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Review

Reducing Carbon Intensity of Food and Fuel Production Whilst Lowering Land-Use Impacts of Biofuels

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Abstract: Science and technology are critical for developing novel and sustainable production of food, fuel, and chemicals in a manner that significantly reduces anthropogenic contributions to climate change. Although renewable energy is gradually displacing fossil fuels for grid energy, oil-based transport fuels remain major contributors to global greenhouse gas emissions. Currently, bioethanol and biodiesel can partially replace petroleum, but these renewables are far from perfect in terms of long-term sustainability and the volumetric expansion needed to fully replace oil. Biofuels made in biorefineries using sugars or oils derived from plants grown on prime food-producing land only partly offset CO_2 emissions relative to petroleum and present problems with respect to land-use change. Here, we provide alternative ideas for lignocellulosic biorefineries that coproduce bioethanol, nutritious protein-rich yeast biomass for animal feeds, and carbon-rich solid residuals that represent green coal or sequestered carbon. A concept of how these biorefineries could be linked to renewable power-to-X, where X can be bioethanol, protein, sequestered carbon, or multiple carbon-carbon based synthetic fuels and chemicals, is presented. We also discuss aspects of the present and future roles for microorganisms in lignocellulosic biorefineries and power-to-X bio/chemical refineries.

Keywords: carbon intensity; greenhouse gases; land use; biofuels; protein; bioethanol; biomass; microorganisms; *Saccharomyces* yeast

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1. Introduction

The combined challenges of global population growth and climate change are placing ever increasing stresses and constraints on our planet and its resources [1–3]. Climate change is disrupting agricultural systems via increasing floods, droughts, and fires at the same time as global demand for food is growing. We therefore face an environmental stress, food supply, and energy supply trilemma [4]. The continued improvement and adaptation of our crops and animals will be critical for a sustainable future as the human population grows [5]. Alternative solutions to provide clean non-fossil energies are also needed [6]. The pressure to achieve net zero greenhouse gas (GHG) emissions by 2050 is increasing. Carbon capture and storage is viewed by many as a direct means of sequestering emissions from heavy industry, fossil fuel energy production, and agriculture. It is estimated that about 5.6 gigatons per annum of CO_2 will need to be captured and stored by 2050 [7]. However, with current rates of deployment, CO_2 storage capacity by 2050 is projected to be only 700 million tons per year, which is only 10% of what is required. The reality is that deployment of technology to capture and store CO_2 underground is challenging and will not likely meet the 2050 requirements. Clearly, new ideas are required to future proof the environment, agricultural productivity, human, animal, and plant health, and the supply of potable water, food, energy, and chemicals.

The fossil fuel-based transportation sector is one of the major contributors to GHG emissions worldwide [8]. About 15% of global GHG and >20% of energy-related CO_2 emissions stem from this sector, leading to the need for renewable non-fossil biofuels. Bioethanol

and biodiesel represent the major biofuels currently available and contribute about four percent of total transport fuels worldwide. Bioethanol represents over 70% of these biofuels produced globally [9]. Bioethanol falls into four categories, namely first-, second-, third-, and fourth-generation. First-generation (1G) bioethanol is a well-established large-scale renewable liquid fuel with over 100 billion liters produced annually by fermentation of sugars derived from human food or animal feed crops such as starchy crops or sugarcane and beet [10,11]. Here, strains of the yeast species *Saccharomyces cerevisiae* are used to convert six-carbon sugars (glucose and fructose) into ethanol that is used directly to replace petroleum. Nevertheless, the reduction of GHG emissions from corn ethanol relative to gasoline is only c.a. 40%, which leaves considerable room for improvement if bioethanol is to become a net zero or negative GHG-emitting fuel [12]. Sugarcane ethanol provides a better GHG reduction, but still does not achieve net zero [13,14]. Reduction in GHG emissions, energy security, and opportunities for rural development are important drivers for renewable fuels, but there are concerns about increasing the production of biofuels. These concerns relate to inflated food prices and the risk of increased GHG emissions due to direct and indirect land-use changes (LUC) [15]. The growing demand for agricultural produce to meet expanding 1G biofuel production risks an increase in deforestation and use of land with a high biodiversity value, as well as associated usage of freshwater, fertilizers, and pesticides, with negative consequences for the environment [15,16].

It is obvious that sustainable alternative approaches to the generation of liquid transport fuels are required moving forwards. Here, second-, third-, and fourth-generation bioethanol will become increasingly important. These processes rely on non-food sources as substrates. Second-generation (2G) ethanol is derived from non-food biomass such as dedicated energy crops (e.g., *Miscanthus*, switchgrass, short rotation coppice, and other lignocellulosic plants), agricultural residues, forest residues, and other waste materials (e.g., corn stover, wheat straw, and sugarcane bagasse). Plant biomass is primarily composed of sugar-rich polymers cellulose and hemicellulose and the phenolic-based lignin [17,18]. Cellulose is a homopolymer of glucose and exists mainly in a highly ordered crystalline form. Hemicellulose is a partially acetylated heteropolymer consisting of five-carbon sugars (xylose and arabinose), six-carbon sugars (galactose, mannose, and glucose), and sugar acids. Xylose is the major hemicellulose component of agricultural residues such as bagasse, corn stover, straw, energy grasses, and hardwoods. Softwoods have a higher proportion of six-carbon sugars such as galactose and mannose. Lignin is a heteropolymer of phenylpropane monomers linked in various ways [19]. In general, lignocellulosic bioethanol from agricultural and forest residues has a greater potential to reduce GHG emissions than bioethanol from energy crops such as *Miscanthus* and switchgrass [15]. Whatever the source of biomass, it must first be depolymerized into its component sugars. This is achieved by various means of pretreatment to make the cellulose and hemicellulose polymers susceptible to enzymatic hydrolysis. The potential range of lignocellulosic biomass is vast and the methods for its depolymerization are varied, as recently reviewed by others [20–22]. The conventional model for producing 2G ethanol is discussed in Section 2. Whereas 1G and 2G ethanol rely on plant biomass, third-generation (3G) bioethanol aims to utilize algal biomass as feedstock. Here, micro- or macroalgae capture CO_2 by photosynthesis and are subsequently used as a source of carbohydrates for ethanolic fermentation [23,24]. Whilst laboratory and pilot scale works show promise for 3G ethanol, the commercial production of biofuels by algae faces many economic and technical bottlenecks, including investment costs, insufficient yields and productivities, high energy input for harvesting, and risks of contamination in open pond systems [25]. The concept of fourth-generation (4G) biofuels is to use synthetic biology to modify algae, cyanobacteria, and other microbes to produce photobiological biofuels and high-value chemicals. It combines photovoltaics and microbial fuel production by synthetic cell factories or synthetic organelles [26]. Although still in its infancy, 4G ethanol is an exciting prospect, suggesting a promising route to GHG negativity in transport fuel generation. For now, 1G and 2G ethanol are the primary sources

of this biofuel, with 1G ethanol being the major contributor representing about 94% of total bioethanol produced.

To significantly replace the role of oil in transport, we need to expand the sustainable production of renewable fuels considerably in a way that does not negatively impact food supplies, land use, or water and does not otherwise adversely affect the environment. Currently, 2G ethanol offers the best way forward, given the land-use limitations of 1G, the relative immaturity of 3G and 4G ethanol, and the urgency of the challenge. Here, we promote the idea of coproducing food and fuel in 2G biorefineries as a means of improving their economics, whilst also addressing the environment, food, and energy trilemma. We also discuss how a 2G food and fuel biorefinery can be linked within a power-to-X concept as we move towards negative GHG fuel emissions. Finally, we also discuss aspects of the present and future roles for microorganisms in lignocellulosic biorefineries and power-to-X bio/chemical refineries.

2. Conventional Second-Generation Bioethanol from Biomass Only Partially Addresses Environmental Issues

The conventional idea of a 2G facility is that it produces ethanol from the six-carbon and five-carbon sugars. A generic 2G process is presented in Figure 1. Whilst fermentation of six-carbon sugars by wild-type strains of *S. cerevisiae* is efficient, the anaerobic fermentation of five-carbon sugars requires genetically engineered strains of *S. cerevisiae* to be employed [27,28]. However, the use of genetic engineering to introduce five-carbon fermentation capability limits the applications of these yeast strains in jurisdictions that reject genetically modified organisms, especially within the food chain. Lignin and other residual or recalcitrant solids are used to cogenerate heat and power for the process. Wastewater streams are rich in organics that are used as substrates for anaerobic methanogenic microorganisms to generate biogas. The cogeneration of heat energy and biogas lowers GHG emissions by replacing the equivalent amount of fossil-fueled grid electricity, which improves the overall decarbonization result of 2G bioethanol processes. Depending on the feedstock used, bioprocess details, and efficiencies, 2G bioethanol can give about a 30% greater reduction in GHG emissions relative to 1G. Even though 2G bioethanol provides advantages over fossil fuels in terms of GHG emission reduction, it can still be argued that direct and indirect LUC are a problem as climate change, population, and economic pressures grow [29]. The conversion of arable land and deforestation to enable energy crop production is of particular concern, given negative impacts on the environment and food availability. Ideally, we would employ biofuel processes that add to, rather than compete with, net food production.

There remain technical and economic challenges for 2G ethanol. The capex for 2G plants is far greater than for 1G. For example, over USD 225 million is required to build a 30-million-gallon 2G plant, whereas a 50-million-gallon 1G corn-ethanol plant requires about USD 80 million [30]. The major cost differences are the requirement for pretreatment and hydrolysis of 2G biomass, relative to the hammer milling of 1G corn kernels. Despite this, several companies have progressed 2G ethanol beyond the laboratory and pilot scales. These companies include GranBio, Raizen, Poet-DSM, Beta Renewables, Synata Bio, DuPont, and Praj [31]. The minimal ethanol selling price (MESP) is the price at which the biofuel needs to be sold to achieve a net project value of zero within a defined period of time (typically 10 to 30 years). In a sugarcane facility using bagasse and assuming yields at 95% of the theoretical for steam pretreatment, enzymatic hydrolysis, fermentation, solid/liquid separation, and anaerobic digestion (biogas production), it was calculated that the most significant reduction in 2G MESP was achieved when pentoses were fermented to ethanol rather than biogas. This was followed, in decreasing order, by higher enzymatic hydrolysis efficiency, increasing water insoluble biomass solids to 30%, and shortening residence time (48 h) in enzyme hydrolysis [32]. In another study, 1G ethanol produced from A-molasses at sugar mills was most cost-effective (MESP of 0.52 cents per liter), while a 1G–2G option produced 98% more ethanol at a MESP of 0.62 cents per liter; 2G-ethanol, from biomass

only, gave a MESP of 0.72 cents per liter [33]. In comparative studies of 1G ethanol derived from corn and 2G ethanol from stover, the MESP for 1G was USD 3.18 per gasoline gallon equivalent, while for the colocated 2G facility it was USD 5.64 [34]. Thus, there continue to be significant economic challenges for producing ethanol from lignocellulosic biomass. One possible way to make 2G ethanol more economical is to produce valuable coproducts in the biorefinery to generate greater value from biomass substrates.

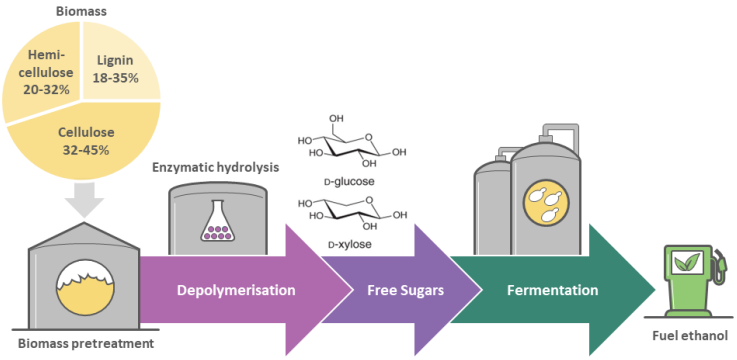

Figure 1. A generic 2G ethanol-only plant. Biomass is depolymerized using physicochemical and enzymatic processes to release sugars (mainly glucose from cellulose and xylose from hemicellulose). The free sugars are fermented anaerobically to ethanol using a microbe such as *S. cerevisiae*. The anaerobic fermentation of five-carbon sugars requires genetically modified yeast strains to be employed. This process produces a single product, namely ethanol. Solid wastes comprising recalcitrant cellulose and hemicellulose, as well as nonfermentables such as lignin, are burned in boilers, thereby reducing reliance on fossil fuel-derived power from the grid. Distillation of ethanol also produces a stillage that is a waste stream containing unmetabolized sugars, acetate, glycerol, furans, phenolics, and other organic compounds and mineral salts (ash). This waste stream requires processing to reduce the biological and chemical oxygen demand. Anaerobic production of biogas by methanogenic bacterial fermentation is one solution for the treatment of the stillage and wastewater.

3. A Food and Fuel Lignocellulosic Biorefinery That Sequesters Carbon

Here, we propose a process that uses renewable energy to drive the coproduction of fuel in the form of bioethanol from cellulose, food in the form of yeast biomass that is high in protein, and sequestered carbon in solid form that can be stored or used as a "green coal" (Figure 2). Not only does this approach provide better economics but it also addresses the environment, food, and energy trilemma. Others have demonstrated the ability of lignin materials to function in comparison with metallurgical coal in the steel-making process, thus indicating the potential for green coal as a feedstock for that industry [35]. In fact, lignin and its derivatives have significant potential in sustainable construction, allowing the partial replacement of petroleum products in, for example, cement composites, rigid polyurethane foams, paints and coatings, phenolic or epoxy resins, and bitumen [36].

Taking a working sugarcane mill as an example [37], a crush of 1.854 million tons would generate about 285,700 tons of sugar and 259,035 tons of bagasse fiber. Sugarcane bagasse comprises cellulose (32–45%), hemicellulose (20–32%), lignin (17–32%), 1.0–9.0% ash, and some extractives [38]. The exact composition will vary from crop to crop based on geography and climate conditions. The washed bagasse from the example mill would comprise on average about 99,728 tons of cellulosic glucose, 67,349 tons of hemicellulose material rich in xylose and acetate, 63,464 tons of lignin, and 12,952 tons of ash. Assuming a 90% release of free glucose from cellulose hydrolysis and a fermentation efficiency of converting glucose to ethanol at 0.46, the process described in Figure 2 would support production of approximately 52 million liters of ethanol. This fermentation would not utilize significant amounts of xylose or other organics. The stillage obtained from the subsequent ethanol distillation process would be rich in xylose, other unfermented sugars, organic acids (acetate stemming from both the bagasse hydrolysis and organic acid byproducts

derived through yeast metabolism) plus glycerol. Assuming an aerobic conversion of yeast biomass at 0.42 g yeast per g carbon substrate, the carbon-rich stillage stream would be used as a substrate to generate about 33,000 tons of nongenetically modified (non-GM) *S. cerevisiae* biomass on a dry basis. This yeast biomass would be 60% *w*/*w* protein, which matches the protein level in fish meal [39]. The process is enabled by *S. cerevisiae* strains derived purely through classical genetic methods to grow aerobically on xylose and many of the other carbon compounds present in stillage, which have a high degree of resistance to inhibitors present in the stillage [40,41]. Because the yeast is non-GM, it would be "generally recognised as safe" and therefore considered as food grade, thereby making it attractive for use in multiple jurisdictions that might otherwise not allow genetically modified organisms in the food chains.

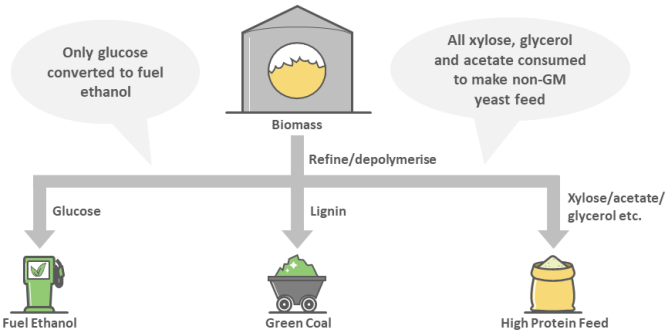

Figure 2. A model lignocellulosic biorefinery that produces food, fuel, and sequestered carbon. Biomass is depolymerized using physicochemical and enzymatic processes to release sugars (mainly glucose from cellulose and xylose from hemicellulose). Only cellulosic glucose is used for anaerobic fermentation by yeast to produce ethanol. The stillage from the ethanol production process is used in a secondary aerobic process to grow a nonrecombinant *S. cerevisiae* that can utilize xylose, glycerol, and organic acids to produce a high protein content dried yeast biomass for feed applications. The nonrecombinant nature of the xylose metabolizing *S. cerevisiae* strain gives it "generally recognised as safe" status needed for use in the food chain. The yeast biomass can be produced with up to 60% protein content comprising the broad range of amino acids needed for human and animal diets. Recalcitrant material, including lignin, is dried and pelletized to provide a carbon-rich mass that represents captured carbon. The pellets can be used as green coal to provide energy for the process or other industrial applications. Alternatively, the pelletized material could be sequestered.

Since the land is already being used for the primary product sugar (and based on data for soybean production [42]), we calculate that feed yeast manufactured from the sugar mill bagasse could replace about 26,000 hectares of soybean production. Although soybean is rich in dietary protein, it contains antinutritional factors, including phytates, tannins, trypsin inhibitors, and oligosaccharides that negatively impact, e.g., iron absorption [43,44]. High protein-containing yeasts can circumvent these antinutritional problems by providing the necessary amino acid balance, together with essential B vitamins and dietary fiber [45]. Moreover, *S. cerevisiae* produces phytase activities that can reduce iron binding issues associated with soybean meal [46]. A third product of this biorefinery would be about 80,000 tons dried pelletized lignin-rich material that could be used as a green coal for provision of power to the overall process or, if sufficient renewable power is already available, could be used as a means of sequestering solid carbon-rich material. Such a process offers a means to substantially reduce the GHG footprint and adds to the sustainability of bioethanol production as a coproduct. Lifecycle analysis indicates that a biorefinery incorporating a yeast biomass production process can result in 29% reduction in GHG (kg CO_2 equivalents), 11% reduced fossil energy use, 108% reduced particulate matter, 50% reduced eutrophication (tons PO_4^{3-} equivalent), 75% less water consumption, and 240% less land use (m^2 per year) relative to a process not producing yeast biomass [47]. Others have shown that indirect land-use effects are minimized by allowing land to be

used for both food and fuel rather than for one or the other [48]. In a sugarcane feed and fuel biorefinery, which produces protein-rich yeast biomass and bioethanol, the yeast can partially substitute for grass in the feed of cattle grazing on pasture and thereby potentially release land for increased sugarcane production, with minimal land use change effects. Applying the concept to the Brazilian ethanol and livestock industry, it would be technically feasible to increase ethanol production threefold without bringing any extra land into agricultural or pastoral use. The ability to achieve partial replacement of feed or fodder crops using microbial protein can also offer advantages in terms of replacing the need for artificial fertilizers, which are significant in terms of their global GHG emissions [49]. The use of live yeasts as a probiotic would offer further advantages in terms of reducing methane expelled from ruminants, thereby providing further potential for reduction in GHG emissions [50]. Furthermore, yeast as a coproduct provides a feasible means of improving the economics of 2G ethanol. It has previously been reported that a 110 million liters per annum 2G ethanol and cofeed yeast facility, with a 70 cents per kg selling price for the yeast, would break even at 68 cents per liter of ethanol [41]. In the past 12 months, ethanol has been sold on the US market for between 57 and 74 cents per liter [51]. Modeling also shows that feed yeast could be sustainably sold between 50 and 90 cents per kg when the ethanol selling price is 55 to 70 cents per liter. This yeast selling price compares favorably with the minimum selling prices given for other microbial proteins (Pekilo (*Paecilomyces variotii*), Torula (*Candida utilis*), and Fusarium (*Fusarium venenatum*)) made from lignocelluloses without ethanol coproduction [52]. In that work, it is modeled that the minimum protein selling prices are between 5 and 10 USD per kg.

4. A Model Lignocellulosic Bio/Chemicals Refinery Delivering Food and Fuel and Power-to-X

Ultimately, to fully address the environment, food, and energy trilemma, we will have to develop GHG-negative technologies that can operate in marginal quality land or even desert areas, where land-use changes will be less critical. If we are to achieve gross zero or negative GHG emissions, we will need to expand biofuel production and concomitantly sequester materials to decarbonize the process. Ideally, processes will valorize CO_2. Here, we link biomass with renewable power-to-X, where X can be bioethanol, synthetic aviation fuel, protein for animal feed, sequestered carbon, or multiple carbon-based synthetic fuels and chemicals (Figure 3). The concept of using renewable power to drive the production of carbon–carbon compounds from CO_2 and H_2 is becoming well established and should be thought of as an important part of our thinking around GHG control and sustainable development, as has been discussed by others [53]. Such a biorefinery would require the capture of CO_2 from anaerobic and aerobic fermentations, the technology for which exists today [54]. Processes for the electrochemical reduction of CO_2 to carbon–carbon products, including methanol, ethylene, and ethanol are also being advanced [55–57]. Recent developments in the generation of synthetic fuels and other chemicals such as dimethyl esters, synthetic natural gas, synthetic gasoline, synthetic diesel, and synthetic aviation fuel from H_2, CO_2, and other gases have been reviewed by others [58]. The possibility to produce urea directly by coupling N_2 and CO_2 in H_2O under ambient conditions using an electrocatalyst has also been demonstrated [59]. Conceptually, a biorefinery operating in the manner described could even provide the fertilizer needed to grow the biomass feedstock or the nitrogen source needed to grow yeast biomass within the process. The potential to grow yeast and other microbes as nutritious protein sources using basic carbon molecules plus urea and salts has recently been discussed by others [60] and demonstrated in our laboratory [45]. These advances open the way to consider biorefineries as an integrated source of microbial protein for food, plus chemicals including those for broader applications as fuels. Such a multiproduct biorefinery could be situated on nonarable marginal land, thereby minimizing primary food crop land-use challenges associated with renewable fuels and overcoming direct and indirect land-use arguments currently used against renewable transport fuels. The combination of a fermentation-based

biorefinery within a green power-to-X bio/chemicals plant is appealing, as it enables the valorization of CO_2 and other carbon compounds that might otherwise be regarded as unwanted byproducts. Moreover, multiple coproduct bio/chemical refineries with the ability to shift the output balance of product types according to market conditions will likely be more sustainable and economically viable than single-product models [61].

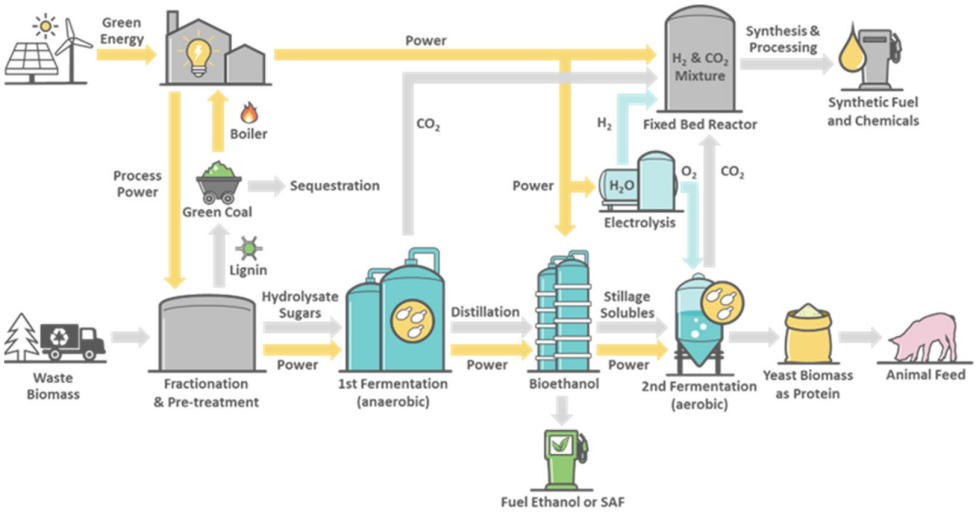

Figure 3. An integrated food, fuel, synthetic bio/chemicals, and carbon sequestering refinery. Renewable green energy is used to provide power for the biorefinery. Biomass is depolymerized using physicochemical and enzymatic processes to release sugars (mainly glucose from cellulose and xylose from hemicellulose). A first fermentation by yeast anaerobically converts cellulosic glucose into ethanol. Ethanol is distilled from the ferment and used directly as replacement for petrol or for generating synthetic aviation fuel (SAF). Stillage from the distillation is used as substrate for a second fermentation using a nonrecombinant yeast able to aerobically convert xylose, glycerol, and other organics into high protein-containing yeast biomass. Aerobiosis of this fermentation is supported by O_2 obtained from electrolyzed water. The green H_2 produced by electrolysis of water is combined with CO_2 captured from the first and second fermentations to produce synthetic fuels or other carbon-based chemicals. Recalcitrant materials, including lignin and other solids, are dried and pelletized to provide a carbon-rich biomass that represents green coal. The green coal can be burned to provide extra power for processing, exported to the grid, used in other industrial applications such as steel manufacture, or can be sequestered as a carbon-rich solid. Yellow arrows represent power generation and flow; grey arrows represent carbon transformation and flow; blue arrows represent green H_2 and O_2 generation.

5. Consideration of Microbial Functions in Lignocellulosic and Bio/Chemical Refineries

Microorganisms and fermentation technology will be of increasing importance for the future provision of fuels, chemicals, and alternative food sources (especially as a source of protein and vitamins) as we move from petrochemical to bio/chemical refineries. Microbes will play multiple roles, including in the enzymatic hydrolysis of biomass to release the monomeric components for anaerobic and aerobic fermentation processes. Various cellulolytic bacteria, yeasts, and mycelial fungal species produce enzymes that are useful in digesting biomass into its different monomer units, making it accessible for bioconversion [62]. Cellulases, hemicellulases, and ligninases are varieties of enzymes expressed by some microorganisms; these are the enzymes broadly responsible for releasing monomer components of biomass. Other so-called accessory proteins that enhance digestion are also produced by microbes [62]. These proteins function either by breaking the hydrogen bonds in cellulose fiber or by oxidative cleavage of glycosidic bonds. Accessory proteins include expansins and swolenins, which swell and loosen lignocellulosic structures, thereby enabling better access and activity of cellulases, hemicellulases, and ligninases.

Notwithstanding the importance of process efficiencies with respect to the hydrolysis of biomass to release utilizable carbon, the yield of any target product per input of biomass-derived substrate, plus the rate of production of that product, will be critical to the economic viability of any biorefinery process. Here, the efficiency of fermentation will be important and will be species and strain dependent. The robustness of microbes will be of critical importance in industrial processes. Regardless of the microbial species and nature of the product being manufactured, the abilities of strains to withstand physicochemical challenges associated with different types of media, fermentation conditions, final product toxicities (e.g., ethanol, organic acids, and other fermentation products can become inhibitory as they accumulate), and downstream treatment procedures are central to process economics. Lignocellulosic hydrolysates are inhibitory environments for many microbial species. They contain high levels of furfural, 5-hydroxymethylfurfural, formic and acetic acids, and phenolics (e.g., syringaldehyde, p-coumaric acid, 4-hydroxybenzaldehyde, vanillin, and ferulic acid) that fermentation organisms must detoxify or resist if they are to grow and thrive [63]. Detoxification of hydrolysates can be achieved chemically by, for example, using overliming (treatment with calcium hydroxide). A more environmentally friendly approach would be to use microbial actions to pre-detoxify hydrolysates prior to inoculation with a primary ethanologen [63]. Bacteria found to have hydrolysate detoxifying actions include *Ureibacillus thermosphaercus*, *Methylobacterium extorquens*, *Pseudomonas* sp., *Flavobacterium indologenes*, *Acinetobacter sp.*, *Arthrobacter aurescens*, and *Desulfovibrio furfuralis*. Some fungi also exhibit abilities to metabolize hydrolysate chemicals into less toxic derivatives. These fungi include *Coniochaeta ligniaria*, *Trichoderma reesei*, and *Aspergillus ascendens* and yeasts such as *Issatchenkia occidentalis* (*Candida krusei*) [64]. Laccase and peroxidase enzymes from the white-rot basidiomycete fungus *Trametes versicolor* and other fungal species such as *Cyathusc stercoreus* have also been shown to detoxify phenolic residues in hydrolysates. Clearly, microbes can have important roles to play in lignocellulosic and power-to-X bio/chemical refineries beyond the obvious function of fermentation. Strain improvements for enhanced hydrolytic enzyme production and applications for detoxification of hydrolysates are important and are key targets for research and optimization.

One intriguing approach to the fermentation of lignocellulosic hydrolysates is to use synthetic microbial consortia. Here, individual microbial strains are engineered to perform only one function toward an overall process. One example is in the fermenting of multiple carbon sources simultaneously, where a consortium of specialized *Escherichia coli* strains (each engineered to specifically consume a single carbon source by deleting gene encoding transporters and enzymes involved in the utilization of other carbon sources) was able to ferment a mixture of arabinose, glucose, xylose, and acetate at rates significantly greater than wild-type strains [65]. As well as concerns over the toxicity of hydrolysates, the conversion of lignocellulosic sugars into high value products is additionally hindered by the competitive inhibition of D-xylose transporters by D-glucose. [66]. A consortium of three strains of *S. cerevisiae,* engineered so that each strain could only ferment one of glucose, xylose, or arabinose, was able to overcome this limitation, resulting in significantly better performance than the wild-type strain [67].

Arguably, *Saccharomyces* yeasts have an advantage over most other microbial species with respect to their proven industrial applications. *Saccharomyces* species have been utilized by humans for millennia and have been at the forefront of research and development for 150 years, significantly impacting microbiology, fermentation, biochemistry, genetics, molecular biology, and synthetic biology/genomics. The production of yeast biomass and its application to respective industrial uses exposes yeast cells to a plethora of stressful conditions that they must withstand in order to perform their required functions well [68]. In this regard, *S. cerevisiae* is the primary candidate microorganism for valorizing lignocellulosic biorefineries, as it is already the proven primary ethanologen on an industrial scale. In addition, industrialized strains of this yeast have a high degree of robustness needed to withstand the toxicity of lignocellulosic hydrolysates [41,61,68]. Over 2 million tons of *Saccharomyces* is produced per annum for applications in baking, winemaking, and alcohol

production, as well as other products such as animal feeds, yeast extracts, biochemicals, enzymes, and pharmaceuticals [68,69]. Substrate costs significantly affect the economics of yeast production and new alternative substrates are sought. Lignocelluloses offer some potential, but to make these substrates economically useful, new yeast strains are needed that maintain all the qualities needed for their niche industrial applications, as well as having the added ability to efficiently utilize lignocellulosic hydrolysate substrates. *S. cerevisiae* can naturally utilize hexose sugars in lignocellulosic hydrolysates but does not ferment pentose sugars under anaerobic conditions. Substantial research and development have been carried out to engineer strains of the yeast able to metabolize xylose and arabinose, as well as to better withstand the toxicity of hydrolysates [70,71]. Breeding and selection strategies also yielded nonrecombinant *S. cerevisiae* able to assimilate xylose efficiently via aerobic metabolism, even within a hydrolysate environment [40,41]. Currently, sulfite liquor waste streams from wood pulping processes are the only lignocellulosic streams available in the volumes needed to support the tonnage scale of industrial *Saccharomyces* production. Recent research has shown that molecular engineering coupled with environmental adaptation can deliver *S. cerevisiae* strains that grow on, and ferment spent sulfite liquors from hardwoods [72,73]. At this stage, we envisage that lignocellulosic substrates would be useful in producing yeast biomass where yeast is being applied in the manufacture of nonpotable ethanol or animal feed applications, but its immediate role in the production of yeast for baking or potable alcohol might be more contentious.

Natural isolates and existing industrialized strains of *S. cerevisiae* provide a valuable resource that we can mine for genetic diversity with respect to lignocellulosic applications [74,75]. The development of new yeast strains with improved existing features (e.g., greater ethanol tolerance, thermotolerance, and resistance to hydrolysate-derived inhibitors) or novel functions for producing different chemicals from lignocelluloses will rely on genetic modifications. Classical genetics can be described as the generation of new organisms using "natural" processes to induce genetic variation and exchange. This can be via breeding (mating or hybridization), cell-to-cell fusion, and/or mutation, followed by selection and screening. The breeding and selection of new yeast strains has been important for developing improved yeast strain varieties for traditional and new biotechnological applications [40,41,76–79]. Classical genetics will continue to be of relevance in the future, whether that be as a standalone technology or coupled with molecular and synthetic biology processes. Synthetic biology is the design and engineering of novel biological entities with new and improved functions. It involves the integration of multiple disciplines, including bioinformatics, omics, robotics, artificial intelligence, systems biology, molecular design, in vitro nucleic acid and protein synthesis, and incorporation of design modifications into functional biological systems using molecular techniques. Synthetic biology is seen by many as having the potential to revolutionize how we address areas of food production, agriculture, environment, energy, chemicals, and human and animal health, because it can introduce radical variations in organism designs. Indeed, synthetic biology is already being used to generate novel bacteria and yeasts (*Saccharomyces* and so-called nonconventional species including *Pichia*, *Kluyveromyces,* and *Yarrowia*) able to generate a vast array of biobased chemicals [80–84]. These chemicals include, for example, astaxanthin, citric acid, glycolic acid, glutaric acid, mesaconic acid, 3,4-dihydroxybutyric acid, monoethylene glycol, 1,4-butanediol, 1,2,4-butanetriol, various medium chain length fatty acids, fatty acid esters, resveratrol, and vitamins A and E. As a cautionary note though, and despite the exciting potential of synthetic biology to advance society, there are sociopolitical issues surrounding genetically modified organisms that need to be confronted [85]. These challenges include questions around biosecurity, biosafety, ethics, and "humans playing God". Arguably, the management of public education, expectations, and fears around synthetic biology and synthetic genomes will prove to be as critical as the technical development of this science if we are to realize promised benefits.

The capture and valorization of CO_2 will be critical to the GHG neutrality or negativity of future refineries; microbes offer great potential in this area. One approach could be to

link 1G and 2G with 3G and, eventually, 4G ethanol, wherein the CO_2 produced by yeast fermentations could be used to drive photosynthesis by algae. The carbohydrates accumulated by the algae could then be recirculated as substrates for further yeast fermentation. Microorganisms will also be important in the direct synthesis of carbon–carbon molecules from gaseous CO, CO_2, and H_2. Species of bacteria can generate carbon compounds direct from CO_2 and H_2, as evidenced by the ethanologenic bacterium *Clostridium ragsdalei* [86,87]. The process to convert gas mixtures of CO_2 and H_2 into ethanol is available on an industrial scale, with companies such as LanzaTech (Skokie, IL, USA) developing a process to commercially convert waste gas streams into ethanol [86,87]. Methanogenic and acetogenic bacteria are also being investigated for their potential to generate different products from CO, CO_2, and H_2. The array of chemicals being produced includes, for example, acetate, butanol, 2,3-butanediol, butyrate, ethanol, formate, fatty acids, lactic acid, methanol, propane, propanol, succinate, and high value compounds such as pigments and ectoine (an active ingredient in skin care and sun protection products), plus many others [88–90]. The discovery and optimization of new microbial species for converting gaseous carbon compounds and H_2 into carbon–carbon precursors of fuels and chemicals will be critical to the economics of power-to X refineries. Synthetic biology can have a key role to play in this regard and recent advances in synthetic biology, genome engineering, and laboratory evolution are enabling the creation of improved synthetic single carbon-utilizing microorganisms [90,91].

6. Final Comments and Recommendations for Future Research

Biological sciences have played a major role in the growth and expansion of human populations and are becoming increasingly important for developing more efficient management of natural resources and improved alternative approaches to supplying society's needs. The scale of liquid fuel that is needed to replace oil is massive and, long-term, it is not sustainable to expand the use of food crops grown on arable farmland to meet the full need of >100 million barrels per day [92]. Given that xylose is the second most abundant sugar in nature [93], this five-carbon sugar provides a largely untapped resource. The fermentation of biomass-derived carbon can play a major role in the manufacture of food, biofuels, and synthetic chemicals. It is anticipated that global biomass demand will double between 2005 and 2050, based on the needs to ensure food security for a growing world population and the requirement for the expanded production of biofuels and biobased materials. This need can be met by improved crop and farmland management [94]. We envisage a scenario whereby lignocellulosic biomass refineries, producing microbial-based food (protein) and biofuels, will be integrated with bio/chemical refineries making fuels and chemicals from methanol and other carbon–carbon substrates that have been synthesized from gaseous carbon (atmospheric and microbially expired) and H_2 that has been derived via electrolysis of water using renewable power. In this way, we can take full advantage of the untapped potential that microorganisms can offer for a biobased future that addresses society's environmental, food, and energy trilemma. Combined lignocellulosic and bio/chemical refineries can also help address GHG emissions and land-use targets. It can be envisaged that such facilities could be built in marginal land areas, keeping prime arable land available for raising food crops and animals. The approaches to microbial protein production discussed herein and in other publications open the possibility to eventually uncouple food production from its strict reliance on photosynthesis-based food chains [45,60,95], which may well be critical in a world with a reduced availability of arable land due to climate change, conflicts, and population growth. Refineries utilizing surplus electricity generated from large-scale renewable power sources could even operate in inhospitable areas such as deserts and, coupled to microbial fermentations, could significantly supplement the human food chain. Thus, by harnessing microbial diversity and new fermentation technologies, we could potentially turn deserts into food bowls.

Despite extensive investment and research, biorefinery systems are not yet significantly displacing oil refinery technologies. Much more development of 2G ethanol remains to be carried out to improve its economics. This includes advancing logistics of biomass

production, harvesting and processing, improving the efficiencies of pretreatment, and reducing the costs of subsequent hydrolysis (more cost-effective enzymes are needed). More efficient organisms are required to improve yields and productivities of fermentations. Targets here include better cofermentation of biomass-derived sugars (e.g., improved concomitant fermentation of cellulosic glucose and pentose sugars) and greater resistance of organisms to hydrolysate inhibitors that are formed during pretreatment and hydrolysis of biomass substrates. To truly attain the goal of GHG negative fuels, we will need to develop multiproduct refinery technologies. Here, we anticipate that 1G, 2G, 3G, and 4G ethanol will all have a part to play (perhaps with 1G being a transitory process until the other technologies mature). The capture and utilization of CO_2 within biorefinery processes offers the best way to achieve GHG negativity. Electrochemical and biological (microbial) conversion of CO_2 and H_2 into carbon–carbon compounds is under development, but more needs to be carried out in this area to bring forward scalable commercial processes. More efficient catalysts are required for electrochemical routes. New and improved strains of microbes are needed that convert CO_2 to carbon–carbon compounds with greater efficiencies than are currently available. Here, synthetic biology offers exciting opportunities to develop organisms capable of manufacturing a vast array of chemicals or chemical precursors needed to replace oil-based chemicals and materials.

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