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Harnessing Nature's Anaerobes for Biotechnology and Bioprocessing

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Abstract

Industrial biotechnology has the potential to decrease our reliance on petroleum for fuel and bio-based chemical production and also enable valorization of waste streams. Anaerobic microorganisms thrive in resource-limited environments and offer an array of novel bioactivities in this regard that could revolutionize biomanufacturing. However, they have not been adopted for widespread industrial use owing to their strict growth requirements, limited number of available strains, difficulty in scale-up, and genetic intractability. This review provides an overview of current and future uses for anaerobes in biotechnology and bioprocessing in the postgenomic era. We focus on the recently characterized anaerobic fungi (Neocallimastigomycota) native to the digestive tract of large herbivores, which possess a trove of enzymes, pathways, transporters, and other biomolecules that can be harnessed for numerous biotechnological applications. Resolving current genetic intractability, scale-up, and cultivation challenges will unlock the potential of these lignocellulolytic fungi and other nonmodel microorganisms to accelerate bio-based production.

INTRODUCTION

Rising global population and economic development have accelerated the demand for chemicals and fuels. The urgent need to develop a strong bio-based economy that sustainably meets these demands is recognized around the world (1–4). Fortunately, microbial bioprocessing has the potential to provide us with many of the resources that are currently delivered by fossil-fueled industries (5, 6). In addition to producing fuels, enzymes, and bio-based chemicals from renewable feedstocks, microorganisms can be used to valorize and reduce industrial, agricultural, and municipal waste and pollution (7, 8).

With few exceptions, industrial application of microbial biotechnology is biased toward model microbes with straightforward culturing requirements, genetic engineering tools, and production scaling. Popular industrial workhorses include the well-characterized bacteria *Escherichia coli* (9) and *Lactococcus lactis* (10), the yeasts *Saccharomyces cerevisiae* (11) and *Yarrowia lipolytica* (12), and the *Aspergillus* spp. filamentous fungi (13, 14). Although the assortment of industrial microbes enables production of a variety of chemicals, there are limits to how radically native metabolism can be rewired to handle novel substrate inputs or product outputs (15).

There is no doubt that nature contains a wide range of microorganisms with bioactivities that remain to be harnessed for biotechnological applications. Adaptation and evolution have allowed life to find its way into a tremendous variety of niches, and consequently the current diversity of organisms and biochemical processes is immense. For practical purposes, organisms can be classified according to their oxygen requirement (16). Aerobic organisms require molecular oxygen for their survival, as it is the terminal electron acceptor in cellular respiration. Facultative anaerobes grow whether oxygen is present or not, whereas anaerobic organisms cannot tolerate an oxygenated environment and have evolved diverse metabolisms for varied electron acceptors. Apart from a lack of oxygen, anaerobic environments are often characterized by extreme physicochemical parameters, such as temperature, pH, salinity, and pressure (17, 18). Consequently, microorganisms in these habitats have evolved a vast diversity of underexplored enzymes and metabolic activities. Facultative anaerobes such as *L. lactis* and baker's yeast have been used for centuries to produce primarily food and beverages, but the diversity of strictly anaerobic microorganisms that are available for industrial use remains particularly limited (19). One exception is the bacteria *Clostridia* spp. that are used to produce organic solvents such as acetone, *n*-butanol, and ethanol (20, 21).

Recent advances in affordable next-generation sequencing technology have accelerated discovery and development of such poorly characterized anaerobes for biotechnological applications (15, 22–24). This review provides a perspective on current and future biotechnology applications of anaerobic microorganisms. The focus is on anaerobic fungi from the phylum Neocallimastigomycota, which are key biomass degraders in herbivore digestive tracts (25–27). The instrumental role of these fungi for the conversion of recalcitrant lignocellulose into digestible sugars in the animal gut was recognized decades ago, but the detailed understanding of the physiology of the fungi and their translation into biotechnology is lacking (25, 28). Recent omics characterization has begun to unravel the biotechnological potential of this cryptic clade for a range of important applications (29–31) (**Figure 1**). Specifically, transcriptomic and genomic analyses have revealed that these specialized fungi possess an exceptional diversity of proteins that are involved in the processing of plant biomass, including biomass-degrading enzymes and membrane-embedded transporters that can be used to feed and modify microbial production platforms (29, 32). Here, we discuss potential applications of anaerobic fungi and challenges to industrial translation and provide an outlook on future development.

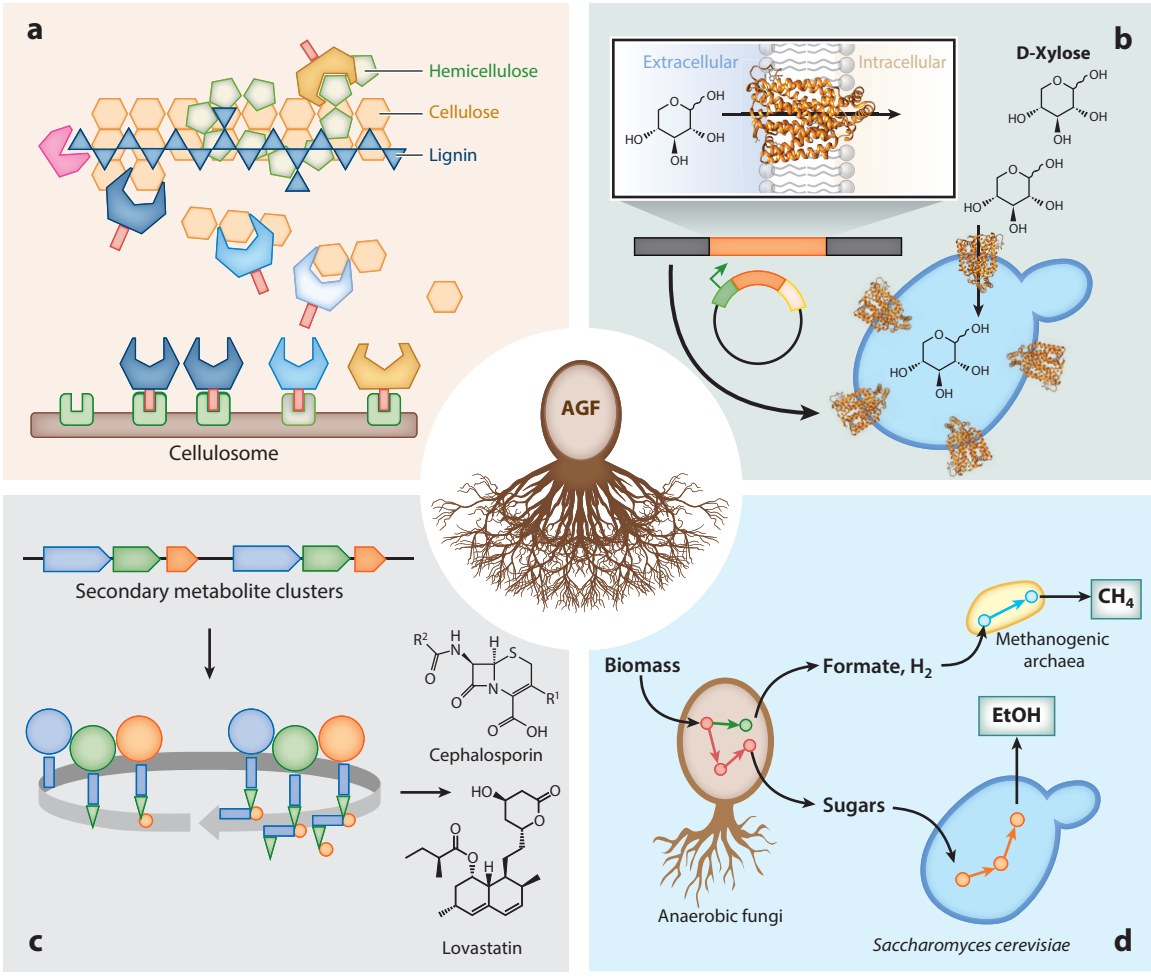

Figure 1

Anaerobic gut fungi (AGF) biotechnological applications exploit unique features: (*a*) robust cellulose-, hemicellulose-, and possibly lignin-degrading enzymes; (*b*) biohydrolysate transporters for heterologous expression; (*c*) novel secondary metabolite clusters; and (*d*) lignocellulose fermentation by consortia that lead to the production of, for example, methane or ethanol. The representative transporter crystal structure in panel *a* was adapted from Reference 234 (PDB: 4GBY).

CURRENT BIOTECHNOLOGICAL USES OF ANAEROBIC MICROBES

Early examples of large-scale industrial processes based on anaerobes, such as acetone-butanol-ethanol (ABE) fermentation by *Clostridium acetobutylicum*, were supplanted by the economically competitive production of chemicals through derivatization of petroleum (33). Consequently, industrial-scale implementation of anaerobes was limited to food production. However, renewed interest in reducing petroleum reliance and developing a sustainable bio-economy has motivated research and development of functionally novel and diverse anaerobes. Anaerobic cell factories now find diverse industrial application in fuel/chemicals fermentation, gas fermentation, waste digestion for biogas production, food production and enrichment, and bioremediation (34).

Inexpensive crude oil continues to limit demand for anaerobe-mediated fermentation of sugars into fuel alcohols and chemicals. Refined ABE fermentation mediated by engineered *Clostridium*

species facilitates *n*-butanol and acetone production, but these processes have sparse implementation (35) or target specialty, green-chemical markets (36). Another example of a commercial use of a strict anaerobe is the production of cyanocobalamin (vitamin B12) by *Propionibacterium freudenreichii* (37). Anaerobes have also been instrumental in the valorization of waste gas and organic sludge streams. For example, the New Zealand–founded LanzaTech has commercialized the fermentation of steel mill flue gas into ethanol using an engineered *Clostridium autoethanogenum* strain (38, 39). Other ventures by Coskata (now Synata Bio) and INEOS Bio sought to ferment reformed syngas and gasified lignocellulose, respectively, for ethanol production, but both companies ceased production after pilot plant operation (40, 41). Production of methane-rich biogas from agricultural or municipal sludge waste via anaerobic digestion has expanded dramatically in Europe (42) and China (43) during the past two decades. In these plants, consortia of predominantly obligate anaerobic bacteria, i.e., *Bacteroides*, *Bifidobacterium*, and *Clostridium*, mediate digestion of organic waste in either continuous or batch configurations (44). Biogas is harnessed for energy production, and the solid digestate is sometimes further processed into nitrogen-rich biofertilizer (45, 46).

Furthermore, anaerobes have been harnessed for denitrification during wastewater treatment. Anaerobic ammonium oxidation (anammox) bacteria have been commercialized as an alternative to aerobic ammonium removal from industrial and municipal wastewater (47, 48). Implementation at treatment plants in Europe has facilitated energy autarky (49) and enhanced the efficiency of biogas generation. Similarly, anaerobes capable of modifying inorganic compounds have been explored for bioremediation applications. Although segregation of waste is typically more economic, bioremediation has been commercialized at a smaller scale, such as the dehalogenation of contaminant organohalides using *Dehalococcoides* spp. developed by Regenesis (50).

Finally, the role of anaerobes in food production has expanded to include flavor enhancement, preservation, and probiotic fortification. *Propionibacterium* spp. used in cheese production have been used to enhance flavor in other fermented foods (51). Further, *Propionibacterium* spp. have been supplemented for vitamin B12 fortification and enhanced preservation through fatty acid production. *Bifidobacterium* spp. live cultures have been developed as whole cell probiotics within food production (52), and formulations using anaerobes have been broadly commercialized as probiotic additives for animal feeds (53).

As shown in **Table 1**, the scope of anaerobic applications extends well beyond what has been realized in industry. Currently, most of these processes lack economic viability yet demonstrate the promise for sustainable manufacturing of chemicals and waste processing. It is important to note that whereas industrial strain development has focused on prokaryotic systems, applications of anaerobic eukaryotes have been poorly explored, despite their potential. For example, ciliates were shown to indirectly enhance methane productivity from anaerobic digesters, but the exact mechanism remains unknown (54). Similarly, anaerobic gut fungi (AGF) within the phylum Neocallimastigomycota exhibit exceptional biomass-degrading capabilities, and their importance for animal husbandry and productivity is well established (55, 56).

ANAEROBIC GUT FUNGI: BEYOND BIOMASS-DEGRADING ENZYMES

A Rich Repository of Carbohydrate-Active Enzymes

The ecological role of fungi is to decompose and recycle biomass, making them attractive tools for bioconversion and valorization of municipal and agricultural waste (57). Plant biomass is a complex mixture predominantly composed of cellulose, hemicellulose, and lignin polymers (**Figure 2**). Cellulose (40–50% of plant cell walls) is a linear polymer of β(1→4)-linked D-glucose units, whereas hemicellulose (20–40% of plant cell walls) is branched and contains

Table 1 Recent examples of anaerobe strain or consortia development

Category	Substrate	Product	Strain(s)	Reference
Sustainable chemicals production	Soy molasses	Propionic acid	*Propionibacterium acidipropionici*	221
	Cellulose	Ethanol	*Caldicellulosiruptor bescii*	222
	Cellulose and hemicellulose hydrolysate	H_2 Ethanol	*Clostridium thermocellum*	223
	Cellulose	H_2	*Desulfurococcus amylolyticus*	224
	Corn husk hydrolysate	Butyric acid	*Clostridium tyrobutyricum*	225
	Model syngas	Ethanol Butanol Hexanol	*Clostridium carboxidivorans*	21
Bioremediation	Cadmium	N/A	*Desulfobacteraceae* and *Desulfobulbaceae*	226
	Hydrogen sulfide biogas	N/A	*Chlorobium limicola*	227
	Uranium (VI)	N/A	*Methanosarcina* spp.	228
	2,4,6-Tribromophenol	N/A	*Clostridium* sp., *Dehalobacter* sp., *Desulfatiglans parachlorophenolica*	229
	Trichloroethane	N/A	*Dehalococcoides* spp.	230
Bioelectrochemical systems	CO_2, electricity	Acetate	*Sporomusa ovata*	231, 232
	Acetate	Electricity	*Geobacter sulfurreducens*	233

many different hexose and pentose sugars, including glucose, mannose, xylose, and arabinose (58, 59). Adding to the recalcitrance of plant biomass, in plant cell walls the cellulose and hemicellulose polysaccharides are surrounded by a layer of aromatic lignin (60), which is particularly resistant to microbial degradation and can vary in content and structure between young and old plants and across different species (61, 62). It follows that complete depolymerization of plant biomass requires the action of several enzymes, including xylanases (EC 3.2.1.8), esterases (EC 3.1.1.6), endocellulases (EC 3.2.1.4), exocellulases (EC 3.2.1.91), and beta-glucosidases (EC 3.2.1.21).

Fungi are the unquestionable masters of biomass degradation in nature, mechanically breaking down plant fibers by burrowing into the material as well as by secreting a vast array of biomass-degrading enzymes (63, 64). Fungi are found in all terrestrial and aquatic habitats on earth where biomass recycling occurs: They thrive in soil; in fresh and marine water; and even in such extreme habitats as animal guts, inside plant tissues, and in the deep ocean crust (65–69). Of particular interest for biotechnological applications are the AGF that inhabit the intestines of a wide range of herbivorous animals, from cattle, goats, and sheep to horses, giraffes, and elephants (27). AGF were first discovered at the beginning of the twentieth century but were not identified as true fungi until the mid-1970s (28, 70, 71). As illustrated in **Figure 3**, their highly unusual lifecycle includes a flagellated, free-swimming zoospore that by unknown chemotactic signals finds and

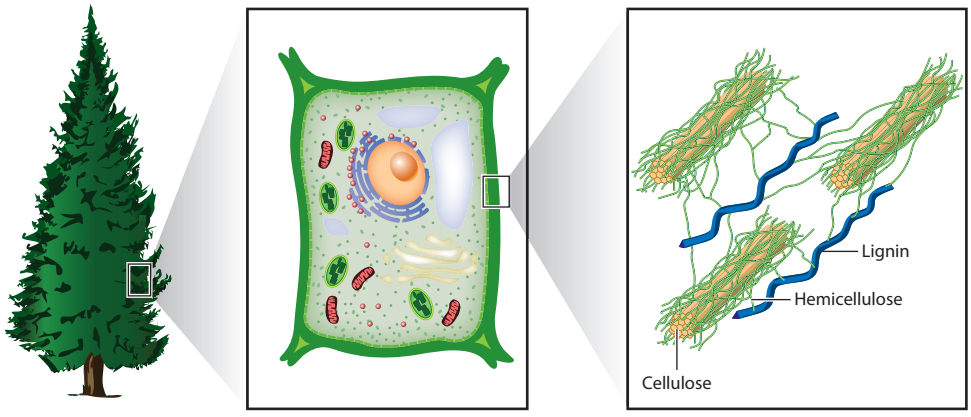

Figure 2

Cell walls in lignocellulosic biomass are primarily composed of cellulose, hemicellulose, and lignin biopolymers.

attaches itself to plant fragments (**Figure 3**). On the plant, the encysted zoospore typically develops a rhizoidal network that burrows into the plant biomass. Inside the growing sporangium, new zoospores are differentiated and eventually released by rupturing of the mature sporangium. Depending on species, the AGF may have one or more flagellum and form a more- or less-developed rhizoidal system (72). Apart from AGF, the herbivore gut microbiome contains cellulolytic bacteria with complementary activity to the fungi, as well as methanogenic archaea that serve as electron sinks to synergistically convert the plant biomass into its constituent sugars (73, 74).

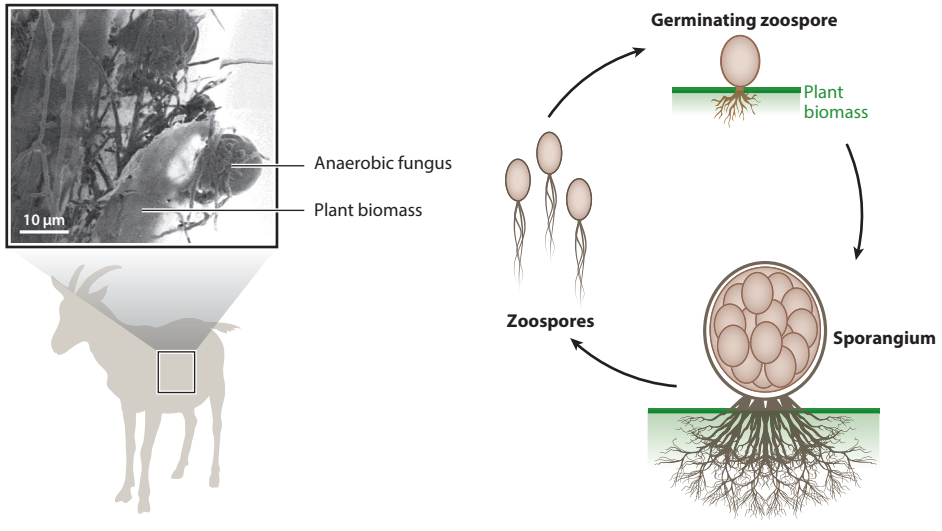

Figure 3

Anaerobic gut fungi (AGF) colonize and degrade ingested biomass within host animals. The helium-ion micrograph depicts *Neocallimastix californiae* colonization of unpretreated reed canary grass. AGF exhibit an unusual life cycle during which they transition from motile zoospores to encysted sporangia.

Although it is well recognized that the AGF are an integral part of the herbivore gut microbiome, they remain understudied and underexplored for biotechnological applications (27, 75). Recent comprehensive transcriptomic and genomic analyses, however, strongly suggest that AGF are a rich source of biomass-degrading enzymes (for a recent review, see 76). Comparative transcriptomics revealed that this clade possesses the largest and most diverse set of biomass-degrading enzymes across the fungal kingdom, and that the exact composition of the secreted enzymes is tuned depending on substrate availability (29, 31). As Seppälä et al. (76) reviewed, several enzymes from AGF have been successfully produced in biotechnological workhorse bacteria and yeast (77–82). Like other fungi, AGF secrete biomass-degrading enzymes to their extracellular milieu, so as to depolymerize biomass into soluble sugars. But as opposed to their aerobic counterparts, many enzymes from anaerobic fungi are organized in large complexes called cellulosomes that likely increase substrate channeling and efficiency of biomass degradation (31, 83). Similar enzyme complexes have previously been reported in cellulolytic bacteria, but the cellulosomes of AGF remain unique among eukaryotes and present an attractive target for downstream engineering (84).

Anaerobic Lignin Deconstruction

Lignin is composed of aromatic noncarbohydrate polymers, and because it is the most recalcitrant fraction of plant cell walls, it is of particular interest to the bioprocessing community (85, 86). Investigations into lignin processing and valorization are driven by the realization that this portion of the plant cell wall must be deconstructed to improve yields from bioenergy feedstocks and economic viability of next-generation biofuels and other bio-based products (87–90). Depolymerization of lignin with ionic liquids and inorganic catalysts has been studied extensively, with significant success (91–94). There is, however, considerable interest in developing more sustainable, bio-based pathways for lignin deconstruction (95–99). Some aerobic fungi, such as white rot basidiomycetes, modify lignin through a complex enzymatic cocktail of laccases, lignin peroxidases, aryl-alcohol peroxidases, and manganese peroxidases (100). Such enzymatic lignin processing complements traditional methods by creating alternative streams of aromatic intermediates and also offers the potential benefits of lowered cost and increased sustainability (96, 101–103).

Although there is currently no described molecular mechanism for anaerobic lignin deconstruction, signs suggest that life has indeed found an oxygen-independent route to break down the most abundant aromatic polymer that exists in nature. Recently, researchers were able to isolate two species of anaerobic bacteria that can use lignin as a sole carbon source (104, 105). In addition, Henske et al. (106) identified transcripts coding for proteins with unknown function coregulating with carbohydrate-active enzyme messenger RNAs in AGF transcriptomes. These findings are important because the vast majority (~60%) of AGF transcriptomes bear no similarity to protein-encoding genes in the National Center for Biotechnology Information database (29, 31). This infers that early-branching fungi contain a wealth of unknown proteins and enzymes for lignocellulose hydrolysis that have yet evaded discovery. Given the high interest level in enzymatic lignin processing, the effectiveness of AGF against crude lignocellulose, and the wealth of novel proteins from AGF that are activated during biomass degradation, the AGF are an obvious source for undiscovered enzymes that anaerobically depolymerize or rearrange lignin (29, 76, 86, 106).

A Potential Source of Carbohydrate and Biohydrolysate Transporters

Cellular membranes and membrane proteins allow cells to control and adjust the chemical composition of the intracellular environment (107). Although often overlooked in biotechnological

applications, membrane proteins contribute greatly to biosynthetic fluxes in individual cells as well as in microbial communities, and there is a growing realization that transporters and receptors are valuable tools for engineering microbial production platforms (reviewed in 108–110). Easily handled yeasts, such as *S. cerevisiae*, remain widely used for the industrial production of fermented food and beverages, and more recently for the production of recombinant proteins, biofuels, and fine chemicals (reviewed in 111). Much effort has been directed toward enhancing the performance of industrial yeasts—both by exploring the genetic diversity of yeasts in nature and by engineering native and/or heterologous enzymes and pathways in existing chassis strains. Meanwhile, renewable plant biomass is emerging as the preferred feedstock for microbial production platforms, yet insufficient biohydrolysate uptake into the cells is a formidable bottleneck in even highly engineered yeast strains (112). The preferred carbon source for *S. cerevisiae* is glucose, and wild-type baker's yeast uses a complex and highly regulated network of ~20 hexose transporters with different affinities and uptake rates, essentially to ensure optimal substrate uptake at all times (113–115). However, plant biomass is rich not only in glucose but also in pentose sugars like xylose and arabinose (58, 59). To fully utilize the various sugar constituents of depolymerized plant biomass, carbohydrate transporters and pathways that can be used for efficient cofermentation in yeast are highly sought after. Apart from engineering endogenous transporters with altered substrate specificities and transport kinetics (116, 117), heterologous transporters sourced from other fungi and plants have been used to enable yeast growth on cellobiose (118–120) and xylose (121–123). Transporter engineering for biomass utilization in yeast was extensively reviewed recently (124).

Efflux pumps extrude a wide range of metabolites from cells and are implicated in solvent tolerance as well as resistance to drugs and antibiotics (125, 126). From a bio-production perspective, efflux pumps may increase the production of hydrophobic biofuels and small molecules by continuously removing product from the cell and thereby increasing flux through the pathway and minimizing toxic effects and feedback inhibition (110). For example, endogenous efflux pumps were massively induced in a yeast strain that was engineered to produce artemisinic acid (127) and carotenoids (128). Similarly, overexpression of endogenous transporters has been shown to increase the tolerance of yeast to ethanol (129) and alkanes (130). Alkane tolerance of yeast was also improved by using transporters originating from the oleaginous *Y. lipolytica* (131).

A recent transcriptomic analysis revealed that AGF possess a wide diversity of membrane-embedded transporters, including pleiotropic drug transporters and a variety of transporters for carbohydrates, amino acids, small metabolites, and metals that are of biotechnological interest (32). Among carbohydrate transporters, the study identified members of the major eukaryotic carbohydrate transporter families [the major facilitator superfamily, solute sodium symporter family, and sugars will eventually be exported transporter (SWEET) family] (32, 132). The role, and abundance, of the SWEETs in the fungal kingdom remains unexplored, but these sugar transporters play important physiological roles in plants (133–136). Furthermore, the study revealed an unexpectedly large variety of putative membrane-anchored substrate-binding proteins that are known to function in concert with carbohydrate uptake systems in prokaryotes (137). These findings suggest that the AGF employ unusual mechanisms for sequestering and transporting carbohydrates, and these could conceivably be transferred to other eukaryotes, such as yeast.

A Cryptic Source of Bioactive Small Molecules

Apart from proteins that are involved in biomass degradation, fungal genomes encode a wealth of enzymes responsible for the biosynthesis of secondary metabolites (SMs), commonly referred to as natural products. Natural products are an extremely diverse class of bioactive molecules that are of great interest for the pharmaceutical industry (138–140). For a thorough review of fungal

secondary metabolism, see Keller et al. (141). Unlike that of Ascomycota and Basidiomycota, the potential of AGF to biosynthesize natural products has not been characterized. However, canonical biosynthetic enzymes for natural products, polyketide synthases (PKSs) and nonribosomal peptide synthetases (NRPSs), were discovered in AGF genomes belonging to strains *Anaeromyces robustus*, *Neocallimastix californiae*, and *Piromyces finnis* (31). Genomic mining using the secondary metabolite unknown region finder (SMURF) algorithm (142), based on hidden Markov model identification of SM gene features, yielded 112 PKS, PKS-like, NRPS, and NRPS-like gene clusters from four other AGF genomes of similar quality from the MycoCosm fungal genomics resource (143). Interestingly, the closely related phylum Microsporidia lacks putative SM clusters. The absence of publications featuring AGF secondary metabolism invites active research in this field. New chemical and evolutionary insights may be gained on the structure and function of these mega-enzymes by studying basal fungi. In addition, AGF SMs may be sources of novel antibiotics, therapeutics, or drop-in biofuels.

Unfortunately, although fungal genomes encode for a wealth of natural products, natural products discovery from fungi has been hindered by the same two major obstacles as for other organisms: (*a*) Silent biosynthetic gene clusters that are not readily expressed under standard laboratory conditions are difficult to identify, and (*b*) orphan or cryptic biosynthetic gene clusters are not easily linked to an actual product (144, 145). Synthetic biology tools, such as heterologous expression of SM biosynthetic gene clusters (146–149) and pathway refactoring (150, 151), provide a means to address these challenges.

Anaerobic Consortia Enhance and Expand Production Capabilities of Cell Factories

Few bioprocesses use cultures of mixed microbial populations, known as consortia (152, 153), despite distinct advantages over clonal bioprocessing. Productivity improves when metabolic pathways are split between microorganisms in a microbial consortium, and tools have been developed to evaluate whether a consortium is capable of outperforming a single community (154–156). Specifically, consortia expand the number of exogenous elements that can be cloned for a process (157–160), enable complex/mixed substrate coutilization (161), and mitigate by-product formation to increase yield (162, 163) and can enhance tolerance to process fluctuations (164–168).

Anaerobic consortia containing AGF are well-suited for biochemical production from lignocellulosic waste, because they possess the greatest variety of biomass-degrading enzymes of all fungi (76). Recently, it was demonstrated that the AGF *N. californiae* and *A. robustus* convert up to 49% of cellulose mass to free glucose in batch cultures (169). Further, growth on crude biomass released excess glucose, mannose, galactose, xylose, and arabinose sugars, sufficient to support subsequent *S. cerevisiae* growth. Transcriptome-derived metabolic maps suggest that galactose and arabinose sugars accumulate owing to a lack of complete, corresponding catabolic pathways. In other words, the sugars that are not catabolized by the fungi can be devoted entirely to the support of other organisms in a coculture. This principle has been applied for two-stage conversion of biomass to ethanol using a batch coculture of anaerobic fungus *Pecoramyces ruminantium* strain C1A and *E. coli* strain K011 (170). Delayed *E. coli* inoculation after preliminary saccharification facilitated 14% dry weight conversion of pretreated corn stover grass to ethanol with a final titer of 28.16 mM. Notably, ethanol yield and biomass conversion using a wild-type anaerobic fungal strain were higher than in engineered monoculture approaches.

The treatment of recalcitrant substrates with anaerobic fungi has been examined as a cost-efficient method for improving the efficiency of biogas production. Neocallimastigomycota

mediate both enzymatic and physical disruption of fiber-rich biomass, providing access to bacterial members for enhanced degradation (171). Hydrolytic pretreatment of hay solids suspended in reactor effluent with *Neocallimastix frontalis* was shown to accelerate degradation and bolster biogas production in batch culture, despite inhibitory volatile fatty acid accumulation (172). Conversely, *Piromyces rhizinflata* YM600 addition augmented hydrogen and methane productivity and volatile fatty acid degradation in a two-stage digestor process (173). Moreover, improvement to biogas fermentation using pig slurry sludge was demonstrated to vary depending on the anaerobic fungal species added (174). Strain selection is of critical importance for effective biogas production, and future formulations of synthetic consortia should explore multiple strains of AGF.

OVERCOMING BARRIERS TO INDUSTRIAL IMPLEMENTATION OF ANAEROBIC GUT FUNGI BIOTECHNOLOGY

Challenges

Development of AGF into industrial production strains is hindered by their strict growth requirements and genetic intractability. AGF isolates cultured under laboratory conditions are typically grown in a complex medium containing up to 15% (v/v) of clarified rumen fluid (175). Although complex media formulations are used for some large-scale bioprocesses (176), the low production volume and high cost of rumen fluid prohibit process development using this formulation. Additionally, lot-to-lot variability of complex media ingredients necessitates quality-control testing and impacts production performance (177). Defined growth media formulations have been described for AGF (178, 179) but do not support the growth of all industrially relevant AGF strains. Isolation of fungi from native consortia in defined media can bias downstream compatibility. Similarly, bioreactor design considerations, such as cell immobilization, heat transfer control, and sensitivity to shear/agitation, are poorly characterized for pure AGF cultures and defined consortia, given the scarcity of studies on developing production processes (169, 170, 174, 180, 181). Furthermore, the low working volumes ($\leq$1 L) used in these studies poorly represent scaled process conditions (169, 170, 174, 180, 181).

Anaerobic culturing conditions and thick cell walls of mature sporangia have hampered the development of genetic methods or transformation of AGF. Strain engineering can be performed without genetic tools by coupling random mutagenesis, mediated by chemical agents or high-energy radiation, with a functional screen for desired mutants. However, this approach becomes laborious for phenotypes that are difficult to screen in a high-throughput manner (182). Moreover, manipulation of the genome is necessary to deepen characterization that is otherwise based on omics analyses and behavior in cell culture. Genetic tools enable tuning of endogenous metabolic pathways and introduction of foreign genes to enhance synthesis of native or non-native compounds. Genetic tool kit development for any organism, including AGF, requires strategies for facilitating exogenous DNA uptake, ensuring that the DNA payload is mitotically stable, and enabling expression of genes encoded on the payload.

To date, only a single study by Durand et al. (183) has described the transient transformation of AGF, specifically the strain *N. frontalis*. Uptake of a plasmid payload into fungal zoosporangia was facilitated using biolistic bombardment and experimentally measured through detection of encoded β-glucuronidase (GUS) reporter system activity under a native AGF promoter (184). However, only transient transformation was reported, as the GUS gene payload was not detected after seven days. Although this study demonstrated that AGF are amenable to transformation and heterologous expression, strategies for stability or targeted manipulation of the genome have not been realized.

Developing Genetic Tools for Metabolic Engineering of Anaerobic Gut Fungi

To establish methods to metabolically engineer AGF, it is inviting to adapt methods that have been developed for other fungi. Two high-efficiency genetic transformation methods are protoplast-mediated and *Agrobacterium tumefaciens*–mediated transformation (for a review of transformation methods, see 185). Unfortunately, narrow cultivation temperatures for AGF (~37–42°C) (178) and poor tolerance to room-temperature conditions (186) lead to incompatibility between the *A. tumefaciens* DNA transfer machinery (187, 188) and AGF. Generation of protoplasts, or cells that lack cell wall, for protoplast-mediated transformation requires significant optimization of preparation parameters that are not always compatible with fungi (189), but it is otherwise simple once established. Given the complicated life cycle of AGF (**Figure 3**), it is important to note that transformation efficiency can differ across cell types of a single fungal strain (190). The zoospore stage of the AGF life cycle is a promising candidate for transformation, as it is surrounded by a thinner (~0.2 μm) cell wall (28, 191), and germinating zoospores have demonstrated natural uptake of small interfering RNA (192). It is even possible that simple electroporation methods, appropriate for yeast, can be adapted for this life stage (193).

To facilitate the detection of DNA uptake, it is also important to identify reporter genes that are compatible with the host. Fluorescent proteins are extremely popular genetic reporters, as they provide a fast and sensitive output, but the widely used green fluorescent protein is not compatible with the anaerobic growth conditions of AGF, as it requires molecular oxygen to fold (194, 195). Conversely, flavin-based fluorescent proteins function in low-oxygen conditions and may become useful tools for AGF transformation (196).

Apart from establishing efficient DNA uptake, it is important to ensure that the strain remains stable over multiple generations. Extrachromosomal plasmids need to be replicated and propagated to progeny, and although plasmids typically encode a gene that gives the cell some kind of advantage (e.g., a gene that allows the cell to grow in selective medium), plasmid loss is still a risk (197, 198). Stability can be engineered through integration of the exogenous DNA onto the genome. Chromosomal integration takes place by homology-directed repair, predominantly homologous recombination (HR), or nonhomologous end joining (NHEJ) DNA repair pathways (199). HR facilitates targeted integration of DNA by using flanking regions that are homologous to the desired integration sites, whereas NHEJ promotes integration into random sites (**Figure 4*a*,*b***). Consequently, random integration restricts classical knockout/knock-in approaches. Although it remains to be shown if it is true for Neocallimastigomycota, nonmodel fungi typically use the random NHEJ pathway (200). HR efficiency can be increased by increasing the length of flanking homologous regions (201), engineering NHEJ-deficient mutants (202, 203), or using a split-selection marker (204) (**Figure 4*c*,*d***).

For efficient gene expression, it is important to identify regulatory elements such as promoters and terminators (205). Assembled genomes and transcriptomes can be leveraged to globally identify and functionally validate native regulatory sequences (206, 207). Although targeted integration and stable plasmid expression enable the manipulation of single genes or gene sets, manipulation is time intensive. Recently developed, versatile genome-editing tools promise to accelerate AGF strain metabolic engineering and translation to industry. The clustered regularly interspaced short palindromic repeats (CRISPR)–CRISPR-associated protein 9 (Cas9) adaptive immune system has been engineered into a broadly used, powerful gene-editing tool (see 208, 209). CRISPR-Cas9 approaches have been applied to rapidly engineer nonmodel fungal strains (210–215), but the function of the associated Cas9 endonuclease is restricted to target sequences containing a NGG protospacer adjacent motif (PAM) sequence (216, 217). Target site selection and therefore genetic manipulation may be severely limited in AT-rich AGF genomes (218). Fortunately, an alternative

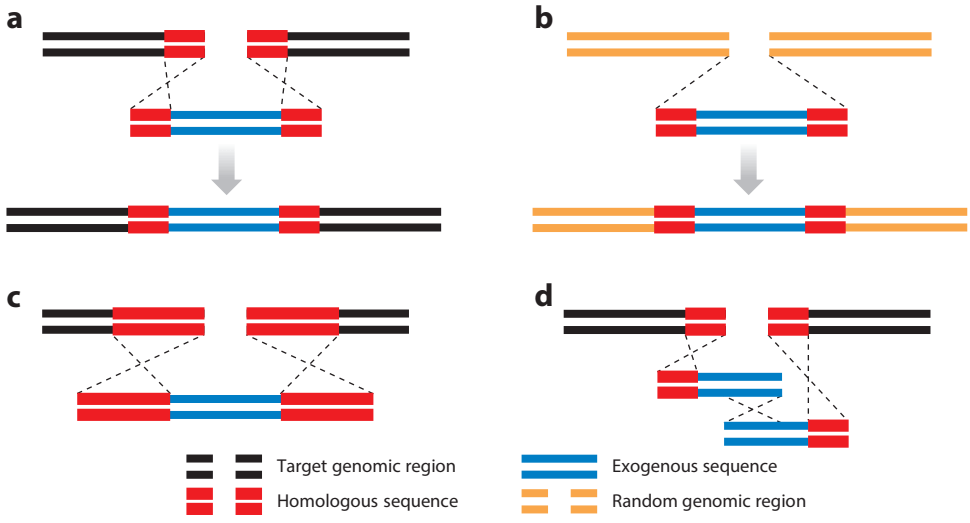

Figure 4

Genomic integration of exogenous DNA is facilitated by either (*a*) homologous recombination (HR) or (*b*) nonhomologous end joining (NHEJ) repair pathways, generating targeted or random integration, respectively. HR can be enhanced by (*c*) designing longer homologous, flanking regions, engineering an NHEJ-deficient mutant, or (*d*) using a split marker. Note, the split marker increases HR efficiency by requiring recombination of the marker itself, which is unlikely to occur at off-target sites.

endonuclease, Cpf1, with a T-rich (TTTV) PAM sequence, has been developed as an alternative genome-editing platform (219) suitable for AGF site selection and rapid strain development.

Anaerobic Gut Fungi Culture Scale-Up

Generally, anaerobic production at scale requires staging of cell culture growth to generate sufficiently dense, large-volume cultures for inoculation of fermentation bioreactors (**Figure 5**). Bioreactor conditions for smaller volumes must be optimized for growth, whereas fermentation conditions may differ to enhance productivity. As inoculation staging lengthens process start-up, continuous bioprocesses are preferred to batch and fed-batch configurations. Anaerobic process design is simplified owing to reduced heat generation during fermentation, lack of aeration concern, and reaction kinetics slower than mass transfer when compared with aerobic processes (220). Ultimately, heat transfer, mass transfer, mixing, and feed loading (solid substrates) must be optimized for proposed AGF processes or any process that relies on anaerobic microorganisms.

Batch studies of AGF enzyme production (180, 181) and biomass saccharification (169, 170) described titers that are insufficient to currently motivate direct scale-up, but these studies can inform future process design. For example, immobilization of AGF within biocompatible calcium alginate beads enabled sequential batch production, resulted in higher initial enzyme titers, and facilitated semicontinuous production of β-glucosidase enzymes with specific activity comparable to nonimmobilized cultures (180). One can easily envision the utility of these immobilized systems to reduce cell mass washout in continuous production schemes and simplify downstream purification of enzyme products. Similarly, AGF pretreatment of lignocellulosic biomass in a two-stage process demonstrated fed-batch approaches to chemicals production that circumvent development of defined microbial consortia (169, 170).

A glaring limitation of all AGF production studies is low reaction volumes (≤100 mL) that may mask mass and heat transfer effects and are currently limited to batch configurations. The

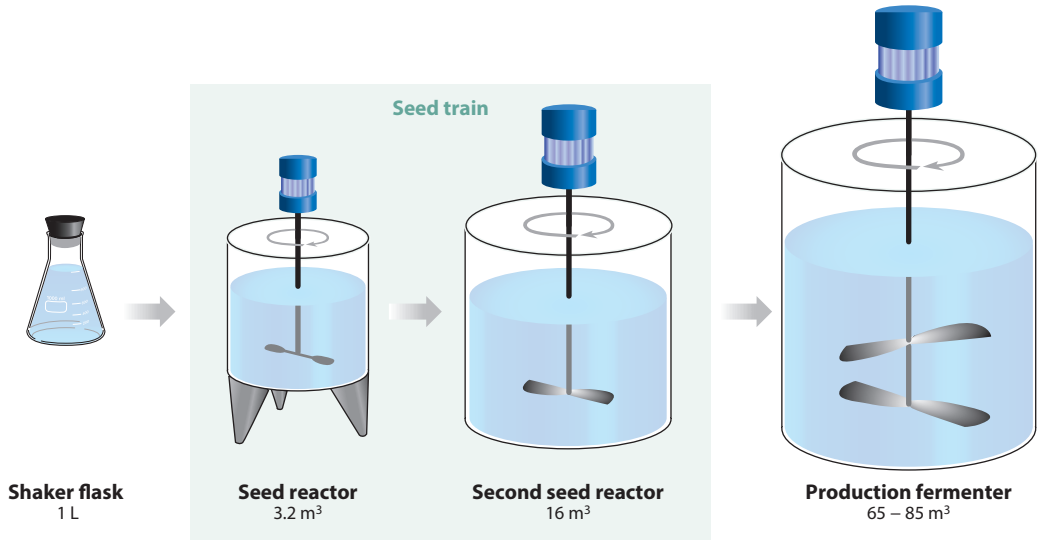

Figure 5

Bioprocess inoculation sequence. The production strain is grown in stages in sequentially larger reactors until inoculation at a desired density in a production fermenter. The inoculation ratio is typically 1:10 to 1:1000, and the number of seed train stages will vary depending on this ratio and the process scale. Example reactor volumes reflect fermentation process parameters for the filamentous fungus *Acremonium chrysogenum* (235).

effects of agitation or mixing regimes and substrate loading have also been neglected. Further characterization of growth and production in larger-volume, bench-top bioreactors that vary these parameters is necessary prior to any true scaling and technoeconomic assessment.

FUTURE OUTLOOK

Biotechnology has the potential to complement and eventually relieve our heavy dependence on oil, coal, and natural gas (5, 6). The biotech industry has targeted anaerobic microorganisms for decades, and the unusual bioactivities of anaerobes have been harnessed for solvent fermentation (35), waste valorization (38, 39, 44–46), and bioremediation (50). Nevertheless, anaerobic eukaryotes remain elusive and underutilized, but recently these organisms have attracted great interest in the industry (29, 31, 74). This is particularly true for the unusual and early-diverging anaerobic fungi (27), which are key species in an interkingdom microbial community that enables the conversion of lignocellulosic plant biomass into digestible sugars. Strict growth requirements and difficulties in maintaining stable laboratory cultures have hampered the exploration of anaerobic fungi. However, the study of unwieldy, nonmodel microorganisms has been greatly facilitated by the development of sophisticated and increasingly affordable omics technologies. Focused sequencing efforts on novel strains are resulting in an ever-growing library of high-quality sequencing data that can be used to deepen our understanding of the physiology and biotechnological promise of these underexplored microorganisms. Sequencing data reveal that anaerobic fungi have a unique array of biomass-degrading enzymes, biohydrolysate transporters, and biosynthetic gene clusters that are likely key to their survival in resource-limited, competitive environments. Cocktails of unmodified AGF biomass-degrading enzymes have demonstrated robust activity on par with industrial formulations (81). We envision that AGF enzymes will soon enhance our capabilities for waste valorization and sustainable chemicals production.

Furthermore, genetic engineering of currently intractable microorganisms like AGF will enable new bioproduction routes and lead to improved chassis strains for industrial-scale fermentations to support a strong and versatile bio-based economy.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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