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Review

Integrating 1G with 2G Bioethanol Production by Using Distillers' Dried Grains with Solubles (DDGS) as the Feedstock for Lignocellulolytic Enzyme Production

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Abstract: First-generation (1G) bioethanol is one of the most used liquid biofuels in the transport industry. It is generated by using sugar- or starch-based feedstocks, while second-generation (2G) bioethanol is generated by using lignocellulosic feedstocks. Distillers' dried grains with solubles (DDGS) is a byproduct of first-generation bioethanol production with a current annual production of 22.6 million tons in the USA. DDGS is rich in fiber and valuable nutrients contents, which can be used to produce lignocellulolytic enzymes such as cellulases and hemicellulases for 2G bioethanol production. However, DDGS needs a pretreatment method such as dilute acid, ammonia soaking, or steam hydrolysis to release monosaccharides and short-length oligosaccharides as fermentable sugars for use in microbial media. These fermentable sugars can then induce microbial growth and enzyme production compared to only glucose or xylose in the media. In addition, selection of one or more suitable microbial strains, which work best with the DDGS for enzyme production, is also needed. Media optimization and fermentation process optimization strategies can then be applied to find the optimum conditions for the production of cellulases and hemicellulases needed for 2G bioethanol production. Therefore, in this review, a summary of all such techniques is compiled with a special focus on recent findings obtained in previous pieces of research conducted by the authors and by others in the literature. Furthermore, a comparison of such techniques applied to other feedstocks and process improvement strategies is also provided. Overall, dilute acid pretreatment is proven to be better than other pretreatment methods, and fermentation optimization strategies can enhance enzyme production by considerable folds with a suitable feedstock such as DDGS. Future studies can be further enhanced by the technoeconomic viability of DDGS as the on-site enzyme feedstock for the manufacture of second-generation bioethanol (2G) in first-generation (1G) ethanol plants, thus bridging the two processes for the efficient production of bioethanol using corn or other starch-based lignocellulosic plants.

Keywords: DDGS; distillers' dried grains with solubles; lignocellulolytic enzymes; cellulase; hemicellulase; 1G + 2G

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1. Introduction

The transportation industry is one of the biggest contributors to greenhouse gas emissions (GHG) in many developed countries [1,2]. Fossil fuels, mainly gasoline, are the main stimuli behind the devastating GHG emissions. The drastic impact on the environment is directly correlated with global anthropogenic activities which have resulted in an increase in the Earth's temperature over the last five decades [3]. Another problem is energy security as fossil fuels are non-renewable on the human time scale and the need for alternative and clean energy sources is ubiquitous all around the globe. Among various proposed solutions, biofuels are one of the most prominent and most implemented solutions especially in the transport industry due to the liquid nature of bioethanol. Bioethanol is currently blended

in various ratios with gasoline across the world to solve energy security and sustainability issues [1].

According to a recent report, 110 billion liters of ethanol was produced in 2019, which was expected to increase but did not due to the COVID-19 pandemic [4]. Typically, bioethanol is produced from sugar-based (sugarcane juice) or starch-based (corn grains) feedstocks [4]. The sugar-to-ethanol conversion is carried out mainly by yeast, *Saccharomyces cerevisiae*, which can be represented by the following chemical equation.

$$C_6H_{12}O_6 \rightarrow 2\ C_2H_5OH + 2\ CO_2$$

However, the breakdown of starch into simple sugars is required in case of starch-based feedstocks:

$$(C_6H_{10}O_5)_n + n\ H_2O \rightarrow n\ C_6H_{12}O_6$$

The two top major producers of bioethanol are the USA (56%) and Brazil (28%), which use starch-based crops (mainly corn grains), or sugar-based crops (mainly sugarcane juice) as the main feedstock for ethanol production, respectively [5]. This type of production process is known as the first-generation (1G) production process. There are several advantages of this process: the feasibility of conversion methods, the C-6 fermentation cycle, and the establishment of the industrial process. Due to these advantages, currently, more than 94% of global ethanol is in the 1G category. The 1G bioethanol production process using corn grains is comprised of several steps: milling, liquefaction, saccharification, fermentation, distillation, and drying. Each of these steps is optimized at industrial scales for the maximum possible production of ethanol [6,7]. However, various issues are still present in 1G ethanol production, such as food vs. fuel issues, the use of land and water resources, and the possible contamination of soils from distillation residues [7].

Recently, second-generation (2G) ethanol has gained research interest due to its capacity to reduce GHG emissions and the availability of more sustainable feedstock. According to a published report, while 1G ethanol can reduce GHG emissions by 39–52% as compared to gasoline, 2G ethanol can further decrease the emissions by 86% [8]. The main feedstock for 2G ethanol is lignocellulosic biomass, which is abundant and inexpensive compared to sugar- or starch-based crops, which are cultivated specifically for fuel generation. This will certainly ease the fuel vs. food concerns. Currently, the annual production of lignocellulosic biomass is 181.5 billion tons and the price is approximately 24 USD to 121 USD per ton [9]. Therefore, lignocellulosic biomass should be preferable over 1G feedstocks for bioethanol production. However, the main disadvantage of lignocellulosic biomass is its recalcitrant nature that comes from lignin, cellulose, and hemicellulose. In any given lignocellulosic biomass, the cellulose content can be 40–60%, the hemicellulose content can be 10–40%, and the lignin content can be 15–30% [10]. These three components are an integral part of a plant cell wall (Figure 1). They support and protect the plant cell as compared to starch or sugars which are the main energy sources. Therefore, the breakdown of such materials as an energy source is naturally more difficult than starch or simple sugars. The enzymes required for the breakdown are known as cellulases, hemicellulases, and lignases or lignin-modifying enzymes [11,12]. These enzymes are currently a major topic of research interest due to their underlying applications in the production of 2G biofuels [11]. However, such enzymes are currently not of industrial standards, and, in addition, they are very expensive as high loading is required. However, various feedstocks and process improvement strategies have been proposed in the literature to improve the quality and production of these enzymes.

A byproduct of starch-based bioethanol, mainly in the USA, is distillers' dried grains with solubles (DDGS). The current global production of DDGS is around 40 million tons, with the USA as the top producer contributing to 58% of global production [13]. Currently, DDGS is used as animal feed or to a much lesser extent as fertilizer. DDGS is a corn residue after the almost complete conversion of starch into bioethanol. One-third of corn is DDGS by the 1G ethanol production process. DDGS is mainly comprised cellulose and

hemicellulose fibers, proteins, and lipids. In the case of corn-based DDGS, the composite fiber content is around 33–40%, the crude protein content is 26–33%, and the fat or oil content can be around 9.1–14.1% [14]. The fiber and protein contents in DDGS make it desirable for the production of microbial products via microbial fermentations such as lignocellulolytic hydrolysis enzymes, which are required to hydrolyze cellulose and hemicellulose fibers in the fermentation media for their efficient utilization [11].

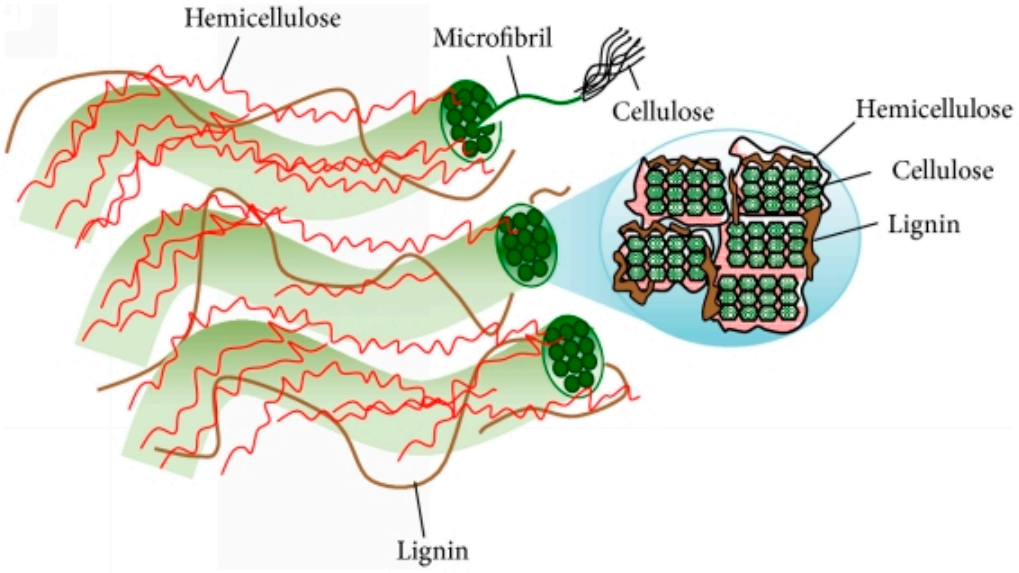

Figure 1. The three main components of a plant cell wall (reprint from [15]).

Therefore, in this review article, a strategy to feasibly establish the 2G ethanol processes with the help of the integration of 1G and 2G bioethanol plants through the production of lignocellulolytic enzymes by using DDGS as the main feedstock is discussed. The potential of such on-site production can only be explored after the research on its crucial individual steps. For this purpose, a special focus is given to the pretreatment, strain selection, media optimization, and the optimization of microbial culture parameters such as pH, temperature, and aeration. In addition to the focus on DDGS as the main feedstock for on-site enzyme production, several other research reports are discussed with the same research focus but with different feedstock or pretreatment strategies. In the end, an integrated biorefinery model is proposed, which can bridge 1G and 2G ethanol production processes via on-site enzyme production from DDGS for a win–win situation.

2. The Main Differences in 1G and 2G Bioethanol Production

Bioethanol production processes are of many kinds, but the main principle is the conversion of simple sugars such as glucose into ethanol via fermentation. The main difference between first-generation (1G) and second-generation (2G) biofuel is the level of *sustainability* in terms of source material or feedstock. The feedstock for 1G ethanol production is mostly pure sugar- or starch-based feedstock [16]. For example, the USA, which is the top producer of bioethanol in the world, uses corn grain as the main feedstock for ethanol production. On the other hand, Brazil, which is the second-largest bioethanol producer uses sugarcane. Currently, 1G bioethanol is the most common type of ethanol in the world with an approximate annual production of 110 billion liters [16]. In the United States, the current production of corn ethanol is approximately 54% of the total global production [16].

During 1G bioethanol production with corn grains, one-third of corn grains are converted into ethanol while the other two-thirds are converted into carbon dioxide and solid residues known as DDGS. Approximately 5% of the glucose is converted into yeast cells which are extracted into thin stillage and approximately 15–30% of the thin stillage is

recycled back into the bioethanol production process while the rest is mixed into DDGS after drying [14,17]. However, there are other parts of corn crops that are wasted or turned into low agriculture products such as corn stover [18]. The main issue with 1G ethanol production is sustainability in terms of making more ethanol from all of the parts of the crops and not just from the grains. On the other hand, the feedstock for the 2G ethanol is lignocellulosic biomass including but not limited to inedible parts of the plants, wood waste, straw, grasses, etc. All such feedstocks are either a waste product or a byproduct of upstream agricultural processes.

The feedstock, however, alone does not characterize all the differences between the two processes. The type and severity of the *pretreatment method* for the breakdown of the complex polysaccharides into simple sugars for their further conversion into ethanol is also a necessary step in both 1G and 2G production processes. In the 1G bioethanol production process, the pretreatment is characterized by the process of liquefaction. In the liquefaction step, the ground corn grains are mixed with water and kept at 85 °C for one to two hours [16]. In the case of 2G ethanol production with lignocellulosic feedstock, the pretreatment methods are more severe and are carried out at temperatures higher than 100 °C (Table 1). These main differences between the two pretreatment steps drastically impact the economics and carbon footprint differences in the two processes.

Table 1. Main differences between 1G and 2G bioethanol productions.

Parameter	1G	2G	Advantage of 1G over 2G or Vice-Versa	References
Feedstock	Sugar or starch based: corn, wheat, sugarcane, beet, etc.	Lignocellulosic biomass: inedible parts of the plant, straw, wood, and sawdust, etc.	Sugar- or starch-based feedstocks are easy to breakdown into simple monosaccharides which are then easily fermented into ethanol.	[19]
Pretreatment	Liquefaction	Dilute acid, steam, AFEX, etc.	Liquefaction requires low to medium process conditions as compared to lignocellulosic pretreatment strategies.	[2,3]
Enzymatic hydrolysis	Saccharification with amylases and glucoamylases	Lignocellulolytic process with cellulases and hemicellulases	Saccharification has high conversion yield as compared to the cellulose and hemicellulose hydrolysis	[16]
Cost of feedstock	40–70%	30%	2G is advantageous as the feedstock is inexpensive.	[16]
Pretreatment cost	Low	High	1G is advantageous as low capital investment is needed.	[16]
Reduction in GHG emissions	39–52%	86%	2G ethanol is more sustainable as compared to 1G ethanol.	[1]
Food vs. fuel issue	Yes	No	2G ethanol is advantageous over 1G for food security.	[20]

The third main difference between 1G and 2G ethanol production is *enzymatic hydrolysis*. Enzymes such as amylases and glucoamylases are added to break down the long starch chains into glucose molecules, which are ready for further fermentation in the 1G ethanol production process. This enzyme hydrolysis step is carried out at 95–107 °C [14]. The enzymes for lignocellulosic hydrolysis and breakdown are known as lignocellulolytic enzymes and the two main contributors are cellulases and hemicellulases. The hydrolysis step is carried out at a temperature similar to that of the 1G ethanol production process. However, the main differences between the two enzymatic processes are the degree of hydrolysis and the cost of the enzymes. It has been confirmed in many studies that the cost

of the enzymes is one of the major bottlenecks in the production of 2G ethanol, and the lower hydrolytic quality of cellulases and hemicellulases than amylases and glucoamylases is another barrier. In addition, the lignin removal step is required which is carried out with the help of different pretreatment methods such as acid hydrolysis or alkaline soaking. In acid hydrolysis, the lignin is dissolved along with hemicellulose fractions and thus lignocellulosic biomass with low lignin content can be treated with such methods. On the other hand, in the alkaline pretreatment methods, the lignin structure is altered and lignin needs to be removed from the pretreated slurries [21]. In addition, such pretreatments also generate toxic byproducts such as furfural, hydroxymethylfurfural, phenols, and organic acids which negatively affect the action of cellulases and hemicellulases [22]. The improvement of such enzymes towards low cost and higher quality is extremely necessary for the commercialization of the 2G ethanol production process [11].

Therefore, the cost of the two production processes plays an important role in the commercialization of bioethanol production at industrial scales. The need for effective pretreatment methods requires high capital investment in the case of the 2G bioethanol production process. This leads to the high cost of lignocellulosic ethanol. The current cost of 1G ethanol from sugar- or starch-based feedstocks is approximately 43% lower than that of 2G ethanol [1]. In another study conducted in the year 2000, it was reported that 2G ethanol can be 60% more expensive than 1G ethanol [23]. Therefore, the cost efficacy of the 1G bioethanol production is one of the main reasons it is currently the only type of commercial bioethanol in the world. The corn ethanol production process in the United States has been optimized through several strategies such as simultaneous saccharification and fermentation [16].

Another and perhaps the most crucial difference between 1G and 2G ethanol production processes is the GHG emissions of the two processes. 1G ethanol was commercialized because it shows lower GHG emissions than gasoline or other conventional fossil-based fuels. In some studies, it was shown that 1G ethanol reduces GHG emissions by 39–52% as compared to gasoline [8]. However, the land use for sugar or starch-based crops for the whole purpose of bioethanol production has raised several concerns related to food security [20]. In addition, the transport of such crops to the site of ethanol production also raises some sustainability issues. On the other hand, bioethanol from lignocellulosic biomass can cause lower GHG emissions than 1G ethanol. Some studies suggest the reduction in GHG emissions from using 2G bioethanol can be as much as 86% lower than gasoline [1].

Even though both 1G and 2G bioethanol production processes are similar to each other, they have very distinctive differences that impact the overall sustainability and cost of industrialization. 2G bioethanol is more sustainable and fed by lignocellulosic feedstocks, which makes it ideal for commercialization. However, the recalcitrant nature of the lignocellulosic biomass makes it difficult for biochemical conversion. Therefore, high capital investment and severe pretreatment methods are needed. This main disadvantage is the reason for 1G ethanol being preferable, and currently, more than 99% of bioethanol is produced through 1G bioethanol production. However, the 1G ethanol production process can be further optimized by integrating the 1G and 2G ethanol production processes at the same biorefinery via strategies such as on-site lignocellulolytic enzyme production.

3. Mechanism of Enzymatic Hydrolysis of 1G Feedstock

1G feedstock in the United States is usually starch-based grains such as corn or wheat. The breakdown of such materials for ethanol production requires the essential steps of liquefaction and saccharification. The enzymatic hydrolysis is carried out with a set of different enzymes such as α-amylases, β-amylases, glucoamylase, and some pullulanases [7]. The most prominent ones, adapted for industrial scales, are amylases and glucoamylases. Starch consists of two main types of glucose polymers: amylose and amylopectin [24]. Amylose is straight linear chains of glucose molecules linked by α-(1, 4) linkages. The glucose molecules in amylopectin are also linked through same α-(1, 4) linkage but it also contains α-(1, 6) linkage, which gives it its signature branched structure.

The α-amylases are glucanohydrolases that hydrolyze the α-(1, 4) linkages at random locations in starch. They release dextrins of varied lengths and oligosaccharides. However, α-amylases have difficulty in hydrolyzing the bonds near α-(1, 6) linkage or branch points in the amylopectin. The β-amylases are hydrolases that removes β-anomeric maltose from α-(1,4) glucans.

Glucoamylases attack starch from the non-reducing end and pullulanases cleave α-(1, 6) linkage in amylopectin [7]. For many years, different formulations and modifications for such enzymes have been performed to obtain the desired results in the 1G ethanol production industry [7]. These strategies have also helped in driving down the price of such enzymes in ethanol production, which has now reduced to approximately 0.30 USD per gallon of ethanol produced [7]. This is one of the many reasons that 1G ethanol is easier to produce as compared to 2G ethanol which requires lignocellulolytic enzymes for the hydrolysis of the feedstock into simple sugars.

4. Mechanism of Enzymatic Degradation of Lignocellulosic Biomass

Lignocellulolytic enzymes are cellulases, hemicellulases, and lignin-degrading enzymes. However, mainly cellulases and hemicellulase are needed for 2G ethanol production, because they are involved in the release of sugar monomers, which can then be converted into ethanol by fermentation (Figure 2). The lignin portion of lignocellulosic biomass is removed with the help of pretreatment usually in the first step of the 2G ethanol production process. There are various pretreatment methods that have extensively been explored for this purpose. The main problem is the recalcitrant nature of the lignocellulosic biomass: cellulose fibers are interlinked with lignin via covalent interactions which makes it hard to remove them from the cellulose and hemicellulose portion of lignocellulosic biomass [25]. The cellulose molecules themselves are bound tightly via hydrogen bonding. All these molecular interactions make lignocellulosic biomass difficult to degrade.

After removal of lignin, cellulase-mediated degradation of cellulose is a multi-step mechanism [26]. Cellulose is insoluble in water. Therefore, the first step is the physical and chemical changes in its solid phase to make it accessible to the enzymes; the second step is the primary hydrolysis which releases small intermediate fragments (essentially soluble in the aqueous phase) from the reactive cellulose surface; the third step is the secondary hydrolysis involving further breakdown of intermediate cellulose fibers into smaller glucose chains or cellodextrins, which are ultimately converted into glucose molecules [27]. This mechanism also shows that enzymes vary in their action based on the freely available cellulose in the aqueous phase which is needed for the enzymatic hydrolysis of cellulose. In addition, some other enzymes such as swollenin are also present which does not have a hydrolytic property but disrupts the cellulose chains through other mechanisms [28]. The rate of hydrolysis is dependent on the complete consumption of cellulose, which means the rate decreases rapidly over time after the initial phase. The cellulases are also heavily influenced by feedback inhibition.

Hemicellulose degradation via the enzymatic pathway is more complex as there are many different types of hemicellulose molecules present in the given portion of lignocellulosic biomass (Figure 2). These molecules are varied lengths of either pentose chains (l-arabinose or d-xylose) or hexose chains (d-galactose, d-mannose, and d-glucose) [29]. The hemicellulose portion also has uronic acids such as d-glucuronic acid, methylgalacturonic acid, and D-galacturonic acid and acetyl groups [30]. Hemicellulose connects lignin with cellulose and is hygroscopic and hydrophilic. It makes hydrogen bonds with cellulose and aromatic esters with lignin to give the recalcitrant nature to the lignocellulosic biomass. Thus, the heterogeneous nature of the hemicellulose requires a synergistic action of various enzymes that are involved in the degradation of hemicellulose.

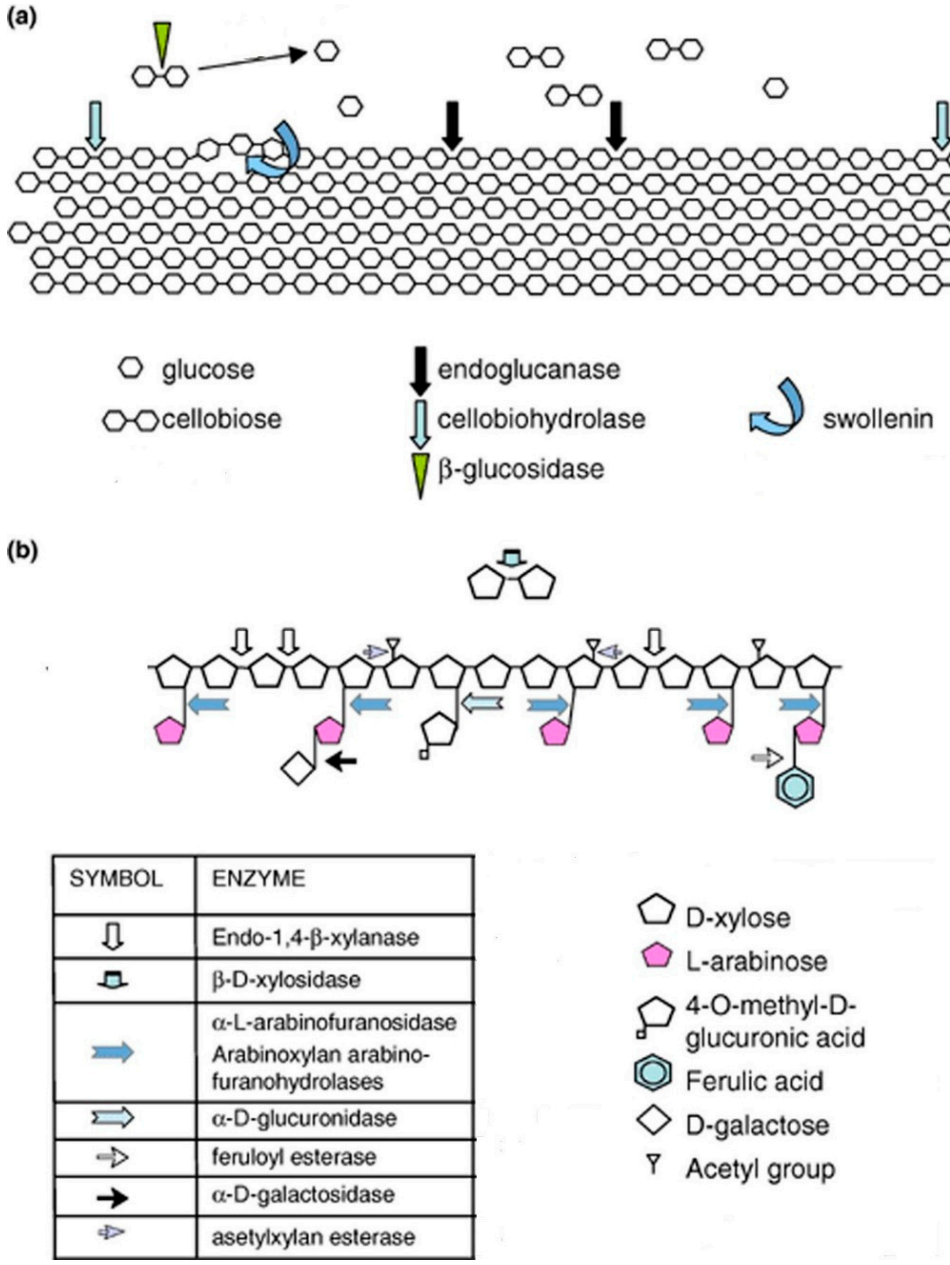

Figure 2. Enzymatic degradation of cellulose and hemicellulose (adapted from [28]). (**a**) Enzymes and molecules involved in the cellulose degradation. (**b**) Enzymes and molecules involved in the hemicellulose (arabinoxylan) degradation.

5. Lignocellulolytic Enzyme Productions

There are various microorganisms capable of producing cellulases and hemicellulases. Two very common examples are fungi and bacteria [26]. Prominent strains in recent pieces of literature are summarized in Table 2. Fungi are more prominent because of their hyperproduction of a diverse range of enzymes. As mentioned earlier, cellulases and hemicellulases are of many types and the complete hydrolysis of cellulose and hemicellulose needs a diverse range of such enzymes. Therefore, the first necessity for the production of such enzymes is an ideal microorganism that can produce as many enzymes as needed for any particular type of lignocellulosic biomass. Among various fungal species which have been recognized for their wood-decaying capability, the four main secretors of cellulolytic enzymes are *Trichoderma*, *Aspergillus*, *Humicola*, and *Penicillium* [31].

Table 2. Recent prominent strains used in the research of lignocellulolytic enzyme productions.

Microorganism	Strain	Reference
Fungi	*Trichoderma reesei*	[32]
	Cladosporium cladosporioides NS2	[33]
	Trichoderma reesei NCIM 1186 and *Penicillium citrinum* NCIM 768	[34]
	Aspergillus flavus	[35]
	Penicillium sp., *Cladosporium* sp., *Fusarium* sp.	[36]
	Trichoderma reesei RUT C30	[37]
	Talaromyces pinophilus EMU	[38]
	Trichoderma reesei	[39]
	Pestalotiopsis microspora TKBRR	[40]
	Talaromyces emersonii	[41]
	Trichoderma orientalis EU7-22	[42]
	Trichoderma reesei	[43]
	Aspergillus niger	[44]
	Trichoderma harzianum EM0925	[45]
	Humicola grisea var. thermoidea	[46]
Bacteria	*Bacillus pseudomycoides*	[47]
	Bacillus velezensis	[48]
	Bacillus amyloliquefaciens FW2	[49]
	Lactobacillus plantarum RI 11	[50]
	Burkholderia sp.	[51]

In the literature, many research articles have been published, which identify and emphasize many fungal species as best producers of cellulases and hemicellulases. Most studies focus on *solid-state fermentation* methods, which are ideal for fungal strains. However, solid-state fermentation is not ideal for industrial applications and the scale-up of this mode of fermentation is problematic. During scale-up, the mass transfer becomes a concern at larger scales. Therefore, many research articles focus on the adaptation of fungal species in the *submerged fermentation* mode. The main problem in cultivating filamentous fungi in submerged fermentation is the formation of mycelial clumps or pallets that hinders the even distribution of oxygen and nutrients. The cells at the center of such clumps or pallets start dying and thus the secretion of enzymes is hindered. To solve such problems widespread solutions including genetic engineering [52] or optimizing the culture conditions for fungal growth and enzyme production are available [11].

The first thing to consider in any fungal fermentation for cellulase and hemicellulase production is the optimization of fermentation media. An ideal microbial media provide all the macro and micronutrients for microbial growth and product formation. Filamentous fungal strains can grow on a variety of carbon sources making them highly adaptable microbial species. These fungal strains can grow on simple sugars as well as complex carbon sources such as wood. However, the main problem is that fungal species secrete enzymes such as cellulases and hemicellulases only when inducers (substrates) for such enzymes are present in the media. Therefore, if there is no cellulose or hemicellulose in the media, these strains will not produce such enzymes or produce a very low amount which would not be industrially adaptable. On the other hand, if these strains are grown on cellulose or hemicellulose fibers, they show better productivity for such enzyme productions. At this stage, another problem arises, which is the solubility of cellulose if the fermentation is submerged culture.

Another problem is that the insoluble cellulose or hemicellulose fibers cannot be evenly distributed in the media thus creating the problem of the uneven distribution of substrate. Furthermore, the difficulty of cellulose utilization by the fungal enzymes, which need soluble substrates for their action in the microbial media, rises. Therefore, microbial media for fungal cellulase and hemicellulase production should contain cellulose and hemicellulose, but they should be in their hydrolyzed form. Additionally, the media and the carbon source should be inexpensive. To solve such problems, one approach is to use agricultural wastes such as corn stover for enzyme production. However, this type of feedstock contains a very limited amount of nitrogen source. Therefore, a feedstock with adequate amounts of nitrogen is preferable. Distillers' dried grains with solubles (DDGS) is one of such feedstocks. However, it needs pretreatment for the release of hydrolyzed cellulose fragments that are easily absorbed by the enzymes into the fermentation media.

6. Pretreatment for Enzyme Production

The word "pretreatment" applies to one or several processing steps that give material or feedstock the desired properties for the final use. In the bioenergy field, pretreatment often refers to the mechanical, physical, chemical, or biological breakdown of the complex biomass into simple components, which can then be used for fermentation into various value-added products. For example, the dry milling of corn grains, before liquefaction and saccharification in 1G ethanol production, can be regarded as a pretreatment step as well as the liquefaction and saccharification steps. Milling is considered as a mechanical pretreatment method while liquefaction and saccharification steps are a combination of physical, chemical, and biological methods. Similarly, for 2G ethanol production, milling of corn stover is a mechanical pretreatment. However, lignocellulosic biomass contains majorly cellulose and hemicellulose instead of starch, thus the pretreatment steps after milling should be more extensive and diverse than the liquefaction and saccharification steps in 1G ethanol production.

Since the realization of commercial bioethanol production and the need for 2G bioethanol, there has been an enormous amount of research dealing with various pretreatment methods and their effectiveness in releasing simple sugars from cellulose and hemicellulose [53–57]. The most common methods to be researched are physical, chemical, and biological methods. Some examples of physical methods include high temperature and pressure, or the action of steam. On the other hand, the chemical treatment includes use of chemicals such as acids or bases. The biological method either employs alive microorganisms and their hydrolytic properties or the enzymes (mainly cellulase and hemicellulases) produced by such microorganisms. The main concern with high temperature or acids and bases is the production of inhibitory compounds, which are detrimental for the subsequent step of fermentation such as furfural and hydroxymethylfurfural [58]. In addition, such treatment methods are capital intensive and detrimental to the environment. Therefore, biological, particularly enzymatic, pretreatment has been proposed in many research articles for 2G biofuel production [56,59–61].

The production of lignocellulolytic enzymes is another topic of interest in many research articles, mainly because of the growing need for such enzymes to produce 2G biofuel [9,32,33]. Various feedstock and pretreatments have been proposed in the last two decades for the production of lignocellulolytic enzymes [62,63]. However, as mentioned in the previous section, an ideal feedstock will be the one which has most of the macronutrients and micronutrients and without the need of extensive pretreatment methods. Nevertheless, it was also identified in the literature that soluble cellulose and hemicellulose induce the production of cellulases and hemicellulases better than simple sugars. Therefore, the fermentation media for the microbial production of such enzymes need to have some soluble cellulose and hemicellulose fibers. One way to obtain such fibers is the mild pretreatment of lignocellulosic biomass, or, more ideally, a feedstock that has both carbon and nitrogen sources.

There have been many studies in the last two decades dealing with different carbon sources and pretreatment methods for cellulase and hemicellulase production [32,36,37]. One example of such studies is the steam pretreatment of corn stover, willow, and spruce for cellulase and hemicellulase production [62]. In another study, rice straw and wheat bran were used where rice straw was chopped and ball milled [64]. Yet in another study, horticultural waste was used as the substrate for enzyme production and two pretreatment methods (steam and soaking in aqueous ammonia) were employed [63]. Many other examples can be found where unconventional carbon sources were pretreated for preparing the feedstock for cellulase and hemicellulase production. The research trends have been prominent since early 2000s and have been going strong in the last two decades. However, the articles about the effect of pretreatment for enzymes production can be traced back to as early as 1984 [65].

In an attempt to find a better pretreatment method for enzyme production, several pretreatment methods were analyzed by the authors [58]. The three main pretreatment methods were dilute acid hydrolysis, steam treatment, and soaking in aqueous ammonia (SAA). At first, the effect of all three methods on sugar release was analyzed. The analyses for glucose, xylose, total reducing sugars, and the inhibitory products were performed by high-pressure liquid chromatography (HPLC) and dinitrosalicylic acid (DNS) methods. It was determined that dilute acid hydrolysis is better than both steam treatment and SAA. The analysis performed through DNS for total reducing sugars also gives insights toward the presence of cellulose and hemicellulose fibers in the dilute acid slurry. This method was then employed further for enzyme production with various fungal and bacterial strains showing promising results for both cellulase and hemicellulase production [66].

7. Optimization of Enzyme Production

There are many ways to increase the microbial productivity of a strain for a specific product and feedstock. One way is genetic modification to enhance the action of product-forming genes. Another way is the optimization of culture conditions and fermentation media in such a way that the desired product formation is increased during the fermentation for the production of cellulases and hemicellulases [67–70]. Some of these studies are centered toward optimization of the carbon source and the addition of nitrogen sources and salts. The media can also be optimized with the help of pretreatment. For example, in the research conducted by the authors, the main outcome of the pretreatment was total sugar release instead of complete hydrolysis [58]. The main reason for this desired outcome was to acquire the induction of cellulase and hemicellulase from DDGS. After the pretreatment, the DDGS-based media were further optimized with the help of the addition of other media ingredients to make it more favorable for enzyme production instead of microbial growth [11].

The addition of nutrients into media has been reported in many research articles for increasing cellulase and hemicellulase production [70,71]. Mainly, the nitrogen sources such as yeast extract, ammonium sulfate, urea, peptone, and many others are optimized along with salts and minerals that are often required for efficient growth and product formation [9,44,47]. In the study conducted by authors on DDGS, the effect of nutrient optimization, mainly ammonium sulfate, peptone, and yeast extract, was evaluated for two *Aspergillus niger* strains [72]. The results show that the addition of a small quantity of these three media ingredients in the dilute-acid hydrolyzed DDGS improved the enzyme production for both cellulase and hemicellulase. Nutrient optimization gives a gateway to increase productivity while also taking into consideration the cost of such media. While DDGS had most of the microbial nutrient elements, the addition of small quantities of nitrogen sources has been proven to increase enzyme activity by 262% [72]. There have been many studies where the effect of nitrogen sources on cellulase production has been evaluated [73].

The optimization of fermentation conditions during the scale-up to the benchtop bioreactors can further increase enzyme production. The culture parameters to consider are

pH, temperature, inoculum size, agitation, and aeration. The optimization of agitation and aeration is particularly important in the case of submerged fungal fermentation because of the shear sensitivity of fungal strains that can greatly affect growth and enzyme production. Fungal cultures under submerged fermentation can form clumps or pallets, which can be problematic for the even distribution of resources. In this regard, the aeration and agitation should be managed in such a way that the optimized size of the clumps or pallets can be achieved. The values of such parameters above the optimized values can also negatively affect growth and product formation by breaking vital mycelial structures. On the other hand, the optimum temperature and pH for product formation for any given strain can be different from the optimum values of growth. Therefore, all such parameters should be looked into before setting up a production process at industrial levels. The optimization of culture conditions (inoculum size, aeration, and agitation) in the benchtop bioreactors with DDGS as the feedstock increased the enzyme production for *Aspergillus niger* strain by many folds for both cellulase and hemicellulase [74]. Many other studies deal with a similar type of optimization strategy, but with a different feedstock [75,76].

8. On-Site Lignocellulolytic Enzyme Production for 2G Bioethanol Production

On-site enzyme production for 2G ethanol production is not a new concept in the research history of 2G bioethanol advancements. There have been several studies reporting various aspects of on-site enzyme production in a 2G bioethanol biorefinery as summarized in Table 3. The main advantage of having on-site enzyme production is to avoid the cost of concentration, stabilizers, and transport. However, recently, it has also been established that on-site enzyme production can also help in the further reduction of greenhouse gas emissions [77]. In addition, on-site enzyme production can reduce the cost of feedstock, according to several studies, if a part of 2G ethanol is conditioned to be used as the enzyme feedstock. However, there are study reports that establish that on-site enzyme production using a fraction of 2G feedstock actually increased the production cost because less feedstock is used to make 2G ethanol [78].

Table 3. Studies conducted to analyze the potential of on-site enzyme production for 2G ethanol.

Research Aspect	Feedstock	Main Results	References
Screening for fungal strains	Wet oxidized wheat straw and filter cake	Twenty-five out of sixty-four fungal strains were selected for their cellulolytic activities.	[79]
Enzyme activities on the substrate and glucose yield	Steam-pretreated sugarcane bagasse	Cellulase activity (1.93 FPU/mL) and β-glucosidase activity (0.37 BGU/mL) were obtained. Glucose yield was 80%.	[80]
Extent of enzyme production	Microwave alkali-pretreated rice straw	Cellulase activity (24 FPU/gds), xylanase activity (258 IU/gds) and β-glucosidase activity (3.8 IU/gds) were obtained.	[81]
Extent of enzyme production	Spent fiber sludge hydrolysates	Cellulase activity of 2700 to 2900 nkat/mL was obtained.	[82]
Production cost and energy analysis of 2G ethanol	Sugarcane bagasse	The minimum selling price of ethanol varies from 4.91 USD to 4.52 USD/gal.	[83]
Greenhouse gas emissions reductions	N/A	On-site enzyme production further decreases the greenhouse gas emissions.	[77]
Extent of enzyme production and hydrolysis	Wheat bran and cellulose	The produced cellulase hydrolyzed alkali pretreated sorghum stover which was fermented to ethanol with approximately 80% efficiency.	[84]
Extent of enzyme production and hydrolysis	Sugar cane bagasse	The fermentation efficiency to ethanol of 78% was achieved with the on-site enzyme blends.	[85]
Technoeconomic analysis	Same as 2G ethanol	Production cost of 2G ethanol decreased by 19% with on-site enzyme production scenario.	[86]
Technoeconomic analysis	Corn stover	The product value (PV) of 2G ethanol was estimated to be 1.42 USD/LGE.	[78]

Nevertheless, it has been well established in many research studies that the cost of the enzymes is one of the major bottlenecks in the establishment of the 2G ethanol industry [87]. The cost of such enzymes can be decreased with in-house enzyme production processes. However, there are multiple facets of this research question, as mentioned in Table 3. The first and most important question is about the ideal microbial strains and the extent of enzyme production [79,80,82]. As mentioned earlier, there are many different cellulases and hemicellulases and only a few microbial strains can produce a wide range of such enzymes [66]. Furthermore, the feedstock analysis is required to check if a particular type of microorganism will produce more enzymes with that type of feedstock. Such questions can be answered through research and analysis.

Another research aspect of on-site enzyme production is the technoeconomic analysis (TEA). TEA is a special technique where a systemic model is designed to understand all the technical and economic details of the underlying processes. The cost of each input stream, technical process, product preparation, and waste management are calculated which gives the unit production cost of the final product. In this particular scenario, the cost of 2G ethanol is required where the base case could be the enzymes purchased from an offsite production facility. On-site enzyme production, however, will need its feedstock, pretreatment step(s), and fermentation. This fermentation would be different from the simultaneous saccharification and fermentation step from the 1G ethanol process as, most of the time, the microorganisms producing cellulases and hemicellulases require different culture conditions than yeast [11].

In the literature, several feedstocks have been proposed for on-site enzyme production process. The ideal feedstock has enough simple sugars for the minimal growth of the fungal strains, but it should also have some cellulose and hemicellulose fibers to induce the production of enzymes [11]. For this purpose, two strategies can be adopted: either have a feedstock different from the main lignocellulosic feedstock for ethanol production or use a fraction of the main lignocellulosic ethanol feedstock for enzyme production. Both strategies have been reported in the literature [78,84]. A separate inexpensive feedstock can reduce the unit production cost, while a fraction of the main feedstock will make the process of transport and pretreatment easier.

Another strategy, that is proposed by the authors, is the use of DDGS as the feedstock for enzyme production. DDGS is an ideal feedstock for enzyme production [14]. However, DDGS is a byproduct of the 1G ethanol process so it would be wiser to make the enzymes when the DDGS is still in its wet distillers' grains with solubles (WDGS) form by establishing an enzyme production process at the 1G ethanol facility. The on-site enzyme production from DDGS has been proven to work with *A. niger* NRRL 330. The further optimization of pretreatment and media has also proven to increase enzyme productivity in submerged fermentations [58,72]. The enzymes are of a wide range and can be used to hydrolyze lignocellulosic biomass to produce 2G ethanol. However, the most prominent advantage of using DDGS is the establishment of a biorefinery where 1G and 2G ethanol can be produced simultaneously, using DDGS as the enzyme feedstock to reduce the cost of fermentation and downstream processing steps. Another reason is the reduction of cost in drying WDGS for its transport to longer distances for its usage as animal feed. Therefore, an integrated biorefinery where 1G and 2G ethanol are produced with a mid step of enzyme formation from DDGS can help in the overall reduction of cost and greenhouse gas reductions.

9. Integration of 1G and 2G Bioethanol Refineries

The integration of 1G and 2G ethanol processes has been proposed in various research articles [88–90]. The main advantage is the use of a single fermentation step with both C-5 and C-6 fermentation strategies [60,61]. Different microorganisms are needed for C-5 and C-6 fermentation as the typical *Saccharomyces cerevisiae* strain that is used for 1G ethanol production is not capable of fermenting C-5 monosaccharides such as xylose. Therefore, other microbial strains such as *Zymomonas mobilis* can be used for the complete fermentation of the pretreated slurry [91]. Other advantages may include low cost, improved energy

utilization, and overall economics [88]. The integration, however, is often talked about in terms of a few or a single step as evaluation of the whole process at an industrial scale and is impossible without the presence of an actual 1G + 2G ethanol industry. These steps have been discussed in the previous sections of this review. Theoretically, the integration of 1G and 2G ethanol processes is very desirable and convenient because of the advantages stated above. Therefore, several research ideas have been presented in the literature in recent years.

Some of these ideas were reviewed by Ayodele et al. [90] and Susmozas et al. [16]. One strategy is the energy efficiency resulting from integrating the input and output energy streams from various processes and thus utilizing the waste heat [90]. Another is the common downstream processing steps along with the fermentation which can further help in the reduction of overall processing costs [90]. Susmozas et al. [16] discussed the advantages of co-locating and retrofitting in the integration of 1G with 2G. With co-location and several common processing steps, the cost of the two processes can be decreased efficiently. For example, the co-location near a corn field can help in the cost reduction of the transport of corn grains and corn stover (as 2G feedstock) can decrease the cost and increase the efficiency of the process. In another study conducted by Erdei et al. [92]., the advantages of mixing the concentrated sugar stream of 1G with the diluted 2G stream are often discussed; the upstream sugar concentration of 1G ethanol production is decreased for acceptable ethanol concentrations in the final stream due to increased toxicity to the microorganisms. On the other hand, the 2G feedstock has low sugar concentration upstream, and therefore combining the two can help in the overall economics and increase energy efficiency. While all the strategies mentioned here can work best theoretically, more research is needed for the evaluation of each of such ideas.

The main idea that is presented in this review is the use of DDGS, which is a byproduct of 1G ethanol, as the feedstock for lignocellulolytic enzyme production, as shown in Figure 3. Among other advantages such as common downstream processing steps and fermentation steps, the feedstock for the enzyme production is readily produced at the site and does not need to be transported from other locations. In addition, since DDGS, before the drying step (wet distillers' grains with solubles or WDGS), is used, a reduction in the heat can be expected in addition to the lack of need for wastewater management at this stage since the water in the WDGS can be used for the enzyme production step. Another advantage is the use of corn stover as 2G ethanol feedstock, thus creating more value from the same amount of corn crop that is cultivated to produce ethanol. This strategy can also help in the adaptation of 2G ethanol at industrial scales, which needs high capital investments. With reduced costs and common processing steps, the adaptation of 2G ethanol plants becomes easier.

The WDGS leaving the 1G ethanol production process can be prepared for enzyme production by using a mild pretreatment strategy such as dilute acid hydrolysis, as mentioned by Iram et al. [58]. The results of this study indicate that a sulfuric acid concentration lesser than 5% (w/w) is desired along with the lowest possible solid load. After pretreatment, a centrifugation step reveals the recovery of remaining DDGS, which can be sold as the byproduct of the process (Figure 3). After the initial upstream processing steps, fungal fermentation can be completed with the optimized media and fermentation conditions [48,50]. For example, in the study conducted by the authors with one of the selected fungal strains, the addition of nitrogen sources (11 g/L yeast extract, 5 g/L peptone and 2 g/L ammonium sulfate) increased the enzyme production by many folds [72]. The optimization of culture parameters (310 rpm agitation rate, 6.5% inoculum size, and 1.4 vvm aeration) further improved the enzyme production for this strain [74]. The enzyme stream from this industrial process can then lead to the 2G feedstock stream for cellulose and hemicellulose hydrolysis into simple sugars. These sugars can then be combined with the upstream of 1G ethanol to produce more ethanol (Figure 3).

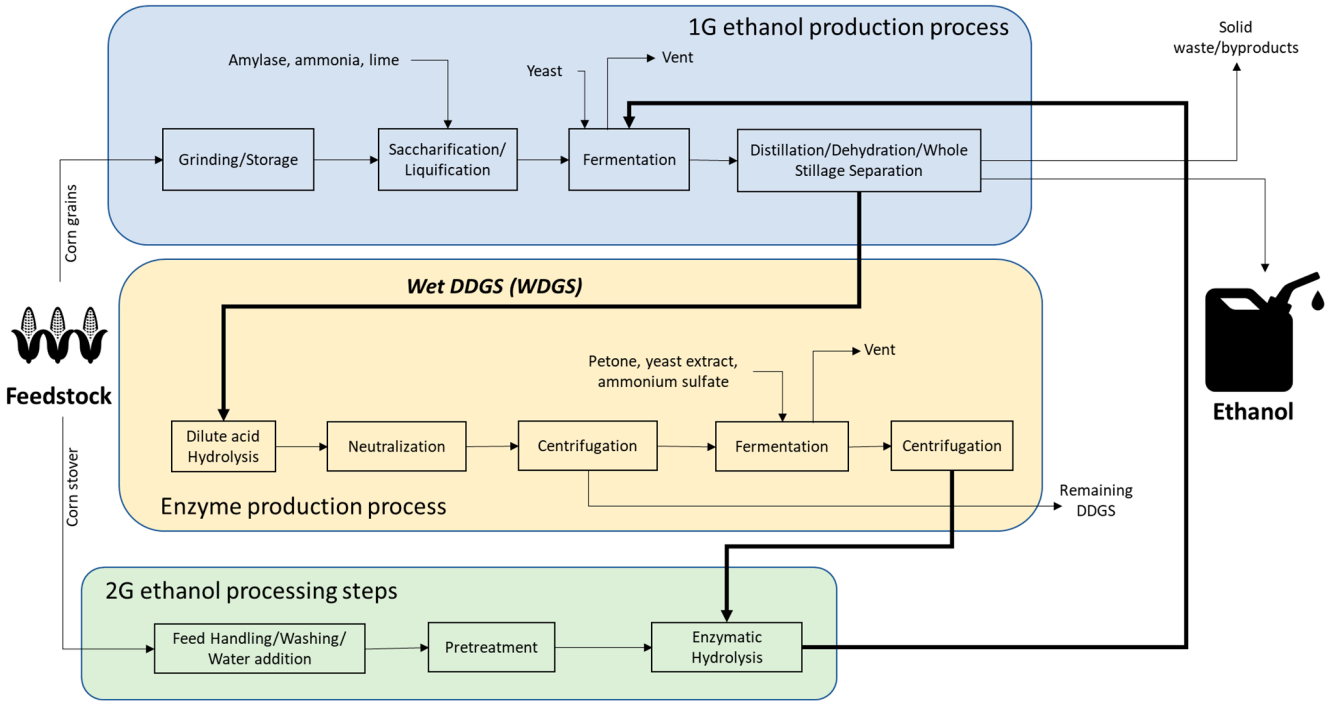

Figure 3. The integration of 1G and 2G ethanol via the production of lignocellulolytic enzymes using DDGS.

Some of the advantages described above have been analyzed with the help of technoeconomic and life cycle analyses. However, there is still a need for several studies to further establish the superiority of such integration to the independent 1G and 2G ethanol processes. The simplified integration model, shown in Figure 3, is a roadmap for a process that is several folds more complex with many steps having more than one input and output stream. Nevertheless, the study of such ideas can further help in the establishment of a bioethanol production system at industrial scales, which can further decrease greenhouse gas emissions and production costs.

10. Concluding Remarks and Future Trends

The integration of the 1G bioethanol production process with the 2G ethanol production process is possible if the enzymes are produced on-site by using DDGS as the feedstock for enzyme production. Major steps in the enzyme production process, however, need to be optimized. For example, a mild pretreatment method such as dilute acid hydrolysis with less than 5% (*w*/*w*) sulfuric acid and the lowest possible solid loads can release sufficient reducing sugars in the media to induce enzyme production. In addition, further media optimization is also required along with the optimization of the scale-up process. There have been several studies reported in this review for such prospects. The media optimization is reached with the help of nitrogen source addition such as yeast extract, peptone, and ammonium sulfate. The culture parameter optimization is conducted and reported in another study where inoculum size (6.5%), aeration (1.4 vvm), and agitation (310 rpm) are optimized for *A. niger* (NRRL 330). The authors then give a comprehensive review of their research about the use of DDGS as the feedstock for lignocellulolytic enzyme production, which can then be used as a bridge for the integration of 1G with 2G ethanol production processes. The evaluation of three pretreatment methods with the optimization of several media elements and culture parameters was conducted to check the suitability of DDGS as the enzyme feedstock. Major technological hurdles include the difficulty of fungal fermentation in submerged fermentation and further optimization at larger scales. In addition, in the next steps, comprehensive studies such as technoeconomic and life

cycle analyses of such integrations should be performed. Several novel techniques for the improvement of the enzyme production process should also be analyzed. Some examples of such techniques are genetic modification, the application of microparticles, and fungal strain improvement strategies. The research is ongoing with regard to these concepts to help improve industrial bioethanol production.

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