Review

Microbial Enzyme Systems in the Production of Second Generation Bioethanol

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Abstract: The primary contributor to global warming has been the careless usage of fossil fuels. Urbanization's threat to the depletion of these resources has made it necessary to find alternatives due to the rising demand. Four different forms of biofuels are now available and constitute a possible replacement for fossil fuels. The first generation of biofuels is generated from the edible portion of biomass, the second generation is made from the non-edible portion of biomass, the third generation is made from algal biomass, and the fourth generation is made using molecular biology to improve the algal strain. Second generation biofuels are extremely important because they are derived from non-edible biomass, such as agricultural and agro-industrial wastes rich in cellulose, hemicellulose, pectin, and starch impregnated with lignin, and are hydrolyzed after delignification by physio-chemical or biological pretreatments using ligninases. There has been a lot of research on their production using chemical and enzymatic hydrolysis, but it has not been economically viable in comparison to first generation biofuels due to the formation of several inhibitors with chemical hydrolysis and the high cost of the enzymes. Furthermore, the need for multiple enzymes due to the various types of carbohydrates in the feedstocks makes the enzymatic process too expensive. This article examines the enzymes involved in the hydrolysis of feedstocks for the production of second generation bioethanol, a highly acceptable biofuel.

Keywords: Biofuels; Bioethanol; Lignocellulose; Cellulases; Amylases; Hemicellulases; Laccase

1. Introduction

Society's advancement has raised the standard of living and made jobs easier, but it has also resulted in environmental issues as a result of excessive use of automobiles, machines, and other items, which has contributed to the depletion of fossil fuel sources. Urban areas house 52.5% of the world's population, with that figure expected to rise to 70% by 2050 [1]. This urbanisation is causing an excess of fossil fuel use in the transportation sector. Cities contribute significantly to CO_2 emissions [2], and the indiscriminate use of fossil fuels has put their reserves at risk. This has prompted researchers all over the world to focus on environmentally friendly alternatives to fossil fuels. Biofuels are one type of such fuel that emits fewer GHGs over their entire life cycle [3]. A biofuel is any fuel that is made from plant biomass and can generate energy for use in a variety of ways [4]. Solid, liquid, or gaseous biofuels are all possible. Wood,

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refuse-derived fuel (RDF), are some of the examples of solid biofuels. Biodiesel, biomethanol, bioethanol, biobutanol etc are the examples of liquid biofuels, while biohydrogen and biomethane are examples of gaseous biofuels. Given the increasing global biofuel consumption trend, major research attention has been directed toward feasible and low-cost biofuel resources, as reported by research publications in the last 20 years around the world, particularly in Asia, Europe, and the United States [5].

According to the proposed sustainable development scenario, biofuels must meet 9% of total transportation fuel demand by 2030, up from 3% in 2018. Biofuel production is not increasing at a rate sufficient to meet this demand, and it grew 6% year on year in 2019, with an average of 3% growth expected over the next five years, leaving total production short of 10% by 2030 to meet the pace required for sustainable development. Food crops account for the majority of biofuels produced. For better sustainability, advanced biofuel production using non-food feedstocks must improve and gain a significant share of total biofuel production. Scaling up the production of these biofuels to a commercial level will require a great deal of effort and innovative research. Bioethanol and biomass-to-liquid synthetic fuels are among the most important advanced biofuels because they can be produced using low-cost, abundantly available feedstocks such as forest waste, municipal solid waste, and agricultural and agro-industrial residues [6-7]. It appears that biologically mediated lignocellulosic biomass conversion is more promising. The primary goal of this article is to review the green approaches used in the production of biofuels, with a focus on the enzymes used in the production of second generation biofuels.

2. Classification of biofuels

Primary and secondary biofuels are the two types of biofuels. Primary biofuels include plants, firewood, animal waste, crop residue, and forest waste. Secondary biofuels are created through processes and technologies that use plants and microorganisms. Secondary biofuels are further classified as conventional or advanced biofuels. Conventional biofuels are made from edible feedstocks such as sugar beets, soybean oil, palm oil, sugarcane, rice, wheat, and so on. These are referred to as first generation biofuels. Advanced biofuels are made from the non-edible parts of plants and are classified into three types including second, third, and fourth generation biofuels, based on the type of substrate used in the production process. Second-generation biofuels make use of waste biomass resources such as agricultural, agro-industrial, municipal solid waste, and forest residue, among others. Third-generation biofuels are primarily made from algal biomass [8, which can be used to make a variety of biofuels and other value-added products]. The fourth generation of biofuels is a newer type that uses synthetic biology tools to produce electro fuels and photobiological solar fuels through direct conversion of solar energy into fuels [9, 10]. Figure 1 depicts a pictorial representation of biofuel classification. The comparison of different generations of biofuels reveals that second-generation biofuels are emerging as the most potent type of biofuel owing to its numerous advantages over the others, and thus the current focus of researchers is to update and improve the already available technologies for the production of fuels from waste biomass.

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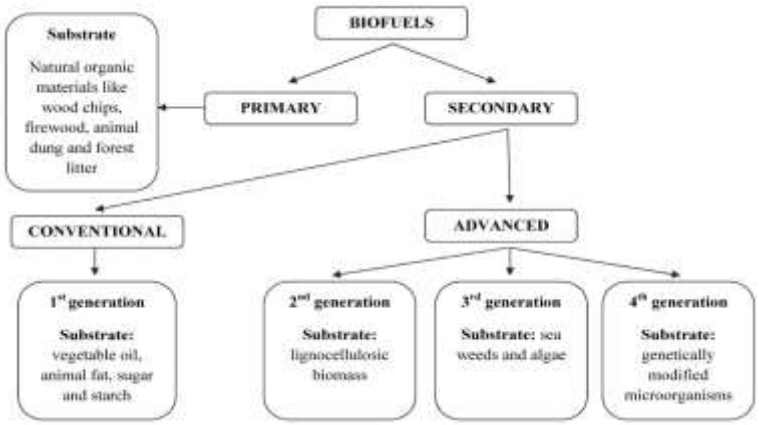

Figure 1: Classification of Biofuels.

A highly acceptable liquid fuel that burns cleaner than gasoline and is simpler to prepare among the various biofuels is ethanol. It is created when the yeast Saccharomyces cerevisiae ferments sugars as part of its oxygen-free metabolism. In many parts of the world, it is used as a legal gasoline blend. The first generation, which is produced using food crops, accounts for the majority of the bioethanol currently produced. Examples include the United States, which makes ethanol from corn, Brazil, which makes ethanol from sugarcane, and Europe, which makes ethanol from wheat and barley [11,12]. Recent concerns about the production of first-generation biofuel brought about by the conflict between food and fuel have prompted field experts to look into alternate paths for biofuel production [13]. Numerous reports attribute the rise in the cost of food ingredients to the production of first-generation bioethanol [14]. The use of waste and inedible agricultural biomass as a substrate for fuel generation is the main justification for choosing second-generation biofuels over first-generation biofuels. Given its abundance and underutilization compared to other natural resources, lignocellulosic biomass is a promising feedstock for the production of fuel [15]. Waste biomass (municipal solid waste, food waste, etc.), agricultural and agro-industrial byproducts (straws, brans, sugarcane bagasse, etc.), and purposefully grown feedstocks are examples of lignocellulosic feedstocks (vegetative grass and other energy crops). The production of second-generation biofuels has the advantage of cheap, readily available raw materials, but these substrates need sophisticated processing procedures to turn them into fuel. The focus has shifted to the production of second-generation biofuels because the feedstock is easily accessible and it has a less significant impact on the food web, water resources, and ecosystem due to the extensive food versus fuel debate associated with first-generation biofuels [16, 17]. The current methods for making second-generation bioethanol are neither cost-effective nor environmentally friendly [12]. Therefore, the entire production process needs to be improved in order for it to be environmentally friendly and for the cost of the fuel produced to be competitive with other fuels already on the market [18,19].

3. Composition of agricultural and agro-industrial waste biomass, the feedstocks for second generation bioethanol

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The majority of plant waste biomass, including cellulose, hemicellulose, and phenolic polymers like lignin, is typically made up of carbohydrates. Other ingredients in varying amounts include starch, pectin, proteins, acids, salts, and minerals [17]. Figure 2 provides a visual representation of the general composition of plant waste biomass, and Table 1 describes the composition of lignocellulosic biomass.

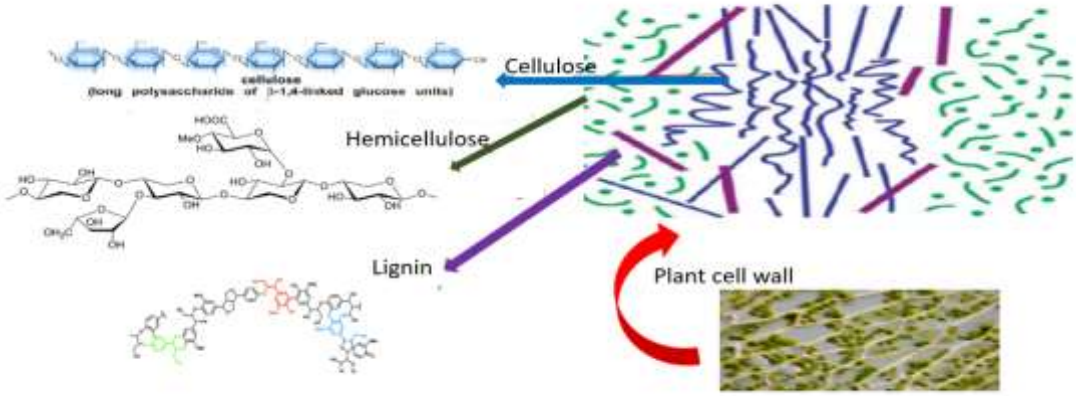

Figure 2: General structure of lignocellulosic biomass [18].

Table 1: Lignocellulosic composition of various agricultural wastes [19].

Substrate	Cellulose	Hemicellulose	Lignin
Rice straw	32-47	19-27	5-24
Rice husk	34.40	29.30	19.20
Wheat straw	35-45	20-30	8-15
Corn straw	42.60	21.30	8.20
Corn cobs	45.00	35.00	15.00
Corn stover	38.00	26.00	19.00
Wheat bran	25.30	14.60	3.20
Sugarcane bagasse	42.00	25-36	19-20
Sweet sorghum	45.00	27.00	21.00
Apple pulp	16.00	16.00	21.00
Coconut shell	14-15	32-35	46-50
Coconut husk	0.52	23.70	3.54
Cocoa pods	41.92	35.26	0.95
Soft wood	36.00	18.50	30.50
Banana empty fruit bunch	8.30	21.23	19.06
Almond shell	32.50	25.50	24.80
Walnut shell	40.10	20.70	18.20
Grasses	25-40	35-50	10-30
Olive stone	14-31	15-17	32-42
Deoiled rice bran	9.80	20.60	3.90

3.1. Cellulose ($C_6H_{10}O_5)_n$

One of the major constituent of plant cell wall which is abundantly available on earth is cellulose that exists as a fibrous structure. It is an unbranched long-chain polymer consisting of several repeated units of cellobiose (Figure 3) which are linked to each other by β-1,4-glycosidic

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bonds [20]. These long chains of cellulose are linked together by Van der Waals and hydrogen bonds packing the cellulose into microfibrils which further bundle together to build cellulose fibers. The straightness of the chain is determined by the hydrogen bonds within these microfibrils. The crystalline and amorphous structures within the cellulose are introduced by interchain hydrogen bonding which imparts order or disorder to the cellulose structure [17].

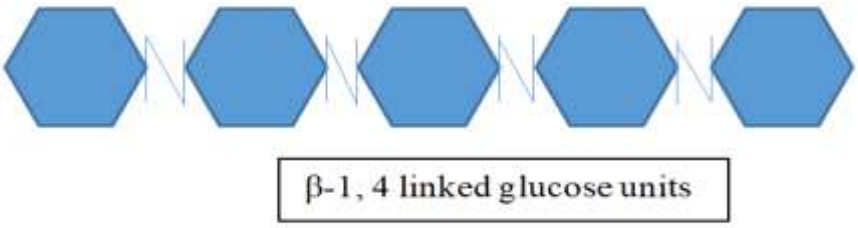

Figure 3: Structure of cellulose.

3.2. Hemicellulose $(C_5H_8O_4)_n$

Hemicellulose is the second notable and prevalent polymer in plant waste. Being chemically heterogeneous sets it apart from cellulose. These pentoses (xylose, rhamnose, and arabinose), hexoses (glucose, mannose, and galactose), and uronic acids (4-o-methyl-glucuronic, D-glucuronic acids) are branching, heterogeneous polymers [20, 21]. In various materials, hemicelluloses have varying proportions. For instance, conifers and hardwoods have widely different proportions and types of xylans and mannan. In conifers, galactoglucomananas (5-8%), arabinoglucouronoxilanes (7-15%), and glucomannan (10-15%) are the primary components, whereas glucomannans (2-5%) and glycoronoxilanes (15-35%) predominate in hardwoods. The primary hemicellulosic components of grass and cereal cell walls are arabinoxylans [17]. The general structure of hemicellulose is depicted in Figure 4.

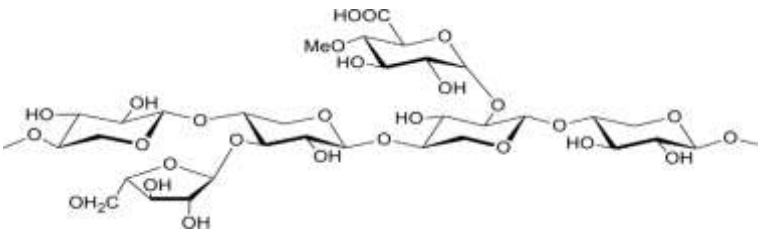

Figure 4: Representative structure for hemicellulose (Xylan type).

3.3. Lignin

Lignin is the third polymer that is widely distributed in nature (Figure 5). This polymer, which is found in plant cell walls, gives the cell wall of the plant a strong defence against any microbial invasion. The major three forms of phenyl propane units found in lignin are guaiacyl propanol also known as coniferyl alcohol, syringyl alcohols also known as sinapyl alcohol, and p-hydroxyphenyl propanol also known as coumaryl alcohol [21]. Lignin is primarily viewed of as the glue that binds the various parts of lignocellulosic biomass together, making it water-insoluble. It is

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extremely challenging to hydrolyze the biomass using enzymatic or microbiological processes because of how tightly lignin is bound to the cellulose structure [17,22].

Figure 5: Structure of lignin [22].

3.4. Pectin

Only a limited amount of pectin can be found in plant cell walls. Pectins are heteropolysaccharides comprised of 1,4-linked units of α-D-galactosyluronic acid residues. Rhamnogalacturonan-I, homogalacturonan, and substituted galacturonans are the three main pectins that have been identified from plant cell walls [23]. The general structure of pectin is shown in Figure 6.

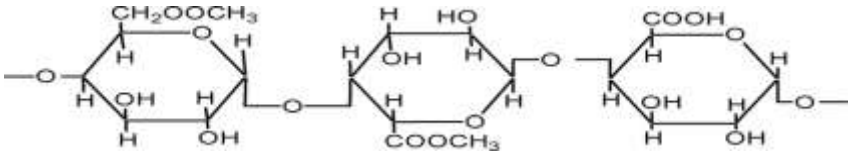

Figure 6: Structure of pectin [23].

3.5. Starch

Glucose units in starch are connected by glycosidic linkages. Figure 7 shows the architecture of the two types of polymeric units that make it up: amylose and amylopectin. By α-1,4 glycosidic linkages, amylose is made up of linearly linked glucose units. Amylopectin is made up of linear glucose chains with a α-1,4 linkage that are joined to the side chains by α-1,6 linkage [24].

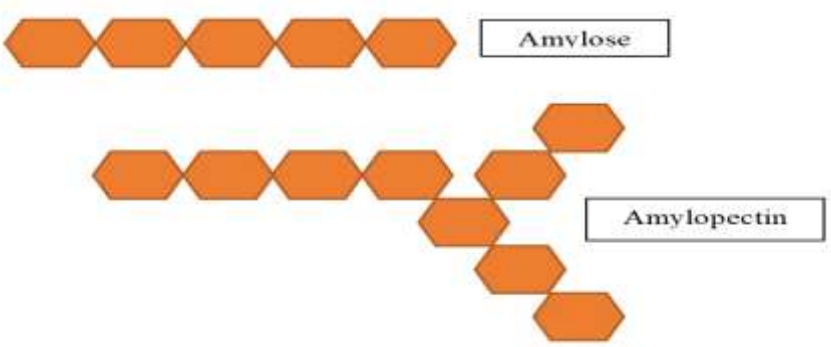

Figure 7: Structure of starch [25].

4. Conversion of agricultural and agro-industrial residues into bioethanol

The development of a biorefinery for the production of numerous value-added products, including second-generation biofuels from plant

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biomass waste, has been the subject of extensive research. Effective cellulose utilisation is crucial for making use of lignocellulosic biomass because it produces sugars that can be fermented further. But because lignin acts as a significant barrier to the existing carbohydrates, pretreatment is an extremely important step in the processing of biomass in order to disrupt lignin and hemicellulose for efficient hydrolysis of cellulose in the following stage. Pretreatment, enzymatic hydrolysis, and fermentation are the three primary processes in the bioconversion of lignocellulosic biomass into bioethanol [25-27].

4.1. Pretreatment

The fundamental obstacle to the generation of biofuels seems to be the resistant and crystalline structure of plant biomass [28]. Enzyme interaction with cellulose is necessary for enzymatic hydrolysis, however cellulose's crystalline structure makes enzymatic attacks difficult. The lignin and hemicellulose matrices are another obstacle because they operate as physical barriers that reduce the accessibility of activated cellulose to enzymes. Additionally, lignin reduces the effectiveness of enzymes by binding cellulase [29]. Therefore, a pretreatment technique is needed to soften the crystalline structure of plant biomass before enzymatic hydrolysis [30]. The pretreatment process alters the structure and composition of the biomass and increases the surface area of the cellulose, making it more porous and more accessible for enzymatic hydrolysis [31-33].

4.1.1. Goal of pretreatment

Different pretreatment techniques have been proposed and put into practise for the maximum release of fermentable sugars from lignocellulosic biomass in order to improve the enzymatic hydrolysis of biomass and fermentation yields [34, 35].

Pretreatment must have the following qualities to be effective: i) it must be economical and environmentally friendly, ii) the most lignin can be eliminated, iii) minimum production of phenols, furals, and furfurals, which prevent fermentation, iv) recovering lignin to create other products with extra value, v) minimal energy required, vi) pretreatment chemicals must be recovered for future use, vii) minimum costs of operation and minimum labour needs.

4.1.2. Factors affecting the choice of pretreatment

Several considerations need to be taken into account when choosing a pretreatment method for a certain feedstock. These variables primarily comprise the biomass's total hemicellulose and lignin contents, cellulose's degree of crystallisation, polymerization, and permeability [36-38].

4.1.3. Types of pretreatments

Different types of pretreatment technologies have been studied so far and basically, four types of strategeis have been categorized including i) mechanical or physical involving mechanical milling and exposure to high temperature using steam ii) chemical/thermochemical involving the use of acids, bases, oxidisng agents or ionic liquids alone or in combination with steam and are energy intensive iii) physicochemical involving steam, CO_2 or ammonia explosion which are also energy intensive, iv) biological involving the microorganisms or microbial enzyme systems for disrupting lignin and hemicellulose. Since this manuscript

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deals with the enzyme systems for the production of second generation bioduels so the biological prereatment is more relevant here and is thus discussed hereafter.

4.1.3.1. Biological pretreatment

Biological pretreatment uses less energy and is less harmful to the environment than chemical and physical procedures. Natural diversity include a variety of cellulolytic and hemicellulolytic microorganisms that can be used for the pretreatment of biomass [38]. Because they destroy lignin and hemicellulose with only a small amount of cellulose, a variety of white, brown, and soft rot fungus have been employed for biological pretreatment [39]. White-rot fungi degrade lignin due to the presence of lignin-degrading enzymes including peroxidases and laccases. With the aid of mediators, laccase can directly target the nonphenolic and phenolic subunits of lignocellulosic biomass, causing structural changes [40].

Some of the white-rot fungal species that have been investigated for the biological pretreatment of biomass include *Pycnoporus cinnarbarinus.Phanerochaete chrysosporium, Cyathus stercolerus, Ceriporia lacerata, Ceriporiopsis subvermispora, Pleurotus ostreaus.* Other basidiomycetes used for biological pretreatment include *Fomes fomentarius, Ganoderma resinaceum, Lepista nuda, Irpex lacteus, Trametes versicolor* and *Pycnoporus sanguineus* [41-46]. The biological pretreatment of the biomass can be accomplished in three different ways, which include the use of enzymes, a consortium of microorganisms, or fungi that can degrade lignin [47]. Ma and Ruan [48] explored simultaneous delignification and hydrolysis of corn stover by co-culturing *Coprinus comatus* and *Trichoderma reesei*. A range of white-rot fungus were investigated in a study to discover the optimum biological pretreatment for corn stover, and *Cyathus stercoreus* NRRL-6573 produced the highest carbohydrate conversion [44]. Although biological pretreatment has advantages, it is not favoured on an industrial scale because it is too sluggish [49]. Therefore, for biological pretreatment to be applied at the industrial level, it is necessary to discover more fungi that can delignify biomass but at faster rates. Rastogi et al. [50] observed that *Pyrenophora phaeocomes* S-1 cultivation on rice straw led to 63 and 51% lignin and hemicellulose breakdown, respectively. Further extraction of these components using a mild alkali revealed that the overall losses for lignin and hemicellulose were 78 and 60%, respectively. An increase in hydrolytic efficiency was seen in a study by Yan et al. [51] by using the *Cupriavidus basilensis* B-8 strain of bacteria in conjunction with diluted acid pretreatment. By forming pores in the biomass and removing the lignin droplets created by the acid treatment, the bacteria increased the surface area available for enzymatic action.

4.2. Hydrolysis to release free sugars for fermentation into ethanol

Pretreatment is followed by hydrolysis of the pretreated substrate to saccharify it leading to the release of monomeric sugars. Hydrolysis can be done by the acid or enzymatic treatments.

4.2.1. Acid hydrolysis

Since a remarkably long time, diverse substrates have been hydrolyzed using acid. The two most frequently used acids are H_2SO_4 and HCl, which can be utilised in both diluted and concentrated forms and at varied concentrations. The dilute acid hydrolysis involves two processes.

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The first step in the process is the saccharification of carbohydrates, and if the reaction persists, sugars will then be converted to furfurals. Because cellulose breaks down more slowly than hemicellulose, a two-stage process is necessary to prevent the formation of furfurals from the sugars released from hemicellulose. The first stage of the process recovers the sugars from the hemicellulose under mild conditions, and the stage two recovers the sugars from the cellulose under harsher conditions. The effective enzyme from *Penicillium* consortium and acid hydrolysis of poplar were also compared by Liang et al. [52], who came to the conclusion that the sugar yield from enzymatic hydrolysis is superior.

4.2.2. Enzymatic hydrolysis

Since it doesn't result in the production of inhibitors, enzymatic hydrolysis of the pretreated substrate is preferred to acid hydrolysis. Furthermore, the enzymes contain no secondary reactions and work in a highly precise manner. By pretreating the substrate, cellulose and hemicellulose's crystalline structure is broken down, allowing the enzymes to attack them and liberate sugars (Figure 8). Cellulases and hemicellulases are needed to break down cellulose and hemicellulose, which are the two main carbohydrates found in the cell wall structure [53]. The pretreated substrate must also include starch and pectin in order for amylases and pectinases, the corresponding enzymes, to fully saccharify the substrate.

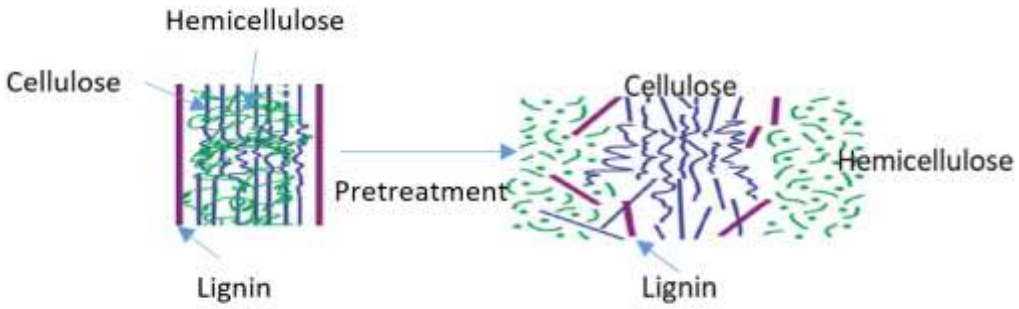

Figure 8: Schematic representation of pretreatment of lignocellulosic residue.

Enzymatic hydrolysis has a number of benefits, such as high specificity, a higher sugar yield, milder reaction conditions, and a reduced formation of undesirable products [54]. Additionally, enzymatic saccharification offers a more cost-effective, environmentally friendly method for releasing sugars from lignocellulosic biomass.

4.2.2.1. Microbial enzymes involved in the hydrolysis of feedstocks for production of second generation bioethanol

Rice straw, wheat straw, corn stover, corn cobs, barley straw, sugarcane bagasse, rice husk, switchgrass, cotton stalks, and poplar biomass, to name a few, all contain between 30 and 48 % cellulose, 15 to 30 % hemicellulose, and 10 to 20 % lignin [55]. Cellulases and hemicellulases are therefore crucial for the effective saccharification of these residues and the creation of free sugars from them. However, in addition to cellulose, hemicellulose, and lignin, other agro-industrial residues such as wheat

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bran, fruit peels, vegetable waste, rice bran, deoiled rice bran, maize bran, apple pomace, and tomato pomace also contain starch and pectin [56-58]. As a result, amylases and pectinases are necessary for the effective hydrolysis of these biomass residues.

4.2.2.1.1. Cellulases

The majority of the time, lignocellulosic biomass requires a combination of numerous enzymes, the most crucial of which are cellulases. Cellulases are classified structurally as glycosyl hydrolases, which hydrolyze cellulose's β-1,4-D-glucan connections to create cellobiose and glucose [59]. To completely dissolve the cellulose framework, three enzymes must act together as depicted in Figure 9 and the role of various enzymes is as follows:

Endoglucanase or Endo-β-1,4-glucanase (EC 3.2.1.4): It makes short-chain oligomers containing non-reducing and reducing tails by randomly cutting the amorphous area of cellulose.

Cellobiohydrolase or Exo-β-1,4-glucanase (EC 3.2.1.91): Endoglucanase's catalytic activity produces non-reducing endings that are hydrolyzed to produce cellobiose, a repetitive unit containing two glucose molecules.

Cellobiase or β-glucosidase (BG) (EC 3.2.1.21): To generate monomeric glucose units, it hydrolyzes cellobiose units.

Cellulose is the primary growth medium needed by the microbes that make cellulases, while they can also use other carbohydrates. Cellulase producing microorganisms include fungi like *Aspergillus Flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus oryzae, Fusarium oxysporum, Trichoderma viride* [60-65].

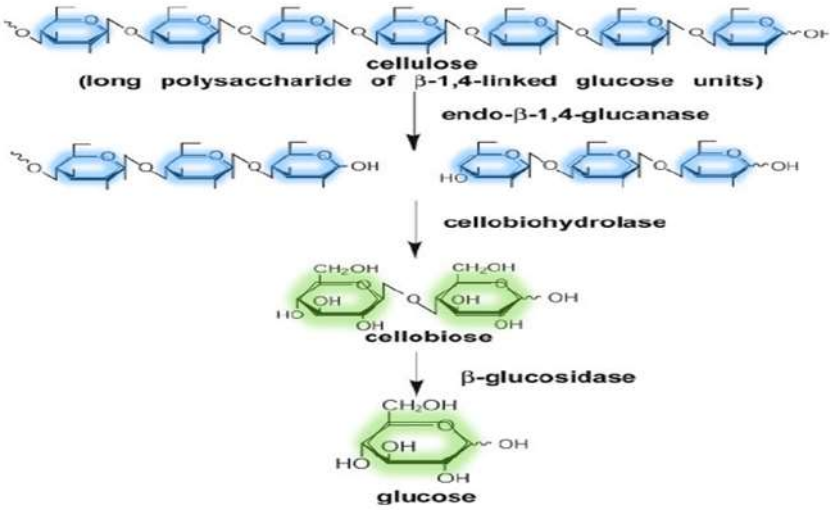

Figure 9: Mode of action of enzymes involved in the breakdown of cellulose.

4.2.2.1.2. Hemicellulases

The second most abundant polymer in nature is hemicellulose that comprises of xylan, mannan, arabinan and galactan. It is soluble in aqueous alkali but not in water or any chelating agent [66]. The enzyme market for hemicellulases is expanding quickly because these enzymes are used

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in a variety of industrial processes. The second-most prevalent carbohydrate in lignocellulosic is called xylan, which is a hetero-polysaccharide made up of 1,4-β-D-xylose monomers with different substituents [67]. Figure 10 shows the several hydrolytic enzymes needed for the complete breakdown of xylan, with xylanase being the most crucial of these. When xylan is hydrolyzed by xylanase, oligosaccharides are produced, which are then hydrolyzed by 1,4-β-xylosidase to produce xylose [68]. For complete hydrolysis of xylans, other enzymes such as ferulic and p-coumaric esterases, xylan esterases, *α*-4-O-methyl glucoronosidases, and *α*-1-arabinofuranosidases work in concert [39].

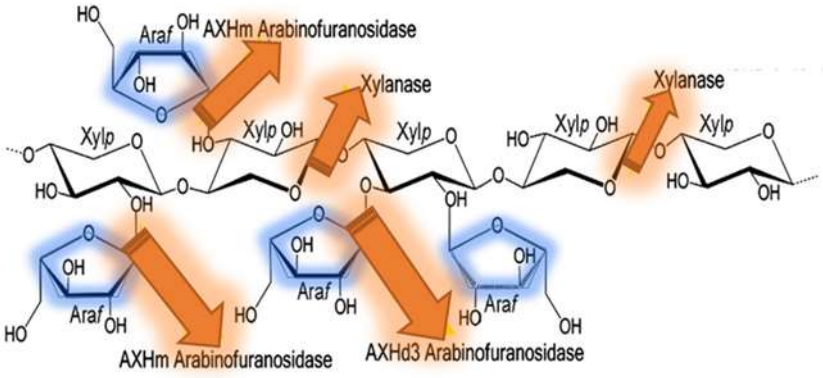

Figure 10: Schematic representation of linkages in arabinoxylan and the enzymes hydrolysing the polysaccharide.

In addition to xylanase, mannans and heteromannanas are additional polysaccharides that are found in the hemicellulose of plant cell walls. D-mannose, a six-carbon sugar, makes up the majority of mannan, but because plant mannans have a complex and heterogeneous structure, it takes a combination of endo-1,4-β-mannanases, exo-mannosidases, and other enzymes to completely break them down [69]. These enzymes can also remove the side chain sugars that are present at various locations on mannans. Following of enzymes are involved in the hydrolysis of different hemicellulosic structures. Xylan degradation is carried out by three different types of xylanases [70].

Endo-β-1,4-xylanase (EC 3.2.1.8): By hydrolyzing glycosidic linkages to release linear and branching oligosaccharides, it randomly splits the xylan chain.

Exo-β-1,4-xylanase or β -1,4-xylan xylohydrolase: It eliminates monomeric xylose units from the xylan polymer's non-reducing terminus.

β-1,4-xylosidase or Xylobiase. (EC 3.2.1.37): This enzyme hydrolyzes disaccharides such xylobiose and the higher xylooligosaccharides that have a lower specific affinity.

The following enzymes, whose modes of action are also shown in Figure 11, are considered to be involved in the hydrolysis of mannan and galactomannans by Moreira and Filho [71].

Endo-β-1,4-mannanase (EC 3.2.1.78): It generates new chain endpoints by randomly cleaving the mannan's β-1,4-linkage internal links.

Exo-β-mannosidase (EC 3.2.1.25): It releases mannose sugar moieties by cleaving β-1,4-linked mannosides from the non-reducing ends of mannan and mannooligosaccharides.

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β-glucosidase (EC 3.2.1.21): This enzyme hydrolyzes the 1,4-β-D-glucopyranose found at the non-reducing ends of the oligosaccharides produced from glucomannan and galactoglucomannan.

α-galactosidase (EC 3.2.1.22): It is a debranching enzyme that breaks down the α-1,6-linked D-galactopyranosyl side chains of galactomannan and galactoglucomannan.

Acetyl mannan esterase: It is a debranching enzyme that causes galactoglucomannan to release its acetyl groups.

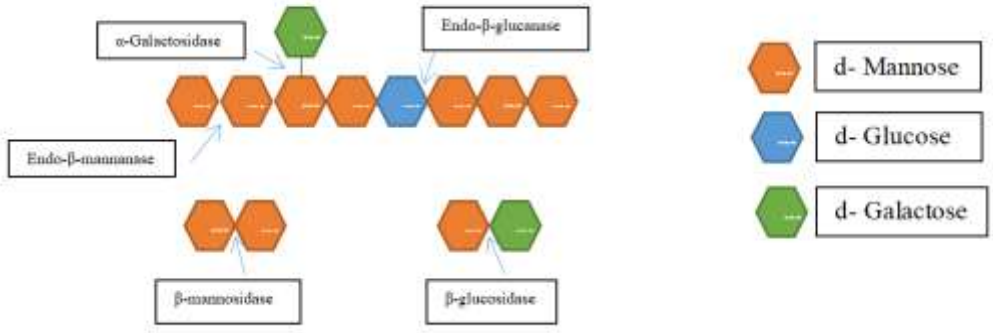

Figure 11: Schematic representation of O-acetylated galacto-glucomannan and enzymes involved in its degradation (a) and the oligosaccharides released (b).

Agaricus [72], *Aspergillus* [73, 74], *Fusarium* [64, 75], and *Trichoderma* [76 - 79] are fungi that have been discovered to breakdown hemicellulose. Hemicellulases are produced mostly by gram-positive bacteria, such as *Bacillus* species [80, 81] and Clostridia species [82, 83]. Among the actinomycetes, some species of *Streptomycetes* group [84].

4.2.2.1.3. Pectinases

Pectinases are the enzymes that hydrolyze pectic polysaccharides into monomers such as galacturonic acids. Pectin is a major component of plant cell walls, so in order to completely break down the lignocellulosic biomass, pectinases are required to completely hydrolyze the pectic materials. This lowers the viscosity of the medium and creates an ideal environment for the other enzymes to act on different polysaccharides. The following are the primary enzymes [85] involved in the hydrolysis of pectic substances:

Protopectinases: To liberate soluble form polymerized pectin, they dissolve protopectin. These are divided into two types: type A, which acts with protopectin at the polygalacturonic acid chain area, and type B, which acts with the polysaccharide chains tying the polygalacturonic acid chain to the components of the cell wall.

Pectin Methyl Esterases (PME) (EC 3.1.1.11): Pectin methyl esterases deesterify the methyl group of pectin, releasing pectic acid and methanol in the process. Prior to pectate lyases and polygalacturonases, which require non-esterified substrates, it catalyses the de-esterification.

Pectin Acetyl Esterases (PAE): In order to liberate pectic acid and acetate, it catalyses the hydrolysis of the acetyl esters found in pectin.

Polymethylgalacturonases (PMG): The pectin backbone's α-1,4-glycosidic linkages are broken down, resulting in the formation of 6-methyl-

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D-galacturonate. It has both endo and exo modes of action. Exo-PMG catalyzes a reaction at the non-reducing end of the substrate while endo-PMG randomly cleaves the substrate.

Polygalacturonases (PG): To create D-galacturonate, it cleaves the polygalacturonic acid's α-1,4-glycosidic linkages. It can act in both endo and exo modes, just as PMG. Exo-PG (EC 3.2.1.67) catalyses the reaction at the non-reducing end of the substrate while endo-PG (EC 3.2.1.15) randomly cleaves the substrate.

Pectate Lyases (PGL): To release α-4,5-D-galacturonate from the glycosidic bonds in polygalacturonic acid, it performs a trans elimination reaction. Exo-PGL (EC 4.2.2.9) cleaves the substrate at the nonreducing end, whereas endo-PGL (EC 4.2.2.2) operates on the substrate at random.

Pectin Lyases (PL): It performs trans elimination of glycosidic connections to randomly break the esterified pectin and create unsaturated methyloligogalacturonates.

Numerous bacteria and fungi that cause plant disease produce pectinolytic enzymes to aid in host invasion. Additionally, they aid in the recycling of carbon ponds in nature by decomposing dead plant materials. Numerous organisms have been shown to generate pectinolytic enzymes, including *Aspergillus* [87], *Fusarium* [88], *Penicillium* [89], *Trichoderma* [90], *Bacillus*, *Erwinia*, and actinomycetes like *Streptomycetes* [91].

4.2.2.1.4. Amylases

The three main categories of amylases, also known as glycosyl hydrolases (GH), according to the International Union of Biochemistry and Molecular Biology (UIBMB), are endo-amylases, exo-amylases, and debranching enzymes. Figure 12 shows how all of these enzymes work to break down starch. The various types of starch degrading enzymes are as follows [92].

Endoamylases or α-amylase (EC 3.2.1.1): It cleaves the α-1,4-bonds present in the inner regions of amylose and amylopectin to break into oligosaccharides and dextrins, decreasing the solution's viscosity.

Exoamylase or β-amylase (EC 3.2.1.2): Only the α-1,4-bonds at the non-reducing ends are broken, releasing limit dextrins and β-maltose.

γ-amylase or Amyloglucosidase or Glucoamylase (EC 3.2.1.3): It functions as a debranching enzyme by cleaving the final α-1,4 links at the non-reducing end of amylase and amylopectin, which releases glucose.

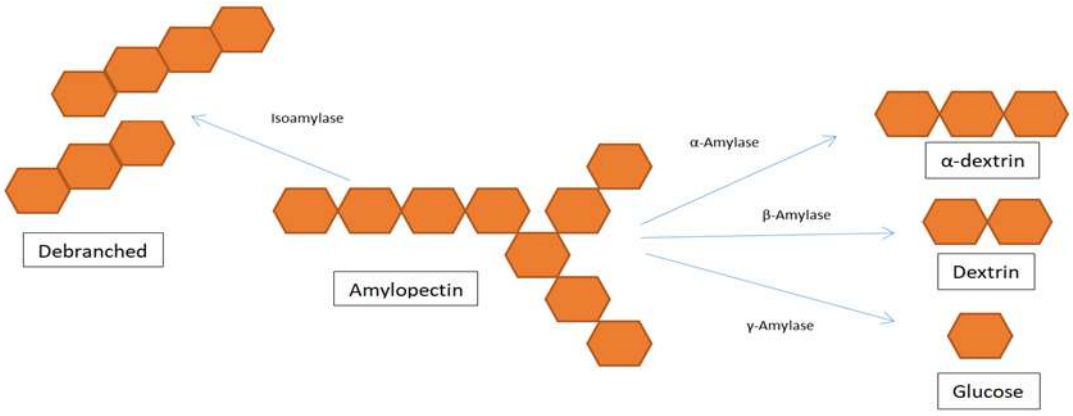

Figure 12: Mode of action of amylases.

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Many fungi, bacteria, and actinomycetes have been found to produce amylases. Several species of the genera *Aspergillus* and *Penicillium* are effective fungal amylase producers. *Aspergillus flavus, Aspergillus fumigatus*, and *Aspergillus niger* are among the fungi that produce amylases [93-95]. *Bacillus* species are the most common types of the many bacteria that produce amylases. *Rhodothermus, Corynebacterium, Geobacillus, Lactobacillus*, and *Pseudomonas* are some more species. *Streptomyces* and *Thermonospora* have been discovered to make amylase among the actinomycetes [96].

5. Production of microbial enzymes for use in the generation of second generation bioethanol

Two different fermentation procedures can be used to produce enzymes at the industrial level while taking production costs and using natural substrates into account. There are two types of fermentation: liquid state fermentation and solid state fermentation.

5.1. Solid state fermentation (SSF)

For the growth of microorganisms, this type of fermentation often uses moist solid substrate. SSF is a fermentation procedure that uses either a natural or inert solid substrate in the absence of freely flowing water [97,98]. A key component of SSF is the choice of solid material, which must be insoluble and serve as both a physical support and a source of nutrition for the bacteria. This imitates their natural environment and promotes the synthesis of enzymes and other useful metabolites for industry [99, 100]. Due to the utilisation of lignocellulosic as a medium or substrate for the development of microorganisms to create cellulases, hemicellulases, pectinases, and amylases, this fermentation is cost-effective. SSF cultures were discovered to produce more enzymes as compared to liquid cultures [57].

SSF might be viewed as a superior method for the industrial synthesis of enzymes while taking into account production costs and employing natural substrates. Higher fermentation productivities, higher product stability, higher product concentration, decreased chances of contamination due to lower water activity need, and development of microorganisms specialised for water-insoluble substrates are all benefits of SSF [101]. Other benefits include the use of straightforward instrumentation, compactness of the fermenter due to a smaller volume of water, lack of foam formation, higher fermentation capacity, decreased catabolic repression, cost-effectiveness, and a reduced need for solvents in the product recovery process [102,103].

Different lignocellulosic feedstocks have been used by bacterial and fungal cultures to produce various hydrolytic enzymes. For achieving the highest enzyme activity, more investigations on enzyme characterisation and optimization of various physical and cultural factors are published in the literature. Examples of how solid-state fermentation can be used to produce hydrolytic enzymes on diverse substrates are shown in Table 2.

Table 2: Examples of the production of hydrolytic enzymes on various substrates by solid-state fermentation.

Substrate	Microorganism	Enzymes	References
Wheat straw, rice straw, corn cobs, wheat bran, oat bran, Arundo donax, Populus tremuloides	*Thermoascus aurantiacus*	Cellulases	[104]

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Rice bran	*Bacillus* sp.	Amylase	[105]
Wheat bran	*Aspergillus awamori Nakazawa* (MTCC 6652)	Glucoamylase, protease	[106]
Wheat bran	*Aspergillus niger NS-2*	Cellulases xylanase, mannanase, pectinase, amylases	[64]
Rice bran, wheat bran, black gram bran soybean	*Aspergillus niger* MTCC-104	Amylase	[107]
Rice bran, wheat bran, black gram bran	*Aspergillus niger*	Amylase	[108]
Papaya waste	*Aspergillus niger*	Amylase	[109]
Deoiled rice bran	*Aspergillus niger, Aspergillus oryzae, Trichoderma reesei*	Cellulase, amylase	[6, 110]
Kitchen waste	*Aspergillus niger* CJ-5	Cellulases, xylanase, mannanase, pectinase, amylases	[56, 111]
Brewer's spent grain	*Fusarium oxysporum* SS-25	Cellulases	[112]
wheat straw, paddy straw, sugarcane waste, maize straw	*Bacillus licheniformis*	α-amylase	[113]
Rice bran, wheat bran, black gram bran	*Achromobacter xylosoxidans*	Amylase, cellulase, xylanase	[114]
Wheat bran, rice bran, sawdust	*Aspergillus niger* RSO-1	Cellulase	[115]
Peanut oil cake	*Aspergillus oryzae*	Cellulase, xylanase, amylase	[116]
Brewer's spent grain	*Aspergillus niger* CECT2088	Cellulase, xylanase	[117]
Orange peel, apple pomace, and rice fiber	Compost from MSW as inoculum	Cellulases	[118]
Coffee husk and wood chips	Compost from MSW as inoculum	Cellulases	[119]
Orange peels and exhausted sugar beet cossettes	*Aspergillus awamori* 2B.361 U2/1	Cellulase, xylanase, pectinase	[120]
Sugarcane bagasse	*Penicillium* sp., *Rhizomucor* sp., *Trichoderma* sp.	Cellulases	[121]
Grape pomace with wheat bran	*Aspergillus niger* 3T5B8	Cellulase, xylanase	[122]
Wheat bran, banana peel, orange peel, rice bran, pine apple peel	*Bacillus subtilis* D19	Amylase	[123]
Mango peels	*Aspergillus tamarii*	Pectinase	[124]
Wheat chaff	*Trichoderma reesei* QM 9414	Cellulases and xylanase	[125]
Rice straw	*Aspergillus niger* P-19	Cellulases, hemicellulases	[126]
Rice straw	*Penicillium* spp.	Cellulase	[52]
Soybean husk and flour mill waste	*Aspergillus oryzae*	Amylase	[127]
Wheat bran	*Bacillus* sp. TC-DT13	Xylanase	[128]
Cassava residue	*Aspergillus niger*	Amylase	[17]
Wheat bran	*Trichoderma reesei, Neurospora crassa*	Cellulases	[129]
Banana peels	*Aspergillus fumigatus*	Pectinase and xylanase	[74]

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5.2. Liquid state fermentation (Submerged and surface)

Under stationary or shaking circumstances, liquid state fermentation involves the development of microorganisms in a liquid medium that contains the necessary nutrients. This type of fermentation is appealing for the development of microbes and the creation of products with added value due to a number of factors, including: a) homogenous distribution of nutrients for the proliferation of microorganisms; b) simplicity of monitoring of variables like moisture, temperature, pH, agitation, oxygen, and nutrient levels; c) powerful technology that has already been best adapted with automatic grade and equipment availability. Cellulolytic enzymes, ligninolytic enzymes, and other beneficial metabolites can all be produced through liquid state fermentation [130].

Liquid type fermentation is divided into submerged and surface culture fermentation depending primarily on whether the incubation is being carried out in stationary or rocking circumstances. In surface culture, fermentation microorganisms develop on the shallow nutritional media's surface, consume the nutrients necessary for their growth, and simultaneously release products into the medium. Since fungi are filamentous in nature and agitation might break their mycelia, segregating biomass from the liquid medium, this mode of fermentation does not call for agitation in the case of fungi [131].

However, surface culture fermentation has a lower bio-reaction rate and longer fermentation periods as compared to submerged fermentation, which involves robust aeration and agitation [132]. Submerged fermentation is preferred over surface culture fermentation as a result of this drawback. Through submerged cultivation, many strains of bacteria, yeast, fungus, and algae have been employed for fermentation. These methods of fermentation can use either synthetically manufactured or lignocellulosic biomass-produced fermentation media.

The fungal hyphae are not desiccated as a result of the continual immersion in liquid medium during liquid state fermentation, which is also the most effective, easiest to sterilise, and most cost-effective approach for producing bioagents in large quantities [133]. With the exception of high-density cultures, microorganisms are exposed to a fixed temperature throughout their life cycle. Additionally, oxygen availability to biomass can be regulated at a specific level of medium saturation. When compared to solid substrates, submerged culture has various benefits, including easier control of fermentation parameters like pH and temperature, improved contamination control, and a lower labour and space demand. The nature and amplitude of forces in a bioreactor are studied using fermenters that offer the organism a low-shear environment. Surface culture fermentation is preferred to submerged fermentation for a number of reasons, including equipment expense, energy usage, aeration breakdown, improved productivity, and yield [134]. Table 3 shows a few instances of liquid state fermentation producing hydrolytic enzymes on various substrates.

Table 3: Examples of the production of hydrolytic enzymes on various substrates by liquid state fermentation.

Type of fermentation	**Substrate**	**Microorganism**	**Enzymes**	**References**
Submerged and surface	Rice bran	*Aspergillus niger*	Pectinase	[135]
Submerged	Solka-Floc cellulose	*Penicillium brasilianum* IBT 20888	Cellulases	[136]
Submerged	Mandarin peels and tree leaves	*Pleurotus dryinus*	Cellulases, xylanase, laccase, manganese peroxidase	[137]

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Submerged	Starch	*Bacillus* sp.	Amylase	[138]
Submerged	Partially delignified cellulignin	*Trichoderma harzianum* IOC-4038	Cellulases	[139]
Submerged	Sugarcane bagasse, corn stover	*Acremonium* sp.	Cellulases, xylanase	[140]
Submerged	Wheat bran	*Aspergillus tamarii* MTCC5152	Amylase	[141]
Submerged	Sugarcane bagasse	*Bacillus* sp.	xylanase	[142]
Submerged and surface	Wheat bran, rice bran, sawdust	*Aspergillus niger* RSO-1	Cellulase	[115]
Submerged	Corn cob	*Aspergillus fumigatus* SD5A	xylanase	[143]
Submerged	Pineapple stem	*Bacillus subtilis* BKDS1	Pectinase	[144]
Submerged	Coffee waste	*Penicillium humicola*	Mannanase	[145]
Submerged	Wheat bran and citrus peel waste	*Bacillus pumilus*	Xylanase and pectinase	[146]
Submerged	Banana peels	*Bacillus subtilis* TYg4-3 and *Bacillus amyloliquefaciens* SW106	Pectinase	[147]
Submerged	Coffee residue powder, date seeds powder, prickly pear seeds	*Bacillus subtilis* US191	Mannanase	[80]
Submerged	Peanut shells	*Bacillus paralichniformis*	Cellulases	[148]
Submerged	Wheat chaff	*Trichoderma reesei* QM 9414	Cellulases and xylanase	[125]
Submerged	Wheat bran, rice husk	*Aspergillus niger*	Amylase	[149]
Submerged	Corn stover	*Phanerochaete chrysosporium* PC2	Cellulases and hemicellulases	[150]
Submerged	Corn bran	*Aspergillus niger*	Xylanase	[151]
Submerged	Wheat bran and citrus peel waste	*Bacillus safensis* M35, *Bacillus altitudinis* J208	Xylanase and pectinase	[152]
Submerged	Banana peels	*Aspergillus fumigatus*	Pectinase and xylanase	[74]

6. Conclusion and future outlook

The scientific community has switched to biofuels that are made from a variety of biomass residues, including municipal and agricultural waste, as a result of the rising cost of fossil fuels, the global warming caused by careless use of these fuels, and the unscientific disposal of agricultural and agro-industrial waste residues. The commercial manufacture of bioethanol, which is now the highest volume industrial fermentation product, generally uses sweet and starchy substrates. However, specialists are careful about their utilisation due to the utility of such starchy residues as human nourishment. Even yet, many nations have established limitations on their permissible usage. Scientists are working to use agricultural, agroindustrial, and municipal solid waste as second generation bioethanol feedstocks as the biofuel industry develops as a result of the rise in ethanol demand. These feedstocks are used by a small number of companies that pretreat and

hydrolyze materials using chemical processes, which results in increased costs and significant chemical loading that eventually enters our life and environment. Enzymatic hydrolysis is advised, despite the fact that it adds between 30 and 50% to the overall cost of producing ethanol from lignocellulosic wastes. Enzymes with higher substrate specificity, lower dose requirements, and improved cost-effectiveness are required. The process economy as a whole can gain from the creation of innovative enzymes that can hydrolyze a variety of substrates, high-titer production of such enzymes, further development using genetic and molecular methods, and lower costs associated with the enzyme production process. Technologies that reuse the enzyme that washed away during hydrolysis can help address the issue of enzyme cost. The development of an effective and environmentally friendly process technology for converting lignocellulosic residues to bioethanol may be made possible by advancements in enzyme technology and commercialization. This technology may prove to be a panacea for pressing global issues like the depletion of fossil fuels and the improper disposal of these priceless resources.

Conflict of Interest

The authors declare no conflict of interest.

Refernces

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