Current and emerging tools of computational biology to improve the detoxification of mycotoxins

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Abstract

Biological organisms carry a rich potential for removing toxins from our environment, but identifying suitable candidates and improving them remain challenging. We explore the use of computational tools to discover strains and enzymes that detoxify harmful compounds. In particular, we will focus on mycotoxins—fungi-produced toxins that contaminate food and feed—and biological enzymes that are capable of rendering them less harmful. We discuss the use of established and novel computational tools to complement existing empirical data in three directions: discovering the prospect of detoxification among underexplored organisms, finding important cellular processes that contribute to detoxification, and improving the performance of detoxifying enzymes. We hope to create a synergistic conversation between researchers in computational biology and those in the bioremediation field. We showcase open bioremediation questions where computational researchers can contribute and highlight relevant existing and emerging computational tools that could benefit bioremediation researchers.

1. Background and Motivation

1.1. Context: detoxifying contaminated food and feed

Fungi that grow on foodstuffs are one of the major sources of contamination in food and feed; these fungi-produced toxins are called mycotoxins. Currently, an estimated 25% of world crops is thought to get contaminated with mycotoxins each year (1, 2), putting a major burden on agriculture and public health. Preventing contamination or detoxifying mycotoxins is a major safety priority (3). In what follows, we briefly describe the threat of mycotoxins, and the potentials of biological organisms to address this threat via detoxifying enzymes. We will then investigate and explore the use of computational approaches to discover and improve such potentials. We will primarily discuss three aspects: (1) the use of bioinformatics tools to search genomic databases for candidate species and enzymes, (2) the use of genetics and genomics data to investigate how the detoxification performance can be improved, and (3) the use of computational tools to improve the detoxifying enzymes. While we discuss established computational methods used in identifying mycotoxin degrading enzymes, we also consider the use of novel, field-adjacent methods that have potential in mycotoxin detoxification.

1.2. Mycotoxins are prevalent and harmful

Mycotoxins are secondary metabolites produced by a variety of filamentous fungi that contaminate common food crops and cause negative health effects in animals and humans. More than 300 types of mycotoxins have been identified so far, all of which would be candidates for detoxification (1). Among these, six major types are of particular interest and the focus of this review because of their detrimental health impact and because they routinely contaminate foods

and animal feed (4, 5): aflatoxin (AF), ochratoxin (OT), zearalenone (ZEA), fumonisin, deoxynivalenol (DON), and patulin.

Aflatoxins, produced by *Aspergillus* species, are one of the most carcinogenic naturally occurring substances and active inducers of mutations, liver cancer, congenital malformations, hormone disorders, and immunodepression (6, 7). Ochratoxin is also produced by *Aspergillus* species, as well as certain *Penicillium* species, and is a nephrotoxin, immunosuppressant, potent teratogen, and renal carcinogen (6, 8, 9). Zearalenone and fumonisins are produced by *Fusarium* species. ZEA acts through estrogen mimicry to dysregulate the hormone receptor and antagonize the estrogen pathway leading to reproductive disorders, hormone imbalance, and breast cancer (6, 10). Fumonisins have been linked to esophageal cancer in humans as well as a variety of health complications in animals such as pulmonary edema and hepatotoxicity (6, 11). Produced by *Fusarium graminearum*, DON is a vomitoxin, causing emetic and nauseous effects after ingestion (12). Finally, patulin is produced by ascomycetes such as *Penicillium*, *Aspergillus*, and *Byssochlamys* species and is commonly found in fruit and vegetable products, especially rotten apples and apple juice (13). Patulin ingestion is linked to a number of health complications, namely immune suppression, ulcers, gastrointestinal inflammation and embryotoxicity (13). There are a variety of food crops that these mycotoxins contaminate, including cereal crops such as wheat, barley, corn, and oats (6, 11). Due to the serious health implications of mycotoxin contamination, economic losses arise from reduction of crop and livestock yields as well as the cost of decontamination efforts. Annually, the United States faces an estimated $932 million economic losses from AFs, fumonisins, and DON alone (14). This sizable economic burden is faced across agriculture and livestock producers globally and requires efficient and cost-effective measures as a solution.

Mycotoxins build up on foodstuff necessitates methods of decontamination in order to supply safe foods for consumption. Currently, decontamination is limited to physical and chemical methods. Physical methods, including sorting and cleaning, have been shown to be effective in some but not all cases of mycotoxin contaminations. Chemical methods, which use chemical agents to reduce or convert mycotoxins into less toxic byproducts, include ozonation and ammoniation. While these physical and chemical methods have been used to reduce mycotoxin contamination, they suffer from high operational costs and limited reliability and may decrease the quality or nutritional value of the food (3, 14–16). These limitations expose the need to look for better solutions.

1.3. Toxin removal by biological processes is a promising solution

Bioremediation, or the use of biological entities to detoxify or remove toxins in the environment, is a promising alternative to current decontamination methods. Bioremediation offers lower costs, fewer undesired environmental side-effects, and potentially higher efficiency and reliability (17–19). The use of microbes is a particularly attractive choice in bioremediation, offering faster activity and the feasibility of strain evolution and engineering for improved performance (20). There are six key factors that make a good bioremediator: 1) fast and efficient at degradation, 2) safe degradation products, 3) non-pathogenic to plants, animals, or humans, 4) not detrimental to the quality of the food/feed, 5) applicable outside of lab settings, and 6) applicable to multiple pollutants (17). Among identified mycotoxin degraders none effectively fit all of these factors, with speed and efficiency often being subpar. Additionally, the mechanisms of degradation by these identified microorganisms are often unknown or understudied, limiting the ability to improve upon the native degradation performance. Therefore, identifying new

88 species that possess mycotoxin degradation ability and elucidating the mechanisms of
89 degradation are beneficial in making this capability effective and commercially viable.

90 **1.4. Modes of biological detoxification**

91 In the context of microbial interventions for removing mycotoxins, the two main modes
92 of detoxification are adsorption and biotransformation. In adsorption, mycotoxins are physically
93 bound to polysaccharides and proteins in the outer cell structures (21, 22). Biotransformation
94 utilizes microbes and their enzymes to convert mycotoxins into non-toxic compounds (23, 24).
95 In this paper we are solely concerned with methods to identify and improve biotransformation
96 processes. Biotransformation can be further broken into two categories (schematically shown in
97 Fig. 1): secretion of enzymes (extracellular degradation) and uptake of the toxin into the cell
98 (intracellular degradation). Intracellular degradation of toxins more closely follows normal
99 metabolic processing of molecules by microbes inside the cell. Microbes that mitigate
100 mycotoxins through extracellular degradation are more likely to produce stable enzymes that can
101 be isolated and used in practice; this has been the strategy for several existing commercial
102 products (25–27). Table 1 shows some of the bacterial and fungal enzymes that have been found
103 to degrade major mycotoxins.

104 Enzymatic degradation has been suggested in a number of studies; however,
105 identification of the degrading enzymes has proven difficult. Sangare *et al.* show a *Pseudomonas*
106 species capable of degrading AFB_1 from cell-free culture supernatant, suggesting that an
107 extracellular enzyme is responsible for the degradation (43). Screening for the effect of common
108 functional cofactors may potentially help identify the enzyme class. Similar extracellular
109 degradation has been reported for *Rhodococcus* spp., *Stenotrophomonas* spp., and *Myxococcus*

spp. (44–46). DON has been observed to be assimilated as a carbon source in some, but not all strains (47). Other extracellular enzymes with mycotoxin degrading abilities include oxidoreductase, dehydrogenase, aldo-keto reductases, and peroxidases (48–50). While there has been less focus on intracellular mechanisms, intracellular enzymatic degradation has been shown by Zhu *et al.* (51).

1.5. Bacteria and fungi carry a rich repertoire of enzymes capable of removing mycotoxins

Biotransformation of mycotoxins into non-toxic products by bacterial and fungal enzymes has already been demonstrated (19, 48, 52, 53). The detoxification performance can be improved by identifying and characterizing the enzymes with degradation/detoxification capability. On one side, uncovering the cellular machinery of degradation (schematically shown in Fig. 1 and explored in Section 2.3) allows us to select conditions to express the enzyme (when searching for candidates) or engineer strains to improve their performance. On the other hand, the enzyme itself can be modified and improved. Structural modelling and design of experiments (DOE) techniques can shed light on the identification of key structural components that contribute to degradation (52).

In the remainder of this work, we will limit the scope to extracellular bacterial and fungal detoxifying enzymes. We make this choice to offer a more focused view on recent developments in computational tools for biological enzymes, but also because deploying enzymes (versus live organisms) in food/feed applications is a more practical approach (23, 52). The use of enzymes for reducing the threat of mycotoxins has reached industrial applications, even if only in a few cases. Mycofix® line of products (27) combine different modalities, including biotransformation and adsorption to remove several mycotoxins from feed. FUMzyme® is a commercially

available fumonisin estrase produced in a genetically modified strain of *Komagataella pastoris* (54) that has shown success in removing the contamination from feed (26). However, more research is still needed to improve the performance of mycotoxin removal.

Several previous reports have cataloged specific enzymes that act on mycotoxins (17, 48, 55) and Table 1 lists representative examples for the major mycotoxins explored in this review. Here instead we focus on current challenges and questions in the field of mycotoxin detoxification that can be addressed by computational tools. In this context, we survey some of the existing tools that have already been applied in this field and then propose emerging tools that have the potential to lead to transformative progress.

2. Current Challenges and Computational Solutions

How can we effectively remove mycotoxins using biological organisms? Conceptually, we break down this search into two steps: (1) finding organisms that have this capability, and (2) optimizing the performance by modifying the environmental conditions, the detoxifying strain, or the target enzymes. We survey existing computational tools that can facilitate this process (Fig. 2). We focus our discussions on genomic and structural biology tools. We acknowledge that there are other useful tools—including proteomics—that can offer additional insights, but are beyond the scope of this mini-review.

2.1. Finding candidate organisms: who can do the job?

Discovering organisms that can degrade mycotoxins poses a number of challenges that can be met both through experimental and computational approaches. In terms of enzymatic

153 degradation, there are three challenges to be addressed. First, organisms must have the genes
154 necessary to produce enzymes and possibly cofactors involved in degradation. Second, the
155 organisms must have favorable regulatory mechanisms for these enzymes. Third, the method of
156 obtaining and isolating the enzymes must be favorable to the end use case. One can describe the
157 search space as being largely defined by these characteristics that may be specific to the use
158 cases, but are still conceptually similar among different cases.

159 From the experimental front, high-throughput screening may be used both to identify
160 candidate organisms as well as explore mutations for optimizing degradation potential.
161 Environmental isolates are a traditional source for identifying mycotoxin degraders. Isolates can
162 be cultivated and tested for degradation, especially when high-throughput screening is possible.
163 As an example, Ciegler *et al.* screened ~1000 organisms, both prokaryotes and eukaryotes, for
164 their aflatoxin-degradation capability (56). Screening can also be used for optimizing the
165 environmental conditions or the enzyme itself. However, unless feasible high-throughput assays
166 are available, this process is resource and time expensive. Therefore, looking to computational
167 methods to screen for new organisms will be beneficial.

168 As an example, there is a known, highly specific two-step enzymatic process in the
169 detoxification of fumonisin, which involves a carboxylesterase and an aminotransferase (34).
170 This becomes a useful bottleneck in the search space, as candidate organisms must contain both
171 enzyme-encoding genes to be viable degraders. Toward this end, tools such as BLASTp (57) can
172 be utilized in cases where genome sequences are available. Simply put, the presence of these two
173 genes largely dictates whether or not an organism is a fumonisin degrader. On the other hand, in
174 the example of AF detoxification, many species can possess hydrolases or oxidases related to
175 those that are known to degrade AF (24, 48, 58). The search space is instead constrained on a

176 separate manifold involving the specificity and affinity of the hydrolase for AFs. That is, the
177 presence of the same hydrolase gene may not be sufficient to identify degradation potential,
178 since it may be optimized for a different substrate. The sequence-to-function relationship then
179 becomes critical, which is not guaranteed to be captured by sequence similarity *à la* BLASTp.
180 This shortcoming can be thought of as a signal to noise ratio, where key amino acids involved in
181 the active site mechanism are sparse signals, and the rest of the sequence functions primarily to
182 provide the correct structural shape and may be noisy in this regard. This is witnessed in the
183 work by Dellafiora and colleagues (59), where two related, AF-degrading oxidases shared only
184 72% sequence similarity, despite using the same mechanism for degradation. In a more extreme
185 example, a recently identified carboxylesterase that degrades fumonisin shows only around 34%
186 sequence similarity to previously reported fumonisin-degrading carboxylesterases (60).

187 Similarity in sequence does not necessarily overlap with similarity in function. Sequence
188 similarity may be used to imply functional similarity; however, such a predicate does not include
189 enzymes that share functional similarity without sequence similarity. High sequence similarity
190 among closely related species might not fully overlap with functional similarity either.
191 Therefore, searches should be conducted on a sequence-to-function relationship model. While
192 this method loses the high-throughput optimizations of BLAST-based sequence similarity, it may
193 be modeled via a reductive filter pipeline to maintain reasonable complexity. It also loses the
194 generalizability of sequence similarity, and instead pipelines must be custom designed for each
195 case. Dellafiora *et al.* have combined an *in silico* screen with an enzymatic assay to address this
196 challenge in search of hydrolyzing enzymes that can degrade ochratoxin A (61). In the example
197 of AFs, initial work has been performed to design a structure-to-function reductive filter model
198 using a number of filters. Furthermore, this model does not necessarily require a labeled, positive

enzyme to seed the search, rather it only requires characteristics to build the filters. Prior research by Risa and colleagues (62) has identified that excreted enzymes can be responsible for degrading AFs. SignalP is able to predict protein excretion in bacteria, and can be used as an initial filter to narrow down proteomes. These sequences can be passed through both size and sequence-based enzyme classification filters based on facile experimental determinations to further reduce the candidate pool. From here, 3D structures may be built, the binding pockets predicted, and AF docked to identify high affinity interactions that then may be confirmed experimentally. These computational processes will be expanded below. The reductive filter model uses low-complexity tools at its head, increasing in complexity towards the tail to ensure efficiency. Similarly, its modular nature allows for easy insertion or upgrading of components as advances occur in each domain.

2.2. Community-level detoxification: when the task needs to be divided

Mycotoxin degradation may require multiple reactions to reach byproducts with complete or significantly decreased toxicity. There are several examples where a single enzyme is insufficient for complete degradation and two or more enzymatic steps are required for the detoxification process. In such cases, we need to better understand how multiple enzymes from the same, or even different, species are required for degradation of a single mycotoxin. While this increases the difficulty and cost of searching for degrading enzymes that can work together, the outcome of complete degradation and reduced toxicity is desirable for application in agriculture where mycotoxin levels must fall under set regulatory limits. For degradation of fumonisin B_1 by *Sphingopyxis* sp. MTA144, Heinl *et al.* found that two enzymes were involved (34). A carboxylesterase facilitated the initial deesterification step to form a hydrolyzed fumonisin $B_{1,}$ which is less active in its known ceramide synthase inhibitory pathway but still

222 possesses significant toxic effect (34, 63). A second enzyme, an aminotransferase, deaminated
223 the hydrolyzed byproduct of the first reaction resulting in complete degradation and loss of toxic
224 effects (34). Similarly, Carere *et al.* elucidated a two component enzymatic pathway involved in
225 the epimerization of DON by *Devosia mutans* 17-2-E-8 (64, 65). The enzymes, designated DepA
226 and DepB, first oxidize DON into 3-keto-DON (DepA) (64) and subsequently reduce 3-keto-
227 DON into 3-epi-DON (DepB) (65), significantly reducing toxicity. These examples highlight the
228 need to understand all the enzymes playing a role in complete degradation.

229 In some instances, mycotoxin biotransformation does not lead to complete detoxification
230 (52); DON degradation above as an example leads to end products that are less toxic than the
231 starting substrate, but still retain some toxicity. In biotransformation of ZEA, there are cases
232 where microbial breakdown results in byproducts, α-zearalenol and β-zearalenol, that are even
233 more toxic than the original compound (39, 66, 67). In such cases, we need to identify additional
234 species or enzymes that can take the byproducts and convert them into non-toxic compounds in a
235 multi-step process.

236 Multi-step degradation underscores the possible need to look beyond single
237 microorganisms and employ microbial consortia to complete the job; as an example, Wang *et al*.
238 discovered a microbial consortium that utilizes multiple species across various taxa working in
239 unison to transform ZEA to non-toxic byproducts (68). Bioinformatic searches for identifying
240 multiple enzymes necessary for a particular case would be an extension of the single-enzyme
241 searches discussed in the previous section, using the similar tools. Of note could be searching for
242 individual organisms that carry two or more necessary enzymes that have previously been
243 identified in multiple species/strains.

2.3. Regulation: even when the detoxification capability exists in an organism, its availability may be under regulation

Even after organisms have been identified that are capable of detoxifying target pollutants, the availability of the relevant enzymes depends on whether the environmental context induces the relevant genes of enzyme production and secretion effectively. These considerations point to the need to explore the internal regulation of the production and secretion of detoxification enzymes. Microorganisms respond to cellular and environmental changes through regulatory decisions that could impact the availability of degradation machinery for target pollutants (69). Production of enzymes is regulated through different mechanisms, such as transcription factors binding in and around promoter regions that contributes to the amount of enzyme produced by the cell. These mechanisms are likely influenced by nutrient availability and overall conditions of the cell (i.e. growth phase) (70). Secreted enzymes have an added layer of regulation due to the high energy cost of secretion. While these enzymes have beneficial effects, often being employed to breakdown macromolecules in the environment for cellular uptake, they also incur an energy/biomass cost (71). Therefore, certain enzymes targeted for secretion are up- or down-regulated by the presence of nutrients in the environment that respectively do or do not require extracellular breakdown.

Here, we primarily emphasize the existing native potential as the starting point, even though ultimately the deployment likely happens in a safe and tractable host organism. Our discussion on regulation and the detoxification machinery in the native context has two purposes. (1) It reveals the preferred conditions for the expression of the detoxification machinery to enable more effective screening for functions of interest. (2) It allows us to better understand the diversity of possibilities and the ideal machinery to be transferred to a host organism.

Understanding the influence of regulation on production and secretion of the enzyme is also necessary for strain optimization to factor in the cost-benefit balance of increased enzyme production and secretion.

Several existing bioinformatic tools can help us uncover aspects of bacterial gene regulation, such as promoter and DNA binding sites, operon regions, and secretion signals, which are touched on in the following sections. The usefulness of these tools in the context of bioremediation is that they allow researchers to uncover possible mechanisms of regulation that control the detoxification process. Insight from regulation, for example similarity to a known catabolic pathway, can also be used to choose suitable environmental conditions or infer the mechanism of degradation.

Promoter Prediction. Identifying promoter regions and DNA binding sites are important in that transcription initiation is the most frequently regulated step in gene expression. Promoters contain an intrinsic strength that governs the amount of transcription a gene undergoes and when transcription occurs according to environmental factors such as nutrient availability (70). It is important to properly regulate gene expression to ensure the degrading enzyme is sufficiently expressed, but only when the particular substrate is present to limit wasteful production of enzymes that are disadvantageous to the cell without the substrate (72). By uncovering promoters associated with genes/enzymes of interest in bioremediation, we can understand how the cell naturally regulates its expression and better manipulate it toward improved expression for application in agriculture. There are several existing tools for predicting and cataloging promoter regions in different organisms, such as phiSITE (73, 74), SAPPHIRE (75), PRODORIC2 (76), BacPP (77), and PPCNN (78). We will expand on the latter three.

PRODORIC2 is a transcription factor binding site (TFBS) database that possesses one of the largest collections of DNA binding sites in prokaryotic organisms (76). In 2018, its most recent update, PRODORIC2 expanded its database to host the genomic information of 2274 bacterial strains and their 5191 replicons (76). This database is curated to only include experimentally validated binding sites, limiting the expanse of bacterial species it contains but ensuring accuracy in its TFBS inventory. De Avila e Silva *et al.* created a bioinformatic tool, BacPP, to predict promoter sequences in *Escherichia coli* strains through neural network simulations (77). BacPP is able to recognize and predict promoter sites with varying levels of accuracy (all above 83%) across the different sigma factors crucial for prokaryotic transcription initiation (77). Additionally, BacPP has 76% prediction accuracy among other enterobacteria species (77). The advantage of this method is in its ability to classify promoter sequences by its sigma factor, an important distinguishing feature that was a shortcoming of previous tools. However, BacPP is currently limited to *E. coli* and, to a lower accuracy, enterobacteria. Another promoter prediction tool is Promoter Prediction Convolutional Neural Network (PPCNN), developed for both eukaryotic and prokaryotic prediction and implemented into the CNNProm program. This approach uses deep learning neural networks for its prediction models (78). For prokaryotes, PPCNN was trained on *E. coli* and *Bacillus subtilis*, offering insight into both Gram-positive and Gram-negative species. A highlight of this method is its applicability to other sequenced species because it predicts promoters without prior knowledge of specific promoter features (78).

Operon Prediction. Metabolically or functionally related genes within prokaryotic genomes are often arranged in contiguous segments called operons and are co-transcribed along the same messenger RNA (79). This organization imparts an added layer of regulation on the genes within

312 the operon. Specifically, in the context of bioremediation, if an enzyme of interest is encoded
313 within an operon, it opens up new genes that could help play a role in degradation, either
314 functionally or through regulation. As an example, Heinl *et al.* identified two fumonisin
315 degrading enzymes that were held within a gene cluster organized in two operons and
316 subsequently determined other genes in the operon held importance to transcriptional regulation
317 and transport of the degrading enzymes, as well as additional enzymes that might play a role in
318 further breakdown on the degradation byproducts (34). Additionally, downstream utilization of
319 the enzyme-encoding gene(s) can be affected by its placement within an operon. For example,
320 Altahli and El-Deeb transferred ZEA degradation capability in *Pseudomonas putida* into *E. coli*
321 via a plasmid encoding detoxification genes (39). Multiple genes were shown to be expressed for
322 detoxification; however, they were unable to separate these genes due to their organization in
323 operons. Therefore, understanding the genomic organization of these genes within operons can
324 aid in their use for degradation. Determining operons computationally has been a field of interest
325 for a number of years, leading to tools such as Operon DataBase (80, 81), OperomeDB (82, 83),
326 Operon Hunter (84), and Operon-mapper (85, 86), with recent advances in *de novo* prediction of
327 operons from genomic data, which is expanded on below.

328 Operon-mapper, a web-based server for operon prediction, was developed in 2018 and is
329 the first publicly available tool for operon prediction that only requires genome sequences as the
330 input (85, 86). Operon-mapper uses a five step procedure: (1) open reading frame (ORF)
331 prediction using *Prokka* software (87, 88); (2) homology gene determination using the
332 *hmmsearch* program based on Hidden Markov Models (85, 88); (3) intergenic distance
333 evaluation using a custom program (85); (4) operon prediction using an artificial neural network
334 with intergenic distance and a score defining functional relatedness of protein products as the

input arguments (85, 89, 90); and (5) gene function assignment using the DIAMOND algorithm (91). The accuracy of this method in predicting operons was ~90% across eight tested genomes with varying size and GC content, and outperformed other algorithms in a recent evaluation of correlation to experimentally validated operons (92). Operon-mapper also has the advantage of providing ORF identification and functional annotation of protein (85).

Secreted Protein Prediction. A signal peptide (SP) is a sequence of amino acids in a newly synthesized protein that targets the protein into or across the membranes in the cell (93). Determining whether and how an enzyme is secreted outside the cell enables better utilization of the degradation machinery (schematically represented in Fig. 1A). To predict secreted proteins, several algorithms to identify SPs within a proteome have been developed: SignalP (94), Psort (95), Pred-Tat (96), and TatP (97).

Of note, SignalP is able to determine these secretion signals and distinguish between the type of secretion pathway. The current version, SignalP 5.0, uses deep neural networks in combination with conditional random field classification and optimized transfer learning to determine SPs in prokaryotes, eukaryotes, and archaea (94). This update builds upon previous versions based on artificial neural networks (98), with added improvements of hidden Markov models (99), enhanced cleavage site predictions (100), and discrimination of signal peptides and transmembrane helices (101). For prokaryotes, there are two main secretion pathways, Sec and Tat, with three enzymes, signal peptidases I-III (SPase I-III), needed to cleave proteins for secretion. SignalP 5.0 is able to distinguish between three types of SPs: (1) Sec substrates cleaved by SPase I; (2) Sec substrates cleaved by SPase II; and (3) Tat substrates cleaved by SPase I (94). Unfortunately, due to limited training data sets, SignalP 5.0 is unable to predict Sec substrates processed by SPase III or Tat substrates processed by SPase II. However, the current

ability to determine between the three secretion pathways is important in understanding how the protein will be secreted and the regulation of the secretion process. SignalP 5.0 is available either through their webserver or as a standalone package, making it an accessible tool for secreted protein prediction. SignalP has already been used in the context of determining mycotoxin degrading enzymes: Carere *et al.* utilized this predictive power in conjunction with an experimental approach to narrow down gene candidates for the identification of DepA in the DON degradation pathway by *D. mutans* (64). This example highlights the application this tool has in aiding mycotoxin degradation research.

2.4. Sub-optimal enzymes: naturally evolved enzymes may not be the best match

Enzymes found capable of degrading mycotoxins may not be naturally optimized for targeting the mycotoxin of interest. Importantly, some of the detoxifying enzymes belong to common categories such as oxidases and hydrolases; however, it is not well understood what features of the particular enzymes separates efficient detoxifiers from nonefficient ones. Thus, there is a need to better understand what aspects determine the efficacy of the enzymes and how they can be improved. Enzyme optimization often involves adaptation of a wild-type isolate to a new substrate or reaction environment. New reaction environments often involve changes of temperature, pH, and solvent conditions, all of which non-trivially affect the structure and activity of the enzyme. One technique that is agnostic to fundamental understanding of these effects is directed evolution (102–104). In directed evolution, genetic diversity is introduced via random mutations and the resultant mutant proteins are screened/selected for improved performance. There is some evidence that restricting directed evolution to residues close to the active site leads to a higher probability of displaying meaningful contributions to its activity (105). However, it remains unclear how such a process is achieved through traditionally

structure-agnostic *in vitro* mutagenesis. Often, directed evolution is applied iteratively to further improve strong performing mutants (106). Though directed evolution conveniently creates a black-box optimization method, it does so at the cost of efficiency, where screening for fitness can become a major bottleneck in the process (107). As an alternative, a variety of computational tools have been developed for targeted enzyme engineering (e.g. those reviewed in (108, 109)).

Protein sequence activity relationship (ProSAR) models can assist the search algorithm by creating a statistical model that links the protein sequence to its activity (i.e., fitness) (110, 111). ProSAR relies on a mutant library generated from mutagenesis with a constraint of constant protein sequence length, along with the corresponding activities of interest (catalytic constant, thermostability, etc.). A statistical model is built that links the presence or absence of individual mutations to a contribution to the activity, from which some subset of the highest contributing mutations can be fixed for the next round of mutagenesis. Unlike the close mutations described earlier by Morely *et al.* (105), this method is able to link individual mutations to activity contributions without explicit knowledge of the 3D structure. The traditional statistical methods for ProSAR involved partial least square regression and genetic algorithm, while more recently traditional statistical methods could be replaced with Recurrent Neural Network architectures (112).

Focused evolution, where targeted mutations are introduced based on rational mutation hypotheses, can increase the efficiency of optimization by narrowing the search space; however, current robust methods require 3D structures of the enzyme. When optimizing for known properties such as thermostability and where reasonable 3D models are available, such as homology models, a small subset of rational mutations can feasibly be explored through computational methods and the final mutations evaluated experimentally. Rational mutation

methods rely on heuristic evaluation methods like FoldX (113) to predict changes in Gibbs free energy from mutations, or predictive methods like DbD2 (114), which predicts mutations to introduce disulfide bonds that potentially have stabilizing effects on the protein for given conditions. Potential mutations identified via heuristic methods are then commonly evaluated as a narrow combinatorial library. Although not strictly necessary, to reduce cost and labor for the *in vitro* experiments, the mutated proteins are often computationally evaluated for stability to further narrow down viable mutations. Because of their heuristic nature, it is always necessary to be able to introduce the mutations *in vitro* and evaluate them experimentally under the target conditions to confirm the mutated protein is improved.

3. Future Outlook

Computational biology tools we have discussed above—although not comprehensive—represent a range of traditional applications for better understanding the mechanisms and ultimately improving the performance of toxin biodegradation. Some of these tools have already been used in this context, whereas others have the potential to yield helpful insights. Table 2 captures the current landscape, using representative examples from the literature. Next, we explore ongoing and future advancements in computational methods that would further facilitate answering pertinent questions in the field of mycotoxin bioremediation.

3.1. Taking the next step: combining machine learning with high-throughput experimentation

Both the use of machine learning and automated, high-throughput laboratory experiments are becoming increasingly prevalent for enzyme optimization. Enzyme engineering may become

a useful tool for the optimization of known degrading enzymes, especially when only sequences, rather than solved crystallographic structures, are known (135). Models for directed evolution can be experimentally realized in parallel and incrementally updated, moving towards an optimal sequence. Like directed evolution, biopanning assays, also known as phage display assays, are a technique often used to determine novel antibodies with high affinity to some known antigen (136, 137). Biopanning involves washing a random peptide library over a target ligand immobilized on some substrate. The non-binding peptides may be washed away, after which the peptides with high affinity remain bound to the ligand and can be separately identified. Like a genetic algorithm, these peptides form the seed for the next round of mutation and panning. While this technique does not offer per-sequence performance metrics, we obtain partitioned sequence datasets resulting from the pannings. Such partitioned datasets have been used in unsupervised, autoregressive sequence models for nanobodies to generate novel sequences that overlap with the high-affinity partition without needing to perform additional physical experiments (138, 139). While further evaluation is needed to obtain specific performance estimates for these novel sequences, the method aims to narrow the search space needed in optimization. Biopanning has been previously shown to optimize TEM-1 beta-lactamase and biotin ligase, indicating it may be feasible to use in optimizing mycotoxin degrading enzymes (140–143).

Complemented by high-throughput assays, machine learning approaches are gradually taking charge to bring out patterns, similarities, and dependencies—for example in sequence-function relation of an enzyme family—that may otherwise be too cryptic. The use of machine learning is in particular expanding in situations when an *a priori* model does not exist.

3.2. Computational chemistry can further advance our understanding of enzymatic processes

Towards the understanding of enzymatic mechanisms, advancements in quantum mechanics (QM) and molecular mechanics (MM, atomistic) studies will be vital for characterizing reaction mechanisms and exploring the chemical space available via mutations (144). Additionally, crystallographic structures can be slow and expensive to solve; therefore, recent advances in protein 3D structure prediction will be instrumental to develop high-throughput pipelines.

Molecular mechanics provides a view of a system at the atomic level. It is often used for molecular dynamics (MD) simulations, where a system (e.g. a protein-substrate interaction) is studied using Newtonian physics, often at nanosecond to microsecond timescales. For some protein systems, this timescale is sufficient to study the relevant mechanisms, such as in the case of using steered MD simulations to characterize an aflatoxin oxidase enzyme isolated from *Armillariella tabescens* as a member of the dipeptidyl peptidase III family of enzymes (145). However, for larger proteins, or proteins involving large conformational shifts, extensive computation may be needed. For these systems, a coarse-grained approach is taken where moieties in the system are combined to reduce the total atom count, reducing the computational cost (146, 147). Some examples are coarse-grained water models, as well as proteins where the side chains are often reduced to a single pseudo-atom. Coarse-grained models face issues in faithfully reproducing the system, and current research is focused in this area (147).

Atomistic models allow some insight into the interaction between the protein and the toxin. Such models are often sufficient to determine if the toxin will sterically fit in the binding

pocket, and may also help to determine pose, electrostatic favorability of the binding, and conformational changes of the protein-ligand complex (148, 149). Unlike the more common use for MM in evaluating non-covalent inhibitors, some difficulty emerges in the inherent covalent nature of detoxification, which cannot be captured by an atomistic view (150). This issue may preclude some energetic effects brought about by the changes in electronic structure, raising concerns about how realistic such a model is. This concern may be partially solved by using QM/MM methods, where part of the system is partitioned into a QM region, and the rest remains in MM views (151). The QM region then can model electronic changes, and the rest can remain in lower cost MM regions. However, the QM region cannot be too large, which precludes cases that require large, complex QM regions (e.g. in metalloenzymes like laccases). Additionally, the QM region adds computational cost and cannot be well-integrated into microsecond timescale calculations.

At a relatively high computational cost, QM calculations provide a detailed and comprehensive view of the electronic state of the system. They can provide information about covalent and electronic changes, often necessary for detoxification studies. An example of this is calculating a Fukui function of a molecule, which describes the change in a frontier orbital as the molecule undergoes a redox reaction. Fukui functions have been used to identify the location of redox in an AF-laccase system (152). QM may also be used to study electron transfer in the protein. As a tool for microbiologists, however, QM remains prohibitively expensive both in computational cost and learning curve, and is often used for fine-grained mechanistic studies in collaboration with a QM expert.

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Figure and Table Legends

Figure 1. Simplified representation of the cellular machinery involved in (A) extracellular versus (B) intracellular detoxification.

Figure 2. Conceptual breakdown of major questions of interest where computational tools can facilitate more efficient removal of toxins.

Table 1. Representative examples of identified bacterial and fungal enzymes with the capability to degrade major mycotoxins are listed. Those hypothesized but not yet confirmed are marked by an asterisk (*).

Table 2. Representative examples of applications of computational biology tools for usages outlined in the previous section are listed. Those used for bioremediation are marked by an asterisk (*).