*fermentation*

MDPI

*Review*

# Enzymes Produced by the Genus *Aspergillus* Integrated into the Biofuels Industry Using Sustainable Raw Materials

**Fernando Enrique Rosas-Vega [1], Roberta Pozzan [2], Walter Jose Martínez-Burgos [1,*], Luiz Alberto Junior Letti [1], Patricia Beatriz Gruening de Mattos [1], Lucia Carolina Ramos-Neyra [1], Gabriel Spinillo Dudeque [1], Gustavo Amaro Bittencourt [1], Gabriela dos S. Costa [1], Luciana Porto de Souza Vandenberghe [1] and Carlos Ricardo Soccol [1]**

[1] Department of Bioprocess Engineering and Biotechnology, Polytechnic Center, Federal University of Paraná, Rua Cel. Francisco H dos Santos—100, Curitiba 81530-000, Brazil; frosasvv@gmail.com (F.E.R.-V.); letti@ufpr.br (L.A.J.L.); patricia.mattos@ufpr.br (P.B.G.d.M.); luciaramosneyra@gmail.com (L.C.R.-N.); gabrielsdudeque@gmail.com (G.S.D.); gustavobittencourt32@gmail.com (G.A.B.); ga.costa13@hotmail.com (G.d.S.C.); lvandenberghe@ufpr.br (L.P.d.S.V.); soccol@ufpr.br (C.R.S.)

[2] Laboratory of Cell Toxicology, Department of Cell Biology, Polytechnic Center, Federal University of Paraná, Rua Cel. Francisco H dos Santos—100, Curitiba 81531-908, Brazil; robertapozzan03@gmail.com

* Correspondence: ing.wjmartinez@gmail.com

**Abstract:** Renewable energy sources, such as biofuels, represent promising alternatives to reduce dependence on fossil fuels and mitigate climate change. Their production through enzymatic hydrolysis has gained relevance by converting agro-industrial waste into fermentable sugars and residual oils, which are essential for the generation of bioethanol and biodiesel. The fungus *Aspergillus* stands out as a key source of enzymes, including cellulases, xylanases, amylases, and lipases, which are crucial for the breakdown of biomass and oils to produce bioethanol and fatty acid methyl esters (FAME). This review examines the current state of these technologies, highlighting the significance of *Aspergillus* in the conversion of energy-rich waste materials. While the process holds significant potential, it faces challenges such as the high costs associated with enzymatic production and final processing stages. Agro-industrial waste is proposed as an energy resource to support a circular economy, thereby eliminating reliance on non-renewable resources in these processes. Furthermore, advanced pretreatment technologies—including biological, physical, and physicochemical methods, as well as the use of ionic liquids—are explored to enhance process efficiency. Innovative technologies, such as genetic engineering of *Aspergillus* strains and enzyme encapsulation, promise to optimize sustainable biofuel production by addressing key challenges and advancing this technology towards large-scale implementation.

**Keywords:** bioethanol; biodiesel; enzymatic hydrolysis; *Aspergillus*; cellulases; xylanases; amylases; lipases; pretreatment



Academic Editors: Xuefeng Zhu and Xuedong Zhang

Received: 9 January 2025
Revised: 24 January 2025
Accepted: 26 January 2025
Published: 1 February 2025

**Citation:** Rosas-Vega, F.E.; Pozzan, R.; Martínez-Burgos, W.J.; Letti, L.A.J.; de Mattos, P.B.G.; Ramos-Neyra, L.C.; Spinillo Dudeque, G.; Amaro Bittencourt, G.; dos S. Costa, G.; Porto de Souza Vandenberghe, L.; et al. Enzymes Produced by the Genus *Aspergillus* Integrated into the Biofuels Industry Using Sustainable Raw Materials. *Fermentation* **2025**, *11*, 62. https://doi.org/10.3390/fermentation11020062



## 1. Introduction

The increasing global energy demand, projected to grow by 49% between 2007 and 2035 [1], combined with the depletion of oil and coal reserves, underscores the urgent need for sustainable alternatives. Biofuels, particularly bioethanol and biodiesel, have emerged as key solutions to reduce dependence on fossil fuels, which currently account for 80% of global energy consumption [2,3]. Within this context, the European Union aims to

replace 10% of its automotive fuel with biofuels by 2030, supported by an investment of $93.67 million in second-generation biofuels [4].

Bioethanol stands out as a prominent alternative fuel due to its high oxygen content, which improves combustion efficiency, and its high-octane number, enabling higher compression ratios in engines [1]. Globally, the United States and Brazil lead bioethanol production, contributing over 85% of the 94 billion liters produced annually. Brazil produces 27.1 billion liters from sugarcane, while the United States produces 59.7 billion liters from corn starch. Additionally, countries such as China and India aim to reduce fossil fuel dependency by 20%, with projected bioethanol consumption levels of 3.8 billion and 1.9 billion liters, respectively [5].

Lignocellulosic biomass, produced in volumes of approximately $1.5 \times 10^{11}$ tons annually, has the potential to yield up to 442 billion liters of bioethanol if fully utilized [5,6]. However, a significant portion of this resource remains underutilized; for instance, in the United States, 90% of corn residues are repurposed for field preparation [7]. Advances in enzymatic hydrolysis, employing xylanases, cellulases, and amylases, have improved the conversion of wheat, corn, and rice residues, as well as municipal solid waste, into fermentable sugars [8–10]. Nevertheless, lignin, comprising 17–32% of sugarcane bagasse biomass, poses a significant challenge due to its chemical recalcitrance [7]. Pretreatment processes designed to reduce lignin content and enhance cellulose and hemicellulose accessibility are critical, albeit costly [11,12].

Biodiesel, primarily produced through transesterification with fungal lipases, is derived from vegetable oils and animal fats. Feedstocks include Honne oil [13], oil palm biomass [14,15], rapeseed oil [13], *Koelreuteria integrifolia* oil [16], *Jatropha* oil [17,18], sunflower oil [19], castor oil [20,21], soybean oil [22,23], *Pongamia* biodiesel [24] and algae and microalgae [25,26]. Unlike bioethanol, biodiesel does not utilize lignocellulosic biomass due to its low lipid content.

Agro-industrial residues represent a key source of biofuel production. Pretreatments (Figure 1), such as physical (milling and grinding) and chemical (alkaline and acid), are essential for enhancing the efficiency of enzymatic hydrolysis and improving the yields of fermentable sugars [12]. However, these processes require specific optimization for each type of biomass, considering its chemical heterogeneity and the generation of inhibitory compounds [11]. This article addresses these strategies within the framework of sustainable technologies for bioethanol and biodiesel production.

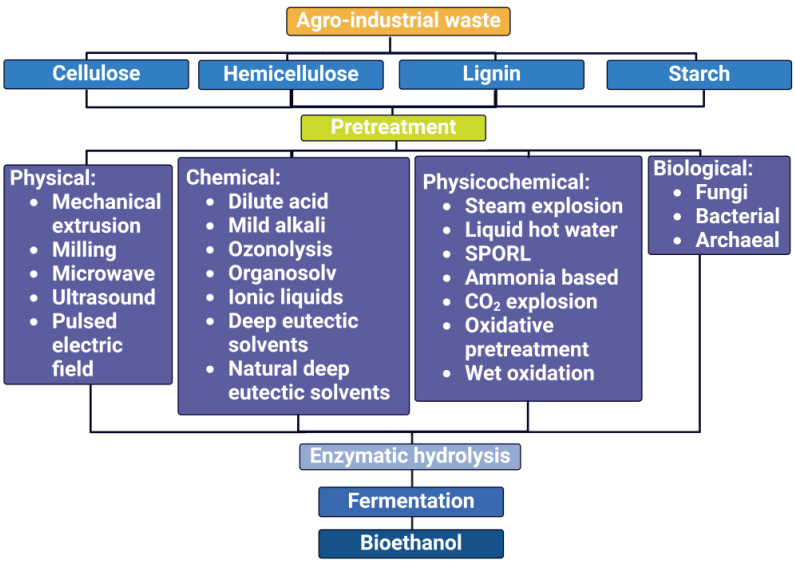


**Figure 1.** Exploration of different agro-industrial waste pretreatments, adapted from [11].

The fundamental principles, advantages, limitations, and performance of these processes in terms of fermentable sugar yields are discussed. This work aims to provide a comprehensive overview of the advances and challenges in biomass pretreatment, offering valuable insights to enhance efficiency and sustainability in biofuel production.

## 2. Renewable Sources for Biofuels Production

In the 21st century, one of the greatest challenges facing humanity is balancing the growing demand for materials, energy, and food with the need to decarbonize the economy to prevent uncontrolled global temperature rise and environmental collapse [27,28]. The transition to renewable sources, such as solar, wind, and biomass, is essential for reducing dependence on fossil fuels and greenhouse gas emissions [29]. Plant biomass, the primary source of carbon on Earth's surface, can be converted into bioproducts such as biofuels, biochemicals, and biomaterials using biorefining techniques that explore the renewability of natural carbon cycles, its sequestration, and subsequent conversion [30].

The bioeconomy can be implemented through the utilization of globally available plant biomass resources. First-generation (1G) biorefineries, based on materials such as sugar (sugar beet, sugarcane, or sweet sorghum), starch (corn, cassava, potato), or vegetable oils (rapeseed, soybean, sunflower) [31], are successful, although concerns exist regarding their environmental and social impacts due to competition for resources like water and arable land. Nevertheless, the residues from these production chains (approximately $5 \times 10^9$ tons annually worldwide) hold significant potential for valorization [32,33]. Lignocellulosic biomass is a promising source of second-generation (2G) biofuels, which not only bypasses the food versus fuel debate but also enables the utilization of industrial residues, generating financial returns from materials that would otherwise incur costs for conventional treatment.

Various types of biomasses have been evaluated for biofuel production, including invasive plants and small-scale industrial residues such as *Corchorus* sp. [34], rose flowers [35], and *Miscanthus giganteus* [36]. Currently, bioethanol production from such plants is gaining global attention due to their advantages in terms of high biomass yield and sustainability. Several studies have demonstrated that *Miscanthus* can be a viable source for bioethanol production owing to its ability to grow on marginal soils and its low input requirements, making it an attractive option for the biofuel industry. Large-scale projects in Europe and the United States are actively exploring their potential to contribute to the transition towards cleaner and renewable energy sources [37,38]. For this reason, continuous technological innovation in its processing is essential, as it is crucial to develop solutions that align with local resource availability and requirements, thereby enhancing economic profitability [39].

As shown in Table 1, numerous emerging biomass sources have been assessed. Bhuyar et al. evaluated the use of *Amorphophallus* sp. tubers, which is a non-edible starchy material generated in tropical regions of the northern part of Thailand. The authors achieved an initial ethanol yield of 8.68 g/L. Although the yield is relatively low, biomass is considered highly innovative due to its novelty in the biofuel production field [40]. Similarly, Sahu investigated the exploration of rose flower waste, which produced 29.5 g/L of ethanol, showing a promising application for these waste streams [35]. More recently, Kabadayi et al. explored the potential of mulberry pomace for bioethanol production by *Hansenispora uvarum*. The authors achieved a 61.3 g/L concentration, further expanding the range of underutilized biomasses for bioethanol production. Also, recent studies have explored the potential of different grasses and, in some contexts, invasive plants for biofuel production [41]. Iyyappan et al. evaluated elephant grass (*Pennisetum purpureum*) using a biological pretreatment with *Trichoderma reesei* and NiO nanoparticles, achieving an ethanol

yield of 14.65 g/L with *Kluyveromyces marxianus* MTCC 1389 [42]. Wongleang et al. studied *T. latifolia* grass with a concentrated acid pretreatment (75% $H_3PO_4$ at 60 °C for 60 min), resulting in an ethanol yield of 8.9 g/L using Saccharomyces cerevisiae TISTR 5339 [43]. Similarly, Kentucky bluegrass (*Poa pratensis*) was used on a humic acid-assisted alkaline pretreatment, achieving an ethanol yield of 15.3 g/L with *S. cerevisiae* YPH499 [44]. These findings highlight the promising potential of these grasses as a way for valorizing non-traditional feedstocks, including invasive species, for sustainable bioenergy production.

Adding to that, residues originating from more conventional agricultural commodities, such as rice husks, wheat, and barley straw, continue to receive great attention for the development of their production technologies, while a biochemical route with the use of specific enzymes is continually evaluated. Jin et al. reported a bioethanol concentration of up to 108.6 g/L using *Aspergillus fumigatus* enzymes in alkaline-pretreated rice straw [45]. Ziaei-Rad et al. achieved 43.1 g/L of bioethanol, with 84.34% yield, exploring wheat straw hydrolysate under the optimized ionic liquid (IL) pretreatment [46]. Similarly, Duque et al. evaluated and alkaline extrusion pretreatment (7.2% NaOH, 100 °C, 3 h, 120 rpm) followed by enzymatic hydrolysis (10 FPU/g, 20% solids) in simultaneous saccharification and co-fermentation on barley straw, resulting in 38 g/L ethanol, corresponding to 15.8 g/100 g raw barley straw [47].

Despite the advances in recent research, the largest examples of successful biofuel production from plant biomass are located in the USA and Brazil, with massive quantities of 1G bioethanol produced annually in both countries. Corn remains the primary feedstock for bioethanol production in the USA, with enzymatic processes playing a crucial role in the efficient conversion of starch to fermentable sugars [48]. The annual production of bioethanol from corn in the USA reached 57 billion liters in 2019, supported by advanced enzyme technologies for starch hydrolysis and fermentation [48]. In Brazil, sugarcane is the main feedstock, driven by its high energy yield and established infrastructure and policy, resulting in 35.6 billion liters in 2020 [49]. However, a significant amount of residual biomass, such as sugarcane bagasse, remains underutilized. This biomass, along with corn residues, such as corncob and corn stover, offers promising potential for 2G bioethanol production [49].

The efficiency of different biofuel production, particularly from 2G sources, hinges on effective pretreatment strategies. These advances are key to optimizing the conversion of lignocellulosic biomass into fermentable sugars, enabling the successful production of biofuels [50]. Table 1 also shows different pretreatment technologies recently evaluated under the context of biofuel production. Physical pretreatments, such as milling and crushing, aim for size reduction and the increase of the surface area for enzymatic action. Zhou et al. applied a drying process at 105 °C for 2 h and crushing after that to size reduction to <2 mm. This enabled effective enzymatic hydrolysis and fermentation, reaching 73.2 g/L of bioethanol with the addition of 1% saponin to act as a surfactant in the bioprocess [51]. On the other hand, chemical methods, such as acid and alkaline pretreatments, are highly effective in biomass fractionation, breaking lignin and hemicellulose bonds; for instance, cassava waste treated with NaOH (0.045 NaOH, 153 °C, 48 min) achieved 93.87% glucose yield [52], while two-stage acid–alkaline pretreatments reduced lignin and improved holocellulose accessibility in mango leaves [53]. Biological pretreatments using fungi like *Pleurotus florida* and *Trichoderma reesei* facilitate lignin degradation and cellulose decrystallization but under a process that could take more than weeks, which represents the major bottleneck of biological pretreatments [42,54]. These approaches, optimized for specific biomasses, must be better understood to be applied on an industrial scale. Further developments in terms of synergies between pretreatment catalysts and enzymes must be developed to significantly enhance the efficiency of biofuel production.

**Table 1.** Different biomasses and bioprocesses explored for biofuel production.

| Biomass | Pretreatment | Biofuel | Strain | Production | Main Finding | Ref. |
|---|---|---|---|---|---|---|
| Rice husk | Alkali, HPAC, and alkali-HPAC | Ethanol | *S. cerevisiae* | 29.9 g/L (85.4%) | Optimized conditions for enzymatic hydrolysis of rice husk resulted in enhanced ethanol production | [55] |
| Rice straw | Alkaline (0.25M $Na_2CO_3$, 121 °C, 15 min) | Ethanol | *S. tanninophilus* | 108.6 g/L | High effective ethanol production using alkaline pretreatment and *A. fumigatus* enzymes | [45] |
| Corn stover | Acid (0.89% $H_2SO_4$, 125 °C, 5 min) | Butanol | *C. saccharobutylicum* DSM 13864 | 9.02 g/L (97.3%) | A complete ABE biorefinery process based on corn stover was developed, including detoxification and gas stripping | [56] |
| Pineapple peels | Ultrasonic (5% biomass loading, LSR 20, 15–45 min at 55 °C, 40 kHz, 50 W) | Ethanol | *S. cerevisiae* | 196.2 g/L | Effective ethanol production from pineapple peels using ultrasonic pretreatment | [57] |
| Deodar sawdust | Thermochemical pretreatment (0.5 M NaOH solution at 80 °C for 2 h) | Ethanol | *P. stipitis* | 14.25 g/L (95.68%) | Optimized conditions significantly increased total reducing sugar concentration | [58] |
| Tobacco waste | Alkaline (10% NaOH, 80 °C, 90 min) | Ethanol | *Mucor hiemalis* | 97% | 90% desilication reached by Alkaline pretreatment | [59] |
| Acacia wood | Acid (0.05% sulfuric acid, 200 °C, 5 min) | Ethanol | *S. cerevisiae* | 4.57 g/L (94.9%) | Soy protein addition improved enzymatic hydrolysis efficiency despite lacking enzymatic activity | [60] |
| Napier grass | DES (1:4 chcl/LA, 80 °C, 5 h) | Ethanol | *S. cerevisiae* | 86.6% | DES pretreatment resulted in 71% cellulose recovery, 68% delignification, and 87.09% glucose conversion | [61] |
| Wheat straw | IL ([TEA][$HSO_4$], 30 °C, 3 h) | Ethanol | *S. cerevisiae* PTCC 5052 | 43.1 g/L (84.34%) | Low-cost ionic liquids can effectively pretreat lignocellulosic biomass for high ethanol yields | [46] |
| Sal sawdust | Acid (1.27% hcl, 10% biomass, 22.43 min) | Ethanol | *S. cerevisiae* MTCC-36 and *P. stipitis* NCIM-3498 | 9.43 g/L (97%) | Sal sawdust from the furniture industry can be effectively transformed into ethanol | [58] |
| Paulownia wood | Two-stage Autohydrolysis (204–222 °C) | Ethanol | *S. cerevisiae* CECT-117 | 37 g/L (100%) | Sequential two-stage autohydrolysis allows for effective recovery of all fractions of Paulownia wood for ethanol production | [62] |
| Pomegranate peel | Hydrothermal (115 °C, 40 min, LSR 10) | Ethanol | *S. cerevisiae* YPH499 | 12.9 g/L (95.1%) | Optimized SSF process achieved significant sugar consumption | [63] |

## 3. Impact of Pretreatment on Enzymatic Production and Conversion to Biofuels

To assess the impact of pretreatment on organic biomass, it is essential to understand its chemical composition and how these processes can alter its molecular structure. The appropriate selection of the pretreatment process directly depends on the composition of the components present in the organic feedstocks. Each type of biomass exhibits a variable proportion of cellulose, hemicellulose, and lignin, which determines its behavior in response to different pretreatment methods [7]. Optimal pretreatment selection allows for maximizing fermentable sugar release while minimizing the formation of toxic by-products, ensuring more efficient processes and the production of high-quality biofuels.

Biomass with elevated cellulose concentrations, such as sugarcane bagasse or cellulose residues, requires pretreatments that enhance the accessibility of hydrolytic enzymes such as amylases and cellulases. In this case, physical pretreatments, such as milling or crushing, increase the contact surface area for these enzymes [64]. Alkaline pretreatments can also be employed; the application of solutions such as sodium hydroxide (NaOH) facilitates the partial removal of lignocellulosic fragments, thereby exposing cellulose and improving its conversion to fermentable sugars [65].

Biomass rich in hemicellulose, such as sorghum bagasse residues and wheat straw, contains a significant proportion of hemicellulose, requiring specific pretreatments for the release of xylose and other sugars. Acid pretreatments, such as diluted acids (sulfuric acid or nitric acid), are particularly effective for hemicellulose hydrolysis. However, it is important to consider that the generation of by-products like furfural may necessitate a detoxification process to prevent adverse effects on bioethanol quality [7]. Ionic liquid pretreatments are an efficient alternative for dissolving hemicellulose without generating inhibitors, resulting in a cleaner process that optimizes the utilization of C5 sugars, increasing conversion efficiency [66].

On the other hand, biomass that contains high levels of lignin, such as rice husks, sawdust, or residues from the wood industry, shows interferent in the digestion of cellulose and hemicellulose. To remove it, alkaline pretreatments, such as sodium hydroxide (NaOH), potassium hydroxide (KOH), or calcium hydroxide ($Ca(OH)_2$), are commonly used due to their ability to significantly reduce lignin, facilitating the accessibility of structural carbohydrates. Lignin reduction improves the digestibility of polymers, promoting the release of fermentable sugars [67,68].

Finally, there are balanced organic materials, such as *Miscanthus giganteus*, which present an equilibrium of cellulose, hemicellulose, and lignin and are receptive to a variety of pretreatments. For these materials, hybrid pretreatments are the most suitable, where the combination of acid and alkaline treatments can optimize the extraction of the compounds of interest. This balanced approach improves biomass-to-bioethanol conversion efficiency, maximizing sugar recovery without compromising biofuel quality [69,70].

### *3.1. Biological Pretreatments with White Rot Fungi (WRF)*

The pretreatment of lignocellulosic biomass using White Rot Fungi (WRF) relies on the action of enzyme-producing fungi, such as laccases, capable of depolymerizing lignin in lignocellulosic materials. This biotechnological approach stands out due to the inherent advantages of enzymes: high affinity for biomass, continuous secretion throughout the mycelium, and enhanced oxygen diffusion, which promotes both mycelial growth and ligninolytic enzymatic activity [71]. WRF fungi grow efficiently in solid-state fermentations (SSF) within low-cost bioreactors with simplified designs that do not require mechanical stirring or intensive aeration [72]. This process is particularly effective at temperatures close to 35 °C and under high humidity conditions, making it feasible for implementation in open and cost-effective systems. However, nitrogen availability is a limiting factor, as lignin degradation is favored under low nutrient concentrations and high C/N ratio conditions. Supplementation with specific salts, such as $NaNO_3$ (4%), KCl (1%), and $MgSO_4 \cdot 7H_2O$ (1.4%), has been shown to increase the recovery of substrates and available carbohydrates in cotton stems [73]. The improvement in enzymatic hydrolysis is attributed to the removal of residual lignin, which reduces the irreversible adsorption of cellulases and favorably alters the physical properties of the substrate. Studies report cellulose recovery rates ranging from 56.74% to 98.4%, and lignin degradation selectivity's between 0.7% and 30.38%, depending on the fungal species and operational conditions [74]. Given the high

potential of these pretreatments, combinations with other techniques have been explored to optimize efficiency.

Ren et al. employed microwave-assisted hydrothermal pretreatment combined with fungal fermentation for enzymatic digestion of cereal straw. This approach resulted in a high yield of fermentable sugars and a significantly superior saccharification efficiency with the combined pretreatment (66.28%) compared to the exclusive use of fungi (25.51%) [75]. Meanwhile, Wang et al. applied a combined process of *Lenzites betulina* C5617 and hot water at high pressure (LHW) to treat poplar wood, achieving a hemicellulose recovery rate of 92.33%, nearly double that obtained with LHW alone. This method enhanced glucose yield by 2.66 times compared to thermal-only pretreatment [76]. Ma et al. investigated the combined pretreatment of the ligninolytic fungus *Echinodontium taxodii* and the brown rot fungus *Antrodia* sp. 5898 with diluted acid on water hyacinth biomass. The results demonstrated increases in reducing sugar yields ranging from 1.13 to 2.11 times compared to the isolated acid treatment, highlighting the synergy of the biochemical approach [77]. Martínez-Patiño et al. performed a sequential pretreatment with *Irpex lacteus* followed by diluted sulfuric acid (2% p/v, 130 °C, 90 min) on olive biomass, observing a 34% increase in enzymatic efficiency compared to the independent acid pretreatment [78]. Additionally, Si et al. reported that the combination of ligninolytic bacteria (*Pandoraea* sp. B-6) and diluted acid increased sugar yield by 40.9%, reaching 772.0 mg/g, demonstrating an effective synergistic mechanism [79]. Zhong et al. used White Rot Fungi (WRF) combined with alkaline solutions at ambient temperature to pretreat corn stover, reducing the biological process time to 15 days. This method resulted in a 50.4% increase in glucose yield (271.1 mg/g) compared to the exclusive use of alkaline solutions [23]. On the other hand, Shen et al. implemented a synergistic treatment of $Na_2CO_3$ and the bacterium *Cupriavidus basilensis* B-8 on rice straw, achieving 799.6 mg/g of reducing sugars, a 285% increase compared to $Na_2CO_3$ alone and 8.15 times compared to raw biomass [80]. Xie et al. evaluated the effect of pretreatment of industrial hemp woody core using *Pleurotus eryngii* combined with alkaline and oxidative (A/O) solutions. This approach increased reducing sugar yields by 1.10 to 1.29 times compared to the fungal-only pretreatment, demonstrating a significant improvement in enzymatic saccharification [81]. Zhuo et al. explored a system based on tetrahydrofuran and water to pre-erosion the surface of corn stover before pretreatment with *Pandoraea* sp. B-6. This approach increased sugar yield by 7.5 times compared to untreated corn stover, attributed to surface modification and substrate porosity, creating a rough and highly accessible structure for enzymes [82]. The bio-coordinated pretreatment using steam explosion (SE) combined with fermentation by *Phellinus baumii* demonstrated increases in sugar yields during enzymatic hydrolysis. The values ranged between 26.3% and 32.3% compared to SE alone and between 6.5% and 78.1% compared to the exclusive use of WRF. This method achieved a glucose yield of 313.31 g/kg, surpassing 2.88 and 1.32 times the yields obtained with raw biomass and SE alone, respectively [75,83].

The enzymatic activity resulting from the treatment of lignocellulosic biomass with WRF depends on several factors, including the origin of the enzymes (cellulases, xylanases, and amylases), biomass loading, temperature, and reaction time. Studies report significant increases in glucose and reduced sugar release after pretreatment with WRF, with increases ranging from 7.5 to 17.6 times compared to untreated materials [71,84].

Pretreatments of lignocellulosic material using WRF, while representing an environmentally friendly option due to their low energy consumption and minimal waste generation, require prolonged timeframes to achieve efficient lignin decomposition, which poses a significant challenge for industrial applicability and scalability of these processes. Additionally, operational costs associated with this technology are increased because of the need to maintain fungal cultures under controlled conditions, directly impacting the

economic feasibility of large-scale implementation. Inherent limitations of this biotechnological approach have been identified [85,86]. WRF exhibits limited growth capacity on species like *Pinus taeda* due to the presence of inhibitory resins. A proposed solution involves a layered approach, where the resins are pre-degraded using recycled organic solvents through distillation, facilitating fungal action on lignin [71]. Pretreating biomass with diluted acid or autohydrolysis aims to remove lignin before fungal action; however, this may reduce the carbohydrates available for fungal consumption and promote delignification by altering the biomass structure [72]. The high viscosity of treated materials limits physical processes like a steam explosion or $CO_2$ supercritical treatment. Using fungi to reduce viscosity, along with $CO_2$ explosions and organic solvents, helps remove inhibitory molecules generated by lignin and improves the structure for enzymatic hydrolysis [73]. Fungal mycelium may hinder the binding of fungal cellulolytic enzymes to the substrate, reducing yield. Washing with ionic liquids or polar solvents can cause the biomass to precipitate with an anti-solvent, breaking down mycelium and microcrystalline cellulose structures and thereby increasing the hydrolysis rate [71,80].

### *3.2. Alkaline and Acid Pretreatment*

Alkaline pretreatment is widely used on lignocellulosic biomass due to its direct action on the lignocellulosic structure, promoting delignification through the breakdown of α- and β-alkyl and aryl ether bonds. This process induces the deprotonation of phenolic groups in lignin, favoring its depolymerization. It also facilitates the removal of uronic acids and acetyl groups from xylan chains, significantly increasing the substrate's susceptibility to enzymatic hydrolysis [87]. Studies related to the production of second-generation biofuels indicate that alkaline pretreatment is a critical initial step. Recent research has evaluated the life cycle of these solutions, demonstrating the economic feasibility of the process through the recovery and reuse of the alkaline agents used [88].

The impact of alkaline pretreatment on enzymatic activity has been evaluated through optimized processes. Saroj et al. applied sodium hydroxide (0.5 M, 10% p/v) at 121 °C for 1 h, achieving increases in total reducing sugar production. Without pretreatment, the concentrations obtained were 27.59 mg/mL and 34.65 mg/mL for cellulases and xylanases, respectively. After pretreatment, these concentrations increased to 32.13 mg/mL and 40.37 mg/mL. By adjusting the lignocellulosic biomass concentration to 2.5% p/v, maximum values of 34.31 mg/mL and 44.03 mg/mL were reached by incorporating enzymatic combinations (cellulases and xylanases) during 48 h of hydrolysis [87].

On the other hand, research employing acid pretreatments has increased in relevance in recent years. This type of pretreatment is based on the use of dilute acidic solutions to modify or remove the molecular structures of hemicellulose and lignin present in the biomass, which are key compounds in biofuel production. Recent studies have focused on the application of such pretreatments in large-scale bioethanol production.

Skiba et al. employed nitric acid at two concentrations (1–4%) to treat *Miscanthus* pulp, a plant that has gained prominence in the last decade due to its chemical composition and potential for conversion into bioethanol. The low acid concentration was used in a pre-hydrolysis phase to remove dust and interfering organic matter, while the higher concentration served as a key pretreatment for the release of cellulose and hemicellulose. Utilizing commercial cellulases, this pretreatment enabled the hydrolysis of up to 90% of the fermentable sugars present, which were then fermented by *Saccharomyces cerevisiae* Y-1693, achieving a total bioethanol production of 260 L per ton of treated *Miscanthus* [70]. Makarova et al. utilized 4% nitric acid as a pretreatment for two variants of cellulose derived from *Miscanthus*, employing commercial cellulose at substrate concentrations ranging from 60–90 g/L. This treatment resulted in a 92% hydrolysis of the present cellulose, yielding

an approximate production of 30.6 to 40.8 g/L of bioethanol after fermentation with *Saccharomyces cerevisiae* [89]. Finally, Skiba et al. used a 4% nitric acid pretreatment at a solid-to-liquid ratio of 1:20 to treat oat husks. After hydrolysis with cellulases, the total reducing sugar yield after 72 h of hydrolysis reached 93%, considering acid-insoluble lignin that did not interfere with enzymatic hydrolysis. Subsequently, the fermentation of these sugars resulted in a production of 0.159 g of bioethanol per gram of treated oat husks, representing a 120% increase compared to processes without pretreatment [69].

Chemical pretreatments, such as acidic and alkaline methods, demonstrate high efficiency in biomass decomposition. However, the by-products generated, including phenolic compounds and residual salts, require proper management, thereby increasing the costs and complexity of the process. Strong acids can produce toxic compounds that must be neutralized, escalating resource consumption and impacting the overall sustainability of the process. For instance, sulfuric acid, commonly used in pretreatment, generates acidic residues or toxic components that are expensive to handle and necessitate additional treatment processes. In terms of costs, acidic and alkaline pretreatments can raise bioethanol production expenses by approximately USD 0.10–0.20 per liter [87]. Sodium hydroxide is the most used alkaline agent, resulting in high operational costs. Additionally, it poses environmental challenges due to the generation of toxic compounds during neutralization and disposal. Implementing chemical recycling systems and strategies to minimize environmental impact is crucial for ensuring the viability of this pretreatment [64]. Although the main goal of the pretreatment is lignin removal, there is a risk of losing soluble sugars and degrading structural components, which not only reduces the total saccharification yield but also generates inhibitory compounds that negatively affect enzymatic activity [90]. The chemical heterogeneity of different lignocellulosic biomasses, such as sugarcane bagasse and agricultural residues, complicates the standardization of the process. Pretreatment conditions need to be adjusted for each type of biomass, increasing the complexity of process design [91]. Enzymes like cellulases, xylanases, and β-glucosidases, often derived from fungi such as *Aspergillus* sp., exhibit limited tolerance to by-products generated during alkaline pretreatment, including phenolic compounds and residual lignocellulose, which inhibit their activity and decrease hydrolysis efficiency [65]. This pretreatment requires elevated temperature conditions and prolonged processing times, significantly increasing energy consumption associated with the process. Implementing alkaline pretreatment at an industrial scale faces both logistical and economic challenges. For successful integration into biorefineries, it is crucial to evaluate not only its technical performance but also its economic sustainability and environmental impact [67].

Chemical pretreatments, such as the use of diluted acids or bases, like sulfuric acid or sodium hydroxide, enable the separation of biomass components. Their impact on the quality of biofuel is significant. Acids, such as nitric acid, are highly effective in solubilizing hemicellulose; however, they can generate inhibitors such as 5-HMF (5-hydroxymethylfurfural) and furfural, which negatively affect fermentative performance and bioethanol quality. Conversely, alkaline agents improve cellulose digestibility and partially remove lignin, but they may increase the ionic load of the medium, potentially affecting fermentation and the final product quality.

### *3.3. Physicochemical Pretreatment*

This pretreatment is one of the most reported due to its impact on lignocellulosic biomass, as it not only removes compounds that inhibit enzymatic hydrolysis but also increases the material's porosity. It begins with mechanical grinding to increase the surface area and remove contaminants. The resulting material is then treated with diluted acid solutions (0.5–5% $H_2SO_4$), which facilitates the denaturation and removal of residual

lignin. However, high acid concentrations can generate inhibitory compounds, such as furfural, salts, and phenolic compounds derived from lignin, which reduce enzymatic efficiency. In the context of biorefineries relying on sugar fermentation, this pretreatment has proven to be efficient, reducing the lignin content in the raw material to below 1% and increasing cellulose availability to levels above 82%. Scanning electron microscopy and X-ray spectroscopy analyses have shown progressive structural damage and a reduction in the crystallinity index of cellulose to values below 13.8% [92].

Ranjan et al. developed a protocol that included grinding the biomass into particles of 2.36 mm, drying it at 50 °C for 12 h, and treating it with sulfuric acid (2%) at 55 °C for 24 h. This approach produced 28.3% p/p fermentable sugars, surpassing the yields obtained from untreated materials (10.2–11.8% p/p). The specific sugar concentrations included 12.5% p/p xylose, 7.4% p/p glucose, 5.4% p/p cellobiose, and 3% p/p arabinose [92]. Nemes et al. performed an acid pretreatment of oat bran by adding 50 mL of an aqueous solution containing 3% sulfuric acid to 10 g of previously ground substrate. The chemical hydrolysis process was carried out for 2 h at room temperature, followed by controlled drying at 40 °C for 24 h. Subsequently, enzymatic hydrolysis of the material was conducted using an enzymatic complex produced by the fungus *Aspergillus niger* (ATCC-6275). This process achieved a yield of 44% in total sugars released after 5 days of reaction under controlled conditions, with the release of maltose (10.43 g/g of substrate), glucose (27.27 g/g of substrate), and fructose (6.543 g/g of substrate) [93]. Finally, Chugh et al. evaluated the effect of combined autohydrolysis and acid pretreatment on the enzymatic hydrolysis of rice bran. The initial process involved a steam pretreatment at 15 psi for 15 min, followed by an acid phase using a 1% $H_2SO_4$ solution. This approach enabled the release of 368.36 mg of reducing sugars per gram of treated substrate, of which 310.88 mg were glucose, an essential carbohydrate subsequently used for bioethanol production [67].

Utilizing lignocellulosic biomass for biofuel production and high-value compounds faces significant challenges due to its recalcitrant structure. Physical pretreatments, such as grinding, steam explosion, or microwave irradiation, have proven effective in improving biomass accessibility. These methods are energy-intensive, and when non-renewable energy sources are employed, the associated costs increase significantly [93]. For instance, grinding is estimated to consume between USD 0.05 and USD 0.15 per kilogram of processed biomass, thereby elevating operational costs in large-scale processes. Physicochemical pretreatments, such as ammonia fiber explosion (AFEX) or carbon dioxide explosion, offer greater control over the generation of by-products. Nevertheless, their industrial-scale implementation remains costly due to the specialized equipment required [92]. Many pretreatment methods fail to fully remove lignin or disrupt cellulose crystallinity, which limits enzymatic accessibility for efficient degradation. A critical challenge is the generation of inhibitors such as furfural, HMF (5-hydroxymethylfurfural), or phenolic compounds derived from lignin [67]. To ensure consistent accessibility to cellulose, it is necessary to ensure that the pretreatment facilitates total biomass disruption, allowing all areas of cellulose to be exposed to enzymatic action [94].

Physicochemical pretreatments combine the accessibility provided by physical pretreatments with the use of chemical agents, such as carbon dioxide explosion or ammonia exposure, to modify or eliminate lignocellulosic structures. Additionally, these processes help to modify the chemical composition of the residues, promoting the conversion of fermentable sugars. However, the formation of degradation products, such as organic acids and phenolic compounds, may decrease the quality of the produced bioethanol, necessitating additional detoxification stages.

### *3.4. Ionic Liquid Pretreatment*

Pretreatment with ionic liquids constitutes a sustainable and efficient alternative for processing lignocellulosic biomass. These solutions, composed of organic cations and anions, exhibit thermochemical stability, low volatility, and a high capacity to dissolve both organic and inorganic molecules. These characteristics allow for improved surface porosity and solubilization of cellulose molecules, facilitating subsequent enzymatic hydrolysis [94]. Furthermore, ionic liquids are recyclable and can be diluted in water, reducing their viscosity and optimizing contact with lignocellulosic biomass [95]. The mechanism of action of these liquids includes interactions with active sites in lignin molecules, such as the structural units of p-coumaryl alcohol and sinapyl alcohol. However, this approach requires prior optimization to tailor it to the specific characteristics of the lignocellulosic material to be processed [96,97].

Sunar et al. developed an ionic liquid composed of isopropyl ethylamine and sulfuric acid in equimolar proportions, which was used as a pretreatment agent for lignocellulosic biomass. A 10% p/v solution of the ionic liquid was prepared, which, after pretreatment, was washed with a water–acetone mixture and subsequently recovered by evaporation. The residual lignin was separated by centrifugation. This method showed a significant positive impact on the enzymatic activity of cellulases and β-glucosidases derived from *Aspergillus* sp., achieving a 76% p/p recovery of fermentable reducing sugars, representing a 470% improvement compared to untreated biomass (16% p/p) [97]. Silveira et al. observed a maximum glucose conversion yield of 70.7% when applying a pretreatment with 1-butyl-3-methylimidazolium acetate in combination with supercritical carbon dioxide and ethanol for 12 h at 180 °C, using sugarcane bagasse (SCB) as the substrate [98]. Brandt-Talbot et al. reported a 77% sugar release through enzymatic saccharification after pretreating *Miscanthus giganteus* with ionic liquids at 120 °C for 480 min [36].

Ionic liquids, while a promising technology due to their ability to efficiently and selectively dissolve biomass, face significant economic challenges. The synthesis and recycling of ionic liquids remain expensive, with costs ranging from $20 to $30 per kilogram of product, hindering their large-scale implementation. Although their efficiency in biomass dissolution surpasses that of other pretreatment methods, their cost remains a substantial barrier to scalability [97]. The high cost and limited recyclability of ionic reagents remain major barriers to scaling ionic liquid pretreatments. Although these liquids have been shown to be recyclable, their recycling efficiency depends on operational conditions and the type of lignocellulosic biomass used [94]. Ionic liquid pretreatment can alter the biomass structure; although lignin removal is the goal, the integrity of cellulose and hemicellulose may also be affected, potentially compromising the performance of subsequent processes [99]. Ionic liquids may directly interfere with enzymatic activity; it has been observed that these compounds can alter the conformation of enzyme complexes, affecting their ability to recognize and degrade glucosidic linkages in cellulose [100]. Combining ionic liquid pretreatment with fermentation systems, such as bioethanol production or biotechnological products, requires careful design. The toxicity and accumulation of inhibitory by-products generated during pretreatment must also be considered, as they may interfere with the recovery and purification of final products [101].

Ionic liquid pretreatments, such as 1-butyl-3-methylimidazolium chloride, have emerged as a promising technology due to their ability to dissolve lignin and hemicellulose without generating significant amounts of inhibitory compounds. Their influence on biofuel quality is notable. One of the main advantages of this pretreatment is that it provides a highly accessible substrate for enzymes, maximizing the production of fermentable sugars and minimizing secondary waste. However, residues of ionic liquids in the biomass can affect fermentation or biofuel quality if not completely removed.

Figure 2 summarizes the metabolic pathways followed by lignocellulosic biomass, microalgal biomass, and vegetable or waste oils to be converted into biofuels.

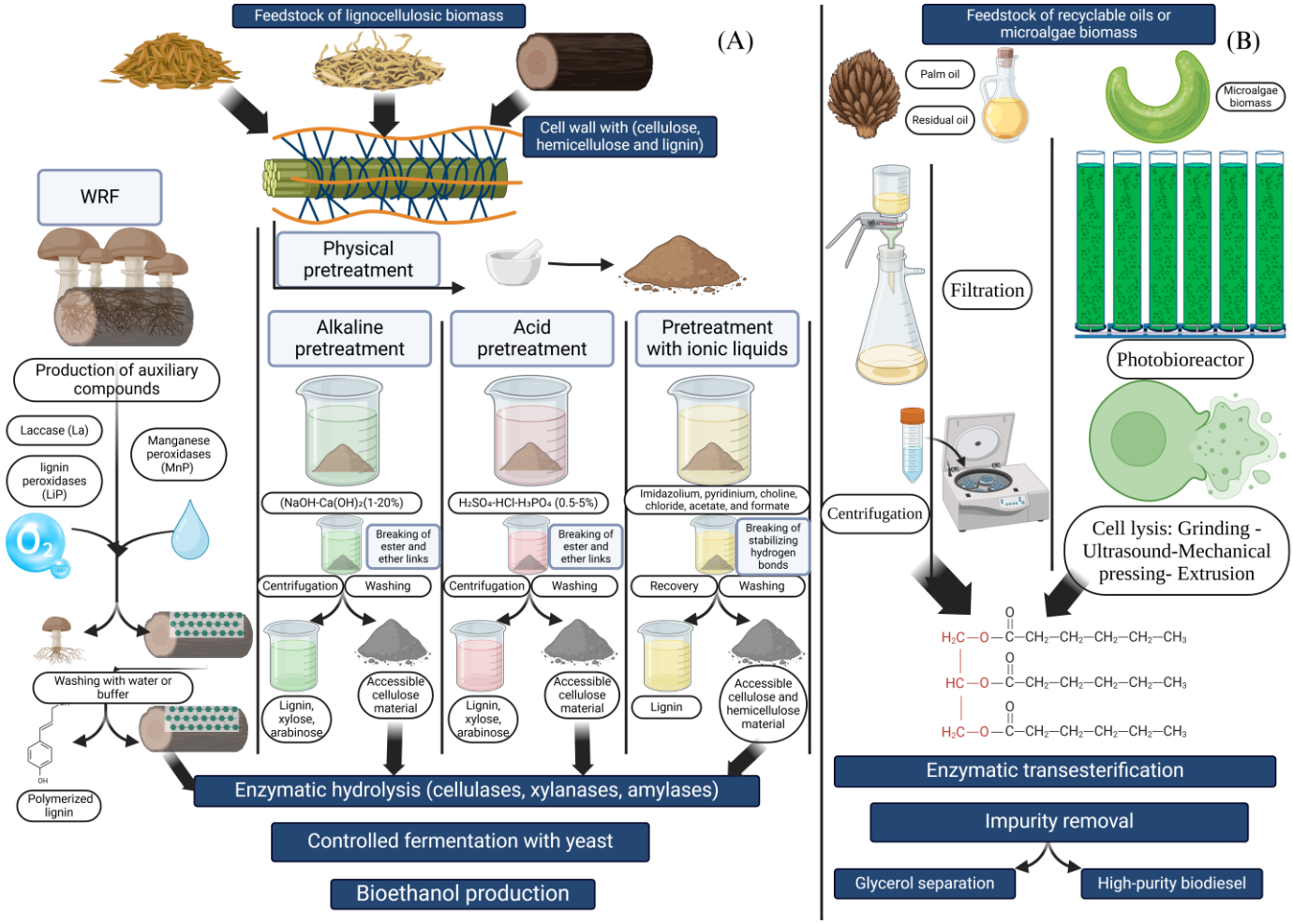


**Figure 2.** Pretreatments for bioethanol and biodiesel: From biomass to energy. (**A**) Pretreatments used in lignocellulosic biomass for bioethanol production. (**B**) Pretreatments in oils, fats, and microalgae biomass for biodiesel production.

### *3.5. Pretreatment for Enzymatic Transesterification*

The release of natural lipids produced by microalgae can be achieved through mechanical, chemical, or combined methods, such as cell rupture by ultrasound or the use of solvents like n-hexane or ethanol. These strategies enhance the release of intracellular lipids, increase the availability of triglycerides and fatty acids for enzymatic reactions, and remove non-lipid compounds, such as proteins and carbohydrates, which may interfere with the enzymatic transesterification process [102].

Raw oils extracted from microalgae often contain impurities such as phospholipids and heavy metals generated during cell lysis or acquired by the microalgae during metabolism. Enzymatic degumming, performed through treatments with hot water or acids, allows for the removal of these impurities. Additionally, oil purification by adsorption with activated carbon is effective in removing trace metals, ensuring an efficient transesterification process [103]. The presence of water in microalgal biomass or extracted oils can interfere with lipase activity during transesterification. To mitigate this effect, drying techniques at controlled temperatures or the use of dehydrating agents such as calcium chloride ($CaCl_2$), silica gel, magnesium anhydride ($MgSO_4$), or aluminum oxide ($Al_2O_3$) are employed.

These dehydrating agents do not interfere with enzymatic reactions and ensure effective conversion [104].

Tien Thanh et al. demonstrated that the release of intracellular lipids from microalgae, such as *Chlorella pyrenoidosa*, is essential for transesterification. High moisture content in the biomass (90%) significantly reduced biodiesel yield (10.3%). However, after subjecting the biomass to a purification process with ethanol and drying at 60 °C for 24 h, the yield increased to 91.4% [102]. Passos et al. reported that degumming with a phospholipase cocktail is a key step in pretreating soybean oil for transesterification using lipases from *A. niger*. A biodiesel yield of 97% was achieved under optimal conditions: an ethanol-to-oil ratio of 4.48:1, a moisture content of 3.41%, and a lipase concentration of 2.43% [103]. Finally, ShenavaeiZare et al. investigated the importance of dehydration in oils extracted from halophytic plants such as *Salicornia persica* and safflower. They employed calcium chloride as a catalyst in a system with a methanol-to-oil concentration of 12.9% and 14% calcium chloride, achieving a 97.01% biodiesel yield after 3 h of reaction [104].

The only way to rigorously assess the impact that pretreatments may have on fungal enzyme hydrolysis and bioethanol production is through the optimization of these processes [65]. To this end, a compilation of research data is presented in Table 2, which outlines studies on physical [105], chemical [66], and ionic [99] pretreatments that influence the release of reducing sugars from various sources of lignocellulosic biomass such as rice straw, soy hulls, and banana peels, among others, after being hydrolyzed using enzymes derived from *Aspergillus*. The considered pretreatments include the use of alkaline (NaOH) [106], acidic ($H_2SO_4$) [107], and ionic solutions [22]. In this context, the amount of sugars released (expressed in mg/g of biomass) is presented, the concentrations obtained from different treatments are compared, and the most significant effects on the efficiency of enzymatic hydrolysis are highlighted. Furthermore, the impact of different pretreatments on the final bioethanol production is discussed, comparing the bioethanol production yields (in g/L) obtained after fermentation with various *Saccharomyces cerevisiae* [65] and *Candida shehatae* [64] strains. Yields are analyzed under standard fermentation conditions (pH 4.5–5.5, 30 °C).

The pretreatment of oils and microalgae biomass, while representing an attractive source of lipids for biodiesel production, also faces significant economic and environmental challenges. The use of organic solvents for lipid extraction can generate waste that is difficult to manage and contributes to environmental contamination if appropriate recovery systems are not implemented. Moreover, the high cost of organic solvents increases operational expenses associated with extraction processes, further complicating their large-scale application [103]. Phospholipids, free fatty acids, and polar compounds in crude oils obtained from agro-industrial by-products or unrefined oils are considered impurities that interfere with the process. Although enzymatic or chemical degumming has proven effective in reducing these impurities, achieving complete removal without affecting essential oil components remains a challenge [108]. Treatment of oils through neutralization or drying can alter the chemical properties of the substrate, impacting the three-dimensional structure of triglycerides and, consequently, the activity of fungal lipases [109]. Water is a key factor in enzymatic transesterification [110]. Drying at high temperatures may induce triglyceride hydrolysis, reducing biodiesel yield; therefore, a low-temperature drying process tailored to the oil characteristics is required, which must be optimized for each type of oil used in the process [111]. The use of low-quality oils, such as residual oils or those from lignocellulosic materials, presents additional challenges. These oils often contain high levels of metallic contaminants or enzymatic inhibitors, necessitating additional purification processes that increase costs and complicate the scaling of the process to industrial levels [112].

**Table 2.** Impact of pretreatments on reducing sugar release and bioethanol production from lignocellulosic biomass and *Saccharomyces cerevisiae* fermentation.

| Pretreatment | Biomass | Enzyme | Microorganism | Sugar Produced | Production of Bioethanol | References |
|---|---|---|---|---|---|---|
| Physical–alkaline | Corncobs | Cellulase: Endoglucanase Exoglucanase β-glucosidase | *A. niger* | 128.20 g/L | 6.4 g/L | [90] |
| | Sugarcane bagasse | cellulases-hemicellulases: β-glucosidase endo-β-glucanase β-xylosidase endo-β-xylanase | *Aspergillus tubingensis* NKBP-55 | 20 g/L | 15.54 g/L * | [64] |
| | Pongamia | Cellulases: endo-β-d-glucanase exo-β-d-glucanase β-glucosidase | *Aspergillus calidoustus* | - | 4.4 g/gds | [106] |
| | Wood waste | | | - | 2.2 g/gds | |
| | Rice straw | Cellulases: CMCase β-glucosidase) | *A. fumigatus* | 557.8 mg/gds | 9.45 g/L | [113] |
| Physicochemical (acid–alkaline) | Sugarcane bagasse | Cellulases | *A. niger* ITV02 | 49 g/L | 22.4 g/L | [65] |
| | Wheat bran and sawdust | β-glucanase | *A. niger* EG-RE (MW390925.1) | 37.5 g/gds | 12 g/L | [91] |
| Physical–acidic | Rice bran | Celullases-Amylases-Xilanase: β-glucosidase CMCase Xylanase α-amylase β-glucoamylase | *A. niger* P-19 | 468 mg/gds | 37.63 g/L–0.41 g/gds | [67] |
| | *Chlorella sorokiniana*, *Tetraselmis* sp. *Skeletonema* sp. | α-amylase β-glucoamylase | *Aspergillus oryzae*-*A. niger* | 464 mg/gds 420 mg/gds 425 mg/gds | 16.512 g/L 7.92 g/L 9.792 g/L | [114] |
| Physical | Banana peel | Cellulase | *A. niger* | - | 4.24 g/L | [107] |
| | Wheat straw | Cellulases | *A. fumigatus* | 889.1 mg/gds | 21.88 g/L | [66] |
| | Watermelon peels | Cellulases, α-amylase, | *A. niger* | - | 57 g/L | [115] |
| | Coffee pulp | Cellulase | *A. niger* MT328516 | 741 mg/gds | 71.39 mg/mL | [116] |
| | Wheat bran | | *Aspergillus flavus* MT328429 | 633 mg/gds | 11.73 mg/mL | |
| | Yam peels banana peels | Cellulases, α-amylase Xylase | *A. niger* | 69.7 mg/gds 65.2 mg/gds | 31.86 g/L 22.72 g/L | [105] |
| | Cassava starch | α-amylase β-glucoamylase | *Aspergillus awamori* | - | 0.46 g/gds | [117] |
| Physical–ionic | Sugarcane bagasse | Xilanases | *A. niger* | 297 mg glucose/gds 236 mg xilose/gds | 10 g/L–0.42 g/gds | [101] |
| | Mango seed starch | α-amylase | *A. niger* | 848 mg/gds | 31.40 g/L | [99] |
| | Starch | alfa-amylasa | *Aspergillus flavus* AUMC10636 | 28.85 g/L | 14.74 g/gds | [100] |

* Fermentation with *Candida shehatae* NCIM 3501 of reducing sugars released after enzymatic hydrolysis with cellulases and hemicellulases from the fungus *Aspergillus tubingensis* NKBP-55.

On another note, to evaluate the impact of pretreatments on the lipase activity of *Aspergillus* fungi, enzymatic transesterification is carried out for biodiesel production, with results summarized in Table 3. This table compares the lipase activities of various *Aspergillus* strains, considering that the raw materials used come from different sources, such as soybean oil, palm oil, Jatropha oil, and microalgal biomass. This implies that their composition requires adequate characterization for pretreatment standardization. Enzymatic performance is expressed as the percentage (%) of conversion of fatty acids to biodiesel. Moreover, the incubation conditions (temperature, pH, and time) that optimize biodiesel production are highlighted. This table summarizes the impact of various fermentation parameters, such as pH, temperature, substrate concentration, and incubation time, on biodiesel production using *Aspergillus* lipases. Additionally, comparisons between free and immobilized lipases are included, emphasizing the improvement in the conversion of oils to biodiesel under optimal fermentation conditions.

**Table 3.** Influence of pretreatments on the yield percentage of enzymatic transesterification catalyzed by *Aspergillus* lipases.

| Pretreatment | Raw Material | Enzyme | Producer | Yield (%) | Conditions | Reference |
|---|---|---|---|---|---|---|
| Gravitational settling | *Scenedesmus obliquus* | Immobilized whole-cell lipase | *A. niger* | 90.8 | 36 h, 35 °C, 5:1 ratio (methanol: oil) | [112] |
| Natural settling followed by lyophilization | *Chlamydomonas* sp. JSC4 | Immobilized whole cell lipase | *A. oryzae* | 97 | 32 h, 30 °C, 7:1 ratio (methanol: oil) | [110] |
| Filtration | Waste cooking oil | Co-immobilized lipases | *A. oryzae* | 98.5 | 24 h, 40 °C, 4:1 ratio (methanol: oil) | [108] |
| | Waste cooking oil | Lipase (1,3-specific) | *A. oryzae* | 98.5 | 9 h, 40 °C, 4:1 ratio (methanol: oil) | [109] |
| Soxhlet extraction. | *Jatropha curcas* seed oil | Lipase immobilized with $TiO_2$ | *A. niger* | 92 | 30 h, 37 °C, 6:1 ratio (metanol:oil) | [111] |

### *3.6. Global Impact of Biofuel Production*

Lignocellulosic biomass pretreatments can generate various negative environmental impacts. Specifically, the chemical pretreatments, both acidic and alkaline, may lead to the formation of problematic by-products. Organic acids such as acetic acid, furfural, and levulinic acid can form during acidic pretreatments, and these compounds are toxic to the microorganisms used in fermentation, reducing biomass conversion efficiency [67,92]. In alkaline pretreatments, compounds such as sodium hydroxide or potassium hydroxide generate alkaline residues that require proper management to avoid soil and groundwater contamination [87,88]. Furthermore, ionic liquid pretreatments, despite their promise of greater sustainability, often require corrosive solvents like hydrochloric acid or sodium hydroxide for residue neutralization, posing risks to both human health and ecosystems. Additionally, ionic liquids are challenging to recover and recycle, exacerbating environmental impacts if not properly managed [97,98]. The energy-intensive nature of enzymatic processes presents a significant environmental challenge. The use of enzymes for biomass conversion requires controlled operating conditions, often involving elevated temperatures and pressures, leading to considerable energy consumption and a larger carbon footprint, especially when energy sources are derived from fossil fuels. Emissions associated with biocatalyst production include greenhouse gases such as carbon dioxide and methane, primarily stemming from fermentation processes used for enzyme production [93]. Moreover, large-scale production of biocatalysts generates industrial waste, including unconsumed substrates, microbial biomass, and intermediate products, which require treatment to prevent accumulation and contamination [95]. A potential solution to mitigate these negative impacts involves implementing circular economy processes and optimizing production pathways. Technologies for recovering and recycling ionic liquids and solvents can minimize waste generation, improving pretreatment processes. Through the use of more efficient catalysts or biocatalysts capable of operating at lower temperatures, energy consumption can be reduced [118,119]. Focusing on reducing industrial waste through the valorization of by-products, such as organic acids or microbial biomass, can contribute to the overall sustainability of the process.

White-rot fungi pretreatments, while promising in terms of sustainability and low environmental impact, face limitations at a large scale due to the slow pace of biological reactions. For instance, fungal fermentation processes require extended timeframes (7 to 10 days), whereas conventional technologies such as acid hydrolysis can be completed in just 4 to 6 h. This discrepancy negatively affects production timelines and cost competitiveness, particularly when considering industrial-scale operating costs, which range between $0.50 and $1.00 per gallon of bioethanol produced [73,78]. Physical and chemical pretreatments, such as acid and alkaline hydrolysis, are widely used in the bioethanol industry due to their speed and optimization for large-scale production, with operational

costs ranging between $0.30 and $0.50 per gallon of bioethanol, acid hydrolysis typically requires large amounts of thermal energy, increasing operational costs and contributing to a higher carbon footprint, with $CO_2$ emissions exceeding 1 ton per 1000 L of bioethanol produced [108,120]. In comparison, ionic liquid pretreatments, although more efficient in terms of selectivity and fermentable sugar generation, require the use of highly corrosive solvents, which increases operational costs, elevating bioethanol production costs to approximately $1.50 per gallon due to additional risks and solvent handling expenses. Transesterification for biodiesel production, one of the most used methods at an industrial scale, demonstrates greater operational efficiency in terms of time and costs, with a biodiesel production cost estimated at approximately $2.50 per gallon [95].

Emerging technologies such as ionic liquid pretreatments have yet to achieve competitive cost standards compared to these mature technologies. Despite the fact that biomass conversion efficiency is higher with advanced pretreatments, the costs of solvents, challenges in recycling, and potential material losses remain significant economic barriers. Although the potential advantages of emerging technologies exist, their scalability remains uncertain. For instance, while the use of commercial enzymes for bioethanol production adds an additional cost of approximately $0.30 per gallon, experimental-phase technologies may require up to three times more in operational costs due to the lack of optimization in biocatalyst use [103,117]. For emerging technologies to become competitive, it is essential to reduce these operational costs, improve energy efficiency, and overcome obstacles related to the safety and management of by-products. The effective integration of these technologies into existing industrial infrastructures will be key to ensuring a smooth transition toward more sustainable and economically viable processes.

## 4. Enzymes Produced by *Aspergillus*

### *4.1. Amylases*

Amylases are classified according to their mechanism of action into four main groups: endoamylases, exoamylases, branching enzymes, and transferases. Endoamylases, such as α-amylase, hydrolyze the α-1,4 glycosidic bonds randomly within starch chains, whereas exoamylases cleave the bonds at the chain ends, with β-amylases acting solely on α-1,4 glycosidic bonds and amyloglucosidase (glucoamylase) also acting on α-1,6 glycosidic bonds [121].

Alpha-amylases (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolase) are extracellular endoenzymes that hydrolyze the internal α-1,4 glycosidic bonds of starch chains [122–124] These enzymes break down long-chain carbohydrates by acting at random sites along the starch chain, ultimately producing maltotriose and maltose from amylose, or maltose, glucose, and limit dextrin from amylopectin. However, these enzymes do not have the ability to break terminal glucose residues or α-1,6 linkages. One of their characteristics is that α-amylases tend to act faster than β-amylases because they can act anywhere on the substrate [125,126].

The two-dimensional structure of typical alpha-amylases and their putative homologs mostly involves three basic domains, denoted as A, B, and C. Domain A is defined as the (β/α) 8 domains with its catalytic residues, where glutamic acid acts as a proton donor and aspartic acid acts as a nucleophile at its catalytic sites [127]. The B domain is a long loop protruding between the β3 strand and α3 helix, while the C domain has an antiparallel beta-sandwich structure consisting of eight strands [128]. As they are calcium-dependent, the absence of these ions could affect the structure, function, and stability of the enzyme, potentially leading to irreversible inactivation [129]. These ions are responsible for maintaining the protein structure in its correct conformation, thereby enabling the enzyme to withstand thermal inactivation. There are also reports suggesting that calcium ions

play a primarily structural role, as the catalytic sites are distant from the calcium-binding sites [130].

β-amylases (EC 3.2.1.2) are exoenzymes belonging to the GH14 family of hydrolases, capable of cleaving α-1,4 glycosidic bonds at the non-reducing ends of starch, producing β-maltose and β-limit dextrins [131,132]. When amylopectin is subjected to the action of this enzyme, the glucan chains α-1,4 of the highly branched molecule are trimmed from the chain end toward the α-1,6 branching points [133]. The term beta refers to the initial anomeric configuration of the released free sugar group and not to the configuration of the hydrolyzed bond [134]. In plants, β-amylases are associated with fruit development, ripening (hydrolyzing stored starch in fruits to maltose, giving ripe fruits their sweet taste), seed germination, and response to abiotic stress [135]. Plants such as sweet potato, soybean, and barley are used as sources of β-amylase. However, due to their disadvantages, such as high production costs and low stability during storage, they are not ideal for industrial processes. The most viable alternative is the use of microbial β-amylases because their production is not affected by the season or climate; they undergo simpler processing, have a uniform nature, are more stable, and are easier to handle [132]. Their structure consists of two peptide domains: a large A domain composed of amino acids from 1 to 417 and a B domain comprising amino acids from 418 to 516. Domain A exhibits a barrel structure (β/α) 8, which is similar in both plant and microbial β-amylases. This structure resembles a pocket where α helices and β sheets associate to form the catalytic site [136].

Glucoamylases (EC 3.2.1.3) are exoenzymes that catalyze the hydrolysis of α-1,4- and α-1,6-glucosidic bonds, with a lower efficiency for α-1,6 cleavage, releasing β-D-glucose from the non-reducing ends of starch, as well as related polysaccharides and oligosaccharides [131,137]. Additionally, they can hydrolyze, at a slower rate, nearly all α-glycosidic bonds, including α, β-(1,1), α-(1,2), and rare α-(1,3) bonds, except for α, α-trehalose [138]. These enzymes have an invertase reaction mechanism, as there is a transfer of protons from a general acid catalyst to the glycosidic oxygen, followed by the nucleophilic attack of a deprotonated water molecule, assisted by a general basic catalyst [139]. Glucoamylase is the primary hydrolytic enzyme used for saccharification in the fermentation process to produce substances with glucose [140]. Glucoamylases are hydrolytic enzymes of particular importance in the food and pharmaceutical industries [141]. From a structural standpoint and according to their origin, glucoamylases can be classified into five types (I, II, III, IV, and V). Glucoamylases obtained from filamentous fungi can be of type I or II. On the other hand, those from yeasts can be of type III and IV, while glucoamylases from prokaryotes are of type V. Type I glucoamylases (Gas) contain a catalytic domain (CD) at the N-terminal end linked to a carbohydrate-binding module (CBM) 20 at the C-terminal end. Type II GAs have a CBM21 attached to the catalytic domain; the β-sandwich structure of the CBM21 domains is similar to that of CBM20, although the CBM21 domains present in type II GAs are always located at the N-terminal end and have two starch-binding sites [142].

In filamentous fungi, differences have been observed in glucoamylases regarding their molecular mass, amino acid sequence, protein stability, glycosylation percentage, and within and outside the starch-binding site. Up to six different forms of glucoamylases have been found. Most of these glucoamylases are multidomain enzymes with a catalytic N-terminal domain and a C-terminal domain containing starch binding sites. However, exceptions include *Rhizopus oryzae*, *A. niger*, *A. oryzae*, and *A. flavus* NSH9, whose glucoamylase lacks the starch-binding domain structure [143]. In type III glucoamylases, the catalytic domain (CD) is not associated with any non-CD or variable region. These enzymes lack additional domains commonly found in other types of glucoamylases. Despite not having a CBM, there are reports of their ability to bind to starch. It is possible that the enzyme uses alternative regions within its CD to interact with starch and carry out its hydrolytic activity.

Type V glucoamylases have a catalytic domain attached to an N-terminal region composed of 18 antiparallel β-strands arranged in β-sheets of a super β-sandwich structure, which would confer thermal stability to the enzyme. The C-terminal catalytic domain is a barrel (α/α)6, lacking the peripheral subdomain of eukaryotic glucoamylases. The binding region between the N-terminal region and the catalytic domain is common to all proteins in the GH15 family of prokaryotes [142].

### *4.2. Cellulases*

Cellulases are enzymes that break down β-glycosidic bonds in carbohydrate molecules. Efficient cellulose hydrolysis requires the coordinated action of a cellulase enzyme complex, which consists of three main types of enzymes: endoglucanase (endo-β-1,4-D-glucan glucanohydrolase, EC 3.2.1.4), exoglucanase or cellobiohydrolase (β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.91), and β-glucosidase or cellobiase (β-D-glucoside glucohydrolase, EC 3.2.1.21) [144].

These three enzymes work together to achieve complete cellulose hydrolysis. Endoglucanase primarily targets the amorphous regions of cellulose, where it randomly breaks internal bonds, creating new chain ends that can be attacked by the other enzymes. This enzyme shows the highest activity against soluble cellulose or acid-treated amorphous cellulose. Exoglucanase, on the other hand, cleaves cellulose chains from the reducing or non-reducing ends, producing glucose or cellobiose units. Finally, β-glucosidase breaks down cellobiose into glucose, but it does not act on either amorphous or crystalline cellulose.

Although the exact mechanism is not fully understood, the early stages of cellulose hydrolysis involve the fragmentation of cellulose aggregates into short fibers, a process known as amorphogenesis, which occurs before any detectable release of reducing sugars [145].

### *4.3. Xilanases*

Xylanases (EC 3.2.1.8) belong to the class of hydrolase enzymes and are responsible for the breakdown of xylan into xylose and xylan oligosaccharides [146]. Xylan is a predominant polymer in hemicellulose, which constitutes the plant cell wall, being the second most abundant natural polymer on Earth after cellulose, accounting for approximately 33% of lignocellulosic biomass [147]. Xylanase includes several subclasses, such as endo-1,4-β-D-xylanases (EC 3.2.1.8), β-D-xylosidase (EC 3.2.1.37), α-L-arabinofuranosidases (EC 3.2.1.55), acetylxylan esterase (EC 3.1.1.72), ferulic acid esterase (EC 3.1.1.73), α-glucuronidase (EC 3.2.1.139), and p-coumaric acid esterase (EC 3.1.1.B10), which primarily act on the β-1,4 linkages present in the xylan structure [148–151]. Among these hydrolytic enzymes, endo-xylanases represent the largest group and are currently applied in four main areas: (i) degradation of agricultural residues; (ii) enzymatic treatment of animal feed; (iii) production of dissolved pulps for cellulose manufacturing used in rayon production; and (iv) pre-treatment of kraft pulp or fiber, promoting lignin removal and altering paper properties [152].

Xylanases with high specific activity play a crucial role in the biodegradation of hemicellulose, making them of significant importance to industry. The biological properties of these enzymes are primarily determined by the active amino acids located in their active sites. According to the CAZy database [153], xylanases (EC 3.2.1.8) are grouped into several families of glycoside hydrolases (GH), such as GH5, GH8, GH10, GH11, GH30, GH43, GH51, and GH98. However, most research has focused on xylanases from the GH10 and GH11 families. Among these, xylanases from the GH11 family are considered true xylanases, characterized by their low molecular weight and the presence of a conserved β-jellyroll structure [152,154]. These enzymes offer several advantages, such as high catalytic efficiency, strict substrate specificity, and stability over a wide range of pH and

temperature conditions, making them valuable for industrial applications in areas such as food production, animal feed, paper pulp processing, and juice clarification [155].

The protein structure of xylanase consists of specific functional units that are associated with the arrangement of active amino acids and their interactions with various biochemical properties of the enzyme. Within the active site, the amino acids play a crucial role in substrate recognition, catalysis, and product release. Hydrogen bonds and stacking interactions are essential forces that facilitate ligand binding, and the active site typically contains several polar and aromatic amino acids. The amino acids interacting within the −2 to +1 subsites of hemicellulose are highly conserved, with the catalytic network centered around two glutamic acid residues, which are critical for the enzyme's catalytic activity. These residues are considered catalytic due to their importance in the enzyme's function. Furthermore, three aromatic amino acids—Y77, W79, and Y171—are located near the −2 subsites of hemicellulose and are believed to play a role in stabilizing the xylanose ring structure, which is essential for the formation of a bond with the substrate [155,156].

### *4.4. Lipases*

Lipases (triacylglycerol acyl hydrolases, EC 3.1.1.3) are enzymes that catalyze the hydrolysis of fats and oils, releasing free fatty acids, diglycerides, monoglycerides, and glycerol. In organic solvents, these enzymes also facilitate synthetic reactions such as esterification, acidolysis, alcoholysis, and interesterification. Lipases operate under mild conditions, exhibit high stability in organic solvents, and possess broad substrate specificity, often demonstrating high regio- and stereoselectivity in catalytic reactions. These features make lipases one of the most widely used biocatalysts in biotechnological applications [157]. They are employed in various industries, including food, detergents, cosmetics, pharmaceuticals, leather, textiles, and paper, as well as in the production of biodiesel, biopolymers and in the treatment of lipid-rich wastewater. These enzymes are found in a wide range of organisms, including animals, plants, bacteria, and fungi, with microbial lipases being particularly attractive due to their versatility and ease of large-scale production [158,159]

Despite exhibiting low sequence identity in their primary structure, lipases share a similar structural fold. Other enzymes, such as esterases, proteases, dehalogenases, epoxide hydrolases, and peroxidases, display comparable structural features and, together with lipases, form the α/β-hydrolase family [160]. The α/β-hydrolase fold consists of a central β-sheet composed of eight parallel β-strands, except for β2, which is antiparallel to the others. This sheet adopts a left-handed superhelical twist, creating a 90° angle between the first and last strands. Strands β3 to β8 are connected by a bundle of helices, where helices A and F are positioned against the concave side of the central β-sheet, while helices B, C, D, and E pack against the convex side [161].

The active site of α/β-hydrolases contains a highly conserved catalytic triad composed of a nucleophilic residue (serine, cysteine, or aspartic acid), a catalytic acid residue (aspartic acid or glutamic acid), and a histidine residue. In lipases, the nucleophilic residue is always serine. The nucleophilic residue in lipases is found within a highly conserved pentapeptide sequence, Sm-X-Nu-X-Sm, where Sm represents a small residue, typically glycine, but occasionally substituted by alanine, valine, serine, or threonine; X represents any amino acid; and Nu corresponds to the nucleophilic residue. This pentapeptide forms a sharp γ-turn between the β5 strand and the αC helix, known as the "nucleophilic elbow". The conformation of this strand-loop-helix motif forces the nucleophilic residue into energetically unfavorable backbone dihedral angles, creating steric constraints on neighboring residues. The "nucleophilic elbow" is considered the most conserved structural feature of the α/β-hydrolase fold [162,163].

### *4.5. Optimal Conditions for Enzyme Production by Aspergillus*

In the past decade, the production of amylases using fungi from the *Aspergillus* genus has gained significant attention due to its importance in industries such as food processing and biofuels. Recent studies have demonstrated that *A. oryzae* can produce α-amylase using low-cost substrates like edible oil cakes, highlighting its potential in sustainable industrial processes [164]. Additionally, a starch-hydrolyzing α-amylase produced by *A. niger* has been characterized, showing high acid tolerance and efficiency in starch hydrolysis, making it suitable for applications in the food industry [122]. Furthermore, optimizing fermentation conditions, such as temperature, pH, and substrate concentration, has significantly enhanced the production of amylases by *Aspergillus terreus*, using pearl millet as a substrate in solid-state fermentation [11]. These findings underscore the importance of *Aspergillus* in efficient amylase production for various industrial applications. Some examples of amylases produced by *Aspergillus* are shown in Table 4.

The production of cellulases by *Aspergillus* species has been a focal point in biotechnological research due to its relevance in the conversion of lignocellulosic biomass into valuable products. Studies have highlighted the efficiency of *A. niger* in utilizing agricultural residues, such as sugarcane bagasse, as substrates for cellulase production under submerged fermentation conditions [165]. Environmental factors like pH have been identified as crucial in enhancing cellulase synthesis, with specific adjustments yielding significant increases in enzymatic activity. Innovations in fermentation strategies, such as employing biofilm-based methods, have shown remarkable improvements in enzyme productivity, with biofilm fermentation increasing cellulase yields by over 50% compared to conventional methods [166]. Some examples of cellulases produced by *Aspergillus* are shown in Table 4.

Recently, various commercial sectors have explored xylanases in processes such as wood pulp biolixiviation, paper manufacturing, food and liquid production, animal nutrition, and bioethanol. Due to their biotechnological properties, xylanases are often produced by microorganisms for industrial applications. Nature is teeming with microorganisms that generate enzymatic complexes capable of degrading cellulose and releasing hemicellulose-derived sugars, which are used in the production of products at highly competitive costs [167]. Microbial xylanases play a critical role in industrial processes. Most commercial enzymes are derived from mesophilic microorganisms, while extremophilic microorganisms, which are capable of surviving and thriving in extreme environments, allow for the use of thermal strategies in the development of industrial processes. Organisms such as bacteria, fungi, and yeasts are known to produce xylanase in natural systems [155,168]. Xylanase enzymes have gained significant popularity in recent decades due to their primary applications in the paper and pulp industry, animal feed processing, beverage clarification, and the production of biofuels from agricultural waste [152,169].

**Table 4.** Enzyme production by different strains of *Aspergillus*.

| Enzyme | Microorganism | Main Substrate | pH | Temperature (°C) | Time of Incubation (days) | Enzymatic Activity | Reference |
|---|---|---|---|---|---|---|---|
| α-amylase | *A. flavus* AUMC10636 | Soluble starch | 5 | 30 | 7 | 22.68 U/mL | [100] |
| | *Aspergillus ochraceus* | Starch | - | 37 | 5 | 1415 U/mL | [170] |
| | *Aspergillus tamarii* MTCC5152 | Wheat bran | 6.7 | 28 | 4 | 519.40 U/gds | [171] |
| | *Aspergillus flavus* S2-OY | Potato peel | 5 | 35 | 3 | 5 U/mL | [172] |
| | *A. terreus* | Pomegranate peel | 6 | 30 | 5 | 340.69 U/ml | [173] |
| | *A. oryzae* | Groundnut oil cake | 4.7 | 32.5 | 4.5 | 9868.12 U/gds | [164] |
| | *A. oryzae* | Soybean husk and flour mill | 6 | 30 | 6 | 47,000 U/gds | [174] |

**Table 4.** *Cont.*

| Enzyme | Microorganism | Main Substrate | pH | Temperature (°C) | Time of Incubation (days) | Enzymatic Activity | Reference |
|---|---|---|---|---|---|---|---|
| Glucoamylase | *Aspergillus wentii* | Starch | 7 | 25 | 3 | 3.5 U/mL | [175] |
| | *A.s flavus* | Wheat straw | 5.5 | 30 | 12 | 13.89 U/gds | [176] |
| Endoglucanase | *Aspergillus uvarum* CBS 121591 | Carboxymethyl cellulose | 7 | 37 | 3 | 2.706 U/mL | [177] |
| | *A. niger* | *Arachis hypogaea* shells | 4 | 40 | 5 | 87.69 U/mL | [178] |
| | *A. fumigatus* JCM 10253 | Ragi husk | 2 | 48.6 | 8 | 97.06 U/mL | [179] |
| Beta-glucosidase | *Trichoderma reesei* | Cellulose | 5 | 30 | 12 | 13.44 U/mL | [180] |

## 5. New Technologies and Innovation of Enzymes Applied to Biofuels

### *5.1. Protein Engineering*

Protein engineering encompasses a range of molecular and computational techniques aiming to modify the amino acid sequence in an enzyme to optimize its activity, expression, stability under different conditions, and substrate specificity [181]. Traditional protein engineering relies on directed evolution or rational design. Directed evolution mimics Darwinian evolution at a higher mutation rate, with enzyme selection based on desirable properties. It involves two main steps: generating genetic diversity through random mutagenesis or gene recombination, followed by screening enzyme activity and selecting the best variants [182]. In contrast, enzyme engineering through rational design involves point mutations in the coding sequence, which requires prior knowledge of protein structure and function. In theory, rational design should be less labor-intensive to perform than directed evolution; however, acquiring knowledge of the relationship between the structure and function of the enzyme may require additional efforts if the information is not promptly available. Directed evolution and rational design can be merged into the semi-rational design, in which structural information is used to select a promising region for generating genetic diversity, producing better-targeted enzyme libraries [183]. Numerous studies have discussed the protein engineering of enzymes applied to biofuel production, most of them focused on cellulases and lipases.

Cellulose is the main carbohydrate in lignocellulosic biomass, and its depolymerization into glucose is a fundamental step in the production of biofuels. This can be achieved by employing cellulases, which are enzymes tailored to hydrolyze glycosidic bonds in cellulose. Because of this, there is a great need to not only develop cellulases with higher and more specific activities but also microbial platforms that express these enzymes in abundance. Researchers at the National Laboratory of Renewables (LNBR) at The National Center for Research on Energy and Materials (CNPEM) in Brazil have developed a solution to this issue [184]. The *Trichoderma reesei* RUT-C30 strain was engineered using CRISPR/Cas9 to produce the highest cellulase and xylanase levels ever reported, reaching 80.6 g/L of extracellular proteins. The enzyme cocktail showed saccharification efficiency comparable to commercial preparations used for sugarcane molasses. This hypersecreting strain was created by introducing recombinant invertase and β-glucosidase, constitutively expressing the cellulase regulator XYR1, and deleting the ACE1 repressor and extracellular proteases SLP1 and PEP1. Other studies have also focused on engineering *T. reesei* for the overproduction of cellulase and xylanase, using techniques like RNAi-mediated gene silencing and inducer-free expression systems. For example, Arai et al. mutated the XYR1 regulator and expressed two cellulase regulators in *T. reesei* E1AB1, generating a strain that did not require inducers to overproduce cellulases and xylanases [185]. Building a productive microbial platform for enzyme production is a strategy that has also been tested in *A. niger*. The antioxidant defense metabolism of *A. niger* was engineered to allow the

overexpression of proteins. Extensive oxidative folding of proteins within the endoplasmic reticulum causes accelerated production of reactive oxygen species (ROS) in *A. niger*, which negatively affects the production of proteins in this species. By integrating different modules for ROS detoxification in *A. niger*, total protein production was augmented by 88%, also increasing the activity of glucoamylases [186].

The same molecular techniques employed to modify *T. reesei* have also been studied in *Aspergillus* sp. For instance, cellulase expression in *A. niger* and *A. nidulans* is under the control of the XIR1 regulator. Gene disruption in XIR1 regulator in these species by homologous recombination increased cellulase and xylanase activities. In another study, CRISPR/Cas9 technology was used to increase endoglucanase activity in *A. fumigatus* by 40%. This was achieved by integrating the eglA gene from *A. niger* into the conidial melanin *pksP* locus, resulting in recombinant albino colonies [187]. Zou G. et al. developed a CRISPR/Cas9 ribonucleoprotein method to edit *T. reesei, Cordyceps militaris*, and *A. oryzae*, overcoming the low efficiency of ribonucleoprotein transformation in these fungi [188]. Another strategy to increase protein production in *Aspergillus* sp. is partly fusing enzyme coding sequences with overexpressed protein-coding sequences so that both genetic codes are expressed at high levels [189,190]. This demonstrates that not only *T. reesei* but also *Aspergillus* sp. has the potential to become an ideal microbial platform for lignocellulose deconstruction.

Xylanases have also been a target of protein engineering to enhance lignocellulose deconstruction for bioethanol production. Almost all native xylanases lose activity under industrial conditions, calling for modifications to increase their stability [191]. Thermal stability and pH stability of xylanase produced by *A. niger* were improved by site-directed mutagenesis. Initially, the amino acids to be modified were selected by visualizing the three-dimensional structure of the enzyme using a computational model. By substituting one glycine and one tyrosine with cysteine at positions 116 and 135, respectively, the engineering xylanase activity was preserved at 70 °C. These modifications also led to stability across a higher range of pH, from pH 4.5–6.0 to pH 5.0–7.0 [192]. Molecular dynamics identified four highly flexible regions (HFR) of acid-resistant xylanase from *A. niger* with the potential to increase thermostability. Iterative saturation mutagenesis was used to modify these regions, resulting in eight mutants. By combining the mutations of HFR III and HFR IV, a thermostable variant was developed, retaining enzymatic activity at 80 °C and 90 °C, making it a robust candidate for bioethanol production [193].

Lipase protein engineering to enhance expression and key properties is crucial for improving enzyme efficiency in biodiesel production. Several studies have focused on increasing lipase expression in *A. niger* by inserting strong promoters into the coding sequence. The glucoamylase PglaA promoter has been shown to induce high expression levels of eight different lipases in *A. niger* [194]. PgpdA is another strong promoter studied to increase lipase synthesis, with the advantage of enabling continuous expression without the need for inducer molecules [195]. Another strategy to increase lipase production is deleting genes for endogenous proteases. Deleting these genes allows for the overexpression of exogenous enzymes, as they are not cleaved after secretion by extracellular proteases. The deletion of aspartyl proteases (*PEPA* and *PEPB*) increased lipase and glucoamylase yield in one study [196]. Another study reported the reduction in protease expression by overexpressing *amyR*, which acts as a repressor of the *PrtT* activator of protease synthesis in *A. niger* [197].

Biodiesel is produced by the transesterification reaction of fatty acids catalyzed by lipases. Triacylglycerols are the most common fatty acids utilized to produce biodiesel, with many advances in protein engineering to improve lipases that catalyze reactions with this substrate [198–200]. However, the engineering of mono- and diacylglycerol lipases (MDGLs) is a prominent field with an urgent need for development once mono- and diacylglycerols are an underexplored source for biodiesel. Lan et al. characterized and modified an *A. oryzae* MDGL to serve as a model for engineering more efficient lipases targeting fatty acids other than triacylglycerols. The authors solved the crystal structure of the *A. oryzae* lipase and compared it to other lipase structures to identify the residue V269 as a catalytically important amino acid. By testing different residue substitutions at that position, it was found that aspartic acid residue increased enzyme activity six-fold by increasing affinity for mono- and diacylglycerols [201].

Although protein engineering is a valuable and established method for optimizing enzyme structure–function relationships, it is a time-consuming and complex process, with an enormous fitness landscape difficult to navigate manually. In biofuel production, this challenge is intensified by the urgent need to develop efficient processes that can compete with fossil fuels and address climate change. A promising approach to accelerate the design-build-test (DBT) cycle in protein engineering is the integration of machine learning (ML) algorithms with automated laboratories [181]. ML models can analyze structure–function relationships of proteins much faster than humans, identifying potential sequences for modification and even generating new sequences with desired properties. Meanwhile, an automated testing station can generate and test enzyme variants based on ML analysis, providing rapid feedback to accelerate the DBT process. Researchers at the University of Wisconsin-Madison developed self-driving autonomous machines for protein landscape exploration (SAMPLE) to optimize the thermostability of glycoside hydrolases [202]. The ML model was built with data on the enzyme structure and catalytic activity, generating a fitness landscape analyzed by a Gaussian process model, which captures patterns from limited experimental data; after 10,000 simulations, 83% of the active sequences were correctly annotated by the ML model, which then proposed optimized sequences using Bayesian optimization [202]. These sequences were sent to an automated robotic laboratory, where DNA fragments were constructed and amplified by PCR reactions. Genetic circuits for expressing the designed genes were built using the Golden Gate methodology [181]. The generated genetic codes were expressed in a cell-free system based on T7 polymerase, and the thermal denaturation of the synthesized enzymes was immediately tested. Each DBT cycle lasted around 9 h, and all four engineered enzymes became more thermotolerant, increasing their denaturation temperature by at least 12 °C. This study demonstrates that ML models and automated laboratories can accelerate the protein engineering process, enabling faster development of optimized enzymes for biofuel production [202].

### *5.2. Enzymatic Immobilization*

Immobilization is a traditional method used to enhance enzyme reusability, recovery, and stability under harsh conditions. Xue et al. developed a bioprocess for integrated aerobic cellulase production from *A. niger* in a synthetic medium, followed by saccharification of NaOH-pretreated corn stover and anaerobic bioethanol fermentation using *S. cerevisiae* in the same gas lift bioreactor. *A. niger* grew and produced cellulases in a wire mesh cylinder inside the bioreactor. After 48 h, oxygen was replaced by N2, and the fermentation broth was pumped into the reactor for bioethanol production by immobilized *S. cerevisiae*. Cellulase activity exceeded 6.28 U/mL over four consecutive batches, and ethanol yield reached 45.9 g/L after 48 h [203]. Directed evolution was used to produce *A. uvarum* cellulases with high activities, which were then immobilized on alginate beads to

enhance reusability. Mutagenesis with ethyl methanesulfonate (EMS) at 12% *v*/*v* resulted in cellulases with 1.4-fold and 1.8-fold higher activities in solid-state and submerged fermentation, respectively. Maximum activity of immobilized cellulase was achieved with 2% *w*/*v* sodium alginate and 0.2 M calcium chloride at 60 °C and pH 7.0. The immobilized enzyme retained 18.5% of its activity after five batches, demonstrating its potential for bioethanol production [177]. Magnetic nanoparticles are a versatile support for enzyme immobilization, enabling control of particle adsorption with a magnetic field. Cellulase from *A. niger* and *T. reesei* and xylanase from *T. longibrachiatum* were immobilized on chitosan magnetic nanoparticles to hydrolyze NaOH-pretreated coconut husks. Tween 80 was added as a surfactant to enhance enzyme stability and prevent denaturation. At 2% (*w*/*v*) of Tween 80, immobilized cellulase from *A. niger* produced 0.412 mg reducing sugar/mL, demonstrating that this immobilization method with surfactant addition is a promising strategy for producing fermentable sugar [204].

Immobilizing xylanases is a key strategy for creating efficient enzyme cocktails for lignocellulosic biomass conversion into biofuels. Calcium alginate is a traditional support for enzyme immobilization, but Jian et al. used a three-dimensional (3D) printer to immobilize xylanase from *A. oryzae* in various particle shapes. The optimized conditions of 1% (*w*/*v*) sodium alginate, 2% (*w*/*v*) $CaCl_2$, and a 10-minute crosslinking time resulted in the highest xylanase concentration inside the particle and minimal mass transfer limitations. Nearly 60% of immobilized xylanase was recovered after seven cycles. Immobilized enzymes produced almost 50% more reducing sugars from corn cob hydrolysis than free xylanase, highlighting the potential of this technology, especially for bioethanol production from corn residues [205]. A biomimetic magnetic nanoparticle was developed to immobilize xylanase from *A. niger*, expressed in *Pichia pastoris*, with excellent reusability and enhanced storage stability. The nanoparticles were synthesized by adding MamC to an iron solution, achieving 87% enzyme immobilization at optimized concentrations of glutaraldehyde and EDC. The immobilized enzymes retained activity after eight cycles of magnetic recovery, demonstrating the potential of this approach as a scalable technology [206].

The integration of starch hydrolysis with immobilized α-amylase and bioethanol production has been widely reported. Recently, α-amylase from *A. flavus* was immobilized on different supports using physical adsorption, ionic bonding, entrapment in gel, and covalent bonding. Immobilization via covalent bonding with 2% glutaraldehyde resulted in the highest starch conversion to reducing sugars (92%) and amylase activity (2522.2 U/mL). The ethanol yield from fermentation of the covalently bound amylase hydrolysate was twice as high as that obtained with free amylase [100]. A novel liquid phase-air phase system was developed to produce amylase and bioethanol from cassava starch hydrolysate. The system consisted of a bioreactor connected to a reservoir by a siphon. *A. awamori* cells were immobilized inside the bioreactor, and the culture broth was siphoned into it, submerging the cells. Once the broth reached a critical level, it was siphoned back to the reservoir, exposing the cells to air. Alternating periods of submerged fermentation and air exposure (12 h submerged and 3 h submerged and 6 h air exposure for raw and cassava starch, respectively) resulted in maximal glucoamylase and α-amylase expression. Simultaneous production of amylases and bioethanol was achieved by immobilizing *S. cerevisiae* in the reservoir, with ethanol yields of 0.46 g/g of starch and 1.73 g/g of starch per hour [117].

Metal–organic frameworks (MOFs) are gaining attention as a promising support for enzyme technology due to their superior stability and catalytic activity compared to traditional supports. The immobilization of *A. niger* lipases in modified zeolitic imidazolate frameworks (ZIF-8), specifically *macroporous* M-ZIF-8, has been investigated to enhance enzyme diffusion. This modification improved enzyme activity, recovery after repeated batches, thermal stability, and reusability compared to ZIF-8. Additionally, a seven-fold smaller concentration of lipase immobilized in M-ZIF-8 achieved the same activity as lipase in ZIF-8 for biodiesel production. The larger pores in M-ZIF-8 allowed more fatty acid methyl esters (FAME) to migrate into the particle, leading to higher biodiesel yields. After 24 h, M-ZIF-8 immobilized lipases produced 80% FAME, while ZIF-8 immobilized lipases reached a plateau at 65% FAME due to enzyme denaturation. The lipases in M-ZIF-8 were protected from denaturation by methanol and glycerol due to the particle's structure [207]. Lipases can also be absorbed onto the surface of ZIF-8. Xia et al. studied the absorption of lipases from *A. oryzae* on ZIF-8, demonstrating that hydrogen bonds, electrostatic interactions, and van der Waals forces did not affect the enzyme's conformation. The immobilization enhanced lipase stability in 10% methanol solution and across a wide pH range. It also improved FAME yield (81.19%) in biodiesel production from used cooking oil and retained 68.46% lipase activity after five consecutive transesterification reactions. This highlights the significant potential of immobilization to enhance biodiesel production processes using lipases from *Aspergillus* species [208].

## 6. World Market of Biofuels' Enzymes

In recent years, the market for enzymes applied in biofuel production has experienced significant growth, driven by advancements in biotechnological processes and the increasing demand for sustainable energy solutions. This market has been shaped by technological innovations, cost reductions, and the optimization of enzymatic formulations tailored to the specific needs of the industry. A notable example is the Beta Renewables biofuel plant in Italy, which integrates patented enzymatic cocktails primarily composed of cellulases and hemicellulases designed for its physicochemical pretreatment process. These cocktails, valued between $5 and $8/mL, are specifically formulated to enhance the hydrolysis efficiency of wheat straw and other lignocellulosic substrates. Beta Renewables collaborates with enzyme producers to develop formulations that address substrate variability and improve yields. These enzymes contribute to an annual production of approximately 30 million liters of bioethanol, highlighting their significance in large-scale processes [209].

In Brazil, companies like Raízen rely heavily on enzyme suppliers to optimize bioethanol production from sugarcane. Enzymatic solutions, particularly cellulases and β-glucosidases, valued at $4 to $6/mL, are employed to maximize cellulose breakdown in sugarcane bagasse. Raízen's investment in enzymatic technologies, combined with physical and chemical pretreatments, supports its annual capacity of 2.5 billion liters of ethanol. The company collaborates with global enzyme manufacturers to ensure a consistent supply and foster innovation in enzymatic formulations, reducing hydrolysis time and enhancing conversion efficiency [210].

GranBio, another Brazilian leader, focuses on second-generation bioethanol production using lignocellulosic residues. Its plant in São Miguel dos Campos incorporates tailored enzymatic blends composed of cellulases, hemicellulases, and β-glucosidases, specifically formulated for the hydrolysis of sugarcane bagasse and straw, with costs ranging from $5 to $9/mL. GranBio's collaboration with enzyme suppliers has enabled the development of high-performance cellulases and hemicellulases that operate effectively under the physicochemical conditions of its processes. These enzymes represent a significant portion of

production costs but are critical for achieving an annual output of over 80 million liters of bioethanol [211].

Amyris, a Brazilian company known for its innovations in biodiesel, employs lipases for the enzymatic transesterification of vegetable oils and microalgal lipids. Its patented enzymatic formulations, composed of lipases specifically designed for triglyceride conversion and valued between $7 and $10/mL, are aimed at improving lipid conversion efficiency while reducing by-product generation. Through investments in enzyme optimization, Amyris has achieved a 10% reduction in biodiesel production costs over the past five years, demonstrating the economic impact of advanced enzymatic technologies in biofuel production [212].

Despite these advancements, the market for biofuel enzymes faces challenges related to production costs, scalability, and market penetration. Enzymes for biofuels account for a substantial portion of operational expenses, with costs ranging from $0.3 to $1 per liter of biofuel, depending on their specificity and activity. Companies must balance enzyme costs with the need for high conversion efficiencies and process stability. Furthermore, the global enzyme market is highly competitive and dominated by key players such as Novozymes, DuPont, and DSM, who are continuously developing customized solutions for different substrates and pretreatment conditions. The future growth of the enzyme market depends on continuous innovation to reduce production costs and enhance enzyme stability under industrial conditions. Strategies such as enzyme immobilization, genetic engineering of enzyme-producing microorganisms, and co-culture systems promise to improve profitability and efficiency, driving the standardization of sustainable energy.

### *6.1. Global Market Analysis of Enzymes Utilized in Biofuel Production*

To comprehensively assess the global market and the growing interest in biofuel production as a sustainable alternative to fossil fuels, it is essential to perform a contemporary patent analysis during periods of peak research activity in this field. For this purpose, the Derwent Innovations Index® database was utilized, applying the following search algorithm: TS = (*ASPERGILLUS*) OR TS = (AMYLASE) OR TS = (XYLANASE) OR TS = (CELLULASE) OR TS = (LIPASE) OR TS = (BIODIESEL) OR TS = (BIOETHANOL) and Derwent Class Code (DC) = (D16) and International Patent Classification (IPC) = (C12P-007/06) AND (C12N-015/80).

This search yielded 913 documents. To refine the dataset, only documents categorized under "Energy Fuels," a keyword provided by the Derwent Innovations Index®, were selected, resulting in a subset of 498 documents. Subsequently, documents with up to eight years of antiquity, corresponding to a period of heightened biotechnological biofuel production, were chosen, reducing the dataset to 111 documents. These records were exported to MS Excel® for manual screening, where titles and abstracts were analyzed to identify patents relevant to the study.

The analysis revealed that the majority of patents for biofuel production via enzymatic hydrolysis were filed predominantly by China and India, accounting for 60.32% and 14.29% of the total, respectively. This dominance can be attributed to the unique socio-economic and environmental challenges faced by these countries. Despite having petroleum reserves, both nations struggle to meet their growing domestic energy demands, which are exacerbated by their expanding populations. Additionally, the large volumes of waste generated in these countries present an opportunity for conversion into biofuels. The pressing need for environmentally friendly and economically viable energy solutions has driven China and India to innovate and protect new technologies for biofuel production (Figure 3).

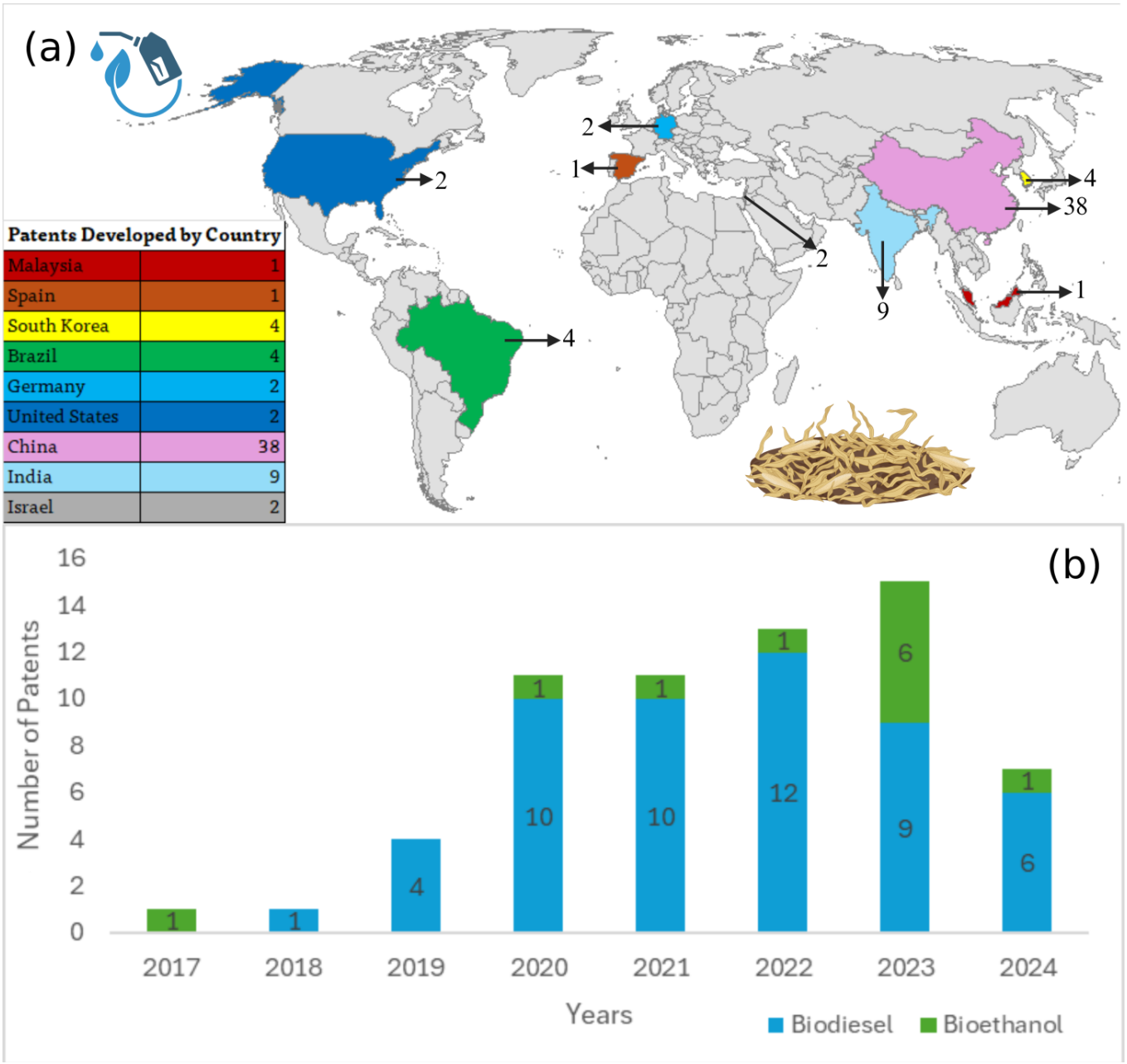


**Figure 3.** Global patent trends: (**a**) distribution of patent filings by country; (**b**) timeline of patents filed per year.

The patents analyzed span an eight-year period, revealing a significant increase in the number of patents filed during 2020, 2021, 2022, and 2023, accounting for 17.46%, 17.46%, 20.63%, and 23.81% of the total, respectively (Figure 3). This trend can be attributed to the global impact of the COVID-19 pandemic, which exposed the critical dependency on fuel resources and highlighted the vulnerability of global supply chains in the face of resource depletion. As activities resumed in 2022, research efforts prioritized the development of new technologies aimed at addressing these challenges and strengthening the global biofuel market.

To facilitate a better classification and understanding of how these technologies are being developed, patents were grouped based on several factors: the type of biofuel produced, the raw materials utilized, the pre-treatments applied to these raw materials, the enzymes used for enzymatic hydrolysis or transesterification, the microorganisms responsible for enzyme production, and the institutions or companies protecting these innovations. All patents analyzed were associated with the "Waste valorization and biofuel production" application area, emphasizing a focus on developing a circular economy for viable biofuel production.

#### 6.1.1. Biofuel Types

The analysis highlighted that 79.37% of the patents filed focused on biodiesel production (Figure 4a). For instance, a patent by Yang J. and Zhang X. reported the immobilization of a fungal lipase from *Aspergillus* sp., used for the enzymatic transesterification of waste cooking oils pre-treated by centrifugation and decantation to remove contaminants. This technology, developed by Shanghai Zhongqi Environmental Protection Technology Co., Ltd. (Shanghai Shi, China, 2022), achieved a production yield of 98.2% [213]. Another patent, by Xu G. et al., focused on engineering a mutant lipase expressed in *E. coli* using genes from *Aspergillus*. This lipase applied to pretreated vegetable oils, improved production yield from 85% to 99%. This technology was developed by Hunan Perfly Biotech Co., Ltd. (Hunan, China, 2021) [214].

Only 20.63% of the patents analyzed addressed bioethanol production (Figure 4a), with limited data available. Of these, two patents utilized enzymes derived from *Aspergillus*. The patent of Yang T. J. et al. employed *Aspergillus* sp. to produce xylanases for enzymatic hydrolysis of agricultural residues pre-treated via milling and steam exposure to enhance surface area and partially remove lignin. This technology was developed by CJ CheilJedang Corporation (Seoul, South Korea, 2021) [215]. Patent of Singh A. and Pandey A. K. from Chhatrapati Shahu Ji Maharaj University (Uttar Pradesh, India, 2024) utilized *A. oryzae* to produce cellulases for enzymatic hydrolysis of sugarcane bagasse. Both patents relied on enzymatic hydrolysis to release fermentable sugars, which were later converted to bioethanol via fermentation with *Saccharomyces cerevisiae* [216].

#### 6.1.2. Raw Materials and Pre-Treatment Methods

Raw materials for biofuel production predominantly consisted of "waste oils or fats" (50.79%), followed by "vegetable oils" (19.05%) and "lignocellulosic waste" (17.46%) (Figure 4b). The pre-treatments applied were primarily physical and thermomechanical methods, including milling, decantation, centrifugation, cavitation, and autohydrolysis, which were reported in 69.84% of the patents analyzed (Figure 4c). A smaller proportion (12.70%) involved physicochemical pre-treatments, combining physical methods with hydrolysis or neutralization processes.

#### 6.1.3. Enzymes and Microorganisms

Enzymes used in biofuel production showed a marked preference for lipases, which accounted for 73.02% of patents, aligning with the dominance of biodiesel-related technologies (Figure 4d). In contrast, amylases, cellulases, and xylanases were collectively reported in only 26.98% of the patents. Microorganism analysis revealed that 30.16% of the patents utilized enzymes produced by *Aspergillus*, while 12.70% used enzymes from *Thermomyces* (primarily for biodiesel production), and 7.94% involved enzymes from *Candida* (Figure 4e). These findings suggest that *Aspergillus* remains underutilized for enzyme production in biofuel technologies, presenting opportunities for further research and optimization.

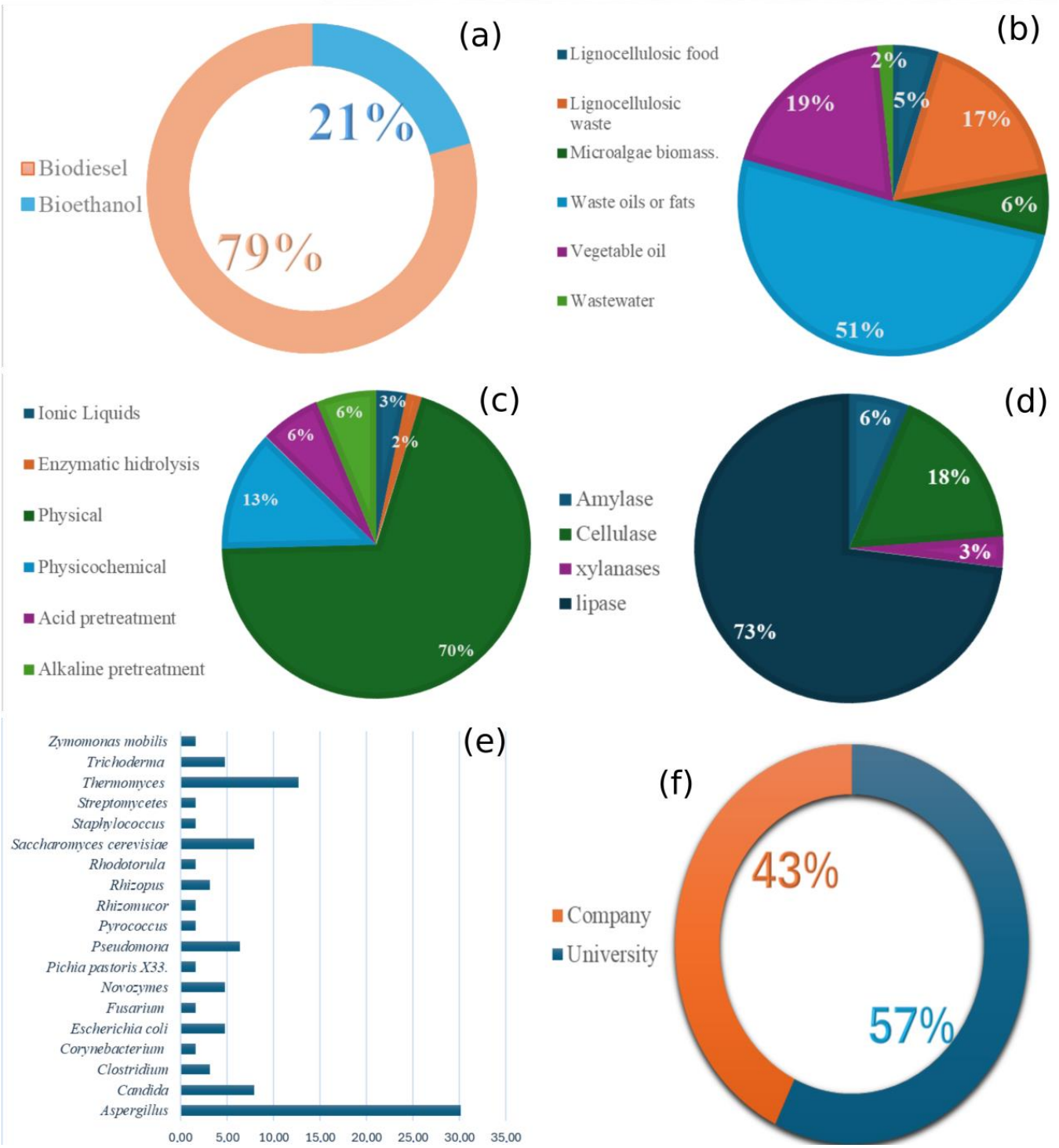


**Figure 4.** Statistical analysis of filed biofuel patents: (**a**) Distribution of biodiesel and bioethanol patents filed. (**b**) Raw material types used in biofuel production. (**c**) Pre-treatment methods are applied to raw materials for biofuel production. (**d**) Enzymes utilized in biofuel production processes. (**e**) Patents reporting microorganism utilization in biofuel production. (**f**) Key entities filing biofuel patents.

6.1.4. Institutions and Companies Filing Patents

A detailed analysis of the entities filing these patents revealed that universities and research institutions were responsible for 57.14% of the filings, while companies accounted for 42.85% (Figure 4f). Notable contributors included the Spanish National Research Council (CSIC), (Madrid, Spain), Perseo Biotechnology S.L., (Madrid, Spain), Shanghai Zhongqi Environmental Protection Technology Co., Ltd., (Shanghai, China), and Shaanxi Haisefu Biological Engineering Co., Ltd., (Xi'an, Shaanxi Province, China). While industrial interest in biofuel technologies is evident, these innovations still require significant optimization to reduce production costs and enhance accessibility for industrial markets.

These findings highlight the critical role of academia and industry in advancing biofuel technologies and the need for continued innovation to develop economically viable and environmentally sustainable alternatives to fossil fuels.

### *6.2. Relationship Between Feedstocks, Pretreatments, and Microorganisms in Biofuel Production*

In response to the growing demand previously mentioned regarding the production of biofuels such as bioethanol and biodiesel, a chart was designed to highlight the various alternatives available to produce these high-energy-value compounds, with the primary objective of evaluating their viability and showcasing the synergy of technologies for industrial scaling. To analyze the current state of biofuel production from fungal enzymes from a bibliographic perspective, an exhaustive analysis of the sources cited in this scientific article was conducted to identify the key points in each investigation that led to favorable results. These references are represented in Figure 5. Following a network analysis, the influence and interrelations among their procedures were measured, and the main current trends in innovation for biofuel production were identified.

The results highlight "Bioethanol" and "Biodiesel" as the most prominent biofuels produced from fungal metabolites, closely linked to microorganisms, organic waste, and production methodologies. The chart shows three main clusters: one for bioethanol (green), another for biodiesel (purple), and a third (orange) representing raw materials and pretreatments that connect the two main clusters. Smaller nodes reflect fewer common methodologies and microorganisms.

The primary challenge in bioethanol and biodiesel production lies in scaling technologies to industrial levels. Optimizing processes to valorize lignocellulosic biomass and residual oils could improve enzymatic complexes for waste treatment and biofuel production. However, this requires alignment with government regulations and institutional support. In Brazil, entities like IBAMA and SABESP oversee waste management and compliance with Law No. 12.305/2010, which promotes waste reduction, reuse, and recycling. Meanwhile, ANP, ANEEL, and APROBIO regulate biofuel production and energy generation from lignocellulosic waste, ensuring adherence to environmental standards and promoting sustainable practices.

According to performance studies, it has been established that the quality of the produced biofuels is closely linked to the type of pretreatment applied to the organic waste used [116]. As illustrated in Figure 5, generated from the published research, it has been shown that physicochemical pretreatments, which combine grinding processes with acid or alkaline hydrolysis, are among the most used methodologies today [106]. For example, Chugh et al. employed a grinding pretreatment followed by acid hydrolysis with 2% $H_2SO_4$ on rice bran, resulting in a significant increase in surface area, enabling a subsequent release of 468 mg of fermentable sugars per gram of hydrolyzed material using amylases and cellulases produced by the fungus *A. niger* P-19. These sugars were then fermented with *Saccharomyces cerevisiae*, producing 37.63 g/L or 0.41 g of bioethanol per gram of hydrolyzed material, which constitutes one of the best results obtained with this methodology [67]. On

the other hand, despite the limited number of performance reports, the pretreatment of materials with ionic liquids, such as imidazole, presents itself as a promising alternative. As seen in Figure 5, this methodology appears somewhat more distanced from the main ones due to its limited bibliography but still maintains a direct interaction with bioethanol production. Awodi et al. used ionic liquids to clean mango seed starch, followed by hydrolysis of these substrates with α-amylase produced by *A. niger*, obtaining a concentration of 848 mg of fermentable sugars per gram of hydrolyzed substrate. This concentration was then subjected to a fermentation process with *Saccharomyces cerevisiae*, achieving a production of 31.40 g/L [99]. Comparatively, it can be stated that the treatment with ionic liquids effectively removed toxic materials for the enzymes and prevented the formation of inhibitors such as furfural while preserving the chemical composition of the areas exposed to hydrolysis, keeping intact their properties for enzymatic release. The optimization of these technologies is crucial to identify the optimal point at which the number of applications of these liquids is maximized, allowing for the highest possible yield and turning this technology into a viable option for industrial scaling.

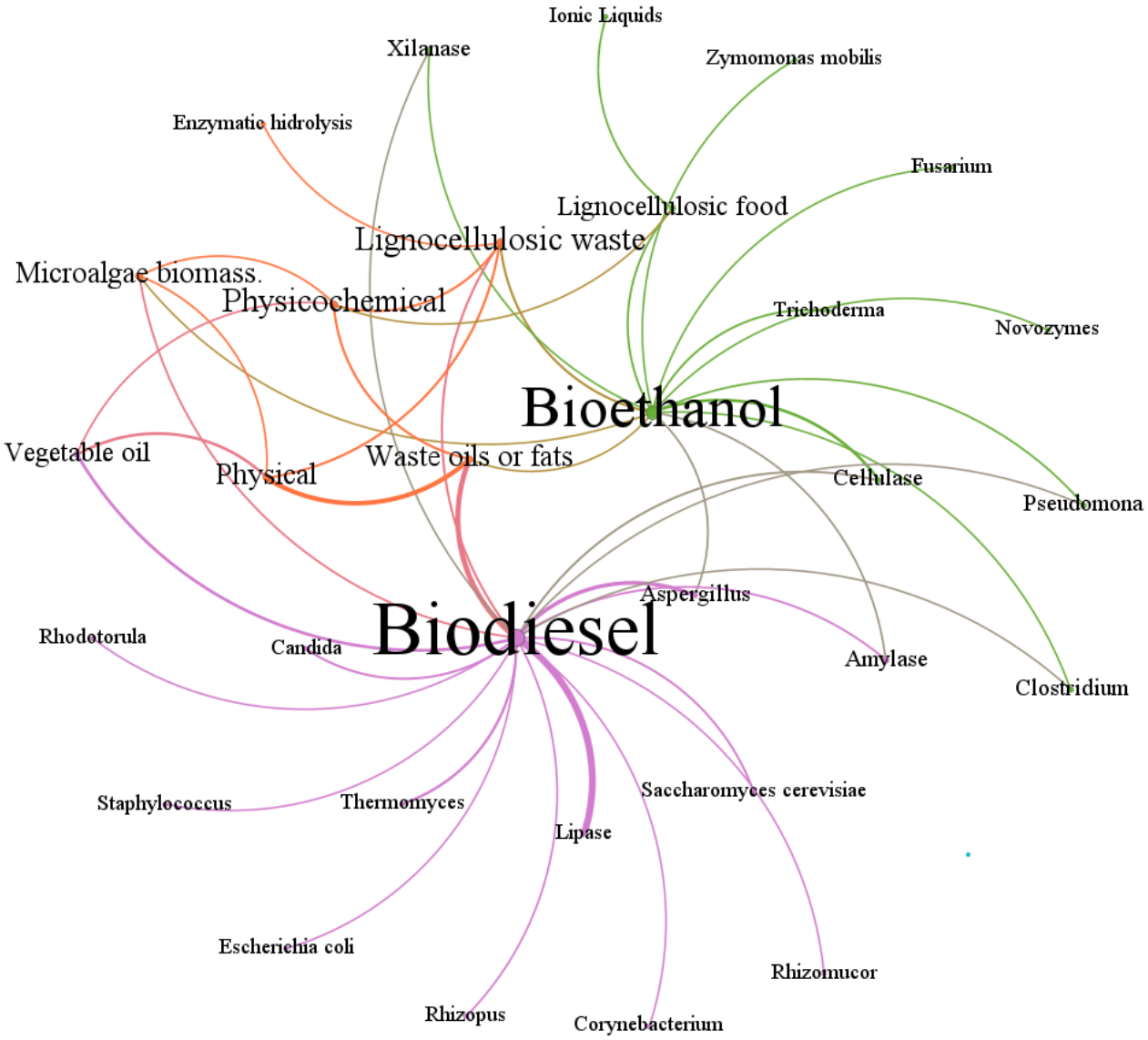


**Figure 5.** Network analysis of scientific terms used to identify emerging innovative trends in the production of bioethanol and biodiesel. The scientific landscape was developed using the network analysis and visualization software, (Gephi 0.10.1).

On the opposite side of Figure 5, the patents and studies analyzed and utilized in this research report the use of waste oils and animal fats. These materials, often discarded without undergoing recovery or neutralization pretreatments, represent a valuable resource for biodiesel production. Closely associated with physical pretreatments, these are the most predominant methodologies for biodiesel production, while physicochemical pretreatments are applied only when neutralization or denaturation of the waste material is necessary. For instance, Wei et al. demonstrated that the filtration of waste cooking oils is sufficient to remove impurities from this type of organic material. Using an immobilized lipase produced by the fungus *A. oryzae*, they achieved a 98.5% yield in the conversion of fatty acids to biodiesel through an enzymatic transesterification conducted at 40 °C for 9 h [108]. This approach represents one of the fastest methodologies reported for biodiesel production. In another case, Amoah et al. performed a natural sedimentation process followed by lyophilization of *Chlamydomonas* sp. JSC4 biomass. Subsequently, the intracellular lipids released were treated through lyophilization and lipases produced by *A.s oryzae*, achieving a 97% yield in biodiesel production. However, this yield was obtained after 32 h at 30 °C, which, while offering a favorable production temperature, limits industrial scaling due to the time required for production [110]. On the other hand, Singh A. and Pandey A. K. used plant oils, animal fats, waste oils, and microbial oils that were pretreated with acids until reaching a fatty acid concentration between 75% and 80%. These materials were utilized in the production of fatty acid methyl esters (FAME) using a lipase, which in this case was derived from the bacterium *Streptomycetes* sp. The enzymatic transesterification was carried out at temperatures of 40–60 °C for a duration of 6 to 10 h at 200 rpm, employing a molar ratio of alcohol to biomass of 3:1 and low-carbon alcohols (C1–C4). This approach achieved a 93% yield, slightly lower than that reported for other enzymatic transesterifications. However, controlling the fatty acid content in the raw material broadens the range of oil sources that can be used, reducing the quality requirements for the feedstock and laying the groundwork for process standardization [216].

The fungus *Aspergillus* is a key microbial producer of industrial enzymes such as cellulases, xylanases, amylases, and lipases, widely used in bioethanol and biodiesel production. Studies show that a single *Aspergillus* strain can synthesize different enzymes depending on cultivation conditions. However, few patents for bioethanol production document the direct use of enzymes from this genus. Instead, the use of other microorganisms is reported, such as *Clostridium* [217], *Fusarium* [218], *Zymomonas* [219], and *Trichoderma* [220], as well as the contribution of enzyme-supplying companies, such as Novozymes [221]. This highlights the need for greater dissemination and optimization in the development of fungal enzymes to ensure their scalability and commercial viability. On the other hand, biodiesel production has shown a greater utilization of lipases produced by the fungus *Aspergillus*. Additionally, other lipase-producing microorganisms have been identified, such as *Rhodotorula* [222], *Staphylococcus* [223], *Thermomyces* [224], *Rhizopus* [225], *Corynebacterium* [226], *Rhizomucor* [227], *Candida* [228], and *Saccharomyces cerevisiae* [229] the latter genetically modified to express optimized lipases. In conclusion, biofuel production demands continuous improvement in processes to ensure both sustainability and efficiency. This challenge is crucial not only to advance toward a cleaner energy matrix but also to preserve the necessary conditions to sustain life as we know it.

## 7. Conclusions

Fungal enzymes, particularly cellulases, xylanases, amylases, and lipases produced by the filamentous fungus *Aspergillus*, play a crucial role in the transformation of lignocellulosic biomass and oils into bioethanol and biodiesel, establishing themselves as key tools in the transition toward a more sustainable biofuel production. Recent advancements, such as the design of more stable enzymes using inorganic supports and the genetic engineering of *Aspergillus* strains, have enhanced the cellulose conversion efficiency by 30%. Additionally, the integration of hybrid pretreatments has reduced conversion times from weeks to days, achieving efficiencies above 90%. Noteworthy examples include the application of these fungi in conjunction with physicochemical technologies to enhance the release of fermentable sugars. Ionic liquids, despite their economic challenges, have shown the potential to reduce costs by up to 30% by facilitating the dissolution of cellulose with high efficiency. Furthermore, recent studies have explored new agroindustrial substrates, such as mango peels, invasive plants, and starchy waste, where these enzymes have demonstrated their versatility in adapting to diverse chemical compositions, achieving bioethanol concentrations of up to 108.6 g/L from rice straw pretreated with alkaline methods. Moreover, the optimization of fermentation and transesterification processes using lipases has achieved biodiesel yields exceeding 97%, even with low-quality oils, underscoring the critical role of these enzymes in overcoming technical and economic barriers. In summary, *Aspergillus*-derived enzymes are not only essential for the valorization of lignocellulosic biomass and oils but also serve as the foundation for recent innovations that have improved efficiency and reduced costs in biofuel production. Future research should focus on customizing biocatalysts for extreme conditions, exploring new substrates, and integrating hybrid systems, aiming to build a more sustainable biofuel industry aligned with circular economy principles.

**Author Contributions:** F.E.R.-V.: conceptualization, investigation, formal analysis, writing—original draft preparation, writing—review and editing; R.P.: conceptualization, writing—original draft preparation, writing—review and editing; validation; supervision; W.J.M.-B.: conceptualization, writing—original draft preparation, writing—review and editing, validation, supervision; L.A.J.L.: conceptualization, methodology, writing—original draft preparation, validation, supervision; P.B.G.d.M.: conceptualization, methodology, investigation, formal analysis, writing—original draft preparation; L.C.R.-N.: conceptualization, investigation, writing—original draft preparation; G.S.D.: conceptualization, investigation, writing—original draft preparation; G.A.B.: conceptualization, investigation, writing—original draft preparation; G.d.S.C.: conceptualization, investigation, writing—original draft preparation; L.P.d.S.V.: conceptualization, resources, supervision; C.R.S.: conceptualization, resources, supervision. All authors have read and agreed to the published version of the manuscript.

**Funding:** This research was supported by the National Council of Technological and Scientific Development (CNPq) (grant number: 440138/2022-1) and the Coordination for the Improvement of Higher Education Personnel (CAPES), PNPD Program (grant number: 88887.931856/2024-00).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** No new data were created or analyzed in this study.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References


1. Cheah, W.Y.; Sankaran, R.; Show, P.L.; Ibrahim, T.N.B.T.; Chew, K.W.; Culaba, A.; Chang, J.S. Pretreatment methods for lignocellulosic biofuels production: Current advances, challenges and future prospects. *Biofuel Res. J.* **2020**, *7*, 1115–1127. Available online: https://www.biofueljournal.com/article_103970.html (accessed on 26 November 2024). [CrossRef]
2. Safarian, S.; Unnthorsson, R. An Assessment of the Sustainability of Lignocellulosic Bioethanol Production from Wastes in Iceland. *Energies* **2018**, *11*, 1493. Available online: https://www.mdpi.com/1996-1073/11/6/1493/htm (accessed on 26 November 2024). [CrossRef]
3. Nazli, R.I.; Aslankaciran, C.; Yang, D.; Liaqat, W.; Polat, M.; Cavdar, A.S.; Tansi, V.; Baloch, F.S. Assessment of Sweet Sorghum Genotypes for Bioethanol Production Potential and Bagasse Combustion Characteristics in a Semi-Arid Mediterranean Climate. *Agronomy* **2024**, *14*, 2626. Available online: https://www.mdpi.com/2073-4395/14/11/2626/htm (accessed on 21 November 2024). [CrossRef]
4. Tayyab, M.; Noman, A.; Islam, W.; Tayyab, M.; Noman, A.; Islam, W.; Waheed, S.; Arafat, Y.; Ali, F.; Zaynab, M.; et al. LinBioethanol production from lignocellulosic biomass by environment-friendly pretreatment methods: A review. *Appl. Ecol. Environ. Res.* **2018**, *16*, 225–249. Available online: https://aloki.hu/pdf/1601_225249.pdf (accessed on 26 November 2024). [CrossRef]
5. Chen, J.; Zhang, B.; Luo, L.; Zhang, F.; Yi, Y.; Shan, Y.; Liu, B.; Zhou, Y.; Wang, X.; Lü, X. A review on recycling techniques for bioethanol production from lignocellulosic biomass. *Renew. Sustain. Energy Rev.* **2021**, *149*, 111370. Available online: https://www.sciencedirect.com/science/article/abs/pii/S1364032121006560 (accessed on 26 November 2024). [CrossRef]
6. Das, N.; Jena, P.K.; Padhi, D.; Kumar Mohanty, M.; Sahoo, G. A comprehensive review of characterization, pretreatment and its applications on different lignocellulosic biomass for bioethanol production. *Biomass Convers. Biorefinery* **2021**, *13*, 1503–1527. Available online: https://link.springer.com/article/10.1007/s13399-021-01294-3 (accessed on 26 November 2024). [CrossRef]
7. Rezania, S.; Oryani, B.; Cho, J.; Talaiekhozani, A.; Sabbagh, F.; Hashemi, B.; Rupani, P.F.; Mohammadi, A.A. Different pretreatment technologies of lignocellulosic biomass for bioethanol production: An overview. *Energy* **2020**, *199*, 117457. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0360544220305648 (accessed on 26 November 2024). [CrossRef]
8. Gronchi, N.; Favaro, L.; Cagnin, L.; Brojanigo, S.; Pizzocchero, V.; Basaglia, M.; Casella, S. Novel Yeast Strains for the Efficient Saccharification and Fermentation of Starchy By-Products to Bioethanol. *Energies* **2019**, *12*, 714. Available online: https://www.mdpi.com/1996-1073/12/4/714/htm (accessed on 26 November 2024). [CrossRef]
9. Nitsos, C.; Rova, U.; Christakopoulos, P. Organosolv Fractionation of Softwood Biomass for Biofuel and Biorefinery Applications. *Energies* **2018**, *11*, 50. Available online: https://www.mdpi.com/1996-1073/11/1/50/htm (accessed on 26 November 2024). [CrossRef]
10. Pradyawong, S.; Juneja, A.; Bilal Sadiq, M.; Noomhorm, A.; Singh, V. Comparison of Cassava Starch with Corn as a Feedstock for Bioethanol Production. *Energies* **2018**, *11*, 3476. Available online: https://www.mdpi.com/1996-1073/11/12/3476/htm (accessed on 26 November 2024). [CrossRef]
11. Kumar, A.K.; Sharma, S. Recent updates on different methods of pretreatment of lignocellulosic feedstocks: A review. *Bioresour. Bioprocess.* **2017**, *4*, 7. Available online: https://bioresourcesbioprocessing.springeropen.com/articles/10.1186/s40643-017-0137-9 (accessed on 27 November 2024). [CrossRef] [PubMed]
12. Maurya, D.P.; Singla, A.; Negi, S. An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. *3 Biotech* **2015**, *5*, 597–609. Available online: https://link.springer.com/article/10.1007/s13205-015-0279-4 (accessed on 27 November 2024). [CrossRef] [PubMed]
13. Srikumar, K.; Tan, Y.H.; Kansedo, J.; Tan, I.S.; Mubarak, N.M.; Ibrahim, M.L.; Yek, P.N.Y.; Foo, H.C.Y.; Karri, R.R.; Khalid, M. A review on the environmental life cycle assessment of biodiesel production: Selection of catalyst and oil feedstock. *Biomass Bioenergy* **2024**, *185*, 107239. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0961953424001922 (accessed on 27 November 2024). [CrossRef]
14. Bhatia, S.K.; Kim, J.; Song, H.S.; Kim, H.J.; Jeon, J.M.; Sathiyanarayanan, G.; Yoon, J.J.; Park, K.; Kim, Y.G.; Yang, Y.H. Microbial biodiesel production from oil palm biomass hydrolysate using marine Rhodococcus sp. YHY01. *Bioresour. Technol.* **2017**, *233*, 99–109. Available online: https://pubmed.ncbi.nlm.nih.gov/28260667/ (accessed on 27 November 2024). [CrossRef] [PubMed]
15. Widayat, W.; Darmawan, T.; Hadiyanto, H.; Rosyid, R.A. Preparation of Heterogeneous CaO Catalysts for Biodiesel Production. *J. Phys. Conf. Ser.* **2017**, *877*, 012018. Available online: https://iopscience.iop.org/article/10.1088/1742-6596/877/1/012018 (accessed on 27 November 2024). [CrossRef]
16. Zhang, H.; Li, H.; Pan, H.; Liu, X.; Yang, K.; Huang, S.; Yang, S. Efficient production of biodiesel with promising fuel properties from Koelreuteria integrifoliola oil using a magnetically recyclable acidic ionic liquid. *Energy Convers. Manag.* **2017**, *138*, 45–53. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0196890417300766 (accessed on 27 November 2024). [CrossRef]

17. Nisar, J.; Razaq, R.; Farooq, M.; Iqbal, M.; Khan, R.A.; Sayed, M.; Shah, A.; Rahman, I. Enhanced biodiesel production from Jatropha oil using calcined waste animal bones as catalyst. *Renew. Energy* **2017**, *101*, 111–119. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0960148116307492 (accessed on 27 November 2024). [CrossRef]
18. Zhou, Z.W.; Cai, C.X.; Xing, X.; Li, J.; Hu, Z.E.; Xie, Z.B.; Wang, N.; Yu, X.Q. Magnetic COFs as satisfied support for lipase immobilization and recovery to effectively achieve the production of biodiesel by great maintenance of enzyme activity. *Biotechnol. Biofuels* **2021**, *14*, 156. Available online: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-021-02001-0 (accessed on 27 November 2024). [CrossRef]
19. Xia, S.; Lin, J.; Sayanjali, S.; Shen, C.; Cheong, L.Z. Lipase-catalyzed production of biodiesel: A critical review on feedstock, enzyme carrier and process factors. *Biofuels Bioprod. Biorefining* **2024**, *18*, 291–309. Available online: https://scijournals.onlinelibrary.wiley.com/doi/abs/10.1002/bbb.2561 (accessed on 27 November 2024). [CrossRef]
20. Badoei-dalfard, A.; Shahba, A.; Zaare, F.; Sargazi, G.; Seyedalipour, B.; Karami, Z. Lipase immobilization on a novel class of Zr-MOF/electrospun nanofibrous polymers: Biochemical characterization and efficient biodiesel production. *Int. J. Biol. Macromol.* **2021**, *192*, 1292–1303. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0141813021022649 (accessed on 27 November 2024). [CrossRef]
21. Baskar, G.; Soumiya, S. Production of biodiesel from castor oil using iron (II) doped zinc oxide nanocatalyst. *Renew. Energy* **2016**, *98*, 101–107. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0960148116301690 (accessed on 27 November 2024). [CrossRef]
22. Valladares-Diestra, K.; de Souza Vandenberghe, L.P.; Soccol, C.R. Oilseed Enzymatic Pretreatment for Efficient Oil Recovery in Biodiesel Production Industry: A Review. *Bioenergy Res.* **2020**, *13*, 1016–1030. Available online: https://link.springer.com/article/10.1007/s12155-020-10132-9 (accessed on 24 November 2024). [CrossRef]
23. Zhong, W.; Yu, H.; Song, L.; Zhang, X. Combined pretreatment with white-rot fungus and alkali at near room-temperature for improving saccharificaiton of corn stalks. *BioResources* **2011**, *6*, 3400–3451. Available online: https://bioresources.cnr.ncsu.edu/resources/combined-pretreatment-with-white-rot-fungus-and-alkali-at-near-room-temperature-for-improving-saccharificaiton-of-corn-stalks/ (accessed on 28 November 2024). [CrossRef]
24. Perumal, V.; Ilangkumaran, M. Experimental analysis of engine performance, combustion and emission using pongamia biodiesel as fuel in CI engine. *Energy* **2017**, *129*, 228–236. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0360544217306837 (accessed on 27 November 2024). [CrossRef]
25. Aghabeigi, F.; Nikkhah, H.; Zilouei, H.; Bazarganipour, M. Immobilization of lipase on the graphene oxides magnetized with NiFe2O4 nanoparticles for biodiesel production from microalgae lipids. *Process Biochem.* **2023**, *126*, 171–185. Available online: https://www.sciencedirect.com/science/article/abs/pii/S1359511323000181 (accessed on 27 November 2024). [CrossRef]
26. Roy, M.; Mohanty, K. Valorization of de-oiled microalgal biomass as a carbon-based heterogeneous catalyst for a sustainable biodiesel production. *Bioresour. Technol.* **2021**, *337*, 125424. Available online: https://www.sciencedirect.com/science/article/abs/pii/S0960852421007641 (accessed on 27 November 2024). [CrossRef] [PubMed]
27. Wallerstein, D. Food-energy-water (FEW) nexus: Rearchitecting the planet to accommodate 10 billion humans by 2050. *Resour. Conserv. Recycl.* **2020**, *155*, 104658. [CrossRef]
28. Shukla, P.D.; Garai, M.; Zafar, K.; Gupta, S.; Shrivastava, S. Process parameters optimization for lipase production by Rhizopus oryzae KG-10 under submerged fermentation using response surface methodology. *Applied Sci. Environ. Sanit.* **2007**, *2*, 93–103. Available online: https://www.researchgate.net/publication/43993022_Process_parameters_optimization_for_lipase_production_by_Rhizopus_oryzae_KG-10_under_submerged_fermentation_using_response_surface_methodology (accessed on 22 November 2023).
29. Usman, I.M.T.; Ho, Y.C.; Baloo, L.; Lam, M.K.; Sujarwo, W. A comprehensive review on the advances of bioproducts from biomass towards meeting net zero carbon emissions (NZCE). *Bioresour. Technol.* **2022**, *366*, 128167.
30. Louw, J.; Dogbe, E.S.; Yang, B.; Görgens, J.F. Prioritisation of biomass-derived products for biorefineries based on economic feasibility: A review on the comparability of techno-economic assessment results. *Renew. Sustain. Energy Rev.* **2023**, *188*, 113840. [CrossRef]
31. Aristizábal-Marulanda, V.; Cardona Alzate, C.A. Methods for designing and assessing biorefineries: Review. *Biofuels Bioprod. Biorefining* **2019**, *13*, 789–808. [CrossRef]
32. Pleissner, D.; Qi, Q.; Gao, C.; Rivero, C.P.; Webb, C.; Lin, C.S.K.; Venus, J. Valorization of organic residues for the production of added value chemicals: A contribution to the bio-based economy. *Biochem. Eng. J.* **2016**, *116*, 3–16. [CrossRef]
33. Ioannidou, S.M.; Pateraki, C.; Ladakis, D.; Papapostolou, H.; Tsakona, M.; Vlysidis, A.; Kookos, I.K.; Koutinas, A. Sustainable production of bio-based chemicals and polymers via integrated biomass refining and bioprocessing in a circular bioeconomy context. *Bioresour. Technol.* **2020**, *307*, 123093. [CrossRef] [PubMed]

34. Singh, J.; Sharma, A.; Sharma, P.; Singh, S.; Das, D.; Chawla, G.; Singha, A.; Nain, L. Valorization of jute (*Corchorus* sp.) biomass for bioethanol production. *Biomass Convers. Biorefinery* **2022**, *12*, 5209–5220. [CrossRef]
35. Sahu, O. Appropriateness of rose (Rosa hybrida) for bioethanol conversion with enzymatic hydrolysis: Sustainable development on green fuel production. *Energy* **2021**, *232*, 120922. [CrossRef]
36. Brandt-Talbot, A.; Gschwend, F.J.V.; Fennell, P.S.; Lammens, T.M.; Tan, B.; Weale, J.; Hallett, J.P. An economically viable ionic liquid for the fractionation of lignocellulosic biomass. *Green Chem.* **2017**, *19*, 3078–3102. Available online: https://pubs.rsc.org/en/content/articlehtml/2017/gc/c7gc00705a (accessed on 30 November 2024). [CrossRef]
37. Low, S.A.; Isserman, A.M. Ethanol and the Local Economy. *Econ. Dev. Q.* **2009**, *23*, 71–88. [CrossRef]
38. Iqbal, Y.; Dai, Y.; Xue, S.; Yi, Z.; Chen, Z.; Li, M.; Cossel, M. Organic Acid-Based Hemicellulose Fractionation and Cellulosic Ethanol Potential of Five Miscanthus Genotypes. *Agronomy* **2024**, *14*, 1389. Available online: https://www.mdpi.com/2073-4395/14/7/1389/htm (accessed on 16 January 2025). [CrossRef]
39. Vaidyanathan, G.; Sankaranarayanan, R.; Yap, N.T. Bridging the chasm—Diffusion of energy innovations in poor infrastructure starved communities. *Renew. Sustain. Energy Rev.* **2019**, *99*, 243–255. [CrossRef]
40. Bhuyar, P.; Trejo, M.; Mishra, P.; Unpaprom, Y.; Velu, G.; Ramaraj, R. Advancements of fermentable sugar yield by pretreatment and steam explosion during enzymatic saccharification of *Amorphophallus* sp. starchy tuber for bioethanol production. *Fuel* **2022**, *323*, 124406. [CrossRef]
41. Kabadayı, H.K.; Demiray, E.; Karatay, S.E.; Dönmez, G. Bioethanol production from mulberry pomace by newly ısolated non-conventional yeast Hanseniaspora uvarum. *Biomass Convers. Biorefinery* **2024**, *14*, 10611–10620. [CrossRef]
42. Iyyappan, J.; Pravin, R.; Al-Ghanim, K.A.; Govindarajan, M.; Nicoletti, M.; Baskar, G. Dual strategy for bioconversion of elephant grass biomass into fermentable sugars using Trichoderma reesei towards bioethanol production. *Bioresour. Technol.* **2023**, *374*, 128804. [CrossRef] [PubMed]
43. Wongleang, S.; Premjet, D.; Premjet, S. Investigating the Potential of Grass Biomass (*Thysanolaena latifolia*) as an Alternative Feedstock for Sugar Platforms and Bioethanol Production. *Energies* **2024**, *17*, 4017. [CrossRef]
44. Kumar, R.; Basak, B.; Pal, P.; Chakrabortty, S.; Park, Y.K.; Ali Khan, M.; Chung, W.; Chang, S.W.; Ahn, Y.; Jeon, B.H. Feasibility assessment of bioethanol production from humic acid-assisted alkaline pretreated Kentucky bluegrass (*Poa pratensis* L.) followed by downstream enrichment using direct contact membrane distillation. *Bioresour. Technol.* **2022**, *360*, 127521. [CrossRef]
45. Jin, X.; Ma, J.; Song, J.; Liu, G.Q. Saccharification and detoxification of Na2CO3 pretreated rice straw with on-site manufactured enzymes secreted by *Aspergillus fumigatus* to enhance bioethanol yield. *Renew. Energy* **2020**, *166*, 117–124. [CrossRef]
46. Ziaei-Rad, Z.; Fooladi, J.; Pazouki, M.; Gummadi, S.N. Lignocellulosic biomass pre-treatment using low-cost ionic liquid for bioethanol production: An economically viable method for wheat straw fractionation. *Biomass Bioenergy* **2021**, *151*, 106410. [CrossRef]
47. Duque, A.; Doménech, P.; Álvarez, C.; Ballesteros, M.; Manzanares, P. Study of the bioprocess conditions to produce bioethanol from barley straw pretreated by combined soda and enzyme-catalyzed extrusion. *Renew. Energy* **2020**, *158*, 263–270. [CrossRef]
48. Lee, U.; Kwon, H.; Wu, M.; Wang, M. Retrospective analysis of the U.S. corn ethanol industry for 2005–2019: Implications for greenhouse gas emission reductions. *Biofuels Bioprod. Biorefining* **2021**, *15*, 1318–1331. [CrossRef]
49. Vandenberghe, L.P.S.; Valladares-Diestra, K.K.; Bittencourt, G.A.; Zevallos Torres, L.A.; Vieira, S.; Karp, S.G.; Sydney, E.B.; de Carvalho, J.C.; Thomaz Soccol, V.; Soccol, C.R. Beyond sugar and ethanol: The future of sugarcane biorefineries in Brazil. *Renew. Sustain. Energy Rev.* **2022**, *167*, 112721. [CrossRef]
50. Bhatia, S.K.; Jagtap, S.S.; Bedekar, A.A.; Bhatia, R.K.; Patel, A.K.; Pant, D.; Rajesh Banu, J.; Rao, C.V.; Kim, Y.G.; Yang, Y.H. Recent developments in pretreatment technologies on lignocellulosic biomass: Effect of key parameters, technological improvements, and challenges. *Bioresour. Technol.* **2020**, *300*, 122724. [CrossRef] [PubMed]
51. Zhou, H.; Jiang, J.; Zhao, Q.; Wang, Z.; Li, L.; Gao, Q.; Wang, K. Performance of high solids enzymatic hydrolysis and bioethanol fermentation of food waste under the regulation of saponin. *Bioresour. Technol.* **2023**, *387*, 129486. [CrossRef]
52. Suriyachai, N.; Imman, S.; Kreetachat, T.; Laosiripojana, N.; Khongchamnan, P.; Champreda, V.; Suwannahong, K.; Sakulthaew, C.; Chokejaroenrat, C.; Kreetachat, N. Enhanced bioethanol production from cassava waste: Optimization of thermochemical pretreatment for maximum sugar recovery and characterisation of potential fractions. *Int. J. Sustain. Energy* **2024**, *43*, 2411833. [CrossRef]
53. Tarrsini, M.; Ng, Q.H.; Teoh, Y.P.; Shuit, S.H.; Ooi, Z.X.; Kunasundari, B. Structural and composition modification of Harum Manis mango (*Mangifera indica*) leaves via chemical pretreatment for bioethanol production. *Biomass Convers. Biorefinery* **2023**, *13*, 3987–3999. [CrossRef]
54. Ranjithkumar, M.; Uthandi, S.; Senthil Kumar, P.; Muniraj, I.; Thanabal, V.; Rajarathinam, R. Highly crystalline cotton spinning wastes utilization: Pretreatment, optimized hydrolysis and fermentation using Pleurotus florida for bioethanol production. *Fuel* **2022**, *308*, 122052. [CrossRef]

55. Song, Y.; Maskey, S.; Lee, Y.G.; Lee, D.S.; Nguyen, D.T.; Bae, H.J. Optimizing bioconversion processes of rice husk into value-added products: D-psicose, bioethanol, and lactic acid. *Bioresour. Technol.* **2024**, *395*, 130363. [CrossRef] [PubMed]
56. Hijosa-Valsero, M.; Garita-Cambronero, J.; Paniagua-García, A.I.; Díez-Antolínez, R. A global approach to obtain biobutanol from corn stover. *Renew. Energy* **2020**, *148*, 223–233. [CrossRef]
57. Casabar, J.T.; Ramaraj, R.; Tipnee, S.; Unpaprom, Y. Enhancement of hydrolysis with Trichoderma harzianum for bioethanol production of sonicated pineapple fruit peel. *Fuel* **2020**, *279*, 118437. [CrossRef]
58. Raina, N.; Slathia, P.S.; Sharma, P. Experimental optimization of thermochemical pretreatment of sal (*Shorea robusta*) sawdust by Central Composite Design study for bioethanol production by co-fermentation using *Saccharomyces cerevisiae* (MTCC-36) and Pichia stipitis (NCIM-3498). *Biomass Bioenergy* **2020**, *143*, 105819. [CrossRef]
59. Sarbishei, S.; Goshadrou, A.; Hatamipour, M.S. Mild sodium hydroxide pretreatment of tobacco product waste to enable efficient bioethanol production by separate hydrolysis and fermentation. *Biomass Convers. Biorefinery* **2021**, *11*, 2963–2973. [CrossRef]
60. Lee, I.; Yu, J.H. The production of fermentable sugar and bioethanol from acacia wood by optimizing dilute sulfuric acid pretreatment and post treatment. *Fuel* **2020**, *275*, 117943. [CrossRef]
61. Panakkal, E.J.; Cheenkachorn, K.; Chuetor, S.; Tantayotai, P.; Raina, N.; Cheng, Y.S.; Sriariyanun, M. Optimization of deep eutectic solvent pretreatment for bioethanol production from Napier grass. *Sustain. Energy Technol. Assess.* **2022**, *54*, 102856. [CrossRef]
62. Domínguez, E.; Nóvoa, T.; del Río, P.G.; Garrote, G.; Romaní, A. Sequential two-stage autohydrolysis biorefinery for the production of bioethanol from fast-growing Paulownia biomass. *Energy Convers. Manag.* **2020**, *226*, 113517. [CrossRef]
63. Mazaheri, D.; Orooji, Y.; Mazaheri, M.; Moghaddam, M.S.; Karimi-Maleh, H. Bioethanol production from pomegranate peel by simultaneous saccharification and fermentation process. *Biomass Convers. Biorefinery* **2021**, 2190–6823. [CrossRef]
64. Prajapati, B.P.; Jana, U.K.; Suryawanshi, R.K.; Kango, N. Sugarcane bagasse saccharification using *Aspergillus tubingensis* enzymatic cocktail for 2G bio-ethanol production. *Renew. Energy* **2020**, *152*, 653–663. [CrossRef]
65. Infanzón-Rodríguez, M.I.; del Moral, S.; Gómez-Rodríguez, J.; Faife-Pérez, E.; Aguilar-Uscanga, M.G. Second-Generation Bioethanol Production and Cellulases of *Aspergillus niger* ITV02 Using Sugarcane Bagasse as Substrate. *Bioenergy Res.* **2024**, *17*, 160–172. Available online: https://link.springer.com/article/10.1007/s12155-023-10640-4 (accessed on 3 December 2024). [CrossRef]
66. Valamonfared, J.; Javanmard, A.S.; Ghaedi, M.; Bagherinasab, M. Bioethanol production using lignocellulosic materials and thermophilic microbial hydrolysis. *Biomass Convers. Biorefinery* **2024**, *14*, 16589–16601. Available online: https://link.springer.com/article/10.1007/s13399-023-03980-w (accessed on 3 December 2024). [CrossRef]
67. Chugh, P.; Kaur, J.; Soni, R.; Sharma, A.; Soni, S.K. A low-cost process for efficient hydrolysis of deoiled rice bran and ethanol production using an inhouse produced multi-enzyme preparation from *Aspergillus niger* P-19. *J. Mater. Cycles Waste Manag.* **2023**, *25*, 359–375. Available online: https://link.springer.com/article/10.1007/s10163-022-01538-y (accessed on 3 December 2024). [CrossRef]
68. Pardo Cuervo, O.H.; Rosas, C.A.; Romanelli, G.P. Valorization of residual lignocellulosic biomass in South America: A review. *Environ. Sci. Pollut. Res.* **2024**, *31*, 44575–44607. Available online: https://link.springer.com/article/10.1007/s11356-024-33968-6 (accessed on 26 November 2024). [CrossRef] [PubMed]
69. Skiba, E.A.; Budaeva, V.V.; Baibakova, O.V.; Zolotukhin, V.N.; Sakovich, G.V. Dilute nitric-acid pretreatment of oat hulls for ethanol production. *Biochem. Eng. J.* **2017**, *126*, 118–125. [CrossRef]
70. Skiba, E.A.; Ovchinnikova, E.V.; Budaeva, V.V.; Banzaraktsaeva, S.P.; Kovgan, M.A.; Chumachenko, V.A.; Mironova, G.F.; Kortusov, A.N.; Parmon, V.N.; Sakovich, G.V. Miscanthus bioprocessing using HNO3-pretreatment to improve productivity and quality of bioethanol and downstream ethylene. *Ind. Crops Prod.* **2022**, *177*, 114448. [CrossRef]
71. Tyagi, A.; Banerjee, B.; Tyagi, S. A holistic approach toward the maximum conversion of lignocellulose into biofuels. In *Biofuels and Sustainability*; Elsevier: Amsterdam, The Netherlands, 2025; pp. 81–103. Available online: https://linkinghub.elsevier.com/retrieve/pii/B9780443214332000141 (accessed on 28 November 2024).
72. Gupta, A.; Sahni, T.; Kumar, S. *Biorefineries: An Analogue to Petroleum Refineries*; Springer: Singapore, 2024; pp. 1–29. Available online: https://link.springer.com/chapter/10.1007/978-981-99-8224-0_1 (accessed on 28 November 2024).
73. Hai, T.T.; Peer, A.; Van Cone, J.W.; Schonewille, J.T.; Baars, J.J.P.; Phung, L.D.; Hendriks, W.H. Incubation temperature affects growth and efficacy of white-rot fungi to improve the nutritive value of rice straw. *Anim. Prod. Sci.* **2024**, *64*, 23403. Available online: https://www.publish.csiro.au/an/AN23403 (accessed on 28 November 2024). [CrossRef]
74. Parmar, M.; Patel, S.A.H.; Phutela, U.G.; Dhawan, M. Comparative Analysis of Ligninolytic Potential among Pleurotus ostreatus and Fusarium sp. with a Spec. Focus. Versatile Peroxidase. *Appl. Microbiol.* **2024**, *4*, 1348–1361. [CrossRef]
75. Ren, H.; Sun, W.; Wang, Z.; Fu, S.; Zheng, Y.; Song, B.; Li, Z.; Peng, Z. Enhancing the Enzymatic Saccharification of Grain Stillage by Combining Microwave-Assisted Hydrothermal Irradiation and Fungal Pretreatment. *ACS Omega* **2020**, *5*, 12603–12614. Available online: https://pubs.acs.org/doi/full/10.1021/acsomega.9b03681 (accessed on 28 November 2024). [CrossRef]

76. Wang, W.; Yuan, T.; Wang, K.; Cui, B.; Dai, Y. Combination of biological pretreatment with liquid hot water pretreatment to enhance enzymatic hydrolysis of Populus tomentosa. *Bioresour. Technol.* **2012**, *107*, 282–286. [CrossRef] [PubMed]
77. Ma, F.; Yang, N.; Xu, C.; Yu, H.; Wu, J.; Zhang, X. Combination of biological pretreatment with mild acid pretreatment for enzymatic hydrolysis and ethanol production from water hyacinth. *Bioresour. Technol.* **2010**, *101*, 9600–9604. [CrossRef]
78. Martínez-Patiño, J.C.; Lu-Chau, T.A.; Gullón, B.; Ruiz, E.; Romero, I.; Castro, E.; Lema, J.M. Application of a combined fungal and diluted acid pretreatment on olive tree biomass. *Ind. Crops Prod.* **2018**, *121*, 10–17. [CrossRef]
79. Si, M.; Liu, D.; Liu, M.; Yan, X.; Gao, C.; Chai, L.; Shi, Y. Complementary effect of combined bacterial-chemical pretreatment to promote enzymatic digestibility of lignocellulose biomass. *Bioresour. Technol.* **2019**, *272*, 275–280. [CrossRef]
80. Shen, Z.; Zhang, K.; Si, M.; Liu, M.; Zhuo, S.; Liu, D.; Ren, L.; Yan, X.; Shi, Y. Synergy of lignocelluloses pretreatment by sodium carbonate and bacterium to enhance enzymatic hydrolysis of rice straw. *Bioresour. Technol.* **2018**, *249*, 154–160. Available online: https://pubmed.ncbi.nlm.nih.gov/29040849/ (accessed on 28 November 2024). [CrossRef] [PubMed]
81. Xie, C.; Gong, W.; Yang, Q.; Zhu, Z.; Yan, L.; Hu, Z.; Peng, Y. White-rot fungi pretreatment combined with alkaline/oxidative pretreatment to improve enzymatic saccharification of industrial hemp. *Bioresour. Technol.* **2017**, *243*, 188–195. [CrossRef]
82. Zhuo, S.; Yan, X.; Liu, D.; Si, M.; Zhang, K.; Liu, M.; Peng, B.; Shi, Y. Use of bacteria for improving the lignocellulose biorefinery process: Importance of pre-erosion. *Biotechnol. Biofuels* **2018**, *11*, 146. Available online: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-018-1146-4 (accessed on 28 November 2024). [CrossRef] [PubMed]
83. Li, G.; Chen, H. Synergistic mechanism of steam explosion combined with fungal treatment by Phellinus baumii for the pretreatment of corn stalk. *Biomass Bioenergy* **2014**, *67*, 1–7. [CrossRef]
84. Sun, W.; Tajvidi, M.; Hunt, C.G.; Cole, B.J.W.; Howell, C.; Gardner, D.J.; Wang, J. Fungal and enzymatic pretreatments in hot-pressed lignocellulosic bio-composites: A critical review. *J. Clean. Prod.* **2022**, *353*, 131659. [CrossRef]
85. Kocaturk, E.; Salan, T.; Ozcelik, O.; Alma, M.H.; Candan, Z. Recent Advances in Lignin-Based Biofuel Production. *Energies* **2023**, *16*, 3382. Available online: https://www.mdpi.com/1996-1073/16/8/3382/htm (accessed on 28 November 2024). [CrossRef]
86. Wan Azelee, N.I.; Mahdi, H.I.; Cheng, Y.S.; Nordin, N.; Illias, R.M.; Rahman, R.A.; Shaarani, S.M.; Bhatt, P.; Yadav, S.; Chang, S.W.; et al. Biomass degradation: Challenges and strategies in extraction and fractionation of hemicellulose. *Fuel* **2023**, *339*, 126982. [CrossRef]
87. Saroj, P.P.M.; Narasimhulu, K. Enhanced reducing sugar production by blending hydrolytic enzymes from *Aspergillus fumigatus* to improve sugarcane bagasse hydrolysis. *Environ. Sci. Pollut. Res.* **2024**, *31*, 48085–48102. Available online: https://link.springer.com/article/10.1007/s11356-024-34246-1 (accessed on 29 November 2024). [CrossRef] [PubMed]
88. Rocha, G.J.M.; Nascimento, V.M.; Silva, V.d.; Corso, D.L.S.; Gonçalves, A.R. Contributing to the environmental sustainability of the second generation ethanol production: Delignification of sugarcane bagasse with sodium hydroxide recycling. *Ind. Crops Prod.* **2014**, *59*, 63–68. [CrossRef]
89. Makarova, E.I.; Budaeva, V.V.; Kukhlenko, A.A.; Orlov, S.E. Enzyme kinetics of cellulose hydrolysis of Miscanthus and oat hulls. *3 Biotech* **2017**, *7*, 317. Available online: https://link.springer.com/article/10.1007/s13205-017-0964-6 (accessed on 17 January 2025). [CrossRef] [PubMed]
90. Winarsih, S.; Siskawardani, D.D. Hydrolysis of corncobs using a mixture of crude enzymes from Trichoderma reesei and *Aspergillus niger* for bioethanol production. *Energy Rep.* **2020**, *6*, 256–262. [CrossRef]
91. El-Shora, H.M.; El-Sharkawy, R.M.; Khateb, A.M.; Darwish, D.B. Production and immobilization of β-glucanase from *Aspergillus niger* with its applications in bioethanol production and biocontrol of phytopathogenic fungi. *Sci. Rep.* **2021**, *11*, 21000. Available online: https://www.nature.com/articles/s41598-021-00237-2 (accessed on 3 December 2024). [CrossRef]
92. Ranjan, A.; Welz, P.J.; Mthethwa, T. Investigation of an effective acid pre-treatment method for the valorisation of *Canola fines*. *Biomass Convers. Biorefinery* **2024**, *14*, 12013–12026. Available online: https://link.springer.com/article/10.1007/s13399-023-03946-y (accessed on 29 November 2024). [CrossRef]
93. Nemes, S.A.; Farcas, A.C.; Ranga, F.; Teleky, B.E.; Calinoiu, L.F.; Dulf, F.V.; Vodnar, D.C. Enhancing phenolic and lipid compound production in oat bran via acid pretreatment and solid-state fermentation with *Aspergillus niger*. *N. Biotechnol.* **2024**, *83*, 91–100. [CrossRef] [PubMed]
94. Acharya, S.; Liyanage, S.; Abidi, N.; Parajuli, P.; Rumi, S.S.; Shamshina, J.L. Utilization of Cellulose to Its Full Potential: A Review on Cellulose Dissolution, Regeneration, and Applications. *Polymers* **2021**, *13*, 4344. Available online: https://www.mdpi.com/2073-4360/13/24/4344/htm (accessed on 29 November 2024). [CrossRef] [PubMed]
95. Buettner, C.S.; Cognigni, A.; Schröder, C.; Bica-Schröder, K. Surface-active ionic liquids: A review. *J. Mol. Liq.* **2022**, *347*, 118160. [CrossRef]
96. Sun, Y.C.; Liu, X.N.; Wang, T.T.; Xue, B.L.; Sun, R.C. Green process for extraction of lignin by the microwave-assisted ionic liquid approach: Toward biomass biorefinery and lignin characterization. *ACS Sustain. Chem. Eng.* **2019**, *7*, 13062–13072. Available online: https://pubs.acs.org/doi/abs/10.1021/acssuschemeng.9b02166 (accessed on 29 November 2024). [CrossRef]

97. Sunar, S.L.; Oruganti, R.K.; Bhattacharyya, D.; Shee, D.; Panda, T.K. Pretreatment of sugarcane bagasse using ionic liquid for enhanced enzymatic saccharification and lignin recovery: Process optimization by response surface methodology. *Cellulose* **2024**, *31*, 2151–2173. Available online: https://link.springer.com/article/10.1007/s10570-024-05768-1 (accessed on 29 November 2024). [CrossRef]
98. Silveira, M.H.L.; Vanelli, B.A.; Corazza, M.L.; Ramos, L.P. Supercritical carbon dioxide combined with 1-butyl-3-methylimidazolium acetate and ethanol for the pretreatment and enzymatic hydrolysis of sugarcane bagasse. *Bioresour. Technol.* **2015**, *192*, 389–396. [CrossRef]
99. Awodi, P.S.; Ogbonna, J.C.; Nwagu, T.N. Bioconversion of mango (*Mangifera indica*) seed kernel starch into bioethanol using various fermentation techniques. *Heliyon* **2022**, *8*, e09707. [CrossRef] [PubMed]
100. Beltagy, E.A.; Abouelwafa, A.; Barakat, K.M. Bioethanol production from immobilized amylase produced by marine *Aspergillus flavus* AUMC10636. *Egypt J. Aquat. Res.* **2022**, *48*, 325–331. [CrossRef]
101. Valladares-Diestra, K.K.; Porto de Souza Vandenberghe, L.; Zevallos Torres, L.A.; Nishida, V.S.; Zandoná Filho, A.; Woiciechowski, A.L.; Soccol, C.R. Imidazole green solvent pre-treatment as a strategy for second-generation bioethanol production from sugarcane bagasse. *Chem. Eng. J.* **2021**, *420*, 127708. [CrossRef]
102. Tien Thanh, N.; Mostapha, M.; Lam, M.K.; Ishak, S.; Kanna Dasan, Y.; Lim, J.W.; Tan, I.S.; Lau, S.Y.; Chin, B.L.F.; Hadibarata, T. Fundamental understanding of in-situ transesterification of microalgae biomass to biodiesel: A critical review. *Energy Convers. Manag.* **2022**, *270*, 116212. [CrossRef]
103. Passos, R.M.; dos Ferreira, R.S.B.; Morgano, M.A.; de Souza, P.T.; Meirelles, A.J.A.; Batista, E.A.C.; Maximo, G.J.; Ferreira, M.C.; Sampaio, K.A. Ethyl biodiesel production from crude soybean oil using enzymatic degumming-transesterification associated process. *Ind. Crops Prod.* **2024**, *222*, 119930. [CrossRef]
104. ShenavaeiZare, T.; Khoshsima, A.; ZareNezhad, B. Production of biodiesel through nanocatalytic transesterification of extracted oils from halophytic safflower and salicornia plants in the presence of deep eutectic solvents. *Fuel* **2021**, *302*, 121171. [CrossRef]
105. Kitson-Hytey, M.; Fei-Baffoe, B.; Sackey, L.N.A.; Miezah, K. Production of bioethanol from plantain and yam peels using *Aspergillus niger* and *Saccharomyces cerevisiae*. *Biomass Convers. Biorefinery* **2024**, *14*, 9087–9095. Available online: https://link.springer.com/article/10.1007/s13399-022-03352-w (accessed on 3 December 2024). [CrossRef]
106. Narendra Kumar, H.K.; Chandra Mohana, N.; Rakshith, D.; Abhilash, M.R.; Harini, B.P.; Satish, S. Bioethanol production by enzymatic hydrolysis from *Aspergillus calidoustus* employing different lignocellulosic wastes. *Biocatal. Agric. Biotechnol.* **2023**, *53*, 102847. [CrossRef]
107. John, I.; Yaragarla, P.; Appusamy, A. Production of Bioethanol from Banana Peel Using Isolated Cellulase from *Aspergillus niger*. In *Global Challenges in Energy and Environment*; Lecture Notes on Multidisciplinary Industrial Engineering; Springer: Singapore, 2020; Part F251; pp. 9–18. Available online: https://link.springer.com/chapter/10.1007/978-981-13-9213-9_2 (accessed on 3 December 2024).
108. Wei, H.; Wang, Q.; Zhang, R.; Liu, M.; Zhang, W. Efficient biodiesel production from waste cooking oil by fast co-immobilization of lipases from *Aspergillus oryzae* and Rhizomucor miehei in magnetic chitosan microcapsules. *Process Biochem.* **2023**, *125*, 171–180. [CrossRef]
109. Liu, M.; Ma, L.; Zhang, Z.; Zhang, X.; Zhang, W. Development of a sustainable co-immobilized lipases on $Co^{2+}$-chelated magnetic metal-organic framework for efficient biodiesel production from waste oil. *Mol. Catal.* **2024**, *563*, 114241. [CrossRef]
110. Amoah, J.; Ho, S.H.; Hama, S.; Yoshida, A.; Nakanishi, A.; Hasunuma, T.; Ogino, C.; Kondo, A. Conversion of Chlamydomonas sp. JSC4 lipids to biodiesel using Fusarium heterosporum lipase-expressing *Aspergillus oryzae* whole-cell as biocatalyst. *Algal Res.* **2017**, *28*, 16–23. [CrossRef]
111. Zulfiqar, A.; Mumtaz, M.W.; Mukhtar, H.; Najeeb, J.; Irfan, A.; Akram, S.; Touqeer, T.; Nabi, G. Lipase-PDA-$TiO_2$ NPs: An emphatic nano-biocatalyst for optimized biodiesel production from *Jatropha curcas* oil. *Renew. Energy* **2021**, *169*, 1026–1037. [CrossRef]
112. Guldhe, A.; Singh, P.; Kumari, S.; Rawat, I.; Permaul, K.; Bux, F. Biodiesel synthesis from microalgae using immobilized *Aspergillus niger* whole cell lipase biocatalyst. *Renew. Energy* **2016**, *85*, 1002–1010. [CrossRef]
113. Jin, X.; Song, J.; Liu, G.Q. Bioethanol production from rice straw through an enzymatic route mediated by enzymes developed in-house from *Aspergillus fumigatus*. *Energy* **2020**, *190*, 116395. [CrossRef]
114. Constantino, A.; Rodrigues, B.; Leon, R.; Barros, R.; Raposo, S. Alternative chemo-enzymatic hydrolysis strategy applied to different microalgae species for bioethanol production. *Algal Res.* **2021**, *56*, 102329. [CrossRef]
115. Olawale, B.I.; Iliyasu, M.Y.; Musa, B.; Abdulrahman, A.; Umar, A.F.; Olawale, B.I.; Iliyasu, M.Y.; Musa, B.; Abdulrahman, A.; Umar, A.F. Production of Bioethanol by Co-Culture of *Aspergillus niger* and *Saccharomyces cerevisiae* using Watermelon Peels as Substrate. *Path Sci.* **2021**, *7*, 6001–6011. Available online: https://pathofscience.org/index.php/ps/article/view/1036 (accessed on 3 December 2024).

116. Alabdalall, A.H.; Almutari, A.A.; Aldakeel, S.A.; Albarrag, A.M.; Aldakheel, L.A.; Alsoufi, M.H.; Alfuraih, L.Y.; Elkomy, H.M. Bioethanol Production from Lignocellulosic Biomass Using *Aspergillus niger* and *Aspergillus flavus* Hydrolysis Enzymes through Immobilized, *S. cerevisiae*. *Energies* **2023**, *16*, 823. Available online: https://www.mdpi.com/1996-1073/16/2/823/htm (accessed on 3 December 2024). [CrossRef]
117. Roble, N.D.; Ogbonna, J.; Tanaka, H. Simultaneous amylase production, raw cassava starch hydrolysis and ethanol production by immobilized *Aspergillus awamori* and *Saccharomyces cerevisiae* in a novel alternating liquid phase–air phase system. *Process Biochem.* **2020**, *95*, 115–121. [CrossRef]
118. Mignogna, D.; Szabó, M.; Ceci, P.; Avino, P. Biomass Energy and Biofuels: Perspective, Potentials, and Challenges in the Energy Transition. *Sustainability* **2024**, *16*, 7036. Available online: https://www.mdpi.com/2071-1050/16/16/7036/htm (accessed on 26 November 2024). [CrossRef]
119. Berwian, G.F.; Rempel, A.; Garda-Buffon, J.; Colla, L.M. Use of residual barley to bioethanol production: Valoration, mycotoxins decontamination and feed production. *Bioresour. Technol. Rep.* **2024**, *26*, 101878. [CrossRef]
120. de C.M. Miranda, R.; Neta, J.; Ferreira, L.F.R. Pineapple crown delignification using low-cost ionic liquid based on ethanolamine and organic acids. *Carbohydr. Polym.* **2019**, *302*, 206–308. Available online: https://www.sciencedirect.com/science/article/pii/S0144861718313122 (accessed on 26 November 2024). [CrossRef] [PubMed]
121. Van Der Maarel, M.J.E.C.; Van Der Veen, B.; Uitdehaag, J.C.M.; Leemhuis, H.; Dijkhuizen, L. Properties and applications of starch-converting enzymes of the α-amylase family. *J. Biotechnol.* **2002**, *94*, 137–155. [CrossRef]
122. Wang, S.; Jeyaseelan, J.; Liu, Y.; Qin, W. Characterization and Optimization of Amylase Production in WangLB, a High Amylase-Producing Strain of Bacillus. *Appl. Biochem. Biotechnol.* **2016**, *180*, 136–151. Available online: https://pubmed.ncbi.nlm.nih.gov/27116321/ (accessed on 12 December 2024). [CrossRef] [PubMed]
123. Aggarwal, R.; Dutta, T.; Sheikh, J. Extraction of amylase from the microorganism isolated from textile mill effluent vis a vis desizing of cotton. *Sustain. Chem. Pharm.* **2019**, *14*, 100178. [CrossRef]
124. Abeleda, H.E.P.; Javier, A.P.; Murillo, A.Q.M.; Baculi, R.Q. Alpha-amylase conjugated biogenic silver nanoparticles as innovative strategy against biofilm-forming multidrug resistant bacteria. *Biocatal. Agric. Biotechnol.* **2020**, *29*, 101784. [CrossRef]
125. de Souza, P.M.; e Magalhães, P.d.O. Application of microbial α-amylase in industry—A review. *Braz. J. Microbiol.* **2010**, *41*, 850. Available online: https://pmc.ncbi.nlm.nih.gov/articles/PMC3769773/ (accessed on 12 December 2024). [CrossRef] [PubMed]
126. Tiwari, S.; Srivastava, R.; Singh, C.; Shukla, K.; Singh, R.; Singh, P.; Singh, R.; Singh, N.; Sharma, R. Amylases: An overview with special reference to alpha amylase. *J. Glob. Biosci.* **2015**, *4*, 1886–1901. Available online: https://www.researchgate.net/publication/312948601_Amylases_an_overview_with_special_reference_to_alpha_amylase (accessed on 12 December 2024).
127. Paul, J.S.; Gupta, N.; Beliya, E.; Tiwari, S.; Jadhav, S.K. Aspects and Recent Trends in Microbial α-Amylase: A Review. *Appl. Biochem. Biotechnol.* **2021**, *193*, 2649–2698. Available online: https://pubmed.ncbi.nlm.nih.gov/33715051/ (accessed on 12 December 2024). [CrossRef] [PubMed]
128. Zhang, Q.; Han, Y.; Xiao, H. Microbial α-amylase: A biomolecular overview. *Process Biochem.* **2017**, *53*, 88–101. [CrossRef]
129. Xian, L.; Wang, F.; Luo, X.; Feng, Y.L.; Feng, J.X. Purification and Characterization of a Highly Efficient Calcium-Independent α-Amylase from *Talaromyces pinophilus* 1–95. *PLoS ONE* **2015**, *10*, e0121531. Available online: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0121531 (accessed on 12 December 2024). [CrossRef] [PubMed]
130. Liao, S.M.; Liang, G.; Zhu, J.; Lu, B.; Peng, L.X.; Wang, Q.Y.; Wei, Y.T.; Zhou, G.P.; Huang, R.B. Influence of Calcium Ions on the Thermal Characteristics of α-amylase from Thermophilic *Anoxybacillus* sp. *GXS-BL. Protein Pept. Lett.* **2019**, *26*, 148–157. Available online: https://pubmed.ncbi.nlm.nih.gov/30652633/ (accessed on 12 December 2024). [CrossRef] [PubMed]
131. Zhu, D.; Wu, Q.; Wang, N. Industrial Enzymes. In *Comprehensive Biotechnology*, 3rd. ed.; Pergamon: Mysia, Turkey, 2011; Volume 3, pp. 3–13.
132. Duan, X.; Zhu, Q.; Zhang, X.; Shen, Z.; Huang, Y. Expression, biochemical and structural characterization of high-specific-activity β-amylase from Bacillus aryabhattai GEL-09 for application in starch hydrolysis. *Microb. Cell Fact.* **2021**, *20*, 182. Available online: https://microbialcellfactories.biomedcentral.com/articles/10.1186/s12934-021-01649-5 (accessed on 12 December 2024). [CrossRef] [PubMed]
133. Taniguchi, H.; Honnda, Y. Encyclopedia of microbiology. In *Encyclopedia of Microbiology*; Academic Press: Cambridge, MA, USA, 2009; pp. 159–173. Available online: https://search.worldcat.org/title/416206211 (accessed on 12 December 2024).
134. Eck, P. Chapter 13 - Recombinant DNA Technologies in Food. *Biochem. Foods* **2013**, 503–556. [CrossRef]
135. Mondal, S.; Mondal, K.; Halder, S.K.; Thakur, N.; Mondal, K.C. Microbial Amylase: Old but still at the forefront of all major industrial enzymes. *Biocatal. Agric. Biotechnol.* **2022**, *45*, 102509. [CrossRef]
136. Nag, M.; Lahiri, D.; Garai, S.; Mukherjee, D.; Ray, R.R. Regulation of β-amylase synthesis: A brief overview. *Mol. Biol. Rep.* **2021**, *48*, 6503–6511. Available online: https://pubmed.ncbi.nlm.nih.gov/34379288/ (accessed on 12 December 2024). [CrossRef] [PubMed]

137. Xu, Q.S.; Yan, Y.S.; Feng, J.X. Efficient hydrolysis of raw starch and ethanol fermentation: A novel raw starch-digesting glucoamylase from Penicillium oxalicum. *Biotechnol. Biofuels* **2016**, *9*, 216. Available online: https://biotechnologyforbiofuels.biomedcentral.com/articles/10.1186/s13068-016-0636-5 (accessed on 12 December 2024). [CrossRef] [PubMed]
138. Kumar, P.; Satyanarayana, T. Microbial glucoamylases: Characteristics and applications. *Crit. Rev. Biotechnol.* **2009**, *29*, 225–255. Available online: https://pubmed.ncbi.nlm.nih.gov/19708824/ (accessed on 12 December 2024). [CrossRef] [PubMed]
139. Marín-Navarro, J.; Polaina, J. Glucoamylases: Structural and biotechnological aspects. *Appl. Microbiol. Biotechnol.* **2011**, *89*, 1267–1273. Available online: https://pubmed.ncbi.nlm.nih.gov/21152915/ (accessed on 12 December 2024). [CrossRef] [PubMed]
140. Zong, X.; Wen, L.; Wang, Y.; Li, L. Research progress of glucoamylase with industrial potential. *J. Food Biochem.* **2022**, *46*, e14099. Available online: https://onlinelibrary.wiley.com/doi/full/10.1111/jfbc.14099 (accessed on 16 September 2024). [CrossRef] [PubMed]
141. Zambare, V. Solid State Fermentation of *Aspergillus oryzae* for Glucoamylase Production on Agro residues. *Int. J. Life Sci.* **2010**, *4*, 16–25. [CrossRef]
142. Wayllace, N.M.; Martín, M.; Busi, M.V.; Gomez-Casati, D.F. Microbial glucoamylases: Structural and functional properties and biotechnological uses. *World J. Microbiol. Biotechnol.* **2023**, *39*, 293. Available online: https://pubmed.ncbi.nlm.nih.gov/37653355/ (accessed on 12 December 2024). [CrossRef] [PubMed]
143. Karim, K.M.R.; Tasnim, T. Fungal Glucoamylase Production and Characterization: A Review. *Bioresearch Commun.—(BRC)* **2018**, *4*, 591–605. Available online: https://www.bioresearchcommunications.com/index.php/brc/article/view/90 (accessed on 12 December 2024).
144. Ilić, N.; Milić, M.; Beluhan, S.; Dimitrijević-Branković, S. Cellulases: From Lignocellulosic Biomass to Improved Production. *Energies* **2023**, *16*, 3598. Available online: https://www.mdpi.com/1996-1073/16/8/3598/htm (accessed on 12 December 2024). [CrossRef]
145. Jayasekara, S.; Ratnayake, R.; Jayasekara, S.; Ratnayake, R. Microbial Cellulases: An Overview and Applications. In *Cellulose*; IntechOpen: London, UK, 2019; Available online: https://www.intechopen.com/chapters/66517 (accessed on 12 December 2024).
146. Chen, Y.; Lu, S.; Ye, Z.; Cai, X.; Wu, S.; Li, P.; Bing, D. New compound probiotic beverage protects against antibiotic-associated diarrhea in mice by modulating the microbiota. *Future Microbiol.* **2022**, *17*, 943–956. [CrossRef]
147. Knob, A.; Beitel, S.M.; Fortkamp, D.; Terrasan, C.R.F.; Almeida, A.F.d. Production, Purification, and Characterization of a Major Penicillium glabrum Xylanase Using Brewer's Spent Grain as Substrate. *Biomed. Res. Int.* **2013**, *2013*, 728735. [CrossRef] [PubMed]
148. Mano, M.C.R.; Neri-Numa, I.A.; da Silva, J.B.; Paulino, B.N.; Pessoa, M.G.; Pastore, G.M. Oligosaccharide biotechnology: An approach of prebiotic revolution on the industry. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 17–37. [CrossRef] [PubMed]
149. Lopes, A.M.M.; Martins, M.; Goldbeck, R. Heterologous Expression of Lignocellulose-Modifying Enzymes in Microorganisms: Current Status. *Mol. Biotechnol.* **2021**, *63*, 184–199. [CrossRef] [PubMed]
150. Singh, R.D.; Nadar, C.G.; Muir, J.; Arora, A. Green and clean process to obtain low degree of polymerisation xylooligosaccharides from almond shell. *J. Clean. Prod.* **2019**, *241*, 118237. [CrossRef]
151. Vázquez, M.J.; Alonso, J.L.; Domínguez, H.; Parajó, J.C. Xylooligosaccharides: Manufacture and applications. *Trends Food Sci. Technol.* **2000**, *11*, 387–393. [CrossRef]
152. Kaushal, J.; Khatri, M.; Singh, G.; Arya, S.K. A multifaceted enzyme conspicuous in fruit juice clarification: An elaborate review on xylanase. *Int. J. Biol. Macromol.* **2021**, *193*, 1350–1361. [CrossRef] [PubMed]
153. *Carbohydrate-Active Enzymes*; CAZy—GH Université d'Aix-Marseille: Marseille, France, 2024; pp. 12–17. Available online: https://www.cazy.org/Glycoside-Hydrolases.html (accessed on 24 December 2024).
154. Tyagi, D.; Sharma, D. Production and Industrial Applications of Xylanase: A Review. *Int. J. Sci. Res. Eng. Trends* **2021**, *7*, 2395–2566.
155. Malhotra, G.; Chapadgaonkar, S.S. Production and applications of xylanases—An overview. *BioTechnologia* **2018**, *99*, 59–72. [CrossRef]
156. Collins, T.; Gerday, C.; Feller, G. Xylanases, xylanase families and extremophilic xylanases. *FEMS Microbiol. Rev.* **2005**, *29*, 3–23. [CrossRef]
157. Moraleda-Muñoz, A.; Shimkets, L.J. Lipolytic Enzymes in Myxococcus xanthus. *J. Bacteriol.* **2007**, *189*, 3072–3080. [CrossRef] [PubMed]
158. Ergan, F.; Trani, M.; André, G. Production of glycerides from glycerol and fatty acid by immobilized lipases in non-aqueous media. *Biotechnol. Bioeng.* **1990**, *35*, 195–200. [CrossRef]
159. Karadzic, I.; Masui, A.; Zivkovic, L.I.; Fujiwara, N. Purification and characterization of an alkaline lipase from Pseudomonas aeruginosa isolated from putrid mineral cutting oil as component of metalworking fluid. *J. Biosci. Bioeng.* **2006**, *102*, 82–89. [CrossRef] [PubMed]

160. Lan, D.; Wang, Q.; Popowicz, G.M.; Yang, B.; Tang, Q.; Wang, Y. The role of residues 103, 104, and 278 in the activity of SMG1 lipase from Malassezia globosa: A site-directed mutagenesis study. *J. Microbiol. Biotechnol.* **2015**, *25*, 1827–1834. [CrossRef] [PubMed]
161. Mead, J.; Irvine, S.; Ramji, D. Lipoprotein lipase: Structure, function, regulation, and role in disease. *J. Mol. Med.* **2002**, *80*, 753–769. [CrossRef] [PubMed]
162. Romero, O.; Rivero, C.W.; Guisan, J.M.; Palomo, J.M. Novel enzyme-polymer conjugates for biotechnological applications. *PeerJ.* **2013**, *1*, e27. [CrossRef] [PubMed]
163. Brocca, S.; Secundo, F.; Ossola, M.; Alberghina, L.; Carrea, G.; Lotti, M. Sequence of the lid affects activity and specificity of Candida rugosa lipase isoenzymes. *Protein Sci.* **2009**, *12*, 2312–2319. [CrossRef]
164. Balakrishnan, M.; Jeevarathinam, G.; Kumar, S.K.S.; Muniraj, I.; Uthandi, S. Optimization and scale-up of α-amylase production by *Aspergillus oryzae* using solid-state fermentation of edible oil cakes. *BMC Biotechnol.* **2021**, *21*, 33. Available online: https://bmcbiotechnol.biomedcentral.com/articles/10.1186/s12896-021-00686-7 (accessed on 12 December 2024). [CrossRef]
165. Bhardwaj, N.; Kumar, B.; Verma, P. A detailed overview of xylanases: An emerging biomolecule for current and future prospective. *Bioresour. Bioprocess.* **2019**, *6*, 40. [CrossRef]
166. Gamarra, N.N.; Villena, G.K.; Gutiérrez-Correa, M. Cellulase production by *Aspergillus niger* in biofilm, solid-state, and submerged fermentations. *Appl. Microbiol. Biotechnol.* **2010**, *87*, 545–551. Available online: https://pubmed.ncbi.nlm.nih.gov/20354693/ (accessed on 14 December 2024). [CrossRef] [PubMed]
167. Belorkar, S.A.; Gupta, A.K. Oligosaccharides: A boon from nature's desk. *AMB Express* **2016**, *6*, 82. [CrossRef] [PubMed]
168. Chakdar, H.; Kumar, M.; Pandiyan, K.; Singh, A.; Nanjappan, K.; Kashyap, P.L.; Srivastava, A.K. Bacterial xylanases: Biology to biotechnology. *3 Biotech* **2016**, *6*, 150. [CrossRef] [PubMed]
169. Gruening de Mattos, P.B.; Porto de Souza Vandenberghe, L.; Valladares-Diestra, K.K.; Ramos Neyra, L.C.; Vieira, S.; Júnior Letti, L.A.; Soccol, C.R. Recent developments in xylooligosaccharides: Sustainable production, characterization, beneficial properties and applications. *Food Res. Int.* **2024**, *197*, 115206. [CrossRef]
170. Devi, R.; Revathi, K.; Arunagirinathan, N.; Yogananth, N. Production and Optimization of α -amylase from *Aspergillus ochraceus* isolated from Marakkanam Saltpans, Tamil Nadu, India. *Bull. Environ. Pharmacol. Life Sci.* **2022**, *1*, 997–1002.
171. Premalatha, A.; Vijayalakshmi, K.; Shanmugavel, M.; Rajakumar, G.S. Optimization of culture conditions for enhanced production of extracellular α-amylase using solid-state and submerged fermentation from *Aspergillus tamarii* MTCC5152. *Biotechnol. Appl. Biochem.* **2023**, *70*, 835–845. [CrossRef]
172. Olakusehin, V.O.; Oyedeji, O. Production of α-amylase from *Aspergillus flavus* S2-OY using solid substrate fermentation of potato (*Solanum tuberosum* L.) peel. *Int. J. Biol. Chem. Sci.* **2021**, *15*, 1950–1967. [CrossRef]
173. Ahmed, N.E.; El Shamy, A.R.; Awad, H.M. Optimization and immobilization of amylase produced by *Aspergillus terreus* using pomegranate peel waste. *Bull. Natl. Res. Cent.* **2020**, *44*, 109. [CrossRef]
174. Melnichuk, N.; Braia, M.J.; Anselmi, P.A.; Meini, M.R.; Romanini, D. Valorization of two agroindustrial wastes to produce alpha-amylase enzyme from *Aspergillus oryzae* by solid-state fermentation. *Waste Manag.* **2020**, *106*, 155–161. [CrossRef] [PubMed]
175. Lago, M.C.; dos Santos, F.C.; Bueno, P.S.A.; de Oliveira, M.A.S.; Barbosa-Tessmann, I.P. The glucoamylase from *Aspergillus wentii*: Purification and characterization. *J. Basic. Microbiol.* **2021**, *61*, 443–458. [CrossRef] [PubMed]
176. Singhal, A.; Kumari, N.; Ghosh, P.; Singh, Y.; Garg, S.; Shah, M.P.; Jha, P.K.; Chauhan, D.K. Optimizing cellulase production from *Aspergillus flavus* using response surface methodology and machine learning models. *Environ. Technol. Innov.* **2022**, *27*, 102805. [CrossRef]
177. Bhati, N.; Shreya, S.A.K. Production and optimisation of cellulase enzyme by *Aspergillus uvarum* CBS 121591 isolated from soil. *Vegetos* **2023**, *36*, 201–209. [CrossRef]
178. Sulyman, A.O.; Igunnu, A.; Malomo, S.O. Isolation, purification and characterization of cellulase produced by *Aspergillus niger* cultured on Arachis hypogaea shells. *Heliyon* **2020**, *6*, e05668. [CrossRef] [PubMed]
179. Saroj, P.P.M.; Narasimhulu, K. Assessment and evaluation of cellulase production using ragi (*Eleusine coracana*) husk as a substrate from thermo-acidophilic *Aspergillus fumigatus* JCM 10253. *Bioprocess Biosyst. Eng.* **2021**, *44*, 113–126. [CrossRef]
180. Shen, L.; Su, Y.; Sun, Y.; Wang, G.; Chen, H.; Yu, X.; Zhan, S.; Chen, G. Establishment of a highly efficient and low cost mixed cellulase system for bioconversion of corn stover by Trichoderma reesei and *Aspergillus niger*. *Biocatal. Agric. Biotechnol.* **2021**, *32*, 101849. [CrossRef]
181. Yang, J.; Li, F.Z.; Arnold, F.H. Opportunities and Challenges for Machine Learning-Assisted Enzyme Engineering. *ACS Central Sci. Am. Chem. Soc.* **2024**, *10*, 226–241. [CrossRef] [PubMed]
182. Wang, Y.; Xue, P.; Cao, M.; Yu, T.; Lane, S.T.; Zhao, H. Directed Evolution: Methodologies and Applications. *Chem. Rev. Am. Chem. Soc.* **2021**, *121*, 12384–12444. [CrossRef]
183. Song, Z.; Zhang, Q.; Wu, W.; Pu, Z.; Yu, H. Rational design of enzyme activity and enantioselectivity. *Front. Bioeng. Biotechnol. Front. Media S.A.* **2023**, *11*, 1129149. [CrossRef]

184. Fonseca, L.M.; Parreiras, L.S.; Murakami, M.T. Rational engineering of the *Trichoderma reesei* RUT-C30 strain into an industrially relevant platform for cellulase production. *Biotechnol. Biofuels* **2020**, *13*, 93. [CrossRef] [PubMed]
185. Arai, T.; Ichinose, S.; Shibata, N.; Kakeshita, H.; Kodama, H.; Igarashi, K.; Takimura, Y. Inducer-free cellulase production system based on the constitutive expression of mutated XYR1 and ACE3 in the industrial fungus Trichoderma reesei. *Sci. Rep.* **2022**, *12*, 19445. [CrossRef] [PubMed]
186. Chen, X.; Pan, B.; Yu, L.; Wang, B.; Pan, L. Enhancement of protein production in *Aspergillus niger* by engineering the antioxidant defense metabolism. *Biotechnol. Biofuels Bioprod.* **2024**, *17*, 91. [CrossRef]
187. Benites-Pariente, J.S.; Samolski, I.; Ludeña, Y.; Villena, G.K. CRISPR/Cas9 mediated targeted knock-in of eglA gene to improve endoglucanase activity of *Aspergillus fumigatus* LMB-35Aa. *Sci. Rep.* **2024**, *14*, 19661. [CrossRef]
188. Zou, G.; Xiao, M.; Chai, S.; Zhu, Z.; Wang, Y.; Zhou, Z. Efficient genome editing in filamentous fungi via an improved CRISPR-Cas9 ribonucleoprotein method facilitated by chemical reagents. *Microb. Biotechnol.* **2021**, *14*, 2343–2355. [CrossRef] [PubMed]
189. Le Gal-Coëffet, M.-F.; Jacks, A.J.; Sorimachi, K.; Williamson, M.P.; Williamson, G.; Archer, D.B. Expression in *Aspergillus niger* of the Starch-Binding Domain of Glucoamylase: Comparison with the Proteolytically Produced Starch-Binding Domain. *Eur. J. Biochem.* **1995**, *233*, 561–567. [CrossRef] [PubMed]
190. James, E.R.; Van Zyl, W.H.; Van Zyl, P.J.; Görgens, J.F. Recombinant hepatitis B surface antigen production in *Aspergillus niger:* Evaluating the strategy of gene fusion to native glucoamylase. *Appl. Microbiol. Biotechnol.* **2012**, *96*, 385–394. [CrossRef]
191. Sürmeli, Y.; Şanlı-Mohamed, G. Engineering of xylanases for the development of biotechnologically important characteristics. *John Wiley Sons Inc.* **2023**, *120*, 1171–1188. [CrossRef]
192. Li, Q.; Wu, T.; Duan, Y.; Pei, J.; Zhao, L. Improving the Thermostability and pH Stability of *Aspergillus niger* Xylanase by Site-directed Mutagenesis. *Appl. Biochem. Microbiol.* **2019**, *55*, 136–144. [CrossRef]
193. Li, R.; Wang, T.; Bo, N.; Wang, Q.; Chen, Q.; Liang, Z.; Guan, Y.; Jiang, B.; Ma, Y.; Zhao, M. The carbohydrate metabolism and expression of carbohydrate-active enzyme genes in *Aspergillus luchuensis* fermentation of tea leaves. *Front. Microbiol.* **2024**, *15*, 1408645. Available online: www.sanshubio.com (accessed on 8 October 2024). [CrossRef]
194. Zhao, P.; Wang, N.; Yao, M.; Ren, H.; Hu, W. Hydrothermal electrodeposition incorporated with CVD-polymerisation to tune PPy@$MnO_2$ interlinked core-shell nanowires on carbon fabric for flexible solid-state asymmetric supercapacitors. *Chem. Eng. J.* **2020**, *380*, 122488. [CrossRef]
195. Kluge, J.; Terfehr, D.; Kück, U. Inducible promoters and functional genomic approaches for the genetic engineering of filamentous fungi. *Appl. Microbiol. Biotechnol.* **2018**, *102*, 6357–6372. [CrossRef] [PubMed]
196. Mattern, I.E.; van Noort, J.M.; van den Berg, P.; Archer, D.B.; Roberts, I.N.A.M.J.J.; van den Honde, P.C. Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. *Mol. Gen. Genet.* **1992**, *234*, 332–336. [CrossRef] [PubMed]
197. Kamaruddin, N.; Storms, R.; Mahadi, N.M.; Illias, R.M.; Bakar, F.D.A.; Murad, A.M.A. Reduction of Extracellular Proteases Increased Activity and Stability of Heterologous Protein in *Aspergillus niger*. *Arab. J. Sci. Eng.* **2018**, *43*, 3327–3338. [CrossRef]
198. Sandaka, B.P.; Kumar, J.; Melo, J.S. Mitigation of methanol inactivation of lipases by reaction medium engineering with glycine betaine for enzymatic biodiesel synthesis. *Fuel* **2022**, *313*, 122637. [CrossRef]
199. Tian, M.; Fu, J.; Wang, Z.; Miao, C.; Lv, P.; He, D.; Li, Z.; Liu, T.; Li, M.; Luo, W. Enhanced activity and stability of Rhizomucor miehei lipase by mutating N-linked glycosylation site and its application in biodiesel production. *Fuel* **2021**, *304*, 121514. [CrossRef]
200. Svendsen, A. Lipase Protein Engineering. *Biochim. Biophys. Acta (BBA) Protein Struct. Mol. Enzymol.* **2000**, *1543*, 223–238. [CrossRef]
201. Lan, D.; Zhao, G.; Holzmann, N.; Yuan, S.; Wang, J.; Wang, Y. Structure-Guided Rational Design of a Mono- And Diacylglycerol Lipase from *Aspergillus oryzae*: A Single Residue Mutant Increases the Hydrolysis Ability. *J. Agric. Food Chem.* **2021**, *69*, 5344–5352. [CrossRef] [PubMed]
202. Rapp, J.T.; Bremer, B.J.; Romero, P.A. Self-driving laboratories to autonomously navigate the protein fitness landscape. *Nat. Chem. Eng.* **2024**, *1*, 97–107. [CrossRef]
203. Xue, D.; Yao, D.; Sukumaran, R.K.; You, X.; Wei, Z.; Gong, C. Tandem integration of aerobic fungal cellulase production, lignocellulose substrate saccharification and anaerobic ethanol fermentation by a modified gas lift bioreactor. *Bioresour. Technol.* **2020**, *302*, 122902. [CrossRef]
204. Tiatira, C.; Hamzah, A.; Sangian, H.F.; Widjaja, A. The effect of surfactant on the hydrolysis of coconut husk using cellulase and xylanase enzyme immobilized on chitosan magnetic nanoparticles. *IOP Conf. Ser. Earth Environ. Sci.* **2022**, *963*, 012020. [CrossRef]
205. Jiang, W.; Pei, R.; Zhou, S.F. 3D-printed xylanase within biocompatible polymers as excellent catalyst for lignocellulose degradation. *Chem. Eng. J.* **2020**, *400*, 125920. [CrossRef]

206. Salem, K.; Jabalera, Y.; Puentes-Pardo, J.D.; Vilchez-Garcia, J.; Sayari, A.; Hmida-Sayari, A.; Jimenez-Lopez, C.; Perduca, M. Enzyme Storage and Recycling: Nanoassemblies of α-Amylase and Xylanase Immobilized on Biomimetic Magnetic Nanoparticles. *ACS Sustain. Chem. Eng.* **2021**, *9*, 4054–4063. [CrossRef] [PubMed]
207. Hu, Y.; Zhou, H.; Dai, L.; Liu, D.; Al-Zuhair, S.; Du, W. Lipase Immobilization on Macroporous ZIF-8 for Enhanced Enzymatic Biodiesel Production. *ACS Omega.* **2021**, *6*, 2143–2148. [CrossRef] [PubMed]
208. Xia, S.; Shen, C.; Lin, J.; Tu, M.; Tan, C.P.; Cheong, L.Z. Enhanced methanol tolerance of ZIF-8-*immobilized Aspergillus oryzae* lipase for biodiesel production from used cooking oil. *Renew. Energy* **2025**, *239*, 122122. [CrossRef]
209. Lozowski, D. Gevo and Beta Renewables sign JDA to develop process for cellulosic isobutanol. *Chem. Eng.* **2012**, *119*, 63–64. Available online: https://go.gale.com/ps/i.do?p=AONE&sw=w&issn=00092460&v=2.1&it=r&id=GALE%7CA301086995&sid=googleScholar&linkaccess=fulltext (accessed on 15 January 2025).
210. Neto, A.C.; Guimarães, M.J.O.C.; Freire, E. Business models for commercial scale second-generation bioethanol production. *J. Clean. Prod.* **2018**, *184*, 168–178. [CrossRef]
211. Dos Santos, L.V.; De Barros Grassi, M.C.; Gallardo, J.C.M.; Pirolla, R.A.S.; Calderón, L.L.; De Carvalho-Netto, O.V.; Parreiras, L.S.; Oliveira, C.E.L.; Drezza, A.L.; Missawa, S.K.; et al. Second-Generation Ethanol: The Need is Becoming a Reality. *Ind. Biotechnol.* **2016**, *12*, 40–57. Available online: https://www.liebertpub.com/doi/10.1089/ind.2015.0017 (accessed on 15 January 2025). [CrossRef]
212. Gray, D.; Sato, S.; Garcia, F.; Eppler, R.; Cherry, J. *Integrated Biorefinery Project Summary Final Report: Public Version*; Amyris, Inc.: Emeryville, CA, USA, 2009. Available online: https://www.osti.gov/servlets/purl/1122942/ (accessed on 15 January 2025).
213. Yang, J.; Zhang, X. Preparing Magnetic Nano-Particle Carrier Immobilized Lipase Useful for Preparing Biodiesel Comprises Preparing Iron (II, III) Oxide Magnetic Nano Particles, Dissolving Chitosan, Forming Uniform Colloidal Solution, Adding Cross-Linking Agent, Separating Solid-Liquid and Washing-Derwent Innovations Index Shanghai Zhongqi Environment Technology (SHAN-Non-Standard); CN114990101(A). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2022C1154N (accessed on 6 December 2024).
214. Xu, G.; Zhao, S.; Zhao, Q.; Zhou, J.; Liu, Y. New Lipase Mutant Useful for Synthesizing Biodiesel Using Vegetable Oil and Fat as Substrate-Derwent Innovations Index Hunan Flag Biotechnology Co., Ltd., (HUNA-Non-Standard); CN113943721(A). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202219254F (accessed on 6 December 2024).
215. Yang, T.J.; Shim, J.; Choi, E.J.; Son, B.; Park, J.Y.; Yangtaejoo; Park, C.; Sun, B.; Cui, Y.; Yang, T. New Variant Polypeptide Having Xylanase Activity, Comprising Mature Polypeptide Coding Sequence, Its Complementary DNA, or Polypeptide Encoded by Polynucleotide that Hybridizes Full-Length Complement Sequence Under Stringency Conditions, Used for Preparing Xylooligosaccharides-Derwent Innovations Index CJ Cheiljedang Corp(CJCJ-C); Cheil Jedang Corp(CJCJ-C); WO2022131798(A1). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2022816394 (accessed on 7 December 2024).
216. Singh, A.; Pandey, A.K. Producing Bioethanol Form Lignocellulosic Biomass Comprises Growing Fungal Strains on Bran, Extracting Crude Secretome Using Sodium Citrate Buffer, Determining Cellulase Activity, Pre-Treating Sugarcane Bagasse Followed by Incubating Biomass Suspension to Hydrolyze Bagasse to Produce Bioethanol-Derwent Innovations Index Univ chhatrapati Shahu Ji Maharaj(UYCH-Non-Standard); IN202411071589(A). 2024. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2024B1209F (accessed on 7 December 2024).
217. Jeon, B.H.; Soo, H.K.; Kurade, M.; Wook, K.K. Producing Biofuel from Microalgae by Performing Pretreatment by Irradiating Microalgal Biomass with Microwaves, Fermenting Pre-Treated Microalgal Biomass with Microorganisms to Produce bioethanol, and Fermenting Bioethanol with Yeast to Produce Higher Alcohol-Derwent Innovations Index Univ Hanyang Iucf-Hyu(UHYG-C); KR2022032368(A). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202241361C (accessed on 9 December 2024).
218. Kuila, A.; Pant, S.; Ritika; Sharma, S. Performing Simultaneous Hydrolysis and Fermentation of Biomass Useful for Obtaining Bioethanol, by Thermo-Chemically Treating Biomass and Placing Pretreated Biomass in Minimal Medium Comprising Inoculum Containing Saccharomyces Cerevisiae-Derwent Innovations Index Univ Banasthali Vidyapith (UYBA-Non-Standard); IN202011051947(A). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2022D46048 (accessed on 9 December 2024).
219. Dubey, S. Producing Biofuels Bio-Ethanol and Biogas from Water Hyacinth in Chhattisgarh Involves Pre-Treating Water Hyacinth with Weaken Sulfuric Corrosive at High Temperature and Tension, So That Reproduction and Monetary Appraisal of Cycle for Additional Enzymatic Saccharification is Contemplated-Derwent Innovations Index DUBEY S; IN202321006069(A). 2023. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202358603R (accessed on 9 December 2024).

220. Marathe, A.B.; Deshmukh, M.P. Synthesizing Blending Fuel Involves Screening Cake, Performing Pretreatment of Jatropha Seed Cakes with Thionyl Chloride, where Complex Structure of Cellulosic Material of Pretreated Cake is Broken by Enzyme Hydrolysis in Presence of Tricoderma, Conducting Fermentation Using Saccharomyces Cerevisiae-Derwent Innovations Index MARATHE A B; Deshmukh M P; IN202321015414(A). 2024. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2024A0520F (accessed on 9 December 2024).
221. Peinado Cebrian, C.; Rojas Munoz, S.; Campos Martin, J.M.; Coll Lozano, C.; Latorre Sanchez, M.; Dura Alvarez, A. Obtaining Bioethylene from Organic Fraction of Municipal Solid Waste Used in Chemical Industry by e.g. Mixing Solid Waste with Inorganic Acid, Adding Enzymatic Cocktail i.e. Cellulase and Amylase and Contacting Bioethanol with Acid Catalyst-Derwent Innovations Index Consejo Superior Investigaciones Cientif (CNSJ-C) Perseo Biotechnology SL (PERS-Non-Standard); WO2024023384(A1). 2024. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:20241310924 (accessed on 9 December 2024).
222. Zhouhou, W.; Zhang, J.; Gong, Z. Producing Oil by Open-Type Fermentation of High-Sugar High-Inhibitor Biomass Hydrolysis Liquid by Subjecting Oil Producing Yeast to Laboratory Directed Evolution for Improving Tolerance of Strain to Biomass Based Inhibitor, Growing and Fermenting Strain in Biomass Hydrolysis Liquid with Inhibitor-Derwent Innovations Index Univ Wuhan Sci & Technology (UWSC-C); CN116479064(A). 2023. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202379299S (accessed on 9 December 2024).
223. Gao, Q.; Wang, H.; Hu, N.; Wang, Y.; Wang, D.; Xue, X. New Recombinant Plasmid Being Plasmid pBT2-SPP-Lipase-Cellulose-Binding Domain-XM Useful for Preparing Biodiesel-Derwent Innovations Index Univ Tianjin Sci & Technology(UYTC-C); CN116396975(A). 2023. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202374888D (accessed on 9 December 2024).
224. Wang, X.; Sun, H.; Liu, X.; Tang, B.; Shan, K. New Immobilized Composite Lipase Obtained by Performing Adsorption and Immobilization of Thermomyces Lanuginosus Lipase, Candida Antarctica B Lipase and Candida Rugosa Lipase with Diatomaceous Earth as Carrier, Embedding and Immobilizing with Sodium Alginate as Carrier, Used for Preparing Biodiesel-Derwent Innovations Index Wuxi Weilan Biotechnology Co., Ltd., (WUXI-Non-Standard); CN117866922(A). 2024. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202440845T (accessed on 9 December 2024).
225. Meerabai, R.S.; Sumathi, R.; Krishnaveni, C. Converting Pongamia Oil into Biodiesel Involves Carrying Out Esterificationof Oil and its Transesterification, where Esterification Process Involves Heating Oil and Adding Methanol-Derwent Innovations Index SUMATHI R (SUMA-Individual) Krishnaveni C (KRIS-Individual); IN201941028903(A). 2021. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202111881G (accessed on 9 December 2024).
226. Liu, D.; Chen, Z. New Recombinant Expression Vector pEC-Cg1554-CALB for Corynebacterium Glutamicum, Useful in the Fermentation Production of Recombinant lipase-Derwent Innovations Index Univ Tsinghua(UYQI-C) Univ Dongguan Shenzhen Tsinghua Res Inst(UYDO-Non-Standard); CN117384935(A). 2024. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2024086409 (accessed on 9 December 2024).
227. Zhang, J.; Yhang, L.; Meng, Y.; Guo, J.; Niu, Y.; Hu, Y. Preparing Composite Catalyst by Preparing Lipase Solution, Adding Glutaric Dialdehyde, Oscillating, Adding Strong Acid Ion Exchange Resin to Lipase, Suction Filtering, Washing Resin, Obtaining Lipase Loaded Strong acid Ion Exchange Resin; and Pre-Freezing-Derwent Innovations Index Shaanxi Heathful Biological Eng Co., Ltd., (SHAA-Non-Standard); CN114657218(A). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:2022868559 (accessed on 9 December 2024).
228. Wang, J.; Ge, M.; Guo, X.; Shao, Y.; Wang, Q. Biocatalyst Using Two-Dimensional Polyamide Immobilized Lipase for Catalyzing Soybean Oil to Prepare Biodiesel, is Obtained by Preparing Two-Dimensional Polyamide Material Using Trimesoyl Chloride and Biphenylenediamine-Derwent Innovations Index Univ Yangzhou (UYYZ-C); CN112980828(A). 2022. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202172275F (accessed on 9 December 2024).
229. Zhang, H.; Chang, J. Producing Biodiesel Comprises Mixing Grease, Acyl Donor and Lipase Catalyst, Reacting, Preparing Transition Metal Catalyst Adding Acyl Donor and the Transition Metal Catalyst, Mixing and Reacting Continuously to Obtain Biodiesel-Derwent Innovations Index Univ Hefei Technology (UYHE-C); CN118064512(A). 2024. Available online: https://www-webofscience-com.ez22.periodicos.capes.gov.br/wos/diidw/full-record/DIIDW:202456062C (accessed on 9 December 2024).