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Review

Advancing sustainable biotechnology through protein engineering

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The push for industrial sustainability benefits from the use of enzymes as a replacement for traditional chemistry. Biological catalysts, especially those that have been engineered for increased activity, stability, or novel function, and are often greener than alternative chemical approaches. This Review highlights the role of engineered enzymes (and identifies directions for further engineering efforts) in the application areas of greenhouse gas sequestration, fuel production, bioremediation, and degradation of plastic wastes.

Highlights

Enzyme biocatalysts can be used to improve industrial sustainability.

Improvements are necessary to reach sustainability targets and reduce the impacts of climate change, plastic pollution, depleting natural resources, and deforestation.

Protein engineering enables the design of enzymes with increased specificity, efficiency, and stability.

Engineered enzymes are being developed and implemented for GHG sequestration, biofuel production, plastic waste treatment, and bioremediation.

A growing awareness for sustainable solutions

Both consumers and producers have recently embraced an increased awareness of sustainable products and manufacturing processes [1]. This trend has been accelerated by many factors such as climate change, plastic pollution, depleting natural resources, and deforestation [2,3]. However, environmental sustainability and/or green manufacturing is a difficult sentiment to convey as it has a wide range of meanings and implications. Moreover, the moniker of sustainable can often be misleading as it may be applicable to one part of the manufacturing process yet not reflect the results of a complete **life cycle assessment** (see Glossary) and greenhouse gas (GHG) analysis, commonly referred to as the carbon footprint, for the entire process and life of the product. In 1987, the World Commission on Environment and Development defined sustainability as meeting the needs of the present without compromising the ability of future generations to meet their own needs [4]. Using this broad definition as a backdrop, this review seeks to demonstrate how industrial processes can move toward sustainable practices through the use of engineered biological **catalysts**, specifically enzymes.

At the outset, it should be stated that simply adopting a biological process does not inherently make an overall process more sustainable (as stated above with the use of the term green manufacturing). However, the potential for improved sustainability is present in biological processes when compared with other chemical industrial processes owing to the generally lower temperatures and reduced (or eliminated) toxic solvents [5]. Likewise, many biological processes exhibit high levels of specificity compared with traditional chemical processes [6]. Thus, biological processes may be more desirable from an environmental perspective in terms of energy consumption and byproducts [7]. Possible biological systems include whole cells, microorganisms, and the use of **purified enzyme systems**. This review will focus on purified enzyme systems as biocatalysts which can enable sustainable catalysis at an industrial scale. An overview of industrially utilized enzymes along with their market share is captured in Table 1. This Review highlights some recent advances in the deployment and engineering of enzyme catalysts for solving industrial sustainability challenges. After starting with a brief overview of enzymes and the means to engineer them, this review highlights the applications of enzyme systems for sustainable **GHG sequestration**, biofuel production, and waste plastic degradation, as well as environmental **bioremediation** (Figure 1). This review is not meant to

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Table 1. Industrial impact of enzyme classes[a]

Enzyme	Uses	Global market for industrial enzymes in 2021 ($ million)[b]	Global market for industrial enzymes in 2021 (%)[b]
Carbohydrase	Catalyze the hydrolysis of complex carbohydrates into simple sugars	2593.7	40.37
Protease	Catalyze the breakdown of proteins into smaller polypeptides or single amino acids	1381.5	21.50
Polymerase and nuclease	Synthesize long chains of polymers or nucleic acids; Catalyze the breakdown of nucleic acids into amino acids	1022.0	15.91
Phytase	Catalyze the hydrolysis of phytic acid into inorganic phosphorus	639.0	9.95
Lipase	Catalyze the breakdown of many types of fats into smaller molecules.	522.0	8.12
Other enzymes	Various functions	266.7	4.15

[a]While not all enzymes mentioned in this article are produced at industrially relevant titers, the application of industrial enzymes make up a very large global market share.
[b]Production of enzymes commonly used industrially and their global market share are listed [111].

be comprehensive in covering all avenues for which enzymes can improve sustainability, but rather serves to highlight key examples of enzyme biocatalysts engineered for enhanced sustainability.

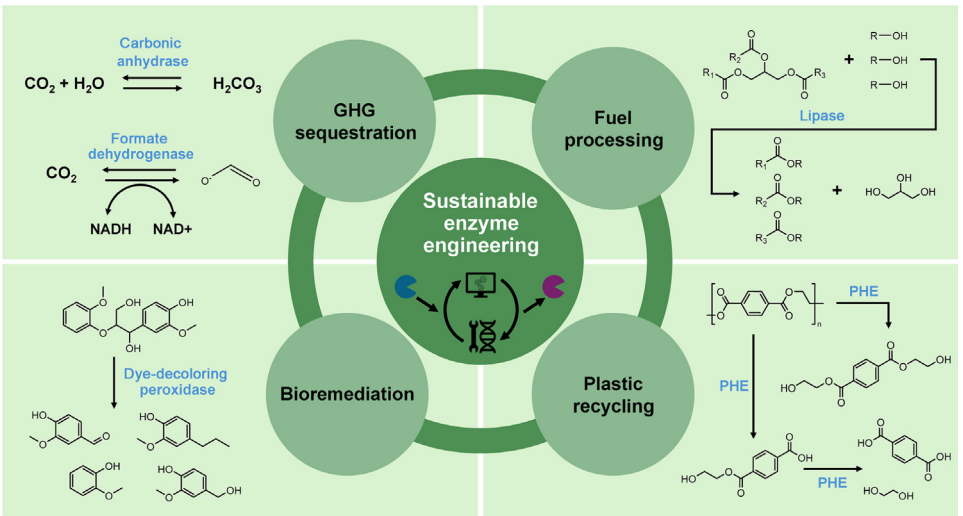

Figure 1. Impact of enzymes on industrial sustainability. Many areas impacting sustainability and green manufacturing can benefit from the use of enzymes. The four highlighted areas and enzymes are not an exhaustive list, but rather highlight how enzymes can enable sustainability. Each section also shows the reactions catalyzed in each section. Abbreviation: GHG: greenhouse gas.

Glossary

Bioremediation: use of microorganisms or other biocatalysts to either break down or remove contaminants from the natural environment, often conducted *in situ*.
Catalysts: materials, either chemical or biological, that lower the activation energy of a reaction, thus increasing the rate without being consumed in the reaction.
Cellulosome: large protein complexes for cellulose degradation usually found in cellulolytic bacterial generally containing scaffoldin, cellulolytic enzymes, and dockerin containing enzymes.
GHG sequestration: process of capturing and storing greenhouse gases, primarily referring to capture/storage of CO_2.
Halotolerant: tolerance of a microorganism or biological component to ionic stress and high salt concentrations.
High crystallinity: polymer crystallinity is a function of the alignment of the polymer chains and can be affected by temperature changes, and mechanical stress. Crystallinity impacts the material properties of the polymer.
Life cycle assessment: analysis done on a process and/or product from raw material to final product to assess the environmental impacts of the process/product.
Protein engineering: sequence-based modification of proteins for a specific purpose, such as increased efficiency, stability, and function through various engineering techniques such as rational/semi-rational design, directed evolution, and computational methods.
Purified enzyme systems: use of enzymes produced and isolated from a microorganism to catalyze a reaction to produce a certain product without the use of a whole living cell.

Enzymes as a powerful set of catalysts

Natively, some enzymes perform very specific reactions with very low error rates and high processivities, whereas other enzymes are far more promiscuous. This promiscuity and general malleability can even be leveraged to catalyze new to nature reactions [8]. **Protein engineering** is a quickly developing field marked by the first engineered proteins being produced in 1982 [9,10] to modern day examples of *in silico*, *de novo* enzyme design [11,12]. The idealized biocatalyst for a given sustainable biotechnology application will likely not be available 'off-the-shelf' and thus will require some form of engineering and design. To this end, there are many methods to engineer proteins, each with their own unique advantages and drawbacks. The topic of protein engineering has been widely studied and reviewed recently [13–17] and this section attempts to only highlight a few of the approaches (with a focus on rational design) used in the applications discussed throughout the rest of this article. A listing of common protein engineering approaches along with recent articles covering these topics is provided in Table 2.

Most initial efforts for protein engineering relied upon creating large libraries of enzyme variants to be screened or selected. To this end, a multitude of techniques, including random mutagenesis, site saturated mutagenesis, gene shuffling, and domain shuffling, have all been employed [18,19]. In each of these cases, increasing the number of enzyme variants leads to an improved probability of discovering an enzyme with the desired phenotype. However, this increase in library size

Table 2. Overview of protein engineering techniques

Technique	Description and uses	Advantages	Disadvantages	Refs
Rational design	An informed design strategy that uses knowledge of the protein structure to create specific mutations to improve enzyme performance. Mutants can be generated via site-directed mutagenesis or synthesis.	Creates a small library for screening	Must have knowledge of the structure	[13,14,112,113]
Semirational design	A semi-informed design strategy that uses knowledge of the structure to create directed mutations to improve enzyme performance. This can be performed using random mutagenesis, site saturation mutagenesis, gene shuffling, and domain shuffling.	Does not require in depth knowledge of the structure	May create a large library for screening	[113–115]
Directed evolution	A method to mimic natural evolution to improve enzyme performance. This can performed using random mutagenesis, site saturation mutagenesis, gene shuffling, domain shuffling, and *in vivo* / continuous gene diversification methods.	Does not require knowledge of the structure	Creates a large library for screening	[15,16,25,116,117]
Computational methods and *de novo* design	Methods which can be used in combination with other design methods, combining structural and sequence data to model beneficial mutations in existing enzymes or to create new enzymes *in silico*.	Less physical laboratory work creating and screening mutants	Must have knowledge of the structure May require large amounts of computational power	[11,25,118–121]

also creates a bottleneck if the screening process for the desired phenotype is difficult or low throughput. Importantly, these directed evolution approaches do not necessarily require a substantial understanding of the enzyme structure and function and thus can be achieved easily through molecular biology techniques such as error prone PCR, DNA shuffling, and staggered extension processes [13].

Moving beyond random selection, many initial rational design-based protein engineering efforts employed site-directed mutagenesis in which a particular amino acid is purposefully changed to another. To be effective (given the lower throughput of this approach), successful rational design requires a deep understanding of the structure and function of the enzyme to be engineered. To this end, modern computational tools and machine learning approaches enable predictable linkages between sequence, structure, and activity, thus leading to more effective rational engineering strategies [20–22]. Specifically, computational tools, including machine learning based structure predictions and neural nets, have been quite effective at predicting favorable mutations [23–25]. Importantly, these computational methods can decrease library size and thus allow for deeper screening and additional rounds of directed evolution [26]. When linked with emerging strategies for *de novo* protein design [27,28] and structure prediction [29,30], these tools can become powerful for protein engineering applications. Certainly, these tools are beginning to change the scope of protein engineering from an exploratory to predictable science. An expanded discussion of this topic can be read in Box 1. Regardless of the method utilized, the goal of enzyme engineering is to improve biochemical activity and stability. In doing so, these engineered enzymes can be used to enable sustainable processing, and such applications are as discussed in the following sections. All of the engineered enzymes discussed in this article along with the method of engineering employed to generate these mutants are provided in Table 3.

Enzymes as a direct means for GHG sequestration

A key component of sustainability is the reduction of GHG emissions as these gases serve as key contributors to climate change and global warming. The three main GHGs often studied are CO_2, methane, and NO, with CO_2 accounting for 64% of the GHG released in 2019 [31]. Thus, carbon capture/storage and carbon conversion/utilization technologies are of strong interest aimed at dealing with high anthropogenic CO_2 levels in the atmosphere. While current industrial methods of carbon capture can reduce global warming potential by up to 82%, they often rely heavily on amine-based solvents that net increase the human health toxicity potential [32] and require additional energy to release the captured CO_2 from the solvent [33]. Alternatively, utilizing enzyme-based technologies alone or in conjunction with current technologies can improve the capture of GHGs while also mitigating the issues related to human health and toxicity and energy usage. As one example, carbonic anhydrase can efficiently catalyze the conversion of CO_2 to

Box 1. Future directions and trends in protein engineering

Rational protein design and engineering relies heavily on both structural and mechanistic knowledge of the enzyme. Collecting the required experimental data (especially crystal structure) used to be (and still can be) a time intensive and costly endeavor. However, substantial advances in sequencing technologies, computational algorithms, protein visualization tools, and machine learning algorithms are rapidly changing the landscape. These tools can not only predict protein structure from sequence, but are beginning to show promise in elucidating mechanism, function, and small molecule interactions [109,110]. From these approaches, it is possible to predict mutations for redesigned enzymes with improved properties and novel functions. Big data mining coupled with these computational tools allow for the collection of data and rapid detection of latent patterns across datasets. We have already seen these approaches applied in many studies (see main text). Automated design and optimization pipelines are helping to provide the data to improve these models further. The exponentially increasing number of protein structures available through the use of protein structure prediction tools is rapidly expanding the database of potential structures[iii]. This effort improves homology modeling efforts. While the use of protein structure prediction tools is still in its infancy, the promise to reshape rational protein design is already being realized.

Table 3. Reference guide of engineered enzymes by section

GHG Sequestration				
Enzyme	Organism	Method	Improvement	Refs
Carbonic anhydrase	*Thermovibrio ammonificans*	Rational design (MD simulations)	Increased enzyme stability at 126°C	[35]
Carbonic anhydrase	*Thermovibrio ammonificans*	Rational design (MD simulations)	Enzyme half-life increased by 3× at 60°C	[36]
Carbonic anhydrase 2	*Bos taurus*	Rational design (surface charge engineering)	Increased activity in salt solutions	[37]
γ-Carbonic Anhydrase	*Halobacterium* sp. NRC-1	Rational design (active site modifications)	Enzyme activity increased 17× in salt solution	[38]
Formate dehydrogenase	*Chaetomium thermophilum*	Rational design (MD simulations)	Enzyme turnover increased 25×	[42]
Biofuel processing				
Enzyme	Organism	Method	Improvement	Refs
Lipase	*Thermomyces lanuginosus*	Rational design (surface charge engineering)	Enzyme half-life increased 6× in 50% methanol solution	[49]
Lipase B	*Candida antarctica*	Semirational design (saturation mutagenesis)	Enzyme activity increased by 31% and biodiesel yield increased to 89%	[50]
Lipase	*Proteus vulgaris*	Directed evolution (error-prone PCR)	Biodiesel yield of >99% and increased stability in methanol 50% solution	[51]
Lipase	*Rhizomucor miehei*	Semirational design (site-directed mutagenesis and optimization of N-glycosylation sites)	Biodiesel yield of >99% with 64% activity retained in 50% methanol after 8 h	[52]
Lipase	*Geobacillus stearothermophilus T6*	Rational design (computational design of disulfide bridges)	Increased activity, yield, and thermostability	[53]
Lipase	*Thermomyces lanuginosus*	Rational design (site-directed mutagenesis)	Increased specific activity, catalytic efficiency, and biodiesel yield	[54]
Lipase	*Serratia marcescens* L1	Rational design	Increased activity, velocity, substrate conversion rate and catalytic efficiency	[55]
Lipase	*Pseudomonas fluorescens*	Directed evolution (error-prone PCR)	Enzyme melting temperature increased by 8°C and methanol tolerance increased	[56]
Lipase	*Rhizopus chinensis*	Rational design (B factor analysis)	Increased thermostability and residual activity after heating.	[57]
Lipase	*Rhizopus chinensis*	Rational design (MD simulations)	Increased thermostability with >40× increase in half-life at 60°C	[58]
Endoglucanase	*Chaetomium thermophilum*	Rational design (N-glycosylation site optimization)	Improved enzyme activity and improved thermostability and extended half-life at 80°C and 90°C	[61]
Cellobiohydrolase	*Penicillium verruculosum*	Rational design (substitution with proline)	Half-life increased by 3.4× at 60°C	[62]
Cellobiohydrolase	*Aspergillus fumigatus*	Rational design (MD simulations)	Catalytic efficiency increased 2× and increased thermostability and substrate affinity	[63]
β-Glucosidase Bgl15	Uncultured bacterium	Directed evolution (error-prone PCR)	Improved glucose tolerance and half-life increased by 225× at 50°C	[64]
Endoglucanase II	*Trichoderma reesei*	Rational design (removal of a disulfide bridge)	Increased enzyme stability at 80°C by 2.4×	[65]
Cellobiose dehydrogenase	*Crassicarpon hotsonii*	Semirational design (site saturated mutagenesis)	Enzyme long-term stability increased by 20%	[76]
Plastic degradation				
Enzyme	Organism	Method	Improvement	Refs
PETase	*Ideonella sakaiensis* 201-F6	Computational neural network	Melting temperature increased to 67.2°C and improved PET degradation at 50°C	[23]

(continued on next page)

Table 3. (continued)

Plastic degradation				
Enzyme	Organism	Method	Improvement	Refs
PETase	*Ideonella sakaiensis* 201-F6	Rational design (active site)	Improved PET degradation at 30°C	[83]
PETase	*Ideonella sakaiensis* 201-F6	Rational design (B factor analysis)	Melting temperature increased by 8.8°C and enzyme activity increased by 14× at 40°C	[84]
Leaf-branch compost cutinase	Unknown prokaryotic organism	Rational design (addition of a disulfide bridge)	Specific activity and melting temperature increased	[85]
PETase	*Ideonella sakaiensis* 201-F6	Directed evolution (saturation mutagenesis and DNA shuffling)	Melting temperature increased to 82.5°C and improved PET degradation at 65°C	[86]
Leaf-branch compost cutinase	Unknown prokaryotic organism	Rational design (MD simulations)	Increased hydrolysis rate by 3.5×	[87]
PETase	*Ideonella sakaiensis* 201-F6	Rational design (computational method)	Melting temperature increased by 31°C and improved PET degradation at 50°C	[88]
Bioremediation				
Enzyme	Organism	Method	Improvement	Refs
Dye-decolorizing peroxidase	*Pleurotus ostreatus*	Directed evolution (error prone PCR)	Increased catalytic efficiency by 2.7× and increased H_2O_2 tolerance by 7×	[99]
Versatile peroxidase	*Pleurotus eryngii*	Directed evolution (error prone PCR)	Increased catalytic rates and increased oxidative stability	[100]
Lactonase	*Sulfolobus solfataricus*	Rational design (engineering of binding pocket)	Increased degradation of organophosphates by over 2000×	[105]
Phosphotriesterase	*Pseudomonas diminuta*	Semirational design	Increased degradation of V agents by over 9000×	[106]
Myoglobin/ peroxidase	*Physeter macrocephalus*	Rational design	Degradation of common personal care products >90%	[108]

bicarbonate, a key step in the process of carbon capture through absorption (Figure 1). However, the wild type versions of these enzymes lack the thermostability and stability in alkaline and/or salt solutions needed for industrial processes [34].

Addressing the deficiencies of carbonic anhydrases would allow for *in situ* enzymatic CO_2 capture from industrial plant flue gas. Successful engineering efforts have used rational design to increase the thermostability of carbonic anhydrases from *Thermovibrio ammonificans*, leading to enzymes with threefold longer half-life than the wild type at 60°C and one mutant that can retain 100% activity at 90°C after 1 h versus the wild type which only retained 30% activity [35,36]. Both studies utilized molecular dynamics simulations in which flexible regions were identified using root mean square fluctuation values and possible mutations were predicted based on their ability to stabilize the flexible regions [35,36]. Engineering a **halotolerant** carbonic anhydrase has been previously attempted using rational design and resulted in four mutant enzymes with increased activity over the wild-type enzyme in increased concentrations of Na_2SO_4, NaCl, $NaNO_3$, and SDS [37]. This was done by increasing the number of acidic residues on the surface of the enzyme; in total, 18 residues were replaced with either aspartic or glutamic acid, causing a more negative surface charge characteristic of a halotolerant protein [37]. An already halotolerant γ-carbonic anhydrase from an uncharacterized Red Sea *Halobacterium* sp. with low activity was rationally engineered for increased activity using insights from other carbonic anhydrases to increase the activity 17-fold in a 3M KCl solution at 40°C [38]. Additional progress in identifying new lead candidates has been made possible using metagenomic searches and whole-

genome sequencing efforts of extremophiles and organisms uncultivable in traditional lab settings [38–40]. Increased halotolerance is important for many enzymes industrially, so the study of halotolerant enzymes has the potential for a wide range of protein engineering efforts, but is especially pertinent when considering carbon capture as the dissolved gases can produce solutes [37].

Once gaseous CO_2 has been converted into bicarbonate, it can be released for storage or can be further transformed through mineralization using either whole cell biological processes or additional enzymatic reactions to create value added chemicals [41]. Utilization of CO_2 and bicarbonate as a sole carbon source is difficult for most microorganisms. Without the improvements made to carbonic anhydrases, upcycling CO_2 to value added products like formate and methanol, or even as biomass is difficult. In this regard, another enzyme for CO_2 utilization is formate dehydrogenase, which reversibly reduces CO_2 into formate by oxidizing NADH to NAD^+ (Figure 1). Towards this point, Tülek *et al.* were able to increase enzyme turnover 25-fold through molecular dynamic simulations and, when combined with enzyme immobilization, could double formic acid production [42]. Collectively, enzymatic carbon capture and usage can be explored as a sustainable process with the potential to also be economically advantageous. The use of carbonic anhydrases is commercially available for industrial processes through a partnership between Saipem and Novozymes[i], which is advertised to have all the benefits described above along with being more cost effective than current amine technologies. While it is encouraging that enzymatic carbon capture is available at industrial scale, for there to be a true impact on sustainability, GHG-producing plants need to either make the switch or add in enzymatic carbon capture to their existing plants.

Another approach for GHG sequestration is using enzymes, such as carbonic anhydrase, to form enzymatic construction materials and thus minimize the CO_2 emissions derived from this industry. The direct addition of the carbonic anhydrase enzyme in a self-healing cement formulation catalyzes a reaction between calcium ions and atmospheric CO_2 to create calcium carbonate crystals [43]. These crystals have mechanical properties similar to that of cementitious material. While this initial example was demonstrated using an unengineered carbonic anhydrase, the examples above point to the strong potential for improving this process through protein engineering. As such, the use of enzymes in construction materials processing could help both reduce and sequester the 8% of CO_2 emission load associated with the creating the cements and concretes in our built environment [44].

Enzymes for processing fuels

Another application of sustainable biotechnology is in the direct displacement of fossil fuels to biofuels. To this end, substantial, long-standing efforts have been made to develop sustainable biofuels, especially for liquid transportation fuels. While biofuels also contribute to GHG emissions, they have the potential to be produced from renewable or waste substrates, thus potentially lowering the impact of fuel production on the environment when compared to traditional fossil fuels [45,46]. While many of these biofuel processes are being explored in the context of whole cells bioconversions, enzymatic biocatalysts can still play a significant role, especially in the pretreatment and post-processing steps. For example, biodiesel produced via an esterification or a transesterification reaction between a free fatty acid and short-chain alcohol can be enzyme catalyzed (Figure 1).

A major issue in the production of biofuels using lipase is enzyme tolerance and reactivity in the presence of short-chain alcohols such as methanol [47,48]. In one case, a lipase from *Thermomyces lanuginosus* was engineered to have a >6 times longer half-life in a 50% methanol reaction buffer compared to its wild counterpart by selecting and replacing hydrophobic residues

with hydrophilic ones [49]. Using B factor analysis, which examines residue displacement, eight surface residues of a lipase from *Candida antarctica* were identified as the targets for saturation mutagenesis. These engineering efforts resulted in 31% higher activity and increased biodiesel production [50]. Directed evolution via error prone PCR was used to increase methanol tolerance of the *Proteus vulgaris* lipase, resulting in a >99% conversion yield of biodiesel [51]. As noted above, selection of successful mutants from a large library requires a high through-put screening method. In this study, a copper soap assay, which produces blue/green colored colonies of positive mutants, was utilized for easy selection of mutants with increased methanol tolerance [51]. Similar enzymatic efficiency increases and improved methanol tolerances have also been reported for the *Rhizomucor miehei* lipase using a combination of mutation and optimization of N-glycosylation sites to achieve 64% activity in 50% methanol for 8 h with a 99.33% conversion yield of biodiesel [52]. While vastly different enzymes and applications, we can see that in the engineering of both lipases and carbonic anhydrases the surface chemistry and enzyme flexibility were identified as targets for improved enzyme function. Other protein engineering efforts have improved the methanol tolerance of other lipases for biodiesel production [53–55] (see Table 3 for an overview of these studies). Outside of improving methanol tolerance, multiple protein engineering methods have also been used to increase the thermostability of lipases for biofuel production, increasing the enzyme melting temperature by up to 8°C [56–58] (see Table 3 for an overview of these studies). Collectively, these efforts have led to more efficient biocatalysts that can improve industrial processing of biodiesel and can readily be incorporated into already existing production plants.

Enzymes have long been used to improve feedstock availability for biofuels, especially in the arena of lignocellulosic biomass conversion to liquid fuels such as ethanol [59]. Specifically, the breakdown of lignocellulosic materials via various isolated enzymes of the **cellulosome** have been explored to work in concert with physical and/or chemical means [60], with a special focus on thermostability. As an example, rational design of the N-glycosylation sites in an endoglucanase gave rise to improved activity (1.85- and 1.64-fold on sodium carboxymethyl cellulose and β-d-glucan, respectively) and improved thermostability at both 80°C and 90°C [61]. Various cellulases have also been engineered for increased stability and activity through an array of protein engineering methods [62–64] (see Table 3 for an overview of these studies). For example, improved stability of an endoglucanase II was engineered through the removal of a disulfide bond, resulting in a 2.4-fold increase in enzyme stability at 80°C [65]. Once again, we see the flexibility of the enzyme structure as a target for engineering, in this specific case the flexibility of the enzyme was increased through the removal of the disulfide bond. For more complete biomass conversion, cellulosome protein complexes can be engineered to breakdown multiple lignocellulosic compounds into fermentable constituents. Specifically, designer cellulosomes have been engineered for high thermostability achieving maintaining activity at 75°C for 72 h [66,67]. While biofuels are considered renewable in comparison to conventional fossil fuels, full life cycle assessments must be completed and take into account the feedstock, the pretreatment necessary, and the overall process efficiency to ensure that the process is in fact sustainable. Nevertheless, more efficient enzymes are critical in pushing these processes to closer to a point of sustainability [68].

Fossil fuels are important for a plethora of applications beyond simply liquid transportation fuels, one of which is energy creation. Many emerging biotechnologies for energy production rely on rewiring the photosynthesis pathways of plants, and while protein engineering is sure to be a key element in creating highly efficient artificial photosynthesis, plant and whole cell technologies lie outside of the scope of this review and have been covered elsewhere [69,70]. After energy production, energy storage is a growing need, and biofuel cells and biobatteries can use enzymes to

catalyze oxidation and reduction reactions instead of traditional metal catalysts [71]. Despite challenges with respect to energy capacity, biological storage alternatives can functions at low temperatures and pH [72]. At the center of many of these applications is the enzyme glucose oxidase which has high specificity for the oxidation of glucose, but is highly restricted in the oxygen sensitivity of the enzyme, the production of H_2O_2, and the location of the redox center that all hinder the activity and stability of this enzyme for bioelectrochemical applications [73]. One novel solution to this issue is the engineering of synthetic active sites which can be ported into alternative enzymes or other proteins [74]. For example, cellobiose dehydrogenase is capable of both direct electron transfer and redox reactions, making it a desirable enzyme for bioelectrochemical applications [75]. Semirational design using site-saturation mutagenesis increased long-term stability of a cellobiose dehydrogenase by 20%, which is an important factor for creating batteries which must have long lifespans [76]. Finding successful mutants did not just result in improved activity but also uncovered the main mechanisms impacting turnover stability, which will inform future studies. While enzymes have the potential to be part of the sustainable solution for electricity generation and storage, there are still many obstacles to overcome before these technologies can move from the lab bench and pilot plant to industrial scale.

Enzymes for degrading waste plastics

Enzymes can also play a role in the conversion of waste products. While consumer plastic products have only been around since the introduction of Bakelite in 1907, they have quickly become a ubiquitous part of modern life, thus creating a significant amount of plastic waste as well. Additionally, plastic waste is generally single use with <10% being recycled and the plastic which is a product of recycling is generally of lesser quality than its virgin counterparts [77]. With all these issues around the production and disposal of plastics, the need for solutions is evident. Many researchers have turned to protein engineering to increase the activity and stability of various enzymes with innate plastic degrading abilities.

Much of the research around polyethylene terephthalate (PET) plastic degradation has focused on two enzymes: PETase, an esterase isolated from *Ideonella sakaiensis 201-F6* in 2016, and leaf-branch compost cutinase (LCC), both of which can degrade PET into its constituent monomers [78,79]. These PET hydrolyzing enzymes (PHEs) generally degrade the amorphous regions of PET, as the highly crystalline regions in PET are resistant to enzymatic degradation as the polymer chains are less available to the enzymes [80] (Figure 1). The glass transition temperature of PET varies from about 60°C to 82°C [81], and elevating enzymatic reactions into this range is thought to help counter the effects of crystallinity on degradation reactions. Therefore, increasing the thermostability of PHEs was thought to be important to developing an industrially relevant PHE; however, recent studies have shown that extended time at or above the glass transition temperature increases PET crystallinity [82].

Regardless, many protein engineering strategies have been used to increase the thermostability and activity of PHEs. Early attempts at engineering PETase utilized rational engineering for incremental improvements to melting temperature and activity [83,84]. More recently, computer-aided rational design was used to determine target amino acids via molecular docking for LCC, resulting in a nearly 10°C increase in melting temperature, as well as increasing the enzyme productivity to productivity of 16.7 g_{TA}/l/h [85]. Beyond rational engineering, directed evolution methods have also been utilized. Over several rounds of saturation mutagenesis and overlap-extension PCR, a PETase variant with improved activity and an increased enzyme melting temperature of 82.5°C was developed [86]. While many of these studies engineered enzymes to ideally function above the glass transition temperature, as previously mentioned, this may actually hinder rather than help depolymerization efforts.

Due to this fact, Pirillo *et al.* rationally engineered LCC using molecular dynamics (MD) simulations to optimally function at 55°C. In <3 days, this mesophilic enzyme completely degraded 1.3 g of untreated postconsumer PET plastic waste using less than mg of enzyme per 1 g of plastic [87]. Compared to enzymes engineered to optimally function at high temperatures, the authors argue that the more mesophilic approach is more environmentally sustainable and prevents polymer aging from extended times at temperatures above the glass transition temperature. Additionally, computational methods for protein engineering of PHEs have been quite successful at increasing both activity and thermostability. For example, DuraPETase, another PETase variant, was engineered using a greedy accumulated strategy for protein engineering (GRAPE) which improved the thermostability by 31°C and activity over the wild-type enzyme by 300-fold at 37°C for 10 days [88]. Another computationally derived PETase variant, FAST-PETase, with a 38-fold higher activity at 50°C over the scaffold enzyme, was designed to have increased thermostability and activity using a deep-learning neural net model [23].

These engineering efforts have improved enzymatic degradation of PET to the point that enzymatic recycling is being used in small scale commercial plants operated by Carbios[ii]. Currently, efforts to degrade **high crystallinity** plastics require that the plastic is preprocessed through either an extrusion or melting/quenching process to lower the crystallinity prior to degradation [23,85]. However, these efforts are too energy intensive to be considered sustainable and thus additional efforts are required for the direct enzymatic degradation of highly crystalline PET without pretreatment. Additionally, this process currently uses a significant amount of sodium hydroxide and water which contribute negatively to life cycle assessments [89]. Engineering enzymes that function at low pH or in low water environments can help to improve sustainability of the process and the efficiency of the downstream terephthalic acid recovery step. Towards this idea, successful PET degradation in low moisture environments has been achieved without the need for excess sodium hydroxide [90]. For these developing moist-solid enzyme systems, additional protein engineering methods will need to be developed to create enzymes to ideally function in this environment as current engineering methods generally assume enzymes are active in an aqueous environment. Along with engineering enzymes that can work in different environments, engineering enzymes that can function alongside several additional enzymes will be necessary for plastics of mixed compositions. Indeed, PET is only one of the many types of plastics produced but can be seen as an exemplification of the work needed for all other plastic types as they will face similar issues [91].

Enzymes for sustainable bioremediation

While plastic is a major environmental pollutant, there are many chemical pollutants of concern such as pesticides, pharmaceuticals, personal care products, industrial chemicals, and their byproducts [92]. Bioremediation using enzymes offers potential *in situ* remediation approach for these pollutants as enzymes generally do not require energy or high temperatures to function as catalysts. Moreover, utilizing purified enzymes for bioremediation can bypass the genetically modified organism release issues. Additionally, *in situ* remediation can provide an ecofriendly way to treat industrial wastewater before it is released into the environment. While many wild-type enzymes possess the ability to degrade certain environmental contaminants, more work is required to engineering these enzymes for desired higher specificity and activity [93,94].

While a large variety of enzymes have been identified to degrade broad classes of environmental contaminants, enzymes such as laccases and peroxidases are often seen as key components of enzymatic environmental remediation [95,96]. Environmental contamination can occur in many ways, with wastewater effluent entering soil and waterways from industrial plants being a prominent example. An example of this contamination is found at textile plants where dyes are found in

effluent streams; these dyes can decrease the dissolved oxygen, decrease visibility, and decrease light penetration into water impacting aquatic flora and fauna [97]. One biological remedy to this problem is the use of a dye-decolorizing peroxidase from *Bacillus amyloliquefaciens,* which was used to treat the effluent from a paper processing plant (Figure 1): a 12-h treatment with the dye-decoloring peroxidase reduced the chemical oxygen demand of the effluent by 82%, the effluent color by 80%, as well as the overall toxicity of the effluent [98]. With the goal of achieving a more efficient enzyme, Alessa *et al.* used a directed evolution method, consisting of three rounds of error prone PCR and one round of saturation mutagenesis, to create a mutant dye-decoloring peroxidase with 2.7-fold higher specific activity and sevenfold higher H_2O_2 tolerance [99]. In this case, activity and tolerance to H_2O_2 are linked as H_2O_2 is used in the reductive decolorization reaction and subsequently enzyme activity will be enhanced with increased H_2O_2 tolerance. Similarly, through error-prone PCR, mutant peroxidase enzymes with increased catalytic rates and oxidative stability have been achieved for the degradation of azo dyes [100]. In this study, a high-throughput method for identifying improved enzyme variants was developed based on flow cytometry/fluorescence activated cell sorting (FACS), and the mutant library members were expressed on the surface of *S. cerevisiae* EBY100 to prevent protein misfolding and sorted using FACS [100]. Current methods of wastewater treatment utilize a combination of physical, chemical, and biological methods to eliminate contaminants, yet some dyes are still resistant to treatment [97]. Engineered enzymes offer a cost-effective, yet specific, method of removal of these various recalcitrant dyes that could easily be incorporated into the already present biological treatment of wastewater.

Additional recalcitrant contamination chemicals of great concern are the so-called 'forever' chemicals polyfluorinated substances (PFASs). These chemicals, first developed in the 1950s, are used in a very large range of industrial and consumer applications [101]. Of greater concern, these chemicals are difficult to degrade by both chemical and biological means since C–F bonds are strong and biological routes are limited due to the scarcity of fluorine chemistry in microbes [102]. Despite these difficulties, there are still identified enzymes which can defluorinated compounds, most notably defluorinase from *Acidimicrobium sp. A6* which demonstrates activity on two different PFASs [103]. With several thousand PFASs to be considered, the area of engineered promiscuity of these defluorinase enzymes is required. While whole cell technologies can play a role in bioremediation, the overall toxicity of these compounds may provide an added benefit of utilizing enzymes to break down PFASs.

Aside from industrial plants, another major contributor to environmental pollution is pesticides, especially organophosphates which are neurotoxic compounds [104]. Fortuitously, enzymes can be used to remediate these compounds. As an example, a lactonase from *Sulfolobus solfataricus* was rationally engineered so that binding pocket resembled a phosphotriesterase thereby increasing the degradation activity on some organophosphates by up to 2210-fold [105]. Another organophosphate, V agent, not used as a pesticide in agriculture but as a chemical warfare agent, could be degraded by an engineered phosphotriesterase with improvements up to 9200-fold [106]. Due to the ongoing threat to human health, organophosphate degrading enzymes can be vital to remediate from continued pesticide usage.

The promiscuity of enzymes can be useful when the exact contaminate is unknown or if there are a large variety of contaminants to be degraded. Increasingly, compounds derived from pharmaceutical and personal care products are being detected in wastewater and can toxify the environment [107]. Through rational design of promiscuous enzymes, two engineered heme enzymes were shown to be highly effective at degrading four common pharmaceutical and personal care product chemicals, degrading diclofenac sodium, 2-mercaptobenzothiazole, paracetamol,

and furosemide at rates higher than 90% for all products tested [108]. In this regard, engineered enzymes can be useful components in active wastewater detoxification.

Concluding remarks

A multifaceted problem requires a multifaceted solution. Process sustainability requires a number of changes to our status quo. At the same time, enzymes and their malleability through efforts such as protein engineering, rational design, random mutagenesis, and computational methods, do offer a multifaceted solution. The incorporation of enzyme technologies into areas such as carbon capture, the production of biofuels, and environmental remediation gives rise to a promise for biotechnologically enabled sustainability (Figure 1). While enzyme technologies are at different stages of development for each of these application spaces, overall goals of increasing enzyme activity, specificity, and stability are critical for implementation. By implementing these technologies and continuing their advancement, we can continue to meet the needs of the present without compromising the ability of future generations to meet their own needs [4]. At the same time, simply using a biological catalyst will not make a process inherently sustainable, thus requiring an entire-system engineering approach (see Outstanding questions).

Outstanding questions

Could computational methods allow for engineered enzymes to compete with current traditional chemical manufacturing processes in a wider range of applications?

Will shifts towards more sustainable manufacturing processes include widespread adoption of enzyme technologies?

Can the process of producing and using enzymes become a universally cost-effective approach?

What additional methods (besides protein engineering and other enzyme stabilization techniques such as immobilization or addition of stabilizing agents) can be used to enhance robustness for industrial processes?

What other fields and applications could benefit from the use of enzymes and biotechnology?

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Declaration of interests

The authors declare no competing interests.

Resources

[i]www.saipem.com/en/media/press-releases/2021-12-13/saipem-and-novozymes-work-together-create-more-sustainable-co2

[ii]www.carbios.com/en/carbios-obtains-building-and-operating-permits/

[iii]https://alphafold.ebi.ac.uk/

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