability, and process cost. These data are essential for determining the most effective enzymatic pretreatment strategy for U. reticulata, rather than relying on evidence extrapolated from terrestrial biomass. Finally, future reviews and experimental studies should adopt transparent and reproducible methodologies. Systematic reviews should clearly report search strategies, screening procedures, inclusion criteria, and quality assessments. At the same time, experimental studies should provide detailed information on biomass characteristics, pretreatment conditions, enzyme loading, analytical methods, and fermentation parameters. Such standardization will facilitate meaningful comparisons and accelerate the development of sustainable *U. reticulata*-based bioethanol technologies.

12. Conclusions

Ulva reticulata is a promising third-generation feedstock for bioethanol production because of its high carbohydrate content and relatively low lignin-like fraction compared with terrestrial lignocellulosic biomass. However, its biomass recalcitrance is governed not only by lignin-like compounds but also by the complex cell-wall matrix composed of ulvan, cellulose, hemicellulose-like polysaccharides, minerals, and other structural components. Consequently, pretreatment remains essential to improve polysaccharide accessibility. In this context, enzymatic delignification should be regarded as a strategy to modify phenolic or lignin-like residues and enhance cell-wall accessibility rather than to remove dominant structural lignin. Enzymatic delignification represents an environmentally friendly pretreatment approach that generates fewer inhibitory compounds than conventional chemical methods. Lignolytic enzymes, including laccase, lignin peroxidase, manganese peroxidase, and versatile peroxidase, have the potential to improve saccharification efficiency by increasing access to fermentable polysaccharides. Among these enzymes, fungal laccases from white-rot fungi are particularly attractive because of their sustainable catalytic mechanism; however, their effectiveness on *U. reticulata* requires further experimental validation. Efficient bioethanol production from *U. reticulata* will depend on integrating pretreatment, hydrolysis, and fermentation processes. Enzyme systems targeting cellulose, hemicellulose-like fractions, and ulvan are required to release fermentable sugars, while advanced fermentation strategies are needed to utilise mixed sugar streams efficiently. Future research should focus on direct comparative evaluations of lignolytic enzymes, optimisation of integrated enzyme cocktails, enzyme immobilisation

and recycling, mixed-sugar fermentation technologies, and techno-economic assessments. Overall, enzymatic delignification offers a promising yet emerging route to enhance the bioconversion of *U. reticulata* into bioethanol. Its successful implementation will require systematic experimental validation and process integration to support the development of sustainable and economically viable macroalgae-based bioethanol production.

13. Authors' Contributions

All authors had equal contributions as the main contributors to this manuscript. PMB-Conceptualization, Resources, Data Curation, Formal Analysis, Writing original manuscript, Writing review editing; DA-Conceptualization, Supervision, Writing review editing; RHBS- Conceptualization, Methodology, Supervision, Writing original manuscript, Writing review editing.

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13. Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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