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# Engineering Microbial Consortia for Bioconversion of Multisubstrate Biomass Streams to Biofuels

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## Abstract

Production of biofuels from nonfood biomass has emerged as a sustainable option to address the problems associated with growing energy demand for transportation, heating, and industrial processes, in the context of diminishing petroleum reserves and global climate change. Biomass resources such as lignocellulose-rich biomass and microalgae, despite being abundant pose several challenges for efficient bioconversion to biofuels. Major challenges that must be addressed are the chemical complexity of the biomass and the associated feedstock variability. In this chapter, the role of microbial consortium-based biocatalysis strategies that are being developed to address these issues are reviewed and discussed. Microbial coculture biocatalysts are systems that are engineered to specialize in the conversion of a general class of substrates present in the biomass hydrolysates into biofuel intermediates, providing the capability of adapting to the variable composition of the feedstock. The techniques being developed to understand the interactions between the members of the bioconversion consortia and the corresponding population dynamics of the engineered cocultures are also discussed.

**Keywords:** biomass feedstocks, feedstock variability, biofuels, multisubstrate, microbial consortia, population dynamics

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## 1. Introduction

The driving forces behind the urgent need for developing sustainable bioenergy and bioproducts include growing concerns over climate change, conflicts over oil supplies, and the desire to stimulate a new bioenergy economy [1]. Biomass is a renewable energy source that can offer a substitute for fossil-based transportation fuels and create economic opportunities and rural development while reducing climate impacts related to greenhouse gas emissions. Biomass

is an energy resource derived from plant and algae-based materials that includes agricultural residues, forest resources, grasses, woody energy crops, algae, wet waste, municipal solid waste, urban wood waste, and food waste [2].

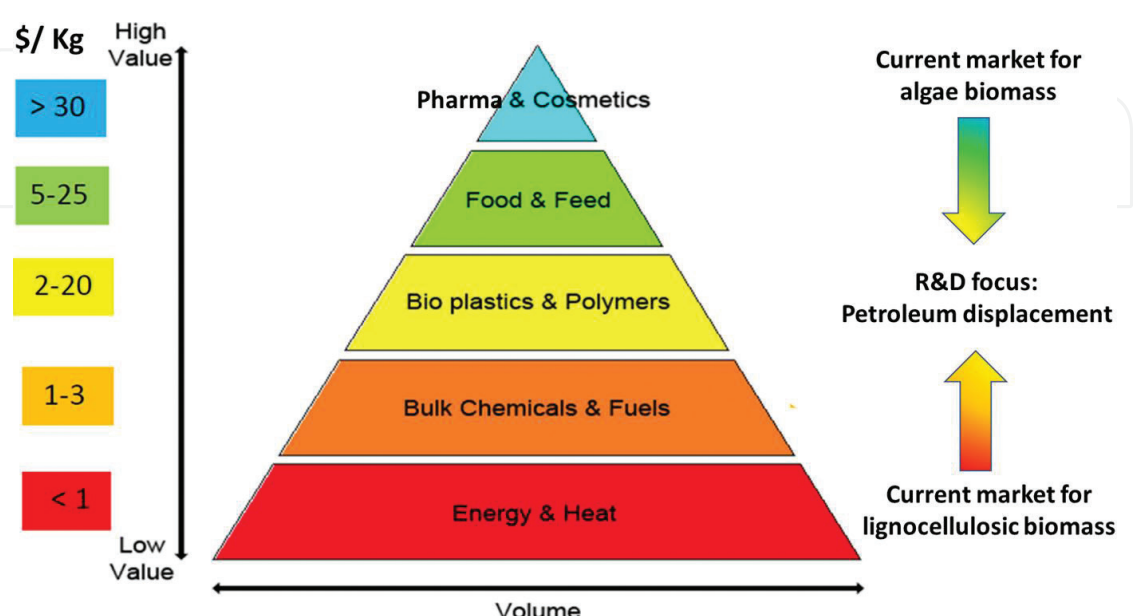
Utilization of industrial biotechnology for production of petroleum displacing products from low cost feedstocks faces major challenges because of recalcitrance of the biomass to hydrolysis as well as the high degree of biochemical complexity and variability in crude biomass hydrolysates [3]. In addition to unintentionally produced fermentation inhibitors from pretreatment, biomass hydrolysates contain a variety of carbohydrates, lignin, protein, lipids, organic acids, and ash, often at nearly stoichiometric ratios of each component. Although significant progress is underway for effective biomass pretreatment and utilization of each of the common types of fermentation substrates [4–6], the diversity of these substrates can result in spiraling costs for upstream separations, supplementation of fermentation media, serial fermentation steps, and product purification. From a technoeconomic perspective, any one of these unit operations can tip the scales against economic viability for a promising breakthrough. In terms of a biochemical or hybrid biorefinery, fractionation of the biomass to generate a spectrum of marketable products is assumed [7]; however, it is very rarely the case that the bulk of the biomass can provide significant or reliable economic return without upgrading. In light of this, process intensification based on single-pot bioconversion of multiple substrates provides a means for achieving dramatic improvements in reaction kinetics, yields, product titers, and separations while minimizing unit operations, thereby reducing cost and investment risk in chemical manufacturing [8]. Furthermore, consortium-based biocatalysis strategies provide the potential for utilizing ecological interactions, such as mutualism or commensalism, to increase biofuel production efficiency and yield [9, 10]. In this chapter, we will review promising biomass feedstocks and the associated feedstock variability challenges paired with the microbial consortia approaches that are being developed for bioconversion of multicomponent biomass hydrolysates to biofuels.

## 2. Biomass feedstocks for biofuel production

Although the current commercial biofuels industry is predominantly focused on feedstocks from harvestable components of food or feed crops (starch, sucrose, and oils), the potential of lignocellulose as an energy feedstock has received significant focus for second-generation biofuel production. Lignocellulosic biomass refers to the lignocellulose-rich materials available from terrestrial plants and crop residues, and is the most abundant renewable form of fixed carbon on earth. Lignocellulosic biomass is primarily composed of three polymers - cellulose, hemicellulose, and lignin. Cellulose, the major component of lignocellulosic biomass, is a linear homopolysaccharide consisting of 500–15,000 anhydrous glucose units that are linked by  $\beta$ -1,4-glycosidic bonds, with cellobiose as the smallest repetitive unit [11]. Hemicellulose is the second most abundant polymer whose role is to provide a linkage between lignin and cellulose. Hemicellulose is a short, highly branched polymer of pentose (e.g. D-xylose and L-arabinose) and hexose (e.g., D-mannose, D-galactose, and D-glucose) which forms a random and amorphous structure [12]. Lignin is the third major component of plant biomass in addition to cellulose and hemicellulose, accounting for 10–40% (w/w) of plant cell wall on the mass basis [13]. Lignin is an amorphous, randomly branched heteropolymer comprising

of phenylpropanoid units [14]. Lignin covalently binds to side groups on neighboring hemicellulose units, forming a complex matrix that surrounds the cellulose micro-fibrils. As the dominant structural polymer in terrestrial biomass, for evolutionary reasons, the lignocellulosic biomaterial is highly recalcitrant to deconstruction and hydrolysis. Because of this recalcitrance, significant effort is required for pretreatment of the material using mechanical, chemical, physicochemical and biological methods or various combinations of these [15] to fractionate lignocellulose into its biochemical constituents which can be converted to biofuels and chemicals using biocatalysis. However, large scale adoption of lignocellulosic biofuels in the market has been slower than expected, and the bulk of lignocellulosic bioenergy production (~3% of the total energy consumed in the U.S.) has been restricted to industrial heat, steam, and electrical power instead of liquid fuels.

Microalgae are another potentially abundant biomass resource for biofuel production. Microalgae comprise a large and diverse group of uni- or multicellular phototrophic and heterotrophic organisms which, because of high growth rate, minimal competition for agriculture land use, the ability to utilize CO<sub>2</sub>, and rapidly improve strains [16], microalgae may provide a dynamic addition to the sustainable fuels portfolio. Current markets for algae derived products have however been restricted to higher value products such as nutraceuticals, cosmetics, and foods. Microalgae have long been touted for their ability to accumulate large quantities of lipids. Oleaginous microalgae have oil contents of 20–50% by weight and several can exceed 80% oil by weight of dry biomass basis [17]; therefore, microalgae are being seriously considered as a feedstock for biodiesel production if the biomass production costs can be significantly reduced. Various species of microalgae produce a host of lipid subtypes, including mono-, di-, and triglycerides, phospholipids, glycolipids, waxes, carotenoids, hydrocarbons among others [18]. Microalgae are also rich in carbohydrates (5–23%) and proteins (6–52%) [19]; different species and cultivation conditions result in variable proportions of protein and carbohydrates. Some species, *Spirogyra* sp. for example, have high carbohydrate content, 33–64% [20], whereas the common nutraceutical algae strain, *Arthrospira*



**Figure 1.** Current markets and the convergent R&D landscape for the general biomass feedstocks.

*platensis* (a.k.a *Spirulina* sp.) have has high protein content, ~67% [21]. During pretreatment of microalgae biomass for biofuels production, the carbohydrate and protein fractions of the microalgae feedstock can be hydrolyzed to soluble monomeric sugars and amino acids, each of which can be converted by a biocatalyst into biofuel products during a microbial fermentation process, thereby increasing the net yield of biofuel intermediates beyond that of the lipids alone. Although algae lack the extreme recalcitrance to pretreatment that is provided by lignin to terrestrial plants, the high biochemical diversity of algae presents similar challenges for consistent pretreatment. In both cases, however, efficient utilization of the bulk of the various biopolymers present in the biomass is vital for development of economically feasible bioconversion routes for biofuel production. **Figure 1** illustrates the current markets as well as the convergent R&D landscape for each of these general biomass feedstocks.

3. Variability of biomass feedstocks

One of the most important challenges facing consortium-based bioconversion technologies is feedstock variability. Changes in biomass composition, even within the same crop in the same geographic location, is an issue that has plagued many industrial bioprocessing plants and has greatly hindered the widespread application of bio-based renewable fuels and chemicals. Since many processing facilities require process optimization around a given biomass source for plant profitability, deviations in biomass composition often require the process to be re-optimized which can cause significant down time in the plant. While the impact on plant operation time has been reduced over time, feedstock variability remains one of the most significant cost drivers for many industrial bioprocessing operations.

While there are many biomass composition variables such as ash and moisture content that are important economic drivers for the larger biorefinery picture, most relevant to the bioconversion process is the biochemical breakdown of the biomass source. **Table 1** highlights the variability of the glucan versus xylan percentages for different crops. The large range of values represents the severity in variation which is most dramatic in corn stover, with a maximum of 66% and a minimum of 39% [22].

One technique that has been deployed to curb these issues is blending of different biomass sources to help with geographic and seasonal variability [1]. This strategy can reduce the impact of feedstock variability, but requires sophisticated biomass logistics management to

	Corn stover	Miscanthus	Wheat
Mean	53	61	50
Maximum	66	65	57
Minimum	39	55	41
Range	27	10	15
Data from [22].			

**Table 1.** Carbohydrate compositions (% xylan + glycan) for various crops.

prioritize variables for maximum operational profitability and is intrinsically limited in its reach due to geographic and seasonal constraints. This strategy is further limited by biomass storage challenges that limit the availability of different sources for blending.

Differences in biochemical composition of various biomass sources have been well documented in the technical literature because biochemical conversion routes often utilize only specific components of the biomass (such as glucose and xylose) and large variations of these components will have major impacts on process efficiency and yields. The majority of this work has focused on the relative biochemical fractions of a particular biomass source that can be classified as carbohydrates, proteins, or lipids, commonly referred to as the proximate composition. In the bioconversion context, these three major biochemical classes have varying importance for different biomass sources, with carbohydrates receiving particular attention for most fermentation-based processes. These variations are explored for three biomass sources with near-term industrial relevance in the section below.

### 3.1. Corn stover

Ethanol production from corn stover has been a significant area of research and development due to the widespread availability of the feedstock from cultivation of maize. The updated Billion Ton Study released by the U.S. Department of Energy in 2016 reports that the base case for corn stover supplied under \$80/dry ton is over 129 million dry tons per year of biomass [1]. The variation of the composition of corn stover from cultivation in different geographic locations is highlighted in **Table 2** [23].

### 3.2. Distillers grains with solubles

Distillers grains with solubles (DGS) is another example of a highly variable biomass source that may play an important role in scale-up of bioconversion technologies. Twenty-three million tons of DGS were produced in 2017 as a coproduct of the corn ethanol process, with significant quantities of similar coproducts such as wet grains and dried grains without solubles also being produced. Because DGS are a coproduct of corn-ethanol fermentation, they suffer from variability of the original crop used as well as differences in different fermentation batches and strategies. These variations are highlighted in **Table 3** [24–28].

	South Carolina	Central Alabama	Northern Alabama
Cellulose	41.13 (0.98)	41.76 (0.18)	41.22 (0.18)
Hemicellulose	31.97 (0.99)	21.98 (0.14)	22.72 (0.16)
Lignin	5.97 (0.48)	6.49 (0.06)	6.56 (0.08)
Holocellulose	73.1 (1.42)	63.74 (0.17)	63.94 (0.15)
Ash	3.97 (0.14)	2.87 (0.02)	3.43 (0.04)

Data sourced from [23].

**Table 2.** Relative % (standard error) for corn stover harvests in the southeastern united states at three different locations.



### 3.3. Microalgae

While microalgae research has generally focused on high lipid strains and cultivation strategies for oil extraction and biodiesel production, recent studies have demonstrated the techno-economic necessity for higher productivity algal systems which generally corresponds to lower quantities of lipids and a higher fraction of proteins and carbohydrates [29]. Because of the wide diversity of potential strains and cultivation conditions, microalgal biomass has a wide range of biochemical possibilities. This is further magnified by the distinct difference in biomass composition at different stages in microalgal cultures growth cycle. As a part of a study to understand the impact of this variation on a dilute acid hydrolysis pretreatment process, the composition of three microalgae strains at different points in their growth cycle was investigated by Peinkos et al. and is summarized in **Table 4** [27].

The challenges caused by feedstock variability provide a unique opportunity for consortium bioconversion strategies. For many biomass sources, techno-economic reports conclude that the cost of the biomass itself is the main economic sustainability driver [29], so a growing field is developing around the use of consortium conversion strategies that enable utilization of the vast majority of the biomass for production of a biofuel or petroleum displacing commodity chemical in an integrated biorefinery scheme. Clearly, if the biomass itself is the key cost driver at scale, then as much value as possible needs to be extracted from of the biomass. Consortium strategies allow for different organisms to specialize in efficient conversion of a particular substrate and collectively convert as much of the diverse and variable biomass-derived biochemical intermediates to a fuel or chemical product as possible. If done effectively, this strategy will

	Spiehs and Whitney [28]	Liu [26]	Belyea et al. [25]	Cromwell and Herkelman [27]
Protein	30.2 (2.9)	27.4 (3.3)	31.4 (2.5)	29.7 (5.7)
Oil	10.9 (1.2)	11.7 (2.2)	12.0 (1.7)	10.7 (5.0)
starch	—	4.9 (2.5)	5.3 (0.7)	—
Total carbohydrate	53.1	56.5	52.1	54.3
Crude fiber	8.8 (1.4)	—	10.2 (1.0)	—

**Table 3.** Biochemical composition of DGS as a dry mass % (range of values) from four different studies as reported by [24].

Species	Growth stage	Lipids	Ash	Carbohydrate	Protein
<i>N. granulata</i>	Early	12.28 (0.16)	14.2	8.92 (0.13)	32.7
	Mid	25.6 (0.20)	13.6	11.12 (0.48)	23.1
	Late	57.33 (0.09)	5.1	10.89 (0.11)	9.4
<i>C. vulgaris</i>	Early	12.07 (0.09)	6.7	11.12 (0.12)	43.2
	Mid	15.02 (0.16)	4.4	35.69 (0.01)	24.0
	Late	23.14 (0.19)	5.3	38.00 (0.36)	15.2

Data from [27].

**Table 4.** Biochemical composition in mass of dry cell weight (Std Dev) for two microalgae species.

be an effective tool to reduce the impact of feedstock variability, as the ratio of growth of the different specialized organisms in the consortium can be engineered to be proportional to the concentration of the different substrates derived from a given biomass source.

## **4. Microbial consortia for biomass conversion and biofuel/chemical production**

Recent advances in synthetic biology, metabolic engineering, and systems biology have enabled rapid progress in developing microbial cell factories [6, 30, 31] and novel enzyme cascade systems [32–34] for the conversion of biomass feedstocks and synthesis of biofuels and other platform chemicals. Although there are some successful examples of developing ‘superbugs’ capable of multiple functions, engineering a single microbe to simultaneously perform multiple tasks is still quite challenging and bioenergetically costly under most situations. Because of the complexity and the multisubstrate nature of the biomass feedstocks, it is especially challenging to engineer a single microbe to efficiently convert the diverse substrates (carbohydrates, proteins, fatty acids, oils, etc.) of the biomass to produce value-added products. In contrast to the ‘superbug’ paradigm, in nature microbes rarely live in isolation, but rather exist in highly diverse and complex communities known as consortia. The microbes in these communities interact in numerous ways ranging from cooperation to competition and are often capable of performing tasks that are far too complex for any single organism to complete themselves [35]. Besides the ability to perform complex biosynthetic tasks, microbial consortia exhibit many other appealing properties such as stability, productivity and functional robustness. Inspired by the powerful features of the natural consortia, there are rapidly growing efforts been undertaken to understand natural consortia and to engineer synthetic consortia for biotechnology applications [36, 37]. Well-designed microbial consortia involving two or more microbes can take advantage of the functions of individual microbes and their interactions to realize synergistic division of labor and more efficient utilization of biochemical substrates than monocultures. Natural and synthetic microbial consortia developed for the conversion of different biomass feedstocks for biofuel and chemical production will be discussed below.

### **4.1. Anaerobic digestion**

When considering consortium-based bioconversion technologies, anaerobic digestion offers a model process due to its long history of industrial application of a complex, albeit unsupervised, microbial consortium. Anaerobic digestion is a biochemical process that converts organic material into a mixture of methane and CO<sub>2</sub> (biogas) in an anaerobic environment. Its widespread use since the middle of the nineteenth century and the extensive research around the details of the process make it an important benchmark technology as well a valuable resource for future consortium conversion technologies [38]. The anaerobic digestion process is generally split into five stages, creating an interconnected web of processes each utilizing naturally adapted microorganisms that play a critical role in the overall conversion process. The stages can generally described as disintegration, hydrolysis, acidogenesis, acetogenesis, and methanogenesis which describes the sequential process of the organic polymers comprising biomass (such as carbohydrates and proteins) being disintegrated,



those polymers being hydrolyzed to their corresponding monomers, the monomers being fermented to organic acids and hydrogen, the long carbon chain organic acids being further broken down into acetic acid and hydrogen, and finally the conversion of acetic acid to the fully reduced methane product.

Anaerobic digestion offers a touchstone example of the technological potential of consortium bioconversion processes because many microorganisms involved are strongly dependent on other organisms in the consortium. This is exemplified by the interspecific transfer of hydrogen, i.e. hydrogenotrophs utilize the hydrogen being produced by syntrophic organisms oxidizing organic acids that require low pressures of  $H_2$  [39]. These syntrophic organisms are not able to grow in pure cultures because of this interdependency. While these symbiotic relationships are in many ways beneficial, they leave the entire process vulnerable to varying environmental conditions. If the temperature, pH, or another important environmental factor becomes unfavorable to an important organism in the anaerobic consortium the whole process is stalled and operates less efficiently. Furthermore, because of the wide variety of microorganisms involved in the anaerobic digestion process and the high level at which conditions can be controlled, it is difficult to engineer the system for any organism or class of organisms. The diversity of microbes involved in anaerobic digestion has minimized the potential roles for including metabolic engineering into the process, with most recent works simply looking to understand the population dynamics of the organisms involved [40]. The robustness of the technology makes it a base case bioconversion route for almost any biomass source, many of which have been investigated to varying levels of detail. Each biomass source presents unique challenges and difficulties to the overall anaerobic digestion process; microalgal biomass is a key example, since cells have been observed to remain intact after a 30 day retention time in a digester [41].

#### 4.2. Consolidated bioprocessing of lignocellulosic biomass

Microbial conversion of lignocellulosic biomass requires multiple biological functionalities, including production of saccharifying enzymes, enzymatic hydrolysis of lignocellulose to soluble fermentable sugars, and metabolism of sugars to desired biofuel products. Cellulose and hemicellulose in lignocellulosic biomass can be hydrolyzed by cellulase and hemicellulase enzymes such as endoglucanase, exoglucanase,  $\beta$ -glucosidase, xylanase, xylosidase, and arabinofuranosidase. Microorganisms that are able to hydrolyze lignocellulosic biomass or secrete cellulolytic hydrolysate enzymes are important for this step. Most biological process employed fungi such as *Trichoderma reesei*, *Penicillium echinulatum*, *Penicillium purpurogenum*, *Aspergillus niger*, and *Aspergillus fumigatus* [4] which secrete cellulolytic enzymes for saccharification. Some anaerobic bacteria such as *Clostridium thermocellum*, *Ruminococcus flavefaciens* and *Clostridium cellulovorans* developed large, extracellular multienzyme complexes called the cellulosomes, which are highly structured and consist of multiple cellulolytic enzyme units that interact with each other synergistically [42]. These anaerobic bacteria are also found to degrade cellulosic substrates efficiently. To reduce the number of unit operations and capital costs, a nascent approach called consolidated bioprocessing (CBP) has been developed for simultaneous enzyme production, hydrolysis, and fermentation by employing the microbial consortia biocatalyst strategy.

The microbial consortia for CBP involve a cellulolytic strain that hydrolyzes hemicellulosic biomass to fermentable sugars and a fermentation strain that utilizes the cellulosic sugars for growth and conversion to biofuel products through the natural or engineered metabolic pathways. Native cellulosic microorganisms such as fungi and some anaerobic bacteria are usually selected as the cellulosic strains in the consortia. For example, the thermophilic anaerobe *Clostridium thermocellum* which is capable of hydrolyzing cellulose through the activity of the cellulosome multiprotein complex was cocultured with the engineered ethanol producing strain *Thermoanaerobacterium saccharolyticum* for the production of ethanol from Avicel [43]. By genetically engineering the two strains to be acetate and lactic acids-deficient, the yield was greatly improved, resulting in the coculture producing 38 g/L ethanol from 92 g/L Avicel. In another study, Minty et al. designed a synthetic fungal/bacterial consortium that involved a cellulolytic fungus *Trichoderma reesei* and an engineered *E. coli* strain for bioconversion of lignocellulosic feedstocks [44]. In the consortium, the cellulolytic fungus secreted cellulase enzymes to hydrolyze pretreated corn stover into fermentable sugars and the *E. coli* converted soluble saccharides into isobutanol with titers up to 1.88 g/L. The *T. reesei*/*E. coli* consortia developed in this study showed a cooperator-cheater dynamics which lead to stable equilibrium population.

The main challenges of using native cellulolytic microorganisms as the cellulolytic strains are the availability of tools for genetic manipulation and the application of these tools to engineer the strains with high yield, titer and robustness under industrial conditions. Industrial strains that are not naturally capable of hydrolyzing cellulose have also been genetically engineered as the cellulolytic strains. For example, *E. coli* was engineered to express cellulase, xylanase,  $\beta$ -glucosidase and xylobiosidase enzymes extracellularly [45]. The resulting two *E. coli* strains were able to grow on cellulose and xylan substrates, respectively. Subsequent incorporation of biofuel production pathways into each cellulolytic and hemicellulolytic strain enabled production of biofuel products such as fatty-acid ethyl ester, butanol and pinene from ionic liquid pretreated switchgrass by the *E. coli* coculture. In this *E. coli* based CBP, cellulose and hemicellulose in the pretreated biomass were hydrolyzed into soluble oligosaccharides by the secreted cellulases and hemicellulases from the two *E. coli* strains. The oligosaccharides were further converted to monomeric sugars by the expressed  $\beta$ -glucosidase enzymes and were eventually metabolized into biofuels via the heterologous pathways of the same *E. coli* strains. The *E. coli* strains developed in this study incorporated the biomass-degrading capability and biofuel-producing ability into one strain, unlike in most typical CBP systems where cellulolytic and fermentation strains are engineered separately. Instead, this *E. coli* coculture divided the labor of producing advanced biofuels from cellulose and hemicellulose fractions of the lignocellulosic biomass between the two engineered strains.

Besides biofuels, valuable chemicals are also produced from nonfood agricultural resources by the CBP approach. For example, Bayer et al. used a synthetic metagenomics approach to identify methyl halide transferase (MHT) genes from various organisms and screened these enzymes in *E. coli* for MHT activity [46]. MHT with the highest activity was cloned in *Saccharomyces cerevisiae* and the resulting strain was able to produce methyl halides from monomeric sugars such as glucose and xylose. The production of methyl halide from unprocessed switchgrass, corn stover, sugar cane bagasse, and poplar was then demonstrated by

the consortium of the engineered yeast and a cellulolytic bacterium *Actinotalea fermentans*. In the coculture, the cellulolytic bacterium produced monomeric sugars from hemicellulosic biomass which were further converted to methyl halide by the engineered yeast.

Inspired by the natural cellulosome in some anaerobic bacteria for the hydrolysis of cellulose, Tsai et al. developed a synthetic yeast consortium displaying an artificial minicellulosome for the synergistic saccharification and fermentation of cellulose to ethanol [47]. In the consortium, one yeast strain displayed a miniscaffolding consisting of three different cohesin domains and three other yeast strains secreted dockerin-tagged endoglucanase, exoglucanase, and  $\beta$ -glucosidase, respectively. Because of the specific interaction between the orthogonal cohesin-dockerin pairs, dockerin-tagged cellulolytic enzymes bound onto the scaffolding and a minicellulosome structure formed on the yeast cell surface. Cellulose was hydrolyzed to glucose by the three cellulases in the yeast displayed minicellulosome and glucose was then utilized by the yeast and fermented to ethanol. In this work, it was found that the ethanol yield could be fine-tuned by adjusting the ratio of the different yeast strains in the consortium. The consortium with the optimized ratio of the different populations produced ethanol up to 93% of the theoretical value from cellulose.

#### 4.3. Microbial consortia for variable sugar mixture feeds

Hydrolysis of lignocellulosic biomass by chemical or enzymatic methods usually results in a mixture of C<sub>6</sub> sugars (glucose, mannose, and galactose) and C<sub>5</sub> sugars (xylose and arabinose) [15, 48]. The relative proportion of these sugars varies among different hemicellulosic biomass sources. Although glucose is the most abundant hexose and xylose is typically the major pentose, the fraction of other sugars found in hydrolysates can also be significant depending on the biomass and the pretreatment process. Natural microbes commonly consume hexose sugars prior to initiating the consumption of a pentose sugar. This phenomenon is known as carbon catabolite repression, in which the presence of a preferred substrate represses the expression of genes in the microbes required for the metabolism of other substrates [49]. Although efforts have been made to develop a single organism that can consume glucose and xylose simultaneously [50–52], sugar mixtures remain inefficiently consumed in these single-organism processes. Moreover, since the sugar composition and proportion vary in different biomass hydrolysates, a single organism has very limited ability to adjust to this variation. Instead, multiorganism coculture systems with each organism selectively consuming only one particular sugar substrate have been developed to eliminate the carbon catabolite repression and convert the mixed sugar feed streams more efficiently and completely. The multiorganism systems also have the ability to adjust the ratio of the different individual populations in the coculture in order to adapt to the fluctuation in the mixed sugar compositions of the feedstocks.

As a recent example, two substrate-selective strains of *E. coli* were metabolically engineered with one strain only able to consume glucose and the other one only able to utilize xylose [53]. To construct the xylose selective (glucose deficient) strain, three genes *ptsG*, *manZ* and *glk* involved in glucose uptake were knocked out from the chromosome and the glucose-selective (xylose deficient) strain has the *xylA* gene encoding for xylose isomerase deleted. This coculture was demonstrated for simultaneous conversion of glucose and xylose for acetate production. Moreover, the relative biocatalyst concentrations in the coculture could be adjusted in order

to optimize the product yield from feed streams with variable sugar compositions. Xia et al. extended the consortium strategy for mixed sugar utilization to a synthetic mixture composed of three sugars-glucose, xylose and arabinose as well as the growth inhibitor acetic acid [54]. In this case one strain engineered to utilize acetate but not sugar was used to selectively remove acetate in the first stage of the bioprocess. In the subsequent stage, three *E. coli* strains which were each engineered to utilize only one sugar were cocultured and consumed glucose, xylose and arabinose simultaneously. Although reduction of the net growth rate of the strains was observed as a result of the chromosomal gene deletions that were required to generate the strains, the consortium showed significant improvement in multiple sugar utilization than any single-organism approach.

Substrate-selective consortia have also been engineered for the production of biofuels from hemicellulose-derived sugar mixtures. In a recent study, the synthetic pathway of n-butanol was engineered into a glucose-selective strain and a xylose-selective strain and the resulting *E. coli* coculture produced n-butanol from the sugar mixture [55]. The system was further improved by distributing the n-butanol production pathway into the two strains for improved redox balance and 5.2 g/L n-butanol was produced by the coculture from a glucose-xylose mixture. In another related study, *Saccharomyces cerevisiae* was genetically engineered to utilize L-arabinose. By coculturing the L-arabinose-utilizing yeast and the yeast that could ferment glucose and xylose, the hexose and pentose sugars present in corn stover hydrolysates was efficiently fermented to ethanol [56].

An *E. coli* coculture system with two substrate-selective strains has also been developed to produce commodity chemical such as *cis*, *cis*-muconic acid from a glucose/xylose sugar mixture [57]. To overcome the high-level intermediate accumulation during the synthesis, the entire muconic acid synthetic pathway was split into the two *E. coli* cells. Therefore, one strain was only able to utilize xylose and convert it to the intermediate 3-dehydroshikimic acid (DHS) which is the precursor for muconic acid. The second strain incorporated a DHS transporter and could utilize glucose and convert DHS to the final product muconic acid through the rest of the split pathway. The *E. coli*-*E. coli* coculture system has shown to use sugar mixtures efficiently even at increased scale, and produce significantly higher amount of muconic acid than previous studies, with a yield of 51% of the theoretical maximum.

#### 4.4. Microbial consortia for sugar and protein mixture

Besides sugar mixtures, many biomass resources are also rich in proteins. For example, distillers grains with solubles (DGS) produced from the first-generation bioethanol process are considered as a rich source of cellulosic polysaccharides (52–57%) and proteins (27–31%) [58]. Microalgae are another example of the biomass resource that is rich in polysaccharides and proteins. Therefore, developing biochemical techniques for simultaneous utilization of the sugar and protein fractions of the biomass is important for efficient bioconversion of these biomass feedstocks.

Microbes normally cannot utilize amino acids as carbon source for growth. However, an *E. coli* strain that was able to use 13 individual amino acids as the sole carbon source for growth was developed after several rounds of chemical mutagenesis. A strategy was subsequently developed to deaminate amino acids and convert the remaining carbon backbones of amino acids to



fusel alcohols through metabolic engineering [59]. In a follow-on study, the engineered *E. coli* was further improved by modifying the cofactor specificity of two enzymes involved in the metabolic pathway and the resulting strain can produce fusel alcohols with significantly improved yield [60].

Liu et al. has demonstrated the feasibility of one-pot bioconversion of the protein and carbohydrate fractions of the DGS hydrolysates and algae hydrolysates into mixed fusel alcohols by an *E. coli* coculture [61]. In the consortia, one strain is dedicated for bioconversion of hexose and pentose sugars in the hydrolysates into isobutanol (C4) and isopentanol (C5) fusel alcohols, whereas the other strain was engineered to convert free amino acids into mixed fusel alcohols. At the optimized inoculation ratio of the two strains, the consortium produced the highest titer of total fusel alcohols, up to 10.3 g/L, including 6.5 g/L isobutanol which comprised 63.1% of the total alcohols. Correspondingly, the consortium with the optimized inoculation ratio consumed the highest amount of DGS carbohydrates and proteins in the hydrolysates, including near complete consumption of the glucose and arabinose and 85.1% of the xylose, as well as 31.3% of the total proteins in the hydrolysates. Evaluation of the biofuel properties of the fusel alcohols produced using this strategy indicates that the higher carbon chain length alcohol mixture (especially C3-C5) provides increased energy densities and a variety of improved physical properties, such as reduced water solubility and corrosivity, than ethanol [16]. Therefore, the mixed fusel alcohols produced in this coculture have promising potential applications as a fuel upgrading feedstock in gasoline, diesel, and jet fuel or as a neat fuel of itself.

Similar *E. coli* cocultures were also developed for the production of terpene mixtures from the carbohydrate and protein fractions of algae hydrolysates [62, 63]. In these works, the caryophyllene biosynthesis pathway was engineered into the carbohydrate conversion strain and the protein conversion strain respectively. The engineered coculture produced up to 507.4 mg/L of total terpene mixture including sesquiterpene, monoterpene, and caryophyllene from algae hydrolysates. Terpenes are considered potential “drop-in” candidates for aviation fuels based on their high energy density. Importantly, the terpene yield produced from the *E. coli* consortia is significantly higher than those from plant tissue.

#### 4.5. Microalgae cocultures

In addition to lignocellulosic biomass, microalgae are an attractive biomass resource for biofuel production. Microalgae can provide several types of biofuels, including methane produced by anaerobic digestion of the algae biomass [64], biodiesel derived from microalgae oil [65], and biohydrogen produced photobiologically [66]. Many microalgae species can accumulate substantial quantities of lipids and contribute to a high oil yield. The average lipid content of microalgae varies between 1 and 70% and some can accumulate up to 90% of dry weight under certain conditions [65]. Furthermore, the lipids extracted from microalgae biomass often consist of triglycerides and can be converted to biodiesel by transesterification reactions in which three fatty acid molecules are esterified with a glycerol molecule. Synthetic consortia with microalgae cocultured with heterotrophs have been developed to take benefit from their mutualistic interactions.

Oleaginous yeast that can also accumulate high account of lipids has been cocultured with microalgae in several studies. In the mixed culture, yeasts use a vast variety of organic matter



and provide CO<sub>2</sub> for photosynthesis by the microalgae. At the same time, microalgae act as an oxygen generator which benefits yeast growth. The mixed cultures usually showed higher yields of lipid production and higher growth rates and biomass concentration of the microalgae. Examples of oleaginous yeast and microalgae coculture include the culture of yeast *Rhodotorula glutinis* with microalgae *Chlorella vulgaris* [67] and yeast *Rhodotorula glutinis* with cyanobacterium *Spirulina platensis* [68]. In these studies, the lipid production and algae growth was reported to be higher than that in the pure cultures. Applying a similar strategy, cocultivation of filamentous fungus *A. fumigatus* and microalgae has also been demonstrated to increase biomass production and lipid yield [69]. Moreover, because filamentous fungi are bioflocculating agents, cocultivation with microalgae assisted the flocculation and therefore the harvesting of microalgae.

In the oleaginous yeast and microalgae coculture, organic carbon feedstocks are usually needed to feed the yeasts to produce biofuel products. A phototrophic sucrose-secreting cyanobacteria *Synechococcus elongatus* was engineered and cocultured with oleaginous yeast strain *Cryptococcus curvatus* or *Rhodotorula glutinis* which is capable of producing biofuel products [70]. In this case, *S. elongatus* utilized sunlight and CO<sub>2</sub> and produced sucrose as a carbon source to yeasts while yeasts assist cyanobacteria growth and survival by eliminating oxidative stress. The oleaginous yeast strain was shown to have increased lipid production in the coculture and could be engineered to produce biofuel products. This synthetic coculture presents a potential sustainable production platform for biofuels production directly from sunlight and CO<sub>2</sub>.

Algae-bacterium-archaeon consortia have also been developed for the production of oil-like mixtures under anaerobic, thermophilic, and atmospheric conditions. Thermostable bacterium *Thermosipho globiformans* and archaeon *Methanocaldococcus jannaschii* were cocultured at 68°C with different species of microalgae under anaerobic conditions, followed by pyrolysis at 300°C and the consortia produced n-alkane rich biofuels and isoprenoids [71]. The composition and quantities of n-alkanes produced by pyrolysis were found to be closely related to the lipid contents and composition of the microalgae.

## 5. Tools for studying microbial consortia

Stability and tunable population compositions are highly desirable for microbial consortia developed for bioprocessing applications, because these properties could expand possible process configurations and improve efficiency. For examples, stability allows the use of continuous reactors and avoids eliminating one strain in the coculture during fermentation, whereas tunability would allow the optimization of the population composition for desired performance. Because of the multisubstrate nature of the biomass, microbial consortia with tunable population compositions are especially important to be adapted to the variable substrate compositions. Understanding the population dynamics and interactions between the members in the microbial consortia is important to develop a coculture with stable population and to tune the composition of the consortia.

Real-time PCR assays have been used to study the population dynamics of the consortia developed for biofuel production. In the study of simultaneous conversion of sugar and protein fractions of the hydrolysates by an *E. coli* coculture, specific primers targeting the unique genes in the chromosome of the two different *E. coli* strains were designed and q-PCR based

quantification method was developed to monitor the temporal profile of cell growth of the two strains in the coculture during fermentation [61]. The results indicated that an optimized coculture population which was tunable by changing the inoculation ratio of the two strains is essential for the consortium to achieve higher biofuel yield. q-PCR was also used to probe the dynamics of the yeast consortium which assembled the mini-cellulosome for ethanol production [72]. The primers were designed to specifically target a unique gene encoding for the endoglucanase, exoglucanase,  $\beta$ -glucosidase and the scaffolding expressed by each of the yeast strain. It was found that the final population ratio of the four yeast species did not change significantly compared with the initial inoculation ratio. By comparing the population of each strain in the assembled mini-cellulosome structure with that in the free enzyme system, the synergistic effect among the cellulases in the mini-cellulosome on cellulose hydrolysis was suggested. In another study, a real-time PCR assay based on the 16S rRNA gene sequence was performed to study the population abundance of each strain in a coculture consist of a cellulolytic bacterium and a noncellulolytic, solventogenic bacterium during the production of butanol [73]. The competition and cooperation relationships between the two strains at different stages of the fermentation was revealed by the population dynamics study; it was found that the population of each strain was readily modulated by culture conditions such as pH and nutrient availability.

Beyond the experimental demonstrations of bioconversion consortia, a modeling framework based on comprehensive ordinary differential equation has been developed to gain insights into the behavior and dynamics of a fungal-bacterial consortium for isobutanol production [44]. The rate expressions for each of the reaction steps were derived and the parameter values were obtained from the literature or by experiment. The concentrations of cellulose, microbial biomass, and isobutanol during the coculture fermentation predicted by the model were validated by experimental data. The model suggested that the competition between the fungal and bacterial strain for soluble saccharides is the key interaction that drives the behavior of two strains in the coculture and the relationship between the two strains was recognized as the cooperator-cheater. The model could also predict the outcomes and stability of the interactions in the microbial consortia, which provided important information for tuning the coculture population and stabilizing the consortia.

Furthermore, genome-based metabolic networks using multispecies dynamic flux balance analysis were developed to build a process model for an open pond system involving the oleaginous yeast and microalgae consortia for biodiesel production [74]. The algal monoculture and yeast monoculture were modeled separately and compared to the algae and yeast coculture with cellulosic glucose and xylose feeds. The model predicted the biomass and lipid productivities of the coculture with results comparable to those reported in literature. The economic analysis of this system was also performed and indicated that the algae and yeast coculture can produce biodiesel at competitive prices.

## 6. Conclusion

The development and implementation of biorefineries using biomass as the raw materials is encouraged by the growth in demand for renewable fuels and chemicals and the need for a reduction of fossil energy derived green-house gas emissions. Challenges facing scale-up of

the general biorefinery concept include technoeconomic hurdles stemming from recalcitrance of the biomass to hydrolysis, feedstock variability, achieving high rate, yield, and titers of bioconversion of the bulk of the biomass to fuels, up- and down-stream separations, and minimizing resource inputs, including water, chemical additives, electricity, and infrastructure. Progress in development of consortium-based bioconversion technologies provides solutions to many of these challenges by consolidating pretreatment and biocatalysis, allowing flexibility for utilization of multiple substrates at variable concentrations, and supporting tunability for targeted end-products. New advances in synthetic biology and metabolic engineering in the context of microbial communities will be required to accelerate adoption and scale-up of these strategies for an economically viable bio-based economy.

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## Conflict of interest

The authors declare that they have no competing interests.

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