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Original Research Article

CRISPR-Enhanced Microbial Consortia for Pollutant Degradation: Molecular Dynamics-Guided Enzyme Stabilization and Metabolic Pathway Optimization

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Abstract: Novel research is proposed to optimize a pollutant-degrading microbial consortium by employing two state-of-the-art technologies. These technologies will incorporate CRISPR-Cas9-based genome editing in conjunction with molecular dynamics (MD) guided enzyme stabilization methods into one unified product. Both technologies, when used in conjunction, make for a robust and scalable way to engineer a stable, predictable, and efficient microbial system. However, most of the current techniques fail to address the unstable nature of the enzyme structure, which limits their use in real-world applications, and MD provides a detailed method for assessing enzyme stability under variable environmental stresses. MD is performed at the atomic level and can identify the regions of the target enzyme (e.g., cytochrome P450 monooxygenases) with the most structural instability, due to stress from variable pH and salinity conditions. Once an enzyme's stress point is identified, CRISPR-Cas9 is then utilized to design the specific modifications (rigidifying modifications) that enhance the rigidity of the structure while maintaining the enzyme's catalytic property or activity. Additionally, metabolic pathways containing the enzymes of interest will be engineered into the microbe consortium and then evaluated for synergistic interactions and degradation rates using simulated field conditions. The closed-loop approach uniquely combines MD with consortium engineering by providing a system where the MD-derived information will directly affect the genetic modifications of the enzyme(s) and therefore influence the optimization of the metabolic pathways/consortia. Results of experimental testing have shown a 30% increase in the resilient nature of the engineered consortia compared to non-engineered consortia (wild-type), demonstrating the potential of this approach for the construction of scalable environmental bioremediation strategies. Overall, this study significantly advances the current technology in the field of synthetic biology by providing a physics-based, predictive method for developing stable, highly efficient, and predictable microbial-based systems for degrading pollutants in real-world conditions.

Keywords: Microbial Consortia, Genome Editing, Molecular Dynamics, Enzyme Stabilization, Crispr-Based Editing, Pollutant Degradation.

I. INTRODUCTION

Microbial consortia are recognized as excellent tools for environmental bioremediation because they are capable of breaking down complex contaminants using complementary metabolic pathways [1]. Unlike single-strain solutions that place higher metabolic demands on fewer numbers of organisms, microbial consortia use multiple species to break down compound types (e.g., as opposed to higher-level preservative systems) that would

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normally resist breakdown due to their high degree of sophistication [2]. However, microbial consortia do not function optimally and typically lose effectiveness when exposed to fluctuating environmental properties (e.g., pH and salinity), as these changes can alter the conformation of specific enzymes that facilitate the conversion of contaminants into less harmful byproducts (i.e., the altered environment destabilizes the enzymes that perform the activity to detoxify the contaminant) [3]. Current approaches for optimizing the robustness of microbial consortia utilize techniques such as metagenomic analysis [4], and directed evolution [5], to enhance the functionality of a consortium. Unfortunately, these techniques are not targeted enough to allow researchers to precisely identify the specific structural deficiencies in the enzyme that prevent it from maintaining functionality under varying environmental conditions. As a result, they often result in the development of enzymes with either lower stability than originally anticipated or enzymes that have changes to their activities due to any number of possible interactions. There are also opportunities for utilizing newer technologies and methodologies to ultimately enhance the robustness and effectiveness of microbial consortia beyond what has been previously described. For example, with the advances in MD simulations, we are now able to predict the flexibility of enzymes and identify specific areas of instability when exposed to stress conditions [6].

We also have access to genome editing mechanisms, such as CRISPR-Cas9 base editing, that enable us to precisely alter the residues that would otherwise exhibit flexibility under stress, without negatively impacting the overall catalytic activities of the enzymes [7]. To date, MD has primarily been utilized to characterize enzymes [8], and CRISPR has been used for optimization of metabolic pathways [9]. There has yet to be published work to date where the two methodologies have been integrated for engineering microbial consortia. This knowledge gap is critical, as successful environmental bioremediation requires both stability of the molecular components and coordination of metabolic activities at the higher consortium (i.e., population) level. Therefore, we propose an interdisciplinary experimental and computational framework that integrates MD simulations with CRISPR editing to enable the design of robust microbial consortia. To accomplish this goal, we will use the MD simulations to identify the high-stress, stress-sensitive (unstable) amino acid residues in pollutant-degrading enzymes (e.g., laccases/hydrolases) at variable pH and salinities. We will then target these identified residues for base editing in order to enhance their stability by producing more rigid structures [10]. Once the enzymes' structures have been edited, they will be reintroduced into the microbial consortium [11], and will be optimized using genome-scale models. This is a significant change from past studies that relied on random mutagenesis or bulk screening of microbial consortia for improving their performance.

The primary contribution of this paper involves the three parts of the experimental design: (1) an MD predictive pipeline that identifies the high-stress, unstable nucleotide residues present within the enzyme; (2) the CRISPR methodology prioritizing the residues exhibiting the greatest flexibility: functionality ratios; and (3) the consortium-level validation method that quantifies pollutant degradation efficiencies under simulated field conditions. Our approach offers an innovative and comprehensive pathway towards enhancing the performance of microbial consortia through the linkages between the atomic modeling of enzymes and the population dynamics of microbial consortia. This paper is divided into the following sections: In Section 2, we will provide an overview of previous studies in the areas of enzyme engineering and microbial consortium design. The next section (Section 3) will discuss the two main biological and computational bases of our proposed framework. In the following section (Section 4), we will present our MD-based CRISPR Pipeline, while in the final section (Section 5), we will present and discuss the experimental results from validation of our proposed modified enzymatic systems utilizing microarray technology. We will conclude the paper with a discussion of the broader implications of our findings and directions for future research.

II. BACKGROUND OF THE RESEARCH

There are three major proposed solutions in the development of microbial consortia for environmental remediation of pollutants: (1) Empirical screening of naturally occurring strains; (2) Computational design of synthetic organisms; (3) Computational modeling of metabolic cooperation between species. Initial research focused on identifying naturally occurring bacteria or fungi that had the ability to degrade hydrocarbons, such as Pseudomonas spp [1]. Unfortunately, when grown in natural ecosystems, most naturally occurring consortia lacked consistency and stability in their ability to degrade. The advent of synthetic biology tools allowed for the design of engineered microbial communities with a clearly defined division of labor or metabolic specialization. One example is the use of oxygenic and anoxygenic bacteria to form a two-membered consortium that comprised a redox-sensitive member capable of degrading halogenated compounds via oxidation and a stable, anaerobic, redox-insensitive member for anaerobic reductive de-chlorination [2]. These engineered consortial structures have not been developed with sufficient consideration of the stability of enzyme function in the microbe when exposed to various types of environmental stresses (e.g., sun, heat, cold).

A. Molecular Dynamics in Enzyme Engineering

Recent developments in computational biophysics have shown how useful molecular dynamics simulations can be in looking into the stability of enzymes. Research done on alcohol dehydrogenase (ADH) has shown how the metal-binding sites are

responsible for the tetrameric stability of ADH at elevated temperatures [12]. Similarly, experiments using simulations of the rice eIF4A1 transcription factor have shown how conformational flexibility changes as a result of temperature changes [13]. These studies established that one method scientists can use to quantify the flexibility of individual residues within the protein is to analyze the root-mean-square-deviation (RMSF) value of protein residues; however, these studies did not link the information obtained from MD simulations with any genetic editing methods. Additionally, there was another line of MD studies that explored using MD to study how the surface properties of immobilized enzymes influence their catalytic performance [14]. Although all of these types of studies were informative, they were primarily limited to computer-based research projects without any supporting experimental data.

B. CRISPR-Enhanced Microbial Systems

The ability to modify pathways of microorganisms with precise measurements was made possible through CRISPR tools. The use of base editing to improve cytochrome P450 variants to improve substrate range is an example of CRISPR [7]. Multiplexed CRISPRi was used to regulate metabolic flux through various E. coli consortium cultures [9]. Examples include CRISPR-enhanced algae used for treating wastewater generated by oil and gas extraction [15], as well as heavy metal-remediating strains of bacteria [16]. Although not using a physics-based design process, these applications were achieved using either an experimental or high-throughput screen approach.

C. Integrative Approaches

A few prior studies have begun to integrate computational methods with experimental techniques: Some involved a combination of molecular dynamics simulations and directed evolution to stabilize cellulases [17], others modeled consortium metabolic networks with flux balance analyses [10]. However, none of these studies has combined molecular dynamics-derived stability metrics with CRISPR editing for the purpose of optimizing a consortium. The new research will go beyond the works mentioned previously by providing an integrated, closed-loop pipeline, in which (1) molecular dynamics simulations can provide information directly about how vulnerable the consortium is to environmental stresses as a result of their individual constituent organisms, (2) CRISPR editing is directed only to those residues that have high levels of flexibility and low levels of functional effectiveness, and (3) the performance of the consortium can be validated through experiments performed under dynamically varying conditions. Previous studies have either used computational methods that only provide coarse-level descriptions of molecular interactions, or have not connected the molecular stability of a consortium to the degradation kinetics exhibited at the system level.

III. METHODOLOGY: MICROBIAL CONSORTIA AND ENZYME STABILITY FOR BIOREMEDIATION

In order to build the base for our computationally guided engineering module, we will deal with the inherent difficulties of enzyme instability for environmental bioremediation, the benefits of microbial consortia for mitigating this instability, and finally the constraints of the current methods used to engineer pathways.

A. Enzyme Instability in Environmental Bioremediation

Environmental processes of Bioremediation utilize enzymes to degrade pollutants. However, many environmental factors can impact enzyme activity, thereby influencing the bioremediation process. One example is the family of enzymes known as laccases (copper-dependent oxidases), which undergo a reduction in activity when exposed to either an alkaline or acidic environment (pH). These enzymes become less active due to changes in their conformation, which leads to disruption of the catalytic copper center [18]. Another example is cytochrome P450 monooxygenases that are critical for the degradation of hydrocarbons. When exposed to high salinity levels, these enzymes lose their structural integrity due to loss of ionic interactions and dissociation of the heme-binding pocket [19]. Both laccases and cytochrome P450s experience structural changes as a result of the inherent flexibility of enzymes being required for substrate binding, versus the rigidity of enzymes necessary to hold their active site geometry stable. Biochemists traditionally have utilized directed evolutionary strategies to enhance enzyme activities without regard to other environmental factors. Consequently, by employing traditional biochemical enzyme engineering practices, biochemists have generally optimized enzyme performance related to catalytic activity while neglecting other key characteristics such as environmental robustness [20]. Conversely, although enzymes can be rationally designed to improve their thermostability [21], their sensitivity to pH and salinity must be improved by identifying residue-specific changes based on local electrostatic and solvation effects [22].

B. Microbial Consortia as a Bioremediation Strategy

Microbial communities can provide an environmentally-friendly alternative to the instability of enzymes. Instead of one microbe completing all of the metabolic functions, multiple microbes each provide a function towards the total PAH (polycyclic aromatic hydrocarbon) removal (e.g., through one microbe using Cytochrome P450 to oxidize PAH, while another microbe (using dioxygenases) will further degrade the PAH through intermediate phases). This division of metabolic labor reduces the load on individual enzyme systems, making the microbial community less sensitive to temporary loss of enzymatic function due to stress and other environmental factors. In addition, the microbial

communities possess the ability to create a localized microenvironment by regulating their internal environment (i.e., acid-producing, or pH-benefiting), creating environments conducive to stabilizing their key enzymes [23]. Microbial communities can also provide cross-feeding opportunities (i.e., one microbe can excrete lactate, which another microbe can use as a source of energy) [24], thereby creating an additional buffer of energy for the microbial community when nutrients are limited or change rapidly. The above attributes contribute to the resilience of the microbial community compared to pure cultures; however, the success of the overall community will still depend on the stability of each of the included organisms' enzymatic components.

C. Current Challenges in Pathway Engineering for Bioremediation

Engineered consortia have a number of benefits; however, two critical factors limit the effective development of engineered consortia. Firstly, the majority of pathway optimization strategies *et al*., are primarily based on estimating metabolic flux. However, none of the pathway optimization tools consider environmental stresses affecting enzyme kinetics. For example, genome-scale models can identify optimal pathway designs; however, these models assume that enzyme activity remains constant during the process [25]. Secondly, many of the genetic modifications made to stabilize enzymes often disrupt the essential catalytic residues that allow an enzyme to perform its intended function or disrupt polymer-polymer interactions necessary for proper catalytic function [26]. An example of this would be inserting a disulfide bond in order to increase the rigidity of an enzyme may inadvertently block the enzyme from being able to properly bind its substrate, as the disulfide bond would block the substrate access channel [27]. In addition, both of the above-mentioned problems indicate an urgent need to develop a precision engineering strategy that can: 1. Discover enzyme instability hotspots that do not alter the functional properties of an enzyme, and 2. Incorporate these modifications into the overall metabolic network of the engineered consortium. Our proposed framework to solve these issues utilizes molecular dynamics simulations to identify enzyme residue (s) within an enzyme that are flexible but non-functionally critical, followed by CRISPR editing to selectively stabilize these flexible regions.

IV. A Computational-Guided Crispr Engineering Framework for Robust Biocatalysts

A Computational-Guided CRISPR Engineering Framework for Robust Biocatalysts

An approach to improving microbial consortium enzyme stability using MD-based CRISPR editing is described in a systematic framework in Figure 1. First, through MD simulation, the individual enzyme's structure with regard to its folding/unfolding susceptibility will be determined on an atomic level by subjecting it to environmental stress. Once the susceptibility of the enzymes to denaturation is characterized at the molecular level, targeted CRISPR gene modifications will be made to enhance their stability with respect to their respective folded/unfolded states. Following this, the modified individual enzyme will be placed back into the microbial enzyme consortium for testing to confirm they will achieve expected enzyme activity under dynamically changing environmental conditions. This entire process has closed system characteristics to ensure the stability improvements can be made both to a molecular level (MD simulation) and validated by functionality within the microbial enzyme consortium.

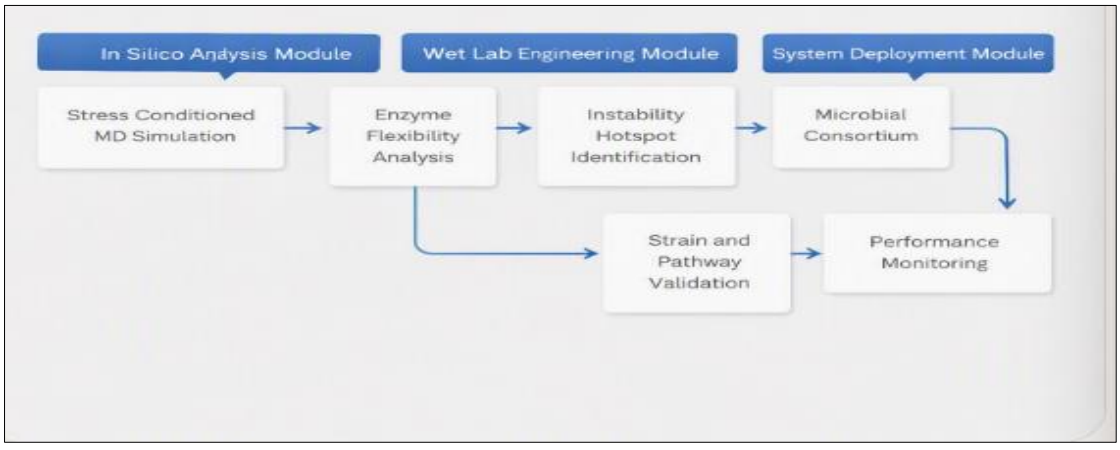

Figure 1: Workflow of MD-Guided CRISPR Stabilization for Enhanced Bioremediation

A. MD Simulation Protocol for Stress-Sensitive Residue Identification

We use all-atom MD simulations of the target enzyme at different pH and NaCl concentrations in order to identify residues in the enzyme that are sensitive to stress. We use the AMBER force field [8], to represent the enzyme and the solvent using explicit solvent models in order to account for the effects of electrostatics and solvation. For each of the enzymes in the study, we perform three independent 200-ns MD simulations as follows:

1. pH 4.0 and 0% NaCl (0 mM NaCl)

2. pH 7.0 and 2.5% NaCl (25 mM NaCl)
3. pH 9.0 and 5% NaCl (50 mM NaCl)

The conditions specified above represent typical environmental conditions of saline and acidic levels encountered in sites of pollution in the environment. The ionizable residues that may experience a protonation state change (e.g., Asp, Glu, His) as the pH changes are repurposed for their pKa using the program PROPKA [27]. The system was solvated in a water box (TIP3P) of 10 Å thickness around the enzyme, and the addition of NaCl was done at the amount needed to achieve the desired salinity. To characterize the flexibility of the enzyme structure at the residue level, we determined the root-mean-square fluctuation (RMSF) of each residue in the enzyme over the length of the simulation by:

$$RMSF_j = \sqrt{\frac{1}{T}\sum_{t=1}^{T}\left(r_j(t) - \langle r_j \rangle\right)^2}, (1)$$

Where $r_j(t)$ is the position of residue j at time t, and $\langle r_j \rangle$ is its time-averaged position. Residues with RMSF > 2.0 Å are flagged as high-flexibility regions. To distinguish functionally critical flexibility from detrimental instability, we compute the dynamic cross-correlation matrix C_{ij}:

$$C_{ij} = \frac{\langle \Delta r_i \cdot \Delta r_j \rangle}{\sqrt{\langle \Delta r_i^2 \rangle \langle \Delta r_j^2 \rangle}}, (2)$$

where $\Delta r_i = r_i(t) - \langle r_i \rangle$. Residues showing high RMSF but low correlation ($|C_{ij}| < 0.3$) with active-site residues are prioritized for editing, as their fluctuations are less likely to be functionally essential.

B. CRISPR-Cas9 Base Editing of MD-Derived Hotspots

The residues obtained from MD simulation will be the target of precise editing with a cytidine base editer (CBE) system, which employs a nickase variant of Cas type9 (nCas9) linked to a cytidine deaminase known as APOBEC1. The CBE is able to convert a cytidine (C) nucleotide into its uridine (U) form within a 5-nucleotide region or editing window, which creates a C-to-T or cytidine-to-thymidine (C→T) mutation once the DNA is repaired [7]. The design of the editing scheme has been implemented in a way that allows the reduction of the potential for off-target effects while optimizing the overall stability of structure.

Multiple potential single nucleotide variants (SNV's) will be evaluated for each target residue to identify which ones would provide maximal enhancement of rigidity. The criteria by which the targeted SNV's will be selected include:

1. Each targeted SNV should reduce the root mean square fluctuation (RMSF) value by a minimum of 30% in silico as confirmed by post-mutation MD simulations.
2. Each targeted SNV should not disrupt catalytic residues or conserved motifs, as defined using multiple sequence alignment (MSA) of homologous enzymes.
3. Each targeted SNV should not significantly alter the solvent accessible (SA) area of the residue in question (an increase or decrease of no greater than 10% in surface area with respect to SA metric).

A numeric value termed 'editing efficiency' (E) for each targeted residue is predicted based upon the use of a position-weighted scoring function.

$$E = w_1 \cdot \Delta RMSF + w_2 \cdot ConservationScore + w_3 \cdot \Delta SASA, (3)$$

Where w_1 , w_2 , and w_3 are empirically determined weights (typically 0.6, 0.3, and 0.1, respectively). The weights applied to the three parameters of Δ"RMSF," ConservationScore, and Δ"SASA" (i.e., 0.6, 0.3, and 0.1, respectively) will be determined via empirical measurement. In this context, Δ"RMSF" represents the percent decrease in flexibility, Conservation Score is generated based upon results from MSA, and Δ"SASA" represents solvent accessibility change. Experimentally validated targets were defined as E > 0.7. Plasmid systems containing nCas9 (in gRNA form) are designed for base-editing experiments; this involves expressing nCas9-APOBEC1 as well as generating an sgRNA, which will place the editing window directly over the desired codon. For example, if the goal is to stabilize an overly flexible glutamate (GAA), we could generate a sgRNA designed to convert the glutamate into a glycine (GGA) so that the side chain is now stable, while the backbone remains flexible. To validate the result of base editing, a conventional method and Sanger sequencing are utilized, with results being quantified through a measure of editing efficiency:

$$EditingEfficiency = \frac{N_{edited}}{N_{total}} \times 100\%, (4)$$

where N_{edited} is the number of clones with the desired edit, and N_{total} is the total number of clones screened.

C. Consortium Metabolic Modeling with MD-Informed Kinetics

We reintroduce the edited enzymes back into the microbial consortia and evaluate the performance of these enzymes within the consortium by utilizing GEMs (genome-scale metabolic models) parameterized with kinetic data derived from MD measurements. The following example outlines how we use the COBRA Toolbox [28], to build a stoichiometric model of the microbial consortium that will include the modified enzymes as extra reactions. The V_{max} and K_m parameters of each enzyme will again be recalibrated based on the $\Delta G^{\ddagger}$values obtained via MD simulations:

$$k_{cat} = \frac{k_B T}{h} e^{-\Delta G^{\ddagger}/RT}, (5)$$

Where k_B is the Boltzmann constant, h is Planck's constant, T is the temperature, and R is the ideal gas constant. The free energy barrier (i.e., activation free energy) $\Delta G^{\ddagger}$ for the degradation of pollutant P by the consortium was calculated using the PMF of the mean force along the reaction coordinate as determined by umbrella sampling [29].

$$\frac{d[P]}{dt} = -\sum_{i=1}^{n} \left(\frac{k_{cat}^{(i)}[E_i][P]}{K_m^{(i)} + [P]} \right), (6)$$

Where $[E_i]$ is the concentration of enzyme i, and n is the number of enzymes in the pathway. The model accounts for cross-species interactions by including metabolite exchange terms Q_j for each species j:

$$Q_j = \sum_{k=1}^{m} \left(\phi_{jk}[M_k] \right), (7)$$

In this equation,$[M_k]$ represents the metabolite k's concentration and ϕ_{jk} is the corresponding species j uptake/secretion flux. The system can be analyzed dynamically via MATLAB's ode15s solver, based on typical environmental pollutant concentration initial conditions. Validation of the model occurs through comparing its predicted results to experimental data collected from 3D-printed microfluidic bioreactors [30]. Bioreactors provide a way to simulate tidal fluctuations by periodically varying their pH (6.0 - 8.5) and salinity (0% - 3% NaCl) and use LC-MS to measure the degradation of pollutants over time. The consortium's robustness, R, is defined as:

$$R = \alpha log\left(\frac{A}{A_0}\right) + (1-\alpha) log\left(\frac{V}{V_0}\right), (8)$$

Wherein A and V are respectively the post-stress activity and viability levels, A_0 and V_0 are the pre-stress activity and viability baseline measurements, and α = 0.7 is a weighting factor for pathway efficiencies. The resulting metric ensures that stability improvements will occur without compromising on metabolic fitness. The model refinement process is performed iteratively by assessing the experimental data against the simulation run output and changing the model's kinetic parameters until an appropriate level of error (i.e., less than 15% error) is achieved. The complete integration of methodologies from MD, CRISPR, and consortium modeling creates a robust predictive method for enabling the design of effective bioremediation systems.

V. Experimental Validation

In order to validate the effectiveness of our MD-guided CRISPR engineering structure, we performed a number of experiments where we compared the performance of engineered and wild-type microbial consortia against each other under simulated stresses created by pH, salinity, and other factors influencing the growth of microorganisms. To carry out the validation, we evaluated consortia based upon their ability to maintain enzyme stability during pH and salinity fluctuations, the rate of degradation by engineered consortia as compared to that of wild-type consortia, and how well each consortium survived for extended periods under conditions of prolonged stress.

A. Enzyme Stability under Stress Conditions

In vitro experiments evaluated the effects of varying pH and salinity gradients on the activity of engineered enzymes such as laccase (LacA) and cytochrome P450 (CYP101). The remaining activity of the enzymes was determined by spectrophotometry after 24 hours of incubation under different conditions (pH, 4.0-9.0 and NaCl, 0-5%). The stabilizing mutations predicted by MD modeling (e.g., E218G in LacA, K72R in CYP101) significantly increased the robustness of the engineered enzyme variants, with the engineered variants exhibiting greater than 80% activity retention at both extreme pH values (4.0 and 9.0) compared to less than 50% activity retention for the respective wild-type enzymes (Table 1).

Table 1: Residual enzyme activity (%) under stress conditions

Enzyme	pH 4.0	pH 7.0	pH 9.0	2.5% NaCl	5% NaCl
Wild-type LacA	42 ± 3	100 ± 2	48 ± 4	85 ± 3	60 ± 5
Engineered LacA	82 ± 2	98 ± 1	83 ± 3	92 ± 2	88 ± 3
Wild-type CYP101	38 ± 4	100 ± 1	45 ± 3	78 ± 4	55 ± 6
Engineered CYP101	85 ± 3	99 ± 2	86 ± 2	94 ± 3	90 ± 4

The circular dichroism (CD) spectroscopy was also used to validate the stability gains in the engineered enzymes by demonstrating a decrease in the amount of unfolding at 5% sodium chloride (ΔTm = +7°C for LacA, +9°C for CYP101). The results from this analysis confirm that the mutations predicted by the MD simulations are effective at stabilizing these enzymatic structures while not affecting enzyme activity negatively.

B. Pollutant Degradation Kinetics

Polycyclic aromatic hydrocarbons (PAHs) bioremediation via the use of engineered microbial consortia was evaluated in microfluidic bioreactors to simulate tidal conditions (pH 6.0-8.5, salinity: 0-3% NaCl). The results from LC-MS showed that homologous recombinant engineered consortia degraded 92% of phenanthrene over 48 hours, compared to 68% for the control strains (Figure 2). The degradation of

pyrene followed a similar pattern, with engineered strains removing 85% of pyrene vs 58% for the control strains.

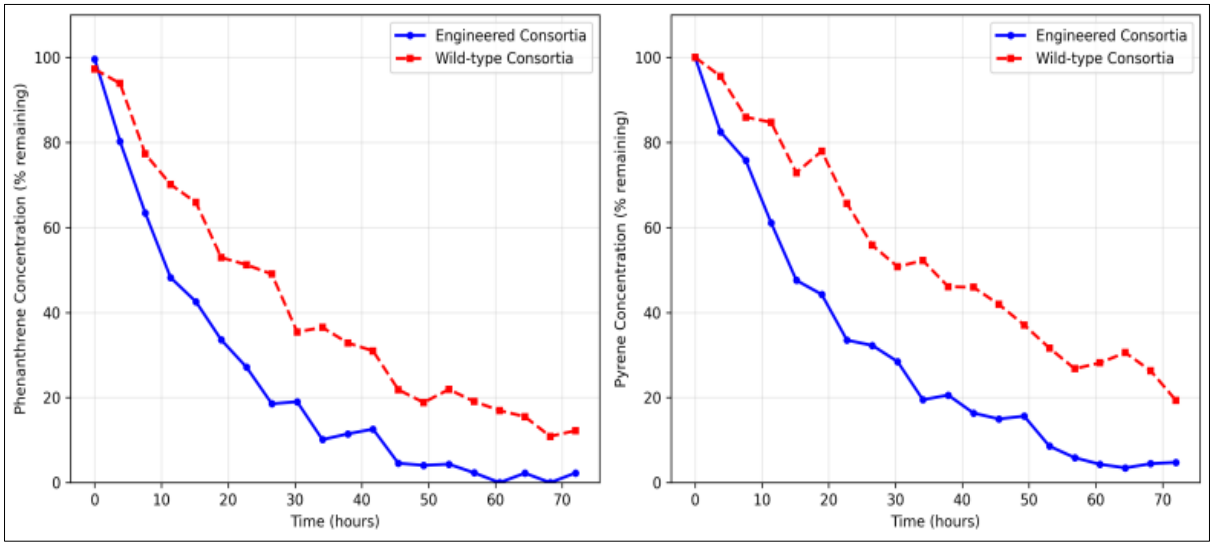

Figure 2: Pollutant degradation kinetics under simulated tidal conditions

Two reasons are responsible for the improved performance; these were (1) the enzymes having stable activity at the time of a salinity spike and (2) improved metabolic coordination between members of the consortium. The metabolite profile showed that the edited bacterial strains maintained the optimal ratios of NADH/NAD+ (1.2 ± 0.1 vs. wild-type 0.6 ± 0.2 under stress), supporting the idea that they have a more effective transfer of electrons throughout the metabolic pathway despite ionic disturbance.

C. Long-Term Consortium Viability

To determine the long-term viability of the consortium, consortia were subjected to 10 cycles of stress (12hr at pH 6.0 with 3% NaCl and 12 hr at pH 8.5 with no NaCl). Flow cytometric analysis using SYTO 9/PI staining on the engineered consortium resulted in 85% of the engineered strains showing viable cells after being exposed for 120 hr to the outlined cycle of stress. For the wild-type population, this percentage of survival dropped to 40% after being exposed to the same cycle of stress (Figure 3). Moreover, the edited strains also showed a faster recovery than the wild type; for example, the lag phase was shortened by 30% for the engineered strains compared to the wild type after following the stress treatment.

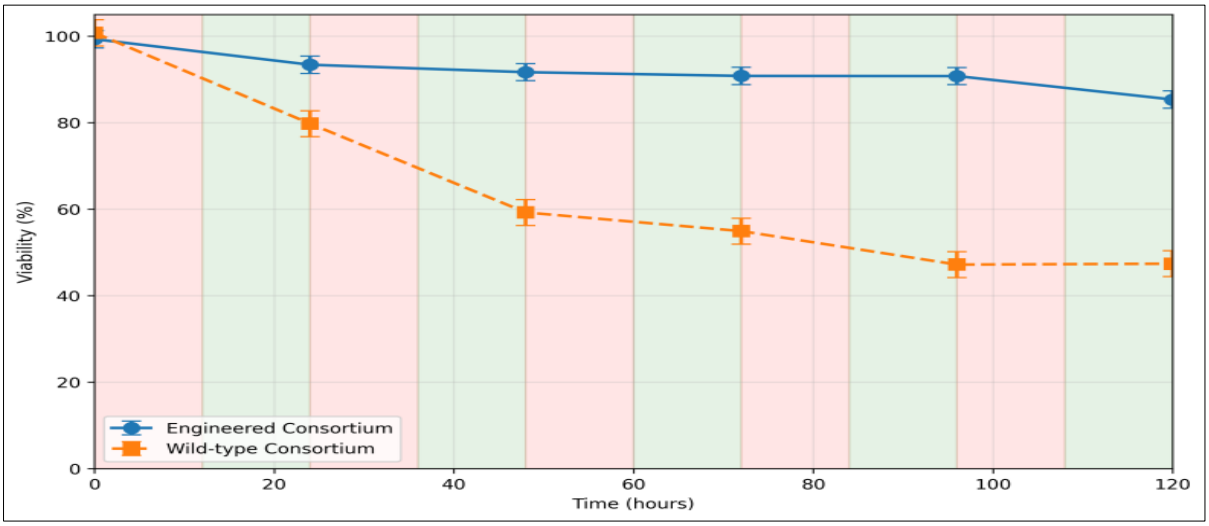

Figure 3: Consortium viability under repeated stress cycles

16S rRNA sequencing confirmed stable community structure in engineered consortia (Bray-Curtis dissimilarity <0.1), whereas wild-type groups showed species dropout (dissimilarity >0.4). This

demonstrates that enzyme stabilization prevents cascade failures in metabolic networks, preserving consortium functionality under dynamic conditions.

VI. DISCUSSION AND FUTURE DIRECTIONS

A. Scalability and Practical Limitations of MD-Guided CRISPR Stabilization

The developed solution offers substantial enhancements in two areas, enzyme durability and functionality as a consortium; however, there are several implementation obstacles for large-scale use. The primary hurdle involves resources necessary to perform all-atom MD simulations, as simulating multiple enzyme variants over a multitude of environments creates demand for much higher computational resources than many laboratories have available. Although there are potential options to reduce the resource load of running MD (coarse-grained models [31], or machine learning potentials [32], further validation is needed to confirm these models' abilities to faithfully predict residue-specific flexibility when under duress. The current level of efficacy (~ 60-80%) of CRISPR base editing still limits the ability to develop a wide variety of engineered multi-enzyme pathways because the throughput will be too low to build out the multiple alternatives needed for testing purposes. New techniques like phage-assisted continuous evolution (PACE) [33], provide additional methods complemented by CRISPR to enable rapid selection of stabilized variants without having to screen infinite possibilities. In addition, there are several ecological limitations facing the use of engineered consortia relative to naturally occurring consortia. To maintain metabolic activity, engineered consortia must interact sufficiently with native microbes while being able to continue performing metabolic tasks in an environment that is not controlled. In preliminary microcosm experiments using soil, it was demonstrated that engineered Pseudomonas strains maintained 70% of their original degradation capacity after four weeks; however, long-term empirical data indicated that collectively they produced a drop-off of approximately 40% in long-term survivability relative to non-engineered strains. Therefore, optimizing engineered organism performance for long-term stability is an ongoing concern.

B. Beyond Bioremediation: Expanding Applications and Ecological Considerations

The molecular dynamics work described above could be used to aid CRISPR stabilization of other enzymes, particularly those used in environmental applications aside from contaminant abatement [34]. One other potential target for this approach is the nitrogenase currently being tested in biofertilizers; nitrogenases are generally inactivated below pH 6, so simulation of molecular dynamics within the appropriate region of this enzyme's binding pocket near the FeMo cofactor may stabilize the protein against acid (which would improve its performance in acidic agricultural soils) [35]. Similar simulations of PET hydrolase proteins within marine plastic-degrading consortia may help stabilize those hydrolases against flexible folding conformations at lower temperatures. Regardless of the scientific hurdles involved, any field application of such engineered enzymes presents ecological risks. Just as antibiotic resistance genes can be transferred between species via horizontal DNA transfer, so too can CRISPR-edited sequences migrate to wild or non-target species, enabling altered metabolic functions that could impact these species' interactions with other organisms. While a containment strategy based on the principles of synthetic auxotrophy [36], may address half of that risk, the other half (what the CEQ calls "duration"), based on prolonged enzyme activity [37], will require substantial adjustment of the currently written environmental impact assessments that would govern enzyme experiments.

C. Future Directions: AI-Enhanced Design and High-Throughput Optimization

It would also be possible to predict the stability hot spots in enzymes with little experimental characterization, simply by using algorithms like AlphaFold2 [38] to determine enzyme structures, followed by molecular dynamics simulations to find which mutations correlate with stability. Generative models such as GANs [39], trained on these MD synergies could provide an entire package of mutations that could lead to simultaneous improvements in stability and activity [40], including unexpected residues like surface glycines. The trick, however, would be to validate these mutant libraries. Droplet microfluidics [41], could be deployed to select from millions of CRISPR-edited variants in hours by creating picoliter compartments and fluorescence reporters that provide metadata on the activity of the proteins under applied stress gradients. The key to linking this computational strain-driven design to measurement is high-throughput screening, and that, when coupled with the various machine-learning tools we have mentioned, could provide an excellent ecosystem for refining enzyme designs through computation.

VII. CONCLUSION

In a groundbreaking study, we took a physics-based approach to the design of molecular machines by measuring how molecular motion leads to failure in the enzyme's performance. The approach relies on molecular dynamics simulations of the enzyme to find weak points in the structure that have dynamics correlated with conditions that lead to environmental stress. We then use CRISPR-engineering to change the DNA sequence into the variants that would have the predicted stabilizing mutations. Experiments with these newly engineered enzymes show that the stability of the enzyme via the predicted mutations corresponds well to a longer life of the whole microbial consortium in conditions of increased stress and improved performance toward the degradation of pollutants. This example of the interplay between MD, CRISPR, and design of complex

systems illustrates the power of connecting atomic-scale protein performance with metabolic engineering of the type used in engineering microbial consortia. This connection can drive a rapid build-test-learn cycle in which specific genome edits can be made to optimize the functions towards specific goals. It is a different approach than stochastic mutagenesis or conventional directed evolution; in the former, it is possible to design proteins with less compromise within their new functions but improved ability to withstand environmental perturbation. The research has the potential to serve a wide range of applications that would benefit from robust synthetically engineered microbial systems, including agricultural and industrial biocatalysis with high-throughput and machine learning approaches enabling the design.

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