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# Proteins in Synthetic Biology with Agricultural and Environmental Applications

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**Abstract:** Synthetic biology tools have become increasingly prevalent as we look to nature for biological approaches to complex problems. With an ever-growing global population, issues of food safety and security, as well as addressing pollution and striving for sustainability are of the utmost importance. In this review, we first highlight synthetic biology techniques such as directed evolution as a toolset for protein engineering and show direct applications for food safety and security. Moreover, we offer an introduction to creative approaches for biosensor design and development and spotlight a few innovative examples. Finally, we address biomanufacturing with direct applications, as well as biomanufacturing to improve natural processes.

**Keywords:** food safety; food security; biosensors; biomanufacturing; protein engineering

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## 1. Introduction

Synthetic biology is a broad, interdisciplinary field leveraging engineering, life sciences, and data science to engineer biological systems with new and useful properties [1]. The growing interest in synthetic biology research is reflected in the substantial increase in the number and diversity of technologies and companies attempting to address challenges in a variety of fields including medicine, agriculture, energy, and consumer products. The synthetic biology market is growing at nearly 20% compound annual growth rate and anticipated to exceed USD 10 billion in the next 5 years [1]. This rapid growth is largely driven by the global challenges resulting from climate change and chemical overaccumulation. Biologically derived technologies and materials often do not contribute to the negative environmental impacts that usually characterize petrochemically derived materials, and therefore can be considered a more sustainable route. This review focuses on a few specific applications within the wide-reaching field of synthetic biology: agriculture, environmental sensing, and sustainability. In particular, we are highlighting subtopics within these areas such as insecticide development, biosensing, and bio-based alternatives to petrochemical products, illustrating how synthetic biology is a leading and critical factor in developing solutions to address environmental challenges.

## 2. Food Safety and Security

According to the USDA, 13.5 million people experienced food insecurity in 2021 [2]. A major contributor to this widespread issue is the overabundance of resource competition from undesirable biomass that ultimately leads to decreased crop yields and increased food prices [3]. While herbicides are an attractive solution, their broad specificity becomes quickly problematic as desirable crops succumb to treatment as well [4]. Additionally, according to the Food and Agriculture Organization of the United Nations, approximately 20–40% of crops worldwide are lost due to insect pests each year, also contributing to food insecurity [5]. We will discuss recent genetic and protein engineering strategies that have

allowed remarkable advancements in herbicide tolerance and detoxification, as well as insect resistance in certain staple crops.

### *2.1. Herbicide Tolerance*

Generating crops with herbicide resistance or tolerance through protein engineering has become an attractive area in agricultural research to address food security. Commonly, the target sites for most herbicides are enzymes involved in plant growth and development, with different herbicides targeting different enzymes. To eliminate weeds, herbicides are attracted to and bind these various enzymes which inhibit their activity leading to altered growth and ultimately plant death [6]. However, the problem then becomes that there is no distinction between these target sites in weeds versus those in food crops, resulting in both weed and crop death and reducing crop yields as well. Murphy and Tranel (2019) published an exhaustive review of herbicide target sites, along with known mutations to confer resistance [6]. Our review focuses on work that has harnessed synthetic biology and protein engineering techniques such as directed evolution, DNA shuffling, site-directed mutagenesis, etc., for the creation of novel herbicide-resistant, insect-tolerant, and environmentally enhanced crops to combat food insecurity.

Enzymes involved in amino acid synthesis or other biosynthesis pathways to support plant growth are major sites of action for herbicides. The target site for common globally used herbicides such as those in the sulfonylurea (SU) and pyrimidinyl-benzoate (PYB) families is acetohydroxyacid synthase (AHAS) [7]. The function of AHAS in plants is to promote growth and development through its role in branched amino acid (BCAA) production [8]. By engineering target sites such as AHAS, decreased affinity for herbicides can be acquired which will allow the properly functioning enzyme to promote healthy plant growth. For example, weed varieties have developed tolerance to herbicides due to mutations in AHAS. This natural phenomenon inspired the engineering of the enzyme based on these mutations for herbicide-tolerant (HT) crops. Rather than experimentally investigating a pool of mutants, Fang et al. (2020) used a molecular docking approach to explore the W548 residue, a common resistance mutation site [8]. Previous studies have only looked at amino acid substitutions, however, through molecular docking validated by in vitro studies, this study found that the deletion of W548 led to multi-family herbicide tolerance and performed better than those of previous substitution mutations [8]. Another enzyme crucial to the production of aromatic amino acids to support plant growth is 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), that is targeted by glyphosate herbicides. Various weed species including *Conyza sumatrensis* and *Eleusine indica* are known to have specific mutations in EPSPS, which are P106T and double-mutant T102I/P106S (TIPS), respectively, making them resistant to glyphosate [9]. The double mutant has proven higher glyphosate resistance than P106T, showing that evolutionary mutations in EPSPS can inform the future design of herbicide-resistant crops [9]. Recently, a study incorporated the TIPS mutation into the EPSPS gene in rice, resulting in glyphosate tolerance [10]. Additionally, Ortega et al. (2018) mutated the EPSPS gene from *Capsicum annum* (chili pepper) to introduce the TIPS mutation and then looked at the use of different promoters to determine which led to the expression of the most highly resistant EPSPS to confer glyphosate resistance in chili peppers [11]. Identifying evolutionary changes in weed species that confer resistance to herbicides and then harnessing synthetic biology techniques to create and express these mutations transgenically is an effective way for food crops to combat herbicide treatment and to maintain global food security.

### *2.2. Insecticidal Activity*

Lepidopteran pests such as *Spodoptera frugiperda* and Coleoptera pests such as *Diabrotica virgifera virgifera* (western corn root worm, WCRW) pose a major threat to crop yields, so providing food crops with the machinery to combat these insects is crucial to addressing food security. *Bacillus thuringiensis* (Bt), a spore-forming bacterium, is known for releasing proteins with insecticidal activity and researchers have found applications for

these proteins in transgenic plants to improve insect resistance. One class of these proteins is the insecticidal crystalline (Cry) proteins which were commercially used to protect food crops from insect pests. However, as the commercialization of these proteins becomes more popular, like antibiotic usage in humans, there is a risk of resistance mechanisms developing. Therefore, continually engineering new insecticidal proteins could help the agricultural world combat potential resistance. Cry proteins have three domains that each provide different functional characteristics to the protein. In recent work, Liu et al. (2022) engineered new Cry proteins through domain swapping. In Liu's work, domain III in the N-terminal, which is responsible for interaction with insect receptors, was swapped with domain III regions from other Cry proteins to create new, novel proteins providing insect resistance [12]. Understanding the functional purpose of the N-terminal domains in combination with domain swapping led to the generation of new, unique Cry proteins such as Cry1Ab-Gc, which provides resistance to two lepidopteran insects in rice and maize [12]. Another Cry protein from Bt, Cry8Hb, was found to be effective against WCRWs [13]. Hou et al. (2019) discovered that fusing maltose binding protein (MBP) to Cry8Hb results in the increased solubility of the protein in the insect midgut, leading to the enhancement of its insecticidal effect. Following these results, DNA shuffling was used on a newly synthesized gene, IP3-1, which closely resembled Cry3 to discover proteins that had increased solubility and therefore higher anti-WCRW activity, but this time without the MBP fusion partner. The mutations generated from DNA shuffling increased the solubility and anti-WCRW activity in the same region where MBP was likely bound to fused proteins. These results suggest that the increased solubility of Cry proteins in the insect gut leads to their enhanced ability to protect food crops from insects and this can be accomplished via MBP fusion or mutations in specific residues [13].

Additionally, Bt produces another category of insecticidal proteins during its vegetative growth phase called vegetative insecticidal proteins (Vip). Like the aforementioned work, Gomis-Cebolla et al. (2020) used domain swapping between Vip proteins to give this category of insecticidal protein greater solubility and stability in the insect gut as well. Vip3A proteins are known to have insecticidal activity, while Vip3B and Vip3C are lacking. By domain swapping, a new Vip protein, Vip3_ch2, was identified to have increased activity against *S. frugiperda* [14]. Furthermore, these studies determined which domains were most important for toxicity and stability to inform the future of engineering novel Vip proteins [14].

### *2.3. Environmental Change Tolerance*

The increasing occurrence of late spring and early fall flash freezes can have damaging effects on crop yields [15,16]. Providing plants with better resistance to cold temperatures will help prepare them for changing climate conditions [17]. Improving the efficiency of photosynthesis is another way in which crops can be enhanced to be better suited for their environment [18].

Crops with better cold resistance offer a wider range of growing locations and seasons while also helping to reduce the amount of crops lost to frost damage [15]. Xu et al. (2020) used a point mutation on the low-temperature tolerance 1 (LTT1) gene of rice for increased seed setting during low-temperature stresses while not hindering crop yields during normal growing conditions. Since previous work has faced difficulties identifying reliable cold tolerance genes, the discovery of LTT1 allows the future hybridization of rice plants with increased cold tolerance using internal mechanisms that do not affect other important plant functions such as the development of pollen and tapetum [17]. Wani et al. (2021) were able to express a cold response protein1 (BOCRP1), which is constitutively expressed in the leaves, roots, and stems of the cabbage species, in a tomato plant susceptible to cold temperatures. The modified tomato plants exhibited increased seed germination and root length in addition to the improved tolerance of cold stresses [19]. These results provide insight for future work in improving the cold tolerance crops while not hampering the normal growth and development of the plant [19].

The photosynthesis process in crops can also benefit from the introduction of new proteins to the crop's chloroplasts. Gomez et al. (2018) showed that two flavodiiron proteins (Flvs) from cyanobacteria, Flv1 and Flv3, were able to increase the photosynthesis performance of tobacco plants during light transition periods, such as sunset or shading caused by passing clouds, while maintaining performance under constant lighting conditions [18]. These Flvs are responsible for relieving the excess energy of the photosynthetic electron transport chain of cyanobacteria and perform a similar function in the *Nicotiana tabacum* chloroplasts [18]. The introduction of Flvs to act as electron sinks during light transition periods could allow other field crops to exhibit photosynthesis protection and efficiency that will lead to improved plant health and yields [18].

## 3. Environmental Sensing

As environmental pollution becomes an increasing concern across the world, a resurgence of interest in environmental sustainability has renewed interest in new methods for the detection and removal of pollutants as a result of more stringent environmental regulations. Specifically, as people come into contact with harmful chemicals through contaminated water and biosolids, both of which pollute agricultural crops, the need for simple and robust pollutant detection methods is rapidly growing.

Traditionally, detection strategies involve analytical chromatographic methods which require extensive pretreatment, expensive machinery, and trained personnel for operation [20–22]. Biosensors, however, provide alternative detection strategies by utilizing biological recognition elements (BREs). While these BREs can consist of any biological element, this review focuses on protein-based recognition units such as binding proteins, antibodies, and enzymes.

Protein-based biosensors are highly valued for their ease of use, low cost, and high specificity [23]. One of the most desirable traits of protein-based sensors, however, is their tunability. Thanks to synthetic biology and its vast toolkit, protein-based sensing scaffolds are quickly being discovered and engineered for various applications [23–27].

### *3.1. Enzyme Based Sensing*

Enzymatic biosensors have been of increasing interest due to their high specificity and sensitivity as well as their abundance and variety in nature [28–30]. While enzyme recognition occurs through either direct analyte metabolism or analyte-specific enzyme activation or inhibition, they can be coupled with a multitude of transducing element types (i.e., electrochemical, piezoelectric, and optical) [31,32]. Electrochemical sensors utilizing oxidizing enzymes, however, have been the most widely developed for the detection of a wide variety of environmental contaminants [25]. While much attention has been given to this mode of detection, it is clear that enzymatic sensing selectivity is currently limited when utilized for non-natural ligands [25,33–36]. While advances in the signal transduction elements as well as enzyme immobilization have been crucial in the recent improvements of these technologies [37–40], the use of synthetic biology to discover new and enhance previous BREs provides a glimpse into the future of biosensing.

One of the best examples of enzymatic biosensing is the use of acetylcholinesterase (AChE) for the detection of organophosphorus (OP) compounds. OP compounds have a long history of use as chemical weapons, medical nerve agents, and as an active ingredient in a wide variety of pesticides [41]. The link between OPs and cholinesterase enzymes has been known since their first use in pesticides, thus, the inhibition of insect AChE enzymes has been studied at length [41–43]. AChE, is a hydrolase present in the cholinergic pathway that catalyzes the conversion of acetylcholine (ACh) into choline and acetic acid. Serine, histidine, and aspartic acid residues located at the active site are implicated in the reaction mechanism, with serine in particular playing an important role in the nucleophilic attack of AChE. When exposed to OPs, phosphorus covalently modifies the serine, permanently inhibiting the enzyme [44]. Comprehensive research has been conducted to optimize the application of AChE for the detection of organophosphorus (OP)

compounds, such as developing different immobilization techniques [33,45,46], introducing mutations to increase specificity and binding affinity [47], and improving the enzyme–electrode interface [45,48]. Recent studies have focused on engineering the AChE biosensor for on-site detection to make the testing of agricultural goods more accessible outside of a laboratory environment.

Tang et al. (2019) investigated how the personal glucose meter (PGM), a ubiquitous and wildly successful personal diagnostic tool, can be used for the detection of OP pesticides. Thiocholine (TCh), one of the products of AChE hydrolysis, can be used as a measure of AChE enzymatic activity [49]. TCh also has enzymatic activity of its own, which can be harnessed for glucose detection. When in the presence of ferricyanide ions, common glucose testing mediators, TCh reduces $Fe(CN)_6{}^{3-}$ to $Fe(CN)_6{}^{4-}$. After this reduction takes place, $Fe(CN)_6{}^{4-}$ can be re-oxidized to $Fe(CN)_6{}^{3-}$ at the electrode within the apparatus, which will provide a read-out on the device. TCh produced accurate PGM results up to 12 mM concentrations, and was concluded to be an effective generator of PGM readout [49]. Paraoxon pesticide was used to test the device for its feasibility as an OP detector and was calculated to have a limit of detection of 0.018 μM, which is a lower concentration than is outlined in EU restrictions [49,50].

### *3.2. Binding Affinity-Based Sensing*

Many successful biosensors for environmental toxins such as hydrocarbon or heavy metal pollutants are based around regulatory and degradation systems found in microorganisms. Specifically, there has been much success in harnessing highly regulated transcription factors and other binding proteins to act as BREs in either whole-cell or cell-free sensing technologies [51–55].

Recently, a nitrogen-regulating class (NtrC) of proteins from prokaryotic soil bacteria has become of increasing interest, specifically in the detection of aromatic hydrocarbons such as phenol [56]. Proteins in this class consist of not only a pollutant-sensing domain, but also a connected AAA+ ATPase readout domain that has been shown to be repressed in the absence of aromatic pollutants [55,57,58]. Armed with new structural insight from the solved crystal structures of the *Acinetobacter calcoaceticus* NtrC protein, MopR, Ray et al. (2018) showed the power of these sensing and read out domains containing regulators as a platform for biosensing [56]. By incorporating in silico-guided mutations into the sensing domain binding pocket, there has since been an enhancement in the phenol selectivity of this protein [56,59]. While research has shown engineered MopR as a feasible construct for chip-based diagnostics [60], further work has also engineered MopR as a successful in vivo universally programmable biosensor platform for a number of hydrocarbon pollutants, including xylene and benzene [61].

The rationale of harnessing microorganisms for sensing scaffolds has also been applied to the detection of heavy metals. Specifically, the development of robust heavy metal biosensors has been achieved with naturally occurring metallic chelating proteins in conjunction with fluorescent proteins capable of signaling binding. For instance, Soleja et al. (2019) used this process to modify a periplasmic mercury-binding protein MerP with two green fluorescent protein variants, enhanced cyan fluorescent protein (ECFP) and venus. The resulting protein, MerFS, is a biosensor for mercury II, producing a concentration-dependent ratiometric fluorescence resonance energy transfer (FRET) with micromolar binding affinity [62]. Other biosensors based on FRET signaling include mApple-D6A3 for cadmium II, copper II, and nickel II [63], as well as LaMP1 for lanthanides [64].

Lee et al. (2019) produced a different fluorescent, split-protein biosensor, modifying metal binding loops (MBLs) within regulatory proteins. Lee and coworkers inserted a specific MBL peptide, chosen for its specificity to cadmium, into ZntR, a protein previously modified for enhanced metal selectivity by Yoon et al. (2017) [65,66]. In association with enhanced green fluorescent protein, ZntR functions as a biosensor for cadmium [65]. Similarly to MerFS, the split-eGFP system uses conformational changes to emit a fluorescent signal in the presence of a metal ion.

The types of engineered protein scaffolds fine-tuned for environmental toxin selectivity and affinity have also extended past microbial regulatory systems. Through a semi-rational mutagenesis array approach, Mann et al. (2022) developed the first protein-based biosensor capable of detecting three different per- and poly-fluoroalkuyl substances (PFASs) in water using acrylodan-labeled human liver fatty acid binding protein (hLFABP) [67]. These "forever chemicals" are resistant to biodegradation and possess a molecular diversity and a large contamination concentration ranges that amplify the need for robust, portable, and flexible detection methodologies such as biosensors. N'Guetta et al. (2020) recently employed a similar method to Mann in their approach to design a biosensor for glyphosate herbicide using a phosphonate-binding protein, PhnD. Site-directed mutagenesis was used to engineer the binding pocket and improve the protein's binding affinity for glyphosate. The protein was labeled with an acrylodan fluorophore via thiol-conjugation near the hinge of the protein, and a significant fluorescence shift was reported upon glyphosate binding [68]. Overall, this collection of work highlights the use of synthetic biology and protein engineering as powerful tools in biosensing scaffold design and enhancement.

## 4. Biomanufacturing

### *4.1. Naturally Occurring Proteins for Biomanufacturing*

Biomanufacturing can be applied to existing systems, helping the environment and reducing costs by implementing biologically derived alternatives to petrochemical products [69]. Protein biosurfactants and biodetergents are two rapidly developing applications of biomanufacturing in a multitude of industries [70,71]. Many of the following examples are still under active study, with opportunities for improvement.

For agriculture, Sheng et al. (2007) demonstrates the bioremediation capabilities of biosurfactants by introducing lipopeptide-expressing *Bacillus* strain J119 into cadmium-contaminated soil [70]. This cadmium-resistant bacterial strain promotes plant growth via the secretion of indole acetic acid, a potent plant growth hormone, and siderophores—metabolites that transfer iron from soil into plants—while also promoting the plant uptake of toxic cadmium via the secretion of a lipopeptide biosurfactant [70,72].

As an alternative to phytoremediation, Lai et al. (2009) utilized the biosurfactant surfactin to solubilize and wash oil and petrochemical wastes out of soil [73]. Surfactin was also utilized by Mulligan et al. (2001) and Singh et al. (2013) to wash heavy metals from soil, providing new methods to increase the access to and utilization of agricultural land [74,75]. These biosurfactants replace and improve upon petrochemical surfactants utilized in identical processes, helping to reduce the toxicity and resistance to biodegradation accompanying these chemicals when added to the soil [73].

Cold active biodetergents are a low-temperature alternative to traditionally high-temperature cleaning methods engineered to help reduce energy expenditures and emissions in multiple industrial areas. These biodetergents contain proteins and biosurfactants originating from organisms native to extremely cold climates.

From the alpine regions of India, Kavitha and Shanthi (2017) isolated and demonstrated the efficacy of a cold active bacterial lipase secreted by *Pseudomonas* sp. VITCLP4, while Sahay and Chouhan (2018) identified a fungal cold active lipase, both with direct applications to biodetergent formulation [69,71]. The bacterial lipase was shown to increase oil removal efficiency when added to existing industrially relevant detergents run at low temperatures [71]. It also demonstrated competitive oil digestion when utilized in isolation, indicating its innate potential as a commercial substitute for high-temperature lipases [71]. Both enzymes were also shown to retain activity under alkaline conditions, a trait typical of commercial detergents [69,71].

Looking at Antarctic bacteria, Park et al. (2018) isolated a protease that was effective down to 0 °C, while Wang et al. (2018) identified an amylase highly functional at low temperatures [76,77]. These enzymes tailored for the digestion of proteins and carbohydrates, respectively, can work together with cold-active lipases in complete biodetergents to break down complex stains and residues containing multiple different macromolecules.

Applications continue to arise for cold active enzymatic biodetergents, and the food, textile, and detergent industries are already capitalizing on cold active enzymes to reduce the energy costs of processes [77].

While these proteins can be directly implemented into biomanufacturing applications, that is not always an option. The natural production and harvesting of these products may be difficult, resource intensive, or environmentally harmful. In some cases, naturally occurring proteins must be tailored for specific biomanufacturing uses. The following examples discuss optimizing protein activity within a pathway, as well as strategically modulating domains within a protein. For a more in-depth review, please see the work by Li et al. from 2020 [78].

### *4.2. Optimizing Protein Activity within a Pathway*

Many natural products are derived from complex biosynthetic pathways. Often the first approach to improve product biomanufacturing targets the overall pathway flux, i.e., modifying the pathway to directly increase production [78]. In most cases, this would entail increasing enzyme concentration, improving enzyme solubility, or otherwise modulating the physical properties of the system. For example, Kang et al. (2019) implemented an enzyme scaffold within the carotenoid biosynthetic pathway to reduce the physical distance between two enzymes, resulting in the greater production of metabolic intermediate and downstream yield by 5.7 fold [79]. A different case focused on the production of phenylalanine derivatives and showed a direct increase in the product occurring with an overexpression of enzyme phenylpyruvate decarboxylase [80]. Both of these cases illustrated a change in overall production yields resulting from a superficial change within the system. However, when this approach still does not yield the desired selectivity or productivity, the intrinsic properties of the protein themselves must be examined.

*Gingko balboa* is a genetically distinct gymnosperm that produces many unique terpenoids of therapeutic interest, including levopimaradiene. Levopimaradiene is one possible product of many in a complex pathway, and the biomanufacturing of this molecule is limited by the lack of specificity and slow kinetics of enzyme levopimaradiene synthase (LPS). Furthermore, the direct chemical synthesis of levopimaradiene is inefficient and costly [81].

Researchers performed site saturation mutagenesis for levopimaradiene synthase, followed by phylogeny-guided analyses which led to the identification of 15 strategic points within the LPS binding pocket. Strategic variations at these 15 residues resulted in a more than 2600-fold increase in levopimaradiene production [82]. A similar strategy to optimize the production of citramalate in *E. coli* identified five binding pocket residues in enzyme citramalate synthase to introduce conservative point mutations, leading to a 125% increase in citramalate yields, again illustrating that increased enzyme specificity and kinetics results in significantly improved product yields [83]. In both cases, changing the physical properties of the enzyme did not increase pathway flux and yields, but engineering intrinsic properties of the proteins did.

Squalene is a terpenoid traditionally extracted from shark liver oil, and is of interest for a variety of cosmetic, pharmaceutical, and biofuel applications. Harvesting squalene from sharks kills millions of sharks every year, however, squalene can also be found in many plants and fungi. Recent work by Bibik et al. (2022) screened orthologous squalene synthase (SQS) proteins from six plant and fungal species with several different farnesyl diphosphate synthase (FDPS) proteins [84]. FDPS performs a preliminary condensation of squalene precursor molecules, which SQS then converts into squalene. Through this special "mix and match" strategy, researchers identified an SDS-FDPS combination that produces 63% more squalene than is natively found [84]. The flux in this pathway was effectively manipulated by fusing SQS-FDPS to a lipid droplet surface protein (LDSP), which has previously been shown to effectively sequester and store terpenes. The co-expression of SQS-FDPS and LDSP resulted in a more than 80% increase in squalene yields, illustrating

that the squalene production at a synthetic organelle (lipid droplet) interface may be an effective approach to optimize enzymatic activity and maintain high pathway flux [84].

### *4.3. Strategically Modulating Structural Moieties within a Protein*

Polyketide synthases are a type of protein that have exceptional potential for manipulation via protein engineering. Polyketide synthases (PKSs) are a class of modular enzymes naturally found in many bacteria, fungi, and plants [85]. They synthesize a wide range of functional natural products that are often of importance to medicine and the pharmaceutical industry [86]. Researchers have identified many PKS natural products, or polyketides, that are used in drug therapies; such as the antibiotic erythromycin [87,88], the anticancer drug leinamycin [86,89], the immunosuppressant rapamycin [85], and so forth. PKSs have also been found to produce unique natural products, such as cinnamoyl lipids [90].

The exceptional diversity of polyketides is largely due to the range of possible structural combinations of not only PKS modules themselves, but also the domains within these modules [86]. The biosynthesis of polyketides is centered around an assembly line of catalysis through multi-domain modules. Intermediate products move through modules that each catalyze a specific reaction in the chain elongation process. The linear nature of the chain elongation process reveals the importance of modular organization and the specificity of module catalysis to the PKS product produced [91]. Understanding polyketide biosynthesis and PKS organization is integral to the generation of novel products and to the optimization of polyketide biosynthesis. Structure-based rational design in PKS systems has been used as early as 1998, when McDaniel et al. engineered the erythromycin PKS to produce a library of novel natural products [87]. They accomplished this by substituting the acyltransferase and $\beta$-carbon processing domains with analogs from the rapamycin PKS that differed in functional activities and substrate specificity [87]. Developing an understanding of PKS pathways and the assembly line of catalysis is key to optimizing polyketide biosynthesis.

In 2020, Koch et al. investigated the importance of individual domains in a PKS module, specifically the thioesterase (TE) domain and its role in substrate processing [92]. They constructed hybrid PKS modules containing TE domains that are both native and non-native to the PKS, then attempted the biosynthesis of polyketides with native and non-native substrates again to the PKS. They found that the hybrid modules containing TE domains from pathways that matched the non-native substrate were the most successful in product biosynthesis, highlighting the importance of the TE domain in polyketide biosynthesis and its potential as a bioengineering target [92].

Another group of researchers explored the significance of particular domain functions, this time in the investigation of a unique natural product called cinnamoyl lipids [90]. Cinnamoyl lipids (CL) represent a small group of natural products that are assembled by a unique type II PKS and have functional effects ranging from antitumor to antibacterial. Deng et al. (2021) were able to characterize the biosynthesis of a CL containing compounds known as youssoufenes using the intracellular-tagged carrier-protein tracking of gene inactivation. This characterization of the youssoufene chain elongation process revealed a dependency on the isomerase domain in benzene ring formation and exemplified that understanding the PKS biosynthesis process can guide bioengineering design [90].

The addition of fluorine molecules to the backbone of polyketides is a remarkable example of unique PKS engineering and could be a powerful tool in the development of synthetic pharmaceuticals with increased clinical effectiveness. Fluorine's high electronegativity and small size make it ideal for optimizing clinical applications because it can form strong polar interactions; however, this same reactivity can make it challenging to incorporate into biomolecules [93]. A recent work by Sirirungurang et al. from 2022 site-selectively incorporated fluorine in PKS via an especially engineered transacylase enzyme that charges the specific module, enabling the incorporation of a fluorine substituent group [94]. These works highlighted a promising future for the creation of fluorinated natural products and

served as a foundation for incorporating similar elements or groups that are not normally found in the nature.

By utilizing and enhancing natural biological systems, the field of synthetic biology looks to solve new and/or difficult problems in creative multidisciplinary ways. The synbio "tool kit" encompasses techniques and methodologies from a vast array of disciplines including many different engineering subsects. In this review, we highlighted some recent work by groups harnessing synthetic biology to produce protein-based advances in several broad fields including agriculture, environmental monitoring, and biomanufacturing.

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