Review

Production of Bioethanol—A Review of Factors Affecting Ethanol Yield

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Abstract: Fossil fuels are a major contributor to climate change, and as the demand for energy production increases, alternative sources (e.g., renewables) are becoming more attractive. Biofuels such as bioethanol reduce reliance on fossil fuels and can be compatible with the existing fleet of internal combustion engines. Incorporation of biofuels can reduce internal combustion engine (ICE) fleet carbon dioxide emissions. Bioethanol is typically produced via microbial fermentation of fermentable sugars, such as glucose, to ethanol. Traditional feedstocks (e.g., first-generation feedstock) include cereal grains, sugar cane, and sugar beets. However, due to concerns regarding food sustainability, lignocellulosic (second-generation) and algal biomass (third-generation) feedstocks have been investigated. Ethanol yield from fermentation is dependent on a multitude of factors. This review compares bioethanol production from a range of feedstocks, and elaborates on available technologies, including fermentation practices. The importance of maintaining nutrient homeostasis of yeast is also examined. The purpose of this review is to provide industrial producers and policy makers insight into available technologies, yields of bioethanol achieved by current manufacturing practices, and goals for future innovation.

Keywords: bioethanol; fermentation; biofuels; solid-state; submerged; very high gravity; yeast

1. Introduction

Continued growth of the global economy has increased both energy consumption and concern regarding the accumulation of atmospheric greenhouse gases, and their effects on climate change. In response, many countries are developing renewable energy, including biofuel production. Biofuels are any fuels produced from biomass, such as organic waste materials [1], and such fuels can have a significantly reduced ecological footprint compared to traditional fossil fuels [2]. One such biofuel is bioethanol, the production of which is projected to surpass 130 billion liters/year worldwide [3], with the United States and Brazil supplying most of the world's ethanol [4]. Bioethanol is ethanol (an alcohol) produced through microbial fermentation of carbohydrates from plants or algae (e.g., corn, sugarcane, wheat, lignocellulosic biomass, etc.).

Microbial fermentation is a natural process used to break larger organic molecules into simpler ones. Prior to alcoholic fermentation, pretreatment processes may be required to prepare the biomass for extraction and fermentation. After preparation, enzymatic hydrolysis can then release fermentable monosaccharide and disaccharide sugars. Yeast then converts these sugars (e.g., glucose, galactose, and fructose) to ethanol, carbon dioxide, and other by-products in metabolic processes that can occur under both aerobic and anaerobic conditions. For example, glucose molecules produce two molecules of pyruvate during glycolysis. The two molecules of pyruvic acid are then reduced to two molecules of ethanol and carbon dioxide [5]. Under anaerobic conditions, pyruvate can be metabolized

to acetaldehyde with the release of carbon dioxide. Subsequently, acetaldehyde can then be reduced to ethanol by alcohol dehydrogenase [6].

Traditional alcoholic fermentation (first-generation bioethanol production) has used food crops as feedstocks (e.g., wheat, corn, potatoes, beets, sugarcane), as these materials are superior sources of easily accessible starch and sugar required for fermentation. However, as the global population grows and the amount of arable land remains limited, there has been increasing concern regarding fuel production from food crops. Therefore, non-edible sources of biomass, such as lignocellulosic materials and algae, are being explored as resources for environmentally sustainable bioethanol production. As a result, bioethanol production can be accomplished using an increasingly wide array of feedstock materials. With improved ethanol production technology, it has become possible to produce ethanol from a greater range of biomass resource materials. Fermentation technology that allows bioethanol production from a previously untapped biomass resource is often designated as a new generation. Approaches for biomass production are also grouped based on factors related to the fermentation conditions. The concentration of water and sugar in the fermentation media and the use of batch or continuous processes are used in grouping fermentation technology. Additional techniques can also be applied to the fermentation media to further optimize ethanol yields. The purpose of this review is to describe current knowledge of fermentable materials and fermentation technologies used in bioethanol production. In addition, this review considers various other factors that influence ethanol yield.

2. Bioethanol Production Processes

Currently, industrial bioethanol production is divided into three generations based on the type of feedstock used (Figure 1) [7]. The processes involved in all biofuel generations include: (1) pretreatment, (2) hydrolysis (although not required in the fermentation of sugar cane), and (3) conversion of sugars to bioethanol via fermentation. Some feedstocks require pretreatment conditions (i.e., lignocellulosic feedstock and algal biomass) to release fermentable sugars into the media. Without pretreatment, fermentation progress can be slowed due to limited availability of fermentable sugars for metabolism. Furthermore, genetics of feedstocks can contribute to variations in sugar content and influence fermentation ethanol yield [8]. Currently, fourth-generation bioethanol production methods are being investigated, which utilize genetically engineered organisms to enhance fermentation efficiency. However, these approaches are not yet implemented at an industrial scale.

First-generation bioethanol is derived through the fermentation of biomass containing high levels of starch (e.g., wheat, corn) and/or sugar (e.g., sugar cane, sugar beet). The industrial production of fuel and potable ethanol using first-generation technology is widely practiced commercially in many countries, although the preferred feedstock varies. The most common feedstock in the United States is corn [9], while in Canada both corn and wheat are widely used [10]. In Brazil, sugarcane is the common feedstock [11], and in Europe, the ethanol industry most commonly uses potatoes, wheat, and sugar beets [12]. These high-quality inputs require little pretreatment to afford relatively high ethanol yields (Table 1). Production of ethanol by first-generation technology and feedstocks is criticized for the consumption of crops which might otherwise be used as food for human or feed for animal consumption [13]. Nevertheless, bioethanol production can offer a means to process crops into useful products and to recover damaged grain that might otherwise be wasted [14]. For example, due to the presence of mycotoxins (e.g., deoxynivalenol), grain infected with Fusarium head blight is potentially toxic to humans or other animals. However, contaminated grain can be detoxified by yeast-based fermentation followed by nutrient recovery using insects (e.g., black soldier fly larvae; *Hermetia illucens*) [15] and lactic acid bacteria [16]. Approaches that reclaim grain products that would otherwise be lost can minimize economic loss, especially due to this fungal disease in cereal grains such as wheat and barley [15,16]. After detoxification, insects and lactic acid bacteria could potentially be used in producing protein feed supplements for domestic animals. Fermen-

tation byproducts from edible crops are also seen as improved feed products. Wet distillers' grain produced from fermenting cereal grains has a much higher protein content on a dry basis than the original grain. Wet distillers' grain can be blended directly into animal feed or mixed with distillers' solubles, another fermentation by-product, and further dried to be sold as an inexpensive feed for livestock.
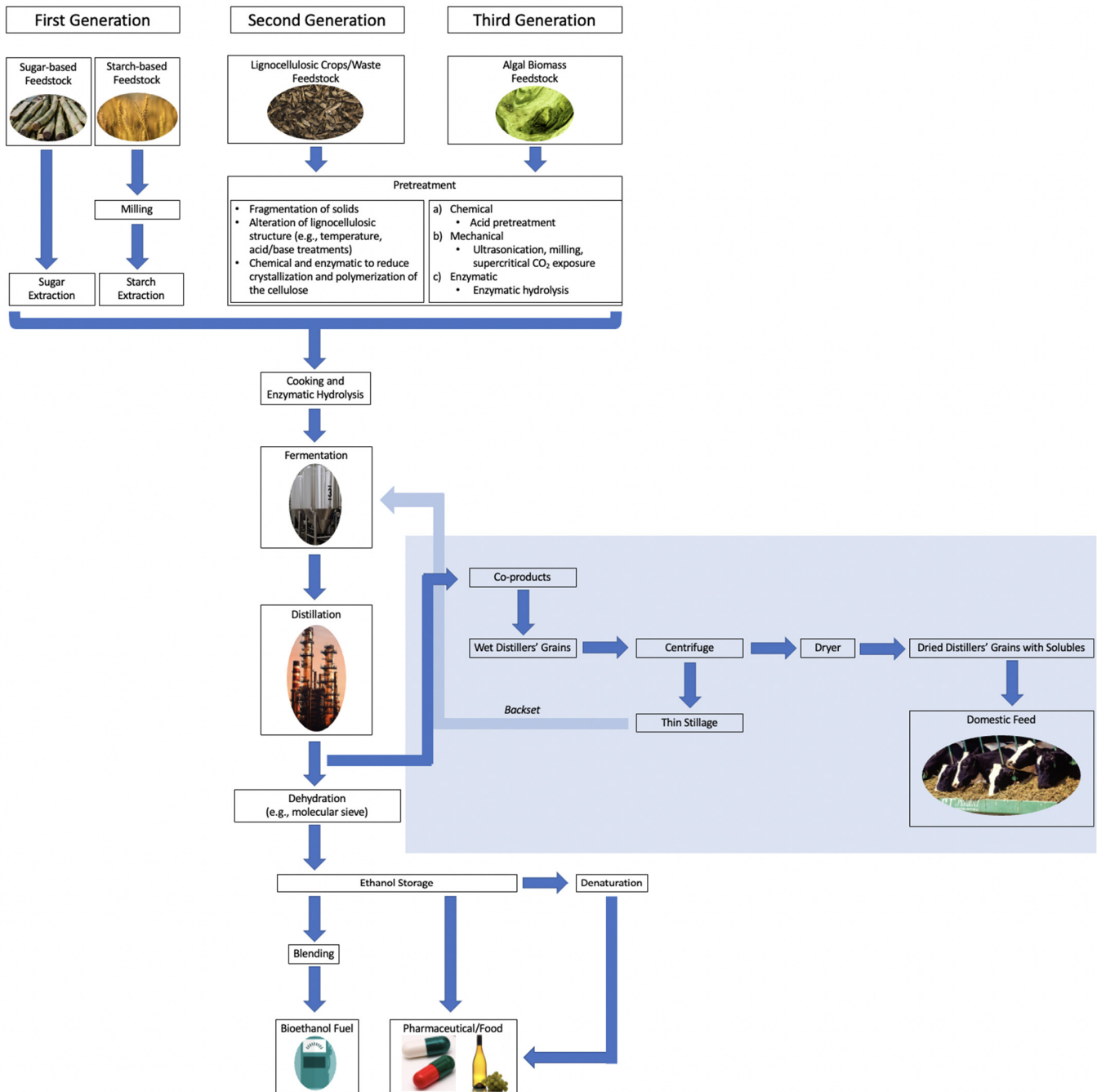
Figure 1. General flowchart of bioethanol production, providing a comparison of the pre-fermentation processing of feedstocks for the first three generations of bioethanol production. The blue highlighted area provides an example of a value-added process that can enhance the value of bioethanol production.

In contrast to the high starch or sugar content found in first-generation feedstocks, second-generation bioethanol typically utilizes non-edible feedstocks [7], such as lignocellulosic materials and agricultural forest residues (e.g., wood) [13,17]. Although the use of these feedstocks for ethanol production does not directly compete with food production,

second-generation feedstocks require more advanced technologies and facilities [16] to process them prior to fermentation [18]. Lignocellulosic biomass sources are predominantly composed of cellulose, hemicellulose, and lignin. These molecules often form highly recalcitrant structures due to their strong covalent bonds and extensive van der Waal and hydrogen bonding [19]. This makes lignocellulosic biomass more resistant to chemical and biological breakdown, and therefore, pretreatment processes must be implemented to disrupt lignocellulose structures prior to beginning biorefinery and fermentation processes [19]. Typical pretreatments can include physical (e.g., milling, temperature, ultrasonication), chemical (e.g., acid and alkaline treatments, organic solvent treatments), physicochemical (e.g., steam or CO_2 explosion treatments), or biological (e.g., enzymatic hydrolysis) processes. Cellulose, hemicellulose, and lignin content vary among feedstocks [19]. This variability might necessitate different approaches for pretreatments [20]. After successful pretreatment, cellulose can be hydrolyzed to sugars and converted to bioethanol via fermentation [21]. Ethanol yield for second-generation bioethanol feedstocks is also highly variable, and feedstock dependent (Table 1).

Third-generation bioethanol utilizes algal biomass for ethanol production [22]. Employing algae as a bioethanol feedstock can be advantageous, as algae can rapidly absorb carbon dioxide, accumulate high concentrations of lipid and carbohydrates, be easily cultivated, and require less land than terrestrial plants [23]. Like second-generation bioethanol, third-generation bioethanol production also requires pretreatment to disrupt algal cells. Such treatments can involve chemical (e.g., acid treatments) or physical (e.g., mechanical forces) pretreatment processes that destroy or disrupt algal cell walls. After pretreatment, complex carbohydrates are more readily converted to fermentable sugars via enzymatic hydrolysis, through a process known as saccharification [24]. However, inadequate pretreatment and saccharification conditions can result in the formation of side products (e.g., formic acid, acetic acid, and furanic compounds) [24–27]. This approach is further complicated by the highly variable composition of neutral sugars, amino sugars, and uronic acids in different algal species [28]. Pretreatment processes are, therefore, highly dependent on the algal species used and their composition. In general, large species of algae (macroalgae), which naturally grow anchored to the seafloor and can be many meters in length, contain fibrous material which requires extensive physical/chemical treatments before starches/sugars are released. In contrast, microalgae are much smaller, often unicellular, and can contain much higher amounts of sugar. However, they cannot easily be recovered from water like macroalgae, which can lead to significant inputs to dewater the algal biomass prior to processing [29]. Ethanol yields per gram algal dry matter are variable, and some have observed lower bioethanol quantities compared to first- and second-generation bioethanol feedstocks. Although production of third-generation bioethanol is less significant than the first two, the technology involved is also more recent, and algal species are being surveyed and engineered to identify and breed more productive species or mixtures of species. Third-generation bioethanol production may continue to grow through future improvements to existing technology, or in combination with other applications such as producing bioethanol from algae also used to treat wastewater.

Technoeconomic evaluations, (e.g., economic benefits, process design, etc.) [30], among the three generations of biofuel processes demonstrated that first-generation biofuels are currently the preferred option for commercial biofuel production [31]. Second-generation biofuel processes are becoming competitive. Further process developments will lower production costs (e.g., lower cost of enzymes) and increase coproduct utilization (e.g., electricity) [32]. Furthermore, integrating both first- and second-generation biofuels can maximize ethanol yields and revenue, and would also require less capital investment to integrate lignocelluloses processes into an industrial design [33]. Finally, third-generation biofuels using microalgae are being considered as the best alternative for biofuel production due to the lower land requirement and high potential to capture CO_2. However, utilization of these feedstocks requires improvements to reduce costs and increase economic sustainability [34].

Currently, fourth-generation bioethanol production methods are in development and utilize genetically engineered organisms (e.g., yeasts and algae) in combination with other methods of improving fermentations such as high-yielding biomass (with low lignin and cellulose contents) [35]. Although fourth-generation methods vary wildly, some fourth-generation bioethanol production methods capture CO_2 emissions throughout the production process using oxy-fuel combustion (a process in which fossil fuels are burned with an oxygen-enriched gas mixture instead of air) [36–38]. This produces flue gas mixtures comprised primarily of CO_2 and H_2O. This method provides an opportunity to capture CO_2 directly by physical compression and cooling (e.g., distillation processes) [38]. Unfortunately, current commercial processes require high energy inputs and are economically infeasible [35]. Another example of fourth-generation bioethanol production is electro-fermentation, in which electrical energy is used to help regulate respiration in genetically engineered algae through the transfer of electrons [36]. These methods are not currently used by industry and represent a substantial shift away from the more traditional bioethanol production processes.

Table 1. Approximate ethanol yields from different feedstocks [39–44].

Bioethanol Generation	Biomass Source	Ethanol Yield (L/t)
First	Sugar beet	110 (L/t) [40]
First	Sugar cane	70–75 (L/t) [40]
First	Cassava	137–180 (L/t) [40]
First	Maize	400 (L/t) [40]
First	Rice	430 (L/t) [40]
First	Wheat	340 (L/t) [40]
Second	Corn stover	362–456 (L/t) [39,41]
Second	Wheat straw	406 (L/t) [39,41]
Second	Sugarcane bagasse	318–500 (L/t) [39,41]
Second	Switchgrass	392–457 (L/t) [39]
Second	Sorghum	268–380 (L/t) [39,41]
Second	Poplar	419–456 (L/t) [39]
Second	*Agave*	347 (L/t) [39]
Second	*Agave Americana*	347 (L/t) [39]
Second	*Agave tequilana*	401 (L/t) [39]
Second	*Agave tequilana* leaves	401 (L/t) [39]
Second	Juice from *Agave americana* leaves	34 (L/t) [39]
Second	Juice from *Agave tequilana* leaves	30 (L/t) [39]
Second	Corn grain	470 (L/t) [39]
Second	Rice straw	416 (L/t) [39]
Second	Cotton gin trash	215 (L/t) [39]
Second	Forest thinnings	308 (L/t) [39]
Second	Hardwood sawdust	381 (L/t) [39]
Second	Mixed paper	439 (L/t) [39]
Third	Microalgae	167–501 (L/t) [42] *
Third	Brown seaweeds (macroalgae)	12–1128 (L/t) [43] **
Third	Seagrass (macroalgae)	747 (L/t) [43] **
Third	Green seaweeds (macroalgae)	72–608 (L/t) [43] **
Third	Red seaweeds (macroalgae)	12–595 (L/t) [43] **

* Denotes conversion of L/ha to L/tonne assuming 8–10% energy conversion efficiency (taken from Benedetti et al., 2018) [44]. ** Denotes conversion from g/g to L/t.

3. Very High Gravity, Solid-State, and Submerged/Liquid Fermentation

There are three main strategies for fermentation used in commercial bioethanol production: submerged/liquid state fermentation, solid-state fermentation, and very high gravity fermentation. A single type of feedstock might be fermented using any of those approaches, however, some strategies are more suited to a particular feedstock than others based on its properties (Table 2).

Table 2. Comparison of three fermentation strategies.

Submerged/Liquid-State Fermentation	Solid-State Fermentation	Very High Gravity Fermentation
• Uses liquid medium to grow microorganisms • Requires larger operational footprint • Increased usage of water and energy • Better monitoring and ease of handling • Shorter fermentation time • High waste generation • High ethanol yield	• Uses solid substrate to grow microorganisms • Smaller vessels • Less water and energy requirements • Not easy to monitor or change parameters • Longer fermentation time • Reduced waste generation	• Uses increased concentrations of sugar substrate to increase final ethanol concentration in the medium • Less water and energy requirements

3.1. Submerged/Liquid State Fermentation

Submerged fermentation refers to processes where the fermentable substrate is substantially liquefied, and microbes are grown in that liquid substrate. This type of fermentation is commonly used in first-generation bioethanol production [45], as ground starch/sugar rich materials can be mixed with water and the starchy materials further liquefied via cooking and enzymatic hydrolysis. This results in a liquid medium in which sugars and various nutrients are either dissolved or suspended as particulate solids. Submerged fermentation is utilized in other bio-industrial processes including enzyme production as this process can quickly afford a high yield of bioactive metabolites (Table 1). Unfortunately, this process can have disadvantages including requirements for inputs of energy and water, requirements for large volume bioreactors and distillation columns, and generation of large volumes of waste or low-value coproducts (e.g., thin stillage and wet distillers' grains). Fortunately, the waste by-product wet distillers' grains can be centrifuged to remove the excess thin stillage, the thin stillage can be dried with modest efficiency to distillers' solubles, and the solids dried to distillers' dried grain. These drying processes lead to three products that are used as feed ingredients: distillers' solubles, distillers' dried grains, and distillers' dried grain with solubles (the latter being a combination of the former two products). Thin stillage can also be provided as a water substitute for cattle in nearby feed lots or be processed via further microbial fermentation to produce a high-quality protein feed. A benefit of this latter technology is the conversion of low-value glycerol to the higher-value compound 1,3-propanediol [46,47].

3.2. Solid-State Fermentation

Solid-state fermentation (SSF) is a process in which organisms grow on non-soluble material or solid substrates in the absence of near absence of free water [48]. Solid-state fermentation is currently used for a wide range of applications in addition to bioethanol, including the production of enzymes, antibiotics, bioactive compounds, organic acids, and biodiesel [49]. The SSF process is affected by many factors including type of microorganism, substrate used, water activity (to prevent the growth of nuisance organisms), temperature, aeration, and bioreactor used [50]. The most common organisms used for SSF are filamentous fungi (e.g., *Trichoderma* and *Aspergillus*), as solid matrices better simulate the natural habitat of some fungi [51]. Nevertheless, SSF is also used with single-celled organisms such as yeast and bacteria [52]. Second-generation bioethanol production often involves solid-state fermentation of waste material and other feedstocks. The second-generation bioethanol feedstocks listed in Table 1 are all fermented using SSF technologies, except for agave.

SSF is frequently used to process large quantities of waste produced by agricultural-based industries [50], which may have poor nutritive value (e.g., low digestibility, crude protein, and mineral content) [53]. These residues are often disposed of via burning or dumping [50], which can lead to greenhouse gas release and other environmental impacts. Many of these substrates contain lignin, cellulose, and hemi-cellulose molecules,

which can be used to produce ethanol when fermented (Table 3). However, due to the complex lignocellulosic structures, saccharification of these materials to make them suitable as substrates for fermentation requires significantly more processing than for starchy materials. Cellulose is derived from linkages of D-glucose subunits which are linked by β-1,4 glycosidic bonds [54], whereas hemi-cellulose is a polysaccharide composed of D-xylose, D-mannose, D-galactose, D-glucose, L-arabinose, 4-O-methyl-glucuronic, D-galacturonic, and D-glucuronic acids linked by β-1,4 and sometimes β-1,3 glycosidic bonds [54]. To make these sugar linkages accessible, the recalcitrant structure of lignocellulosic must be disrupted via mechanical or physiochemical pretreatment processes (e.g., steam explosion and acid/alkaline treatments). Acid prehydrolysis followed by enzymatic hydrolysis is then required to saccharify the substrate. Implementation of these pretreatment processes is feedstock dependent as the composition of cellulose, hemi-cellulose, and lignan depend on the agro-industrial waste used [50].

Table 3. Examples of fermentable agro-industrial residues.

Agricultural Residues		Industrial Residues
Field Residues	**Process Residues**	
Straw	Husks	Potato peels
Stalks	Seeds	Orange peels
Leaves	Bagasse	Cassava peels

Another difference between submerged fermentation and SSF is related to enzyme use. Submerged fermentations typically rely on large initial doses of enzymes for saccharification, whereas SSF processes releases reducing sugars continuously through enzymatic cellulose hydrolysis. Reducing sugars are fermented to ethanol in a process referred to as simultaneous saccharification and fermentation, where enzymatic hydrolysis and fermentation occur in a single step, thereby increasing ethanol yields by minimizing product inhibition and reducing the need for separate saccharification and fermentation reactors. However, the optimum temperature for enzymatic hydrolysis is typically greater than the fermentation temperature; thus, to fully incorporate this hybrid method it is important to identify a temperature range that is compatible with both hydrolysis and fermentation [55].

To achieve simultaneous saccharification and fermentation, a combination of filamentous and thermotolerant fungi (e.g., *Trichoderma* and *Aspergillus*) or bacteria (e.g., *Streptomyces*) [56] and yeast (e.g., *Saccharomyces cerevisiae*) is often utilized [57]. Thermotolerant yeasts and bacteria are compatible with higher temperatures needed to improve enzymatic hydrolysis [58], which is often the rate-limiting step during the SSF process [59]. Microbial saccharification and simultaneous fermentation can reduce the need for expensive enzymes, although longer incubation times may be required and monitoring the internal temperature and maintaining the appropriate process conditions can be challenging. Solid-state fermentation strategies show great promise in utilizing agricultural wastes for bioethanol production [60], with simultaneous saccharification and fermentation helping to decrease costs and improve SSF ethanol yields for many feedstocks. Solid-state fermentation has been accomplished without supplementary nutrients [61,62].

Another hybrid approach is simultaneous saccharification and cofermentation. This technology primarily involves simultaneous consumption of two different substrates by some microorganisms [55]. However, this approach is challenging, as many organisms utilize substrates sequentially [63]. For example, a microorganism grown in the presence of both xylose and glucose might initially metabolize glucose more readily than xylose and will only begin consuming xylose when glucose concentrations are depleted. The sequential depletion of substrates can slow fermentation. Methods to alleviate this phenomenon include initial acclimatization of the microorganism to low glucose substrate and forcing the microorganism to utilize both substrates simultaneously [64]. Genetic engineering has also been investigated to explore this avenue in biofuels production [65].

Nonetheless, sequentially conducting solid-state fermentation for enzyme generation followed by hydrolysis on a second medium for submerged/liquid state fermentation is also being explored [66]. Combining these two technologies (SmF and SSF) can result in synergistic advantages as elaborated in López-Gómez and Venus (2021) [67].

3.3. Very High Gravity Fermentation

Very high gravity (VHG) fermentation is an emerging strategy using high-sugar musts or feedstock to increase ethanol production while minimizing production costs [68]. In general, sugar concentrations for ethanol production can be divided into normal gravity (<180 g/L total sugars), high gravity (180–240 g/L of total sugars), and very high gravity ($\geq$250 g/L of total sugars) [69,70]. In VHG fermentations, more than 30% of solids are consumed to achieve high ethanol concentrations [71]. Very high gravity fermentation can achieve more than 15% (*v/v*) of ethanol, compared to the average of 10–12% (*v/v*) that is observed in most distilleries [72]. The benefits of VHG fermentation include decreased waste production, energy consumption, and water consumption, resulting in reduced production costs as well as improved environmental sustainability. However, under VHG fermentation conditions, yeasts undergo multiple stresses due to increased metals and sodium ions, nutrient stresses (e.g., nutrient limitations, such as free amino nitrogen and dissolved oxygen), increased temperatures, acidic conditions, osmotic stress, and increased ethanol concentrations [72,73]. The increased osmotic pressure exerted on the yeast cells can result in intracellular ethanol accumulation, leading to decreased production efficiency and yeast-cell viability as fermentation progresses [72]. Although some yeasts (e.g., *S. rouxii*) are more tolerant to these osmotic stresses, their ability to produce ethanol is lower than *S. cerevisiae* [72]. Very high gravity fermentation usually involves feedstocks used in first- and third-generation bioethanol production and has the greatest potential for high ethanol yields compared to SmF and SSF (Table 1).

4. Batch, Fed-Batch, and Continuous Fermentation Modes

Another factor affecting fermentation outcomes is the batch type, which includes batch, fed-batch, and continuous fermentations (Table 4). The optimal batch type for a fermentation depends on the kinetics of the microorganisms used and the feedstock.

In batch fermentation, the microorganisms are typically inoculated to a fixed volume of medium in the fermenter. As the nutrients are consumed and the microorganisms reproduce, by-products accumulate. Once the nutrients are depleted, the fermentation is complete. As a result of the fixed initial nutrient input and continuous consumption of nutrients by microorganisms, the culture environment is continuously changing [74]. This type of fermentation typically produces a standard growth curve consisting of a lag phase, exponential phase, stationary phase, and death phase. The lag phase is the first major phase of microbial growth in batch fermentation when the organism begins to adapt to the new environment. During the exponential phase the organisms reproduce at a constant rate, resulting in an exponential increase in microbial growth (logarithmic growth phase). The cell growth rate is often substrate limited and can be due to products missing from the media (e.g., nutrient imbalance) or high substrate concentrations (e.g., excess sugar) [75], resulting in prolonged fermentation times and reduced ethanol yields [76]. Following the exponential phase, the microorganisms will enter the stationary phase where the number of cells reproducing and those dying reach an equilibrium, due to depletion of nutrients in the media (e.g., sugar) or accumulation of toxic by-products (e.g., ethanol toxicity) [77]. Once fermentation completes, a death phase may occur as the density of viable cells decreases. However, some industries avoid lag phases by pre-growing yeasts in smaller tanks with favorable conditions, then adding a large innoculum to the main fermentation [78]. This truncates the exponential phase and can improve fermentation efficiency. Nonetheless, batch fermentation has the advantages of typically being inexpensive, having low risk of contamination, and easier sterilization and management of feedstocks than other fermentation types. However, compared to fed-batch and continuous fermentations, batch

fermentations exhibit lower cell density [74], as nutrients are not supplemented during the exponential growth phase. There is also increased downtime due to frequent cleaning and sterilization of the vessels between subsequent fermentation batches. Batch fermentation is most used in long-term, small-scale, or solid-state fermentation processes [75].

Table 4. Comparison between batch, fed-batch, and continuous fermentation under a submerged/liquid state [74,79].

Batch	Fed-Batch	Continuous
Microorganisms are provided with a fixed volume of medium (nutrients and other ingredients). Culture environment is consistently changing as nutrients are consumed.	Media is inoculated with microorganisms which then grow under a batch regime for a certain amount of time, then nutrients are added incrementally throughout the fermentation.	Fresh media is continuously added to the fermenter, replacing the consumed nutrients. Ethanol, used media, and toxic metabolites are continuously removed.
Advantages:	**Advantages:**	**Advantages:**
• Low cost • Low risk of contamination • Less control required • Easier sterilization	• Maintenance of maximum viable cell concentration • Extended lifespan of cells • Higher ethanol accumulation • By-product accumulation is limited • Control of factors (e.g., pH, temperature, dissolved oxygen)	• Less downtime for vessel cleaning • Increased productivity • Lower cost • Higher degree of control • Ability to automate, more cost-efficient and less sensitive to human error.
Disadvantages:	**Disadvantages:**	**Disadvantages:**
• Lower cell densities, ethanol production • Longer downtime between batches due to cleaning, vessel setup, and sterilization	• Increased costs for process control • Longer downtime between batches due to cleaning, vessel setup, and sterilization	• Less control for non-growth-related products • Cell aggregation can prevent optimum steady-state growth • Long growth periods can increase risk of contamination • Can be difficult to maintain filamentous organisms due to viscosity and heterogeneity of the medium

Fed-batch fermentation is like batch fermentations, except nutrients are incrementally added to the fermenter throughout the fermentation [74]. The consistent addition of nutrients results in increased cell density during the exponential phase and thus enhances product yields. For example, continuous supply of sugars to yeast cells in the stationary phase can maximize ethanol yield. However, the maximum working volume of the fermentation vessel can limit the amount of fresh media/nutrient input. Yeast alcohol production is maintained by nutrient additions, which also reduce the risk of overflow metabolism or the risk of excreting metabolic by-products that could otherwise be used for catabolism or anabolism [77]. This type of fermentation is exceptionally useful when the desired product is correlated with microbial growth, such as bioethanol [74].

Finally, during continuous fermentation, fresh media/nutrients are continuously added to the fermenter at the same rate as ethanol, by-products, and toxic metabolites are removed from the culture [74,80]. During these processes, yeast is often recovered and returned to the fermentation vessel. If the medium is continuously fed at a suitable rate, a steady state is eventually achieved in the fermentation broth [80]. As a constant volume is achieved in continuous fermentations, the maximum working volume of the bioreactor does not limit the amount of fresh medium which can be added to the culture over the duration of the fermentation [74], unlike in fed-batch fermentations. Cultures in a steady state can last for extended periods of time (up to months) [74], thus reducing equipment downtime between batches, and improving economic yields by keeping the culture at an ideal state for ethanol production. However, due to the long fermentation time, continuous fermentation methods are prone to contamination and downstream processing can be difficult. Traditional methods of distilling ethanol from fermented media through heating

would result in the destruction of the microbial culture, so methods such as settling and/or filtering of yeast from the product stream before distillation are employed [75].

5. Yeast Stress

Most yeasts can convert a range of hexose sugars to ethanol via glycolysis. However, *Saccharomyces cerevisiae* is by far the most used yeast organism for alcoholic fermentation due to its robustness and tolerances. *S. cerevisiae* has several advantages over other yeasts as it is a facultative anaerobe capable of growing under both aerobic and anaerobic conditions in the presence of glucose [81] and is tolerant of elevated ethanol concentrations [82]. Under anaerobic conditions, *S. cerevisiae* will produce acetaldehyde, which is further reduced to ethanol [82].

During inoculation and fermentation, yeast cells are subjected to several stressors that can affect bioethanol yields, including biological (e.g., cellular ageing, microbial competition), chemical (e.g., toxicity from ethanol and its metabolites, pH), and physical stressors (e.g., temperature shock, osmotic pressure) [83]. Stress can result in increased mutations, microbial contamination, altered yeast flocculation, increased glycerol production, decreased ethanol production, and production of undesired compounds (e.g., flavor and aromatic compounds in fermented beverages) [84,85]. Poor activity and declines in yeast viability from stress can also cause stuck or sluggish fermentations. Fortunately, several methods have been developed to reduce these stresses including increasing fermentation temperature and pitching rate [86–88], nutritional supplementation [89–92], using mutant yeast strains [93], immobilizing yeast [94–98], and enhancing aeration efficiency [90,99]. Fermentation success is also influenced by various additional factors, including nutrition imbalances (e.g., nitrogen, vitamins, mineral deficiencies), medium composition (e.g., sugar concentration), and inoculum size.

Biotic stress factors (e.g., microbial contamination) can also affect fermentation efficiency. These factors primarily involve the presence of contaminating microorganisms, such as lactic acid bacteria (LAB) [100]. Lactic acid bacteria can not only compete to utilize available sugar, but also produce lactic acid, and other metabolites that can suppress fermentation [83]. These LAB are also capable of producing naturally antimicrobial compounds called bacteriocins. Bacteriocins can suppress the growth of other bacteria by disrupting transmembrane potential and forming pores in the membranes of sensitive cells [101]. This can provide LAB with competitive advantages against other bacterial organisms. There are also risks associated with competing yeasts producing toxins (e.g., ionophore-acting compounds) [83], which can contaminate the fermentation broth. Microbial contamination in an industrial fermentation can be highly problematic requiring extended shutdown of facility operations for cleaning and sterilization before the next fermentation.

Yeasts have developed various mechanisms that help them adapt to chemical and physical stresses. For example, in response to temperature stress yeast cells will produce the disaccharide trehalose to help stabilize their plasma membrane [84,102]. In high-sugar or -salt environments, yeasts will produce glycerol as an osmoprotectant, to reduce osmotic stress and protect the cells against lysis [103–105]. Glycerol is also produced to maintain the balance between the NAD^+/NADH ratio during cell growth [106]. Production of these metabolites can reduce ethanol synthesis efficiency, as more time is required for acclimatization to the fermentation media. Therefore, minimizing the acclimation period, by providing optimal growth media, can maximize ethanol yield [107].

The composition of the media and nutrients (e.g., concentration and type of sugars) can also influence fermentation efficiency. *Saccharomyces cerevisiae* is more effective at using glucose than fructose [108–110]. The presence of sugars that are slowly metabolized can affect the fermentation ethanol yield [111]. Accumulation of fructose can result in stuck or sluggish fermentations. Problems with fructose concentrations are more common with sugar cane and fruit-based feedstocks, such as in wine fermentation [112]. To address stuck fermentations, reinoculation of non-*Saccharomyces* yeast [113,114] (e.g., *Zygosaccharomyces bailii*)

capable of utilizing fructose [115,116] and tolerating elevated ethanol concentrations is typically employed [117].

As yeast consume medium nutrients, the concentration of ethanol increases. This increase in ethanol can result in physiological impairment. In the presence of excess ethanol (>10–20% *v/v*) [83,118–120], yeast can exhibit reduced cell viability and growth, such as a decrease in cell volume [121–123]. There can also be effects on yeast metabolism (e.g., stress-response proteins, lowered protein levels and denaturation) [124–128], cell structure, and membrane function (e.g., inhibition of endocytosis, loss of electrochemical gradients) [128–134]. Ethanol toxicity to yeast is primarily due to cell membrane damage [83]. However, maintaining an ion balance (e.g., magnesium and potassium) can provide the membrane with protective effects from ethanol toxicity and temperature changes [135–139].

Stress in yeast can be mitigated *via* physiological and genetic strategies. These can include maintaining nutrient availability and balance during fermentation through adaptive evolution or genetic modification [85,140]. For example, temperature and ethanol stress resistance in yeast can be enhanced by prolonged serial culture at elevated temperatures or with higher concentrations of ethanol [141]. Enhancing ethanol tolerance in yeast *via* genetic modification can be more difficult, as stress tolerant phenotypes are influenced by more than one gene [142], and the genetic background of *S. cerevisiae* can be complex. Yeasts can be aneuploids or polyploids, making genetic strategies for improvement challenging [143]. However, gene editing (e.g., clustered regularly interspaced palindrome repeats, CRISPR, and CRISPR-associated protein-9 nuclease, cas9) [144] and recombinant DNA techniques [85,142,145] can be utilized to circumvent these constraints and lead to greatly improve yeast strains. In fact, CRISPR-cas9 has successfully been utilized to increase yeast tolerance to ethanol [146], acetic acid [147,148], and temperature changes [149]. A commercially available strain of genetically modified *S. cerevisiae* has also been developed to secrete a heterologous glucoamylase that enables starch saccharification in SSF processes. This reduces the dependency on exogenous enzymes, while simultaneously enhancing the rate of fermentable sugar release for yeast metabolism [150]. Another genetic approach has been to attempt to construct new *S. cerevisiae* hybrids with improved stress tolerances (e.g., acetic acid tolerance) through hybridization, protoplast fusion, rare mating, and mutagenesis [151].

Nutrient and growth factor imbalances are also factors that affect fermentation efficiency and can result in stuck or sluggish fermentations. For example, *S. cerevisiae* requires oxygen for the biosynthesis of key membrane constituents such as sterols (e.g., ergosterol) and unsaturated fatty acids (e.g., oleic acid) [142], and help with stress tolerance. Therefore, lack of oxygen can reduce the ability for yeast to synthesize membrane components, reducing inoculum efficiency and leading to unsuccessful or incomplete fermentations.

Other typical imbalances that can occur include deficiencies in free amino nitrogen, vitamins, and minerals (e.g., zinc or magnesium). For example, it has been reported that insufficient free amino nitrogen (<150 mg/L) [152] and trace metals (e.g., zinc <0.1 ppm) can result in stuck fermentations [153]. This is because the terminal enzyme of fermentation, alcohol dehydrogenase, is a zinc-dependent enzyme [142]. This enzyme is responsible for reducing acetaldehyde to ethanol during glucose fermentation [154]. The bioavailability of these trace metals can be affected by the feedstocks' physicochemical properties, sometimes leading to precipitation, chelation, or absorption within the medium [142].

In addition, magnesium is another key element required for efficient fermentation. Deprivation of this essential metal can result in adverse cellular physiological effects (e.g., loss of protein conformation) [155]. This divalent metal is responsible for the activation of several enzymes involved in metabolic bioenergetic and biomolecular pathways (e.g., DNA duplication) [156]. Magnesium is also required in the maintenance of cellular structural integrity, yeast functionality, heavy-metal detoxification, and stress protection [122,157]. During fermentation, magnesium ions can improve cell viability by promoting tolerances to dehydration [138], elevated ethanol conditions [136], and heat shock [136,158], during the exponential and stationary growth phases [156]. These tolerances result from the re-

pression of stress-protein synthesis [158]. Increased biomass and ethanol yields have also been observed in the fermentation of lignocellulosic material in the presence of Mg^{2+} [159]. Although Mg^{2+} is an essential factor for yeast performance, it has also been observed to be an antagonist for Ca^{2+} and can influence and destabilize calcium complexes [160–162].

Other trace elements that are important for yeast physiology include Ba^{2+}, Fe^{3+}, Co^{2+}, Mo^{2+}, Ni^{2+}, and Cu^{2+} [156]. These trace elements are often required in small amounts (<10 mM) and are essential for the activation and modulation of several metabolic processes involved in yeast performance and survival [156]. Altogether, free amino nitrogen, minerals and trace metal elements, vitamins, and accessory growth factors are required for optimizing *S. cerevisiae* fermentation for ethanol production [142,156].

6. Conclusions

As the global demand for energy increases, bioethanol produced from renewable feedstock is a valuable and eco-friendly alternative to non-renewable fuels. However, with growing concerns over the global food supply, lignocellulosic non-edible biomass (second-generation bioethanol) and algal sources (third-generation bioethanol) are increasingly attractive feedstocks for bioethanol production. Pretreatment conditions are required for second-generation and third-generation feedstock to disrupt the recalcitrant lignocellulosic structure and algal cell wall, to make the fermentable sugars accessible. Fermentation efficiency and bioethanol yields are dependent on the feedstock, cultivar, and organism used. Biotic (e.g., microbial contamination) and abiotic factors (e.g., nutrient, trace metal, and vitamin deficiencies) must also be addressed to ensure optimum fermentation rate and extent. Different modes of fermentation can be utilized to address some of these concerns (e.g., fed-batch and continuous fermentation modes) and help alleviate yeast stress. Furthermore, supplemental additions (e.g., Mg^{2+} and other micronutrients) and adaptive responses can increase stress tolerance (e.g., heat shock and ethanol shock) on yeast organisms and improve fermentation performance. Altogether, it is important for industrial bioethanol producers to investigate requisite pretreatment conditions when determining a feedstock candidate, incorporate the different fermentation technological designs, and determine potential adversities that may occur during fermentation. When taken together, these steps optimize fermentation performance and maximize ethanol yield. Technoeconomic aspects should also be evaluated to investigate the feasibility, and economic impacts of implementing these technologies in the future production of biofuels, especially in analyzing and promoting the use of third-generation biofuels.

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