Rational Bioengineering of LadA Monooxygenase in Polyethylene Biodegradation Pathway

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Abstract

Polyethylene, a stable and common commercial plastic, presents a costly and persistent environmental problem. We are currently engineering a polyethylene biodegradation pathway by modifying an alkane degradation pathway in which a monooxygenase enzyme, LadA, performs the initial terminal oxidation step converting the alkane into an alcohol. The alcohol can later be processed into a fatty acid to be consumed for energy in a natural fatty acid metabolic pathway. Using Molegro Virtual Docker, we computationally analyzed the substrate specificity of LadA and identified a sub-region of Insertion Region 4 (IS4), comprising of residues 300-349, as the primary residues responsible for hindering polyethylene binding. We have isolated the wild-type gene from *Geobacillus thermodenitrificans* and are currently engineering a LadA mutant capable of binding to polyethylene through a combination of rational design and directed evolution. A large library of mutant LadA genes, that vary in the IS4 subregion, will be synthesized, spliced into bacteriophage vector and amplified in *E. coli* BLT5403 host to screen for a polyethylene-binding mutant through a phage display assay. In further research, the selected mutant will be combined with enzymes in the natural alkane biodegradation pathway to form an *in vitro* process that degrades polyethylene.

1 INTRODUCTION

1.1 Polyethylene

Polyethylene is a thermoplastic formed from the polymerization of ethylene molecules, constituting a chemically inert compound that resists both chemical and biological degradation. Polyethylene is compositionally an alkane [1], though different from those that comprise fuels in two major regards: polyethylene molecules are far larger in size (ranging from 70 carbons in length in micronized powder form to hundreds of thousands of carbons in length in high density polyethylene). Polyethylene's size causes it to have strong London dispersion forces that augment the already strong carbon-carbon bonds of its backbone, rendering it resistant to depolymerization (figure 2) [2]. As one of the most common commercial plastics, polyethylene comprises a wide range of products from plastic bags to artificial limbs. Due to the combination of its chemical stability and prevalence in society, polyethylene has become a significant environmental concern: accumulating in landfills at approximately 25 million tons per year [3, 4].

Currently, the most widely employed countermeasure to this environmental threat is pyrolysis, a process in which polyethylene is heated—in the absence of oxygen—to about 400°C, inducing de-polymerization to yield radicals and/or smaller hydrocarbon oils and gases [5]. Due to the strength of the carbon-carbon bonds in polyethylene, pyrolysis of polyethylene involves significant investments in equipment and energy, resulting in even more waste and limiting its feasibility as a method for efficiently disposing of polyethylene.

An alternative to pyrolysis and the method of choice in this study is the microbial degradation of the polymer. Fungi and bacteria such as *Sphingomonas macrogoltabidus* [6], *Brevibacillus borstelensis* [7], *Rhodococcus ruber* [8], *Phanerochaete chrysosporium* [9], and *Trametes versicolor* [9] have been found to degrade polyethylene. However, mechanisms proposed by cur-

rent research into bioremediation of the plastic waste have indicated that microbial degradation of polyethylene is hindered by necessary pretreatments of UV radiation and/or addition of pro-oxidants, and overall is a slow and inefficient process [1].

Furthermore, other barriers to the degradation of polyethylene center on its excessively large molecular size. Unable to pass through a cell's membrane, the polymer eludes existing alkane degradation pathways. Moreover, unlike other more reactive polymers, polyethylene is too chemically inert to be hydrolyzed into monomers [2].

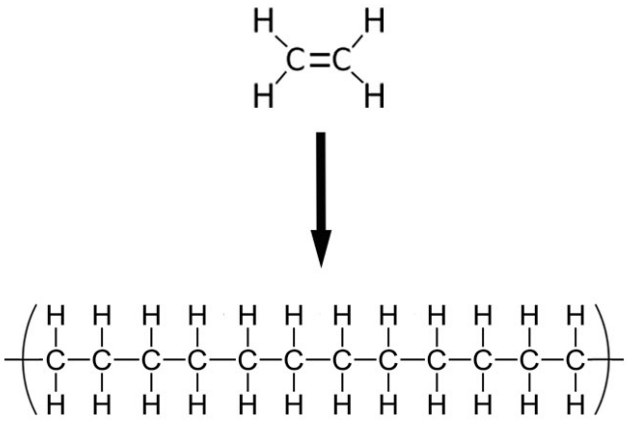


Figure 1. The alkene ethylene polymerizes into polyethylene, a chemically stable, very large hydrocarbon

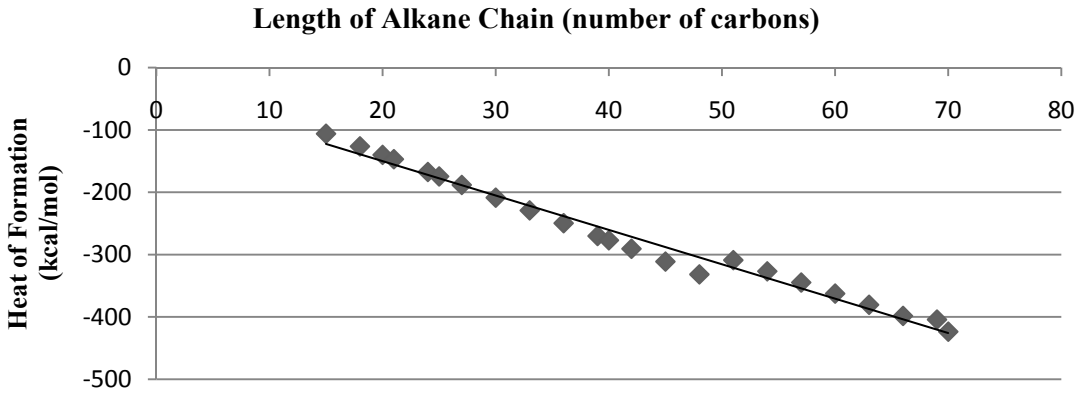


Figure 2 As the number of carbons in an alkane increases, the heat of formation becomes increasingly negative, affirming that longer alkanes, such as polyethylene are highly stable and inert. These data were calculated using the Mopac AM1 method [10].

1.2 The Pathway

Since polyethylene is structurally a very large, branched alkane, the mechanism of polyethylene degradation should mirror the known pathway for alkane degradation—terminal oxidation followed by β-oxidation [1]. In this pathway, the terminal methyl group of the alkane is oxidized by replacing a hydrogen with a hydroxyl group (figure 3). The resultant alcohol is then converted into an aldehyde by an alcohol dehydrogenase and subsequently into a fatty acid by an aldehyde dehydrogenase. The fatty acid can then be decomposed in the beta-oxidation cycle [1, 11, 12].

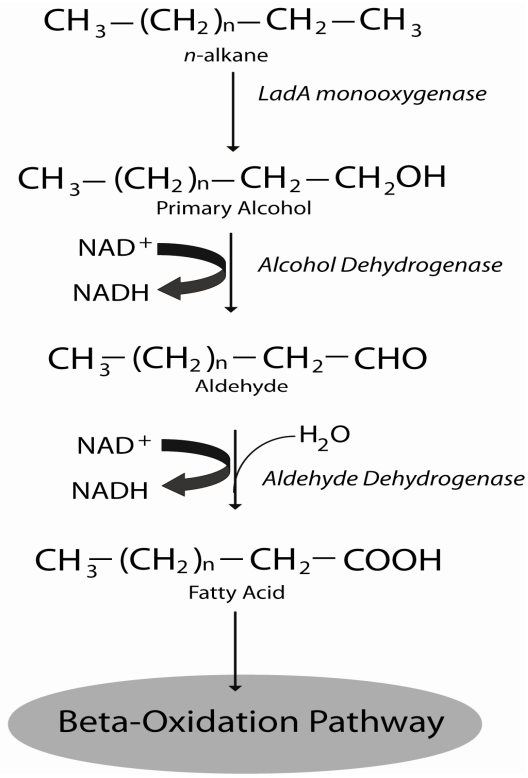


Figure 3 Schematic of alkane degradation pathway (adapted from [13-15]). An alkane monooxygenase hydroxylates the terminal carbon of the alkane, forming an alcohol. The alcohol is converted to an aldehyde by an alcohol dehydrogenase and further into a fatty acid by an aldehyde dehydrogenase. The fatty acid is degraded recursively in the beta-oxidation pathway in which two carbons are removed at a time in the form of acetyl-CoA.

Of the steps in the pathway, the terminal oxidation of polyethylene is the most critical and difficult, performed either by the aforementioned photo-oxidation/chemical oxidation methods or by enzymatic hydroxylation. Oxidation of these inert, hydrophobic polymers increases

their reactivity and hydrophilicity, which in turn increases accessibility by microorganisms [2]. Of the enzymes known to catalyze the oxidation of alkanes, those of the AlkB family are the most well known. AlkB is an alkane monooxygenase originally found in *Pseudomonas putida* on a plasmid (labeled the OCT plasmid) containing not only AlkB but also the enzymes for the subsequent dehydrogenation steps [16]. Though promising in that it has many homologs with largely conserved activities across many different organisms, AlkB (and others in its family) is a membrane-bound enzyme and must catalyze the hydroxylation of alkanes within the cell—a requirement which precludes activity on polyethylene molecules since they are far too large to enter cells even through active transport [17].

Because of the limitations of AlkB, we opted to engineer our pathway around the more versatile long-chain alkane degradation enzyme LadA.

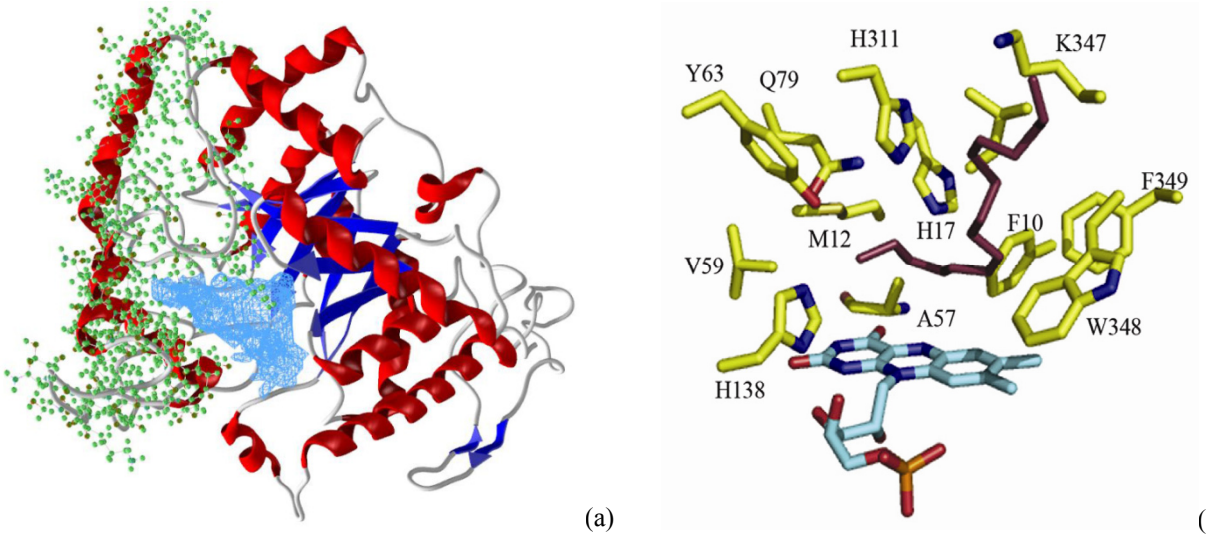
1.3 LadA Monoxygenase

Considering the enzymes available for our purposes, we chose LadA for the advantages it offered over AlkB mentioned above. LadA is an enzyme that occurs naturally in a homodimer form, found in the bacteria *Geobacillus thermodenitrificans* which was isolated from an oil reservoir in northern China [13, 15]. LadA largely conforms to a TIM-barrel structure, though with five insertion regions at the C-terminus located at the junctions between β-strands and their corresponding α-helices. These five insertion regions flank a deep cavity in the surface of the enzyme that forms the active site, which consists of residues including Met12, His17, Ala57, Val59, Tyr63, Gln79, His138, and His311 [15]. LadA is not a membrane-bound protein, and has a flavin mononucleotide (FMN) coenzyme that catalyzes the oxidation of the terminal alkane and which can be removed to facilitate our phage display assay by allowing LadA to bind to but not catalyze and hence be unable to release its substrate [13, 15]. Normally, FMN enters the active

site pocket and is protonated by His138 in the presence of O_2 to form a C4a-hypdroperoxyflavin intermediate. This unstable oxidizing agent can then hydroxylate the terminal carbon. In our case, by not providing the coenzyme FMN which is not encoded for on the LadA gene, we can induce LadA to dock to but be unable to hydroxylate its substrates, forming a permanent LadA:alkane/polyethylene binary complex which we can then assay for binding affinity [15].

LadA has a native substrate specificity of alkanes from 15 to 36 carbons in length [17]. The orientation of LadA's substrates when docked is such that the head of the alkane enters a pocket containing the active site and catalytic residues while the tail of the chain lies along the surface of Insertion Region 4 (IS4), identified in green in figure 4(a) [15]. Because of this specific orientation, the active site only recognizes either of the ends of the alkane chains, and therefore cannot be responsible for the substrate specificity of LadA. This conjecture is later affirmed by our computational modeling. Our *in silico* docking simulations prove the impediment to favorable binding of polyethylene to LadA can be attributed to steric, electrostatic, or polarity incongruities of the residues along IS4 with polyethylene.

Due to these advantages, LadA has the greatest potential to be combined with other enzymes found in existing alkane degradation pathways to form a novel polyethylene degradation mechanism to be expressed extracellularly in *in vitro* bioremediation of polyethylene waste.



(a) (b)

Figure 4 (a) A ribbon structure diagram of one of the LadA monomers. IS4 and the active site pocket is highlighted in green and light blue respectively. (b) obtained from [15]—identifies the active site residues (in yellow). The blue molecule on the bottom is FMN, LadA's coenzyme, and the purple molecule represents an alkane substrate [15].

2 METHODS AND MATERIALS

2.1 Computational Modeling of the Active Site

According to Li et al (2008), five insertion regions—IS1, IS2, IS3, IS4, and IS5—constitute the wall of the substrate docking site and IS4 bulges out from the deep active site pocket (figure 4a). While all five insertion regions are responsible for binding coenzyme FMN and the terminal portion of the alkane substrate, IS4 in particular may be in greatest contact because the tail of the substrate lies along its surface. Using the computational algorithm MolDock in the Molegro Virtual Docker [18], we attempted to identify a region of the sequence of the gene that may be mutated so that LadA may bind to a non-native substrate, polyethylene, rather than the natural 15-carbon to 36-carbon alkane substrates. MolDock was preferred over other docking algorithms for its higher docking accuracy [18].

MolDock employs a guided differential method in which a pool of offspring solutions, or substrate poses (a specific orientation of the ligand of interest), derived from parent solutions are

selected for the lowest energy score [18]. Energy score is calculated from the sum of the intermolecular and intramolecular energy values (equations are found in [18]) that quantify the electrostatic interactions between heavy atoms in protein, cofactor and water atoms and the substrate and is given in kcal/mol. Each offspring solution's energy score is evaluated and becomes the parent to a new pool of offspring solutions if it has a lower energy score than the parent that gave rise to it [18]. Offspring solutions are derived by slightly modifying the dihedral angles of the parent solution. These calculations are repeated for a specified number of iterations per run for a specified number of runs. Poses with the lowest energy scores are retrieved from each run. This *in silico* evolution and differentiation selects for a pose that exhibits the lowest total energy of interaction between substrate and enzyme.

In calculating the interaction energies of residues involved in the binding of alkanes of varying lengths, we applied the MolDock Simplex Evolution algorithm [18] which is more suitable for the large number of flexible bonds in our alkane chains and used a docking template to screen for solutions that exhibit similar orientation to that described in [15]. The terminal carbon lies directly under the four catalytic residues—His17, Tyr63, Gln79 and His311—and above the coenzyme FMN. The rest of the alkane consists of a straight tail that exits the binding pocket. The template was initially created from the 15-carbon alkane pose that best conformed to this requirement and used for the next longest substrate; the template was extended for longer substrates by creating a new template from the substrate that best conformed to the previous template in an *in silico* directed evolution fashion. All poses were selected from solutions that conformed to the final docking template. Alkanes of chain lengths less than 36 carbons conformed well to the docking template, but alkanes of over 50 carbons in length often did not. Poses that were not oriented with the terminal carbon located under the four catalytic residues were dis-

carded. The analysis consisted of 3000 iterations per run for 20 runs for each of the 20 substrates over three repetitions to give a total of 132 poses hand-selected from 1200 solutions derived from 2.4 million iterations. We chose to run alkane chains of up to 70 carbons length, which is the size of the lowest molecular weight of polyethylene available to us.

3 RESULTS AND DISCUSSION

3.1 Docking Results

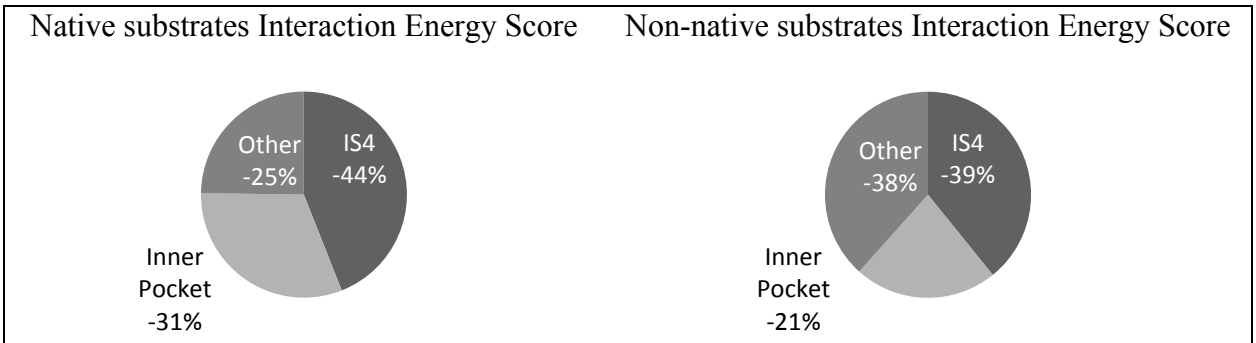


Figure 5 shows the relative percentage of the energy contributed by IS4, inner pocket, and other residues to the overall interaction energy between the substrate and LadA enzyme. The inner pocket consists of Phe10, Met12, His17, Ala57, Val59, Tyr63, Gln79, His138, His311, Trp348, and Phe349. The inner pocket and IS4 interact 10% less and 5% less respectively with nonnative substrate than with native substrate while other residues increase interaction by 13%. The fact that the inner pocket was more affected by substrate length than IS4 supports our previous assumption that longer alkanes are blocked from entering the binding pocket by residues along the outer rim.

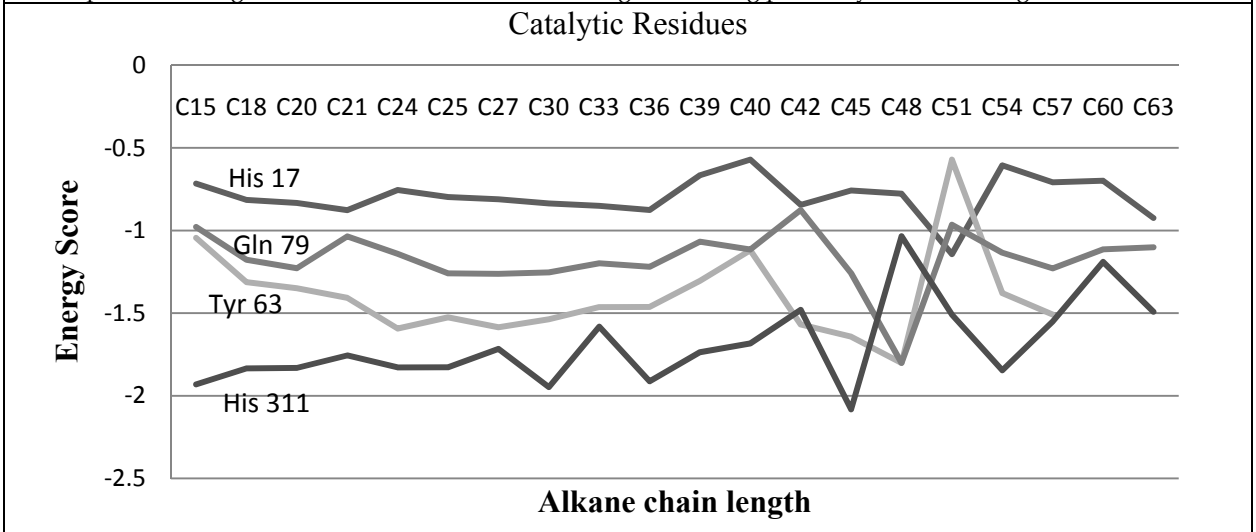
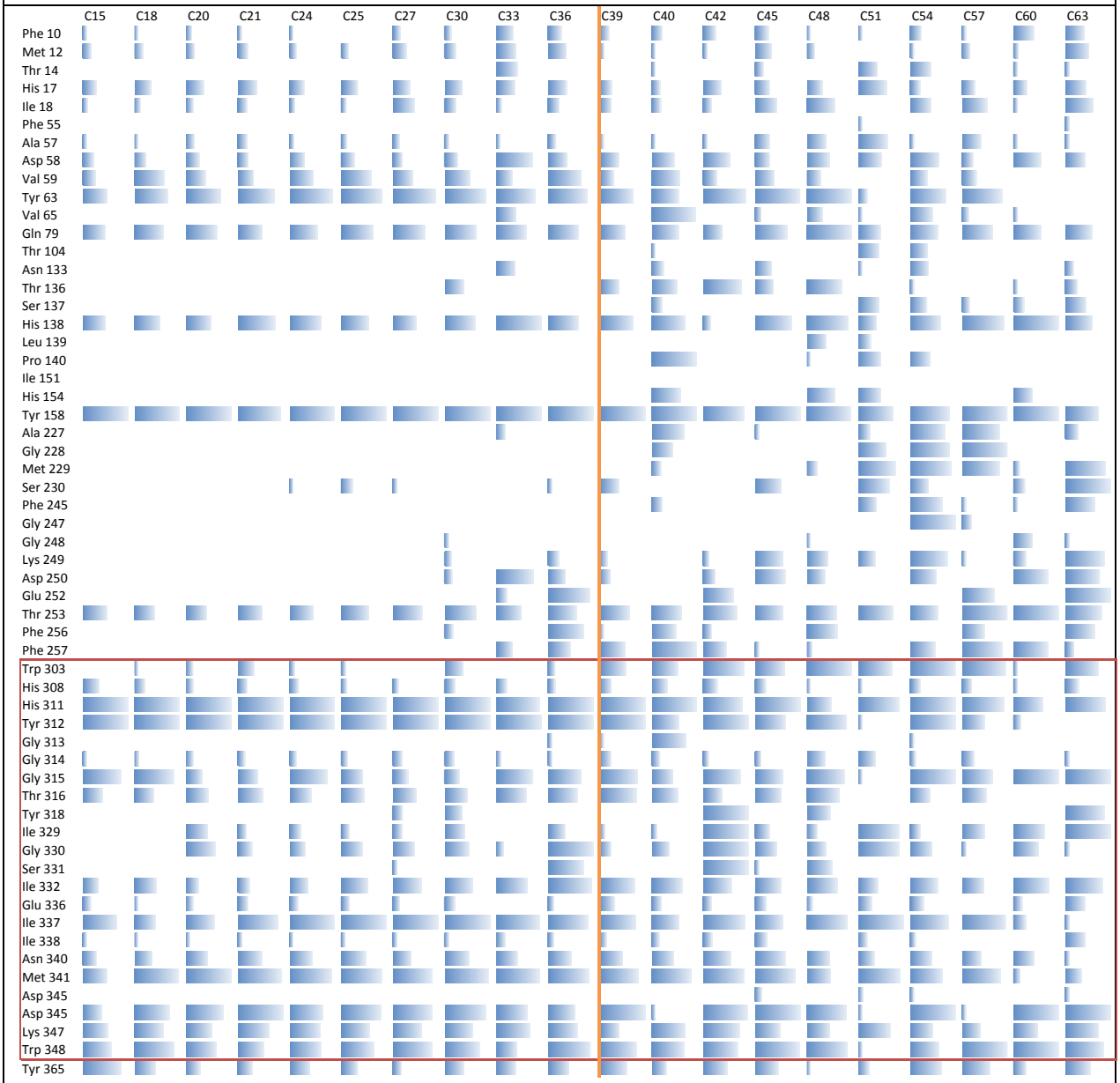


Figure 6 The energy scores of four catalytic residues' --His17, Gln79, Tyr63, and His311-- interactions with alkanes from 15 to 63 carbons in length.

Table 1



The above table lists the average total energies of interactions between 58 residues and 20 substrates of 132 poses selected from 1200 runs and 2.4 million iterations. Longer bars indicate more negative interaction energies and greater relative binding affinities while shorter bars indicate more positive interaction energies or lesser relative binding affinities. The orange vertical line separates LadA's native substrates, alkanes of 15 to 36 carbons, from its non-native substrates, alkanes of over 36 carbons. The red box outlines the residues that lie within IS4. Only residues with significant interactions are listed in the table and residues that appear in the table that only interact with one or two of the twenty substrates were discarded to account for the stochastic nature of the MolDock algorithm [18]. Data from this table were used to create graphs in figure 5 and 6

Catalytic residues His17, Tyr63, Gln79 and His311 exhibit predicted interaction patterns with substrates of longer carbon chain lengths (figure 6). While showing relatively consistent interaction energies with substrates of native carbon chain lengths (15 carbons to 36 carbons), the activities of the four catalytic residues are much less stable beyond 36 carbon chains, though they still maintain relatively similar average total energies of interaction compared to those of their interactions with native substrates. These data suggest catalytic residues may still function normally if longer alkanes are held with the correct orientation, asserting that LadA's inability to bind the longer alkanes is the sole factor inhibiting its effectiveness in catalyzing polyethylene. Furthermore, His311, a residue lying within IS4, began displaying inconsistent interactions with the substrates starting with chains of 25 carbons in length, reinforcing the aforementioned prediction that IS4 is most antagonistic of insertion regions towards long carbon chains. However, this graph overlooks the fact that though His311 is hindering binding, it is essential for the oxidation of the terminal carbon.

To identify the region to mutate, we primarily looked at three groups: IS4, the inner pocket residues and other residues that interacted with the substrates. Inner pocket residues include Phe10, Met12, His17, Ala57, Val59, Tyr63, Gln79, His138, His311, Trp348, and Phe349. While the three residues His311, Trp348, and Phe349 lie within IS4, we grouped them with the inner pocket residues. As seen in figure 5, residues lying within IS4 show greatest interactions with both native and non-native substrates, confirming the fact that IS4 is in direct contact with the substrate. Specifically, the residues 300 – 349, a subregion of IS4, contribute on average approximately 51.1% of the interaction energy with all 20 substrates, expressed in kilocalories per mole (Table 1). Though many of the residues residing in the subregion exhibit a lower energy of interaction with the long chain alkanes, we cannot exclude the possibility that these interactions

are contributing to the hindrances that are prohibiting longer alkanes and polyethylene from attaching to the binding pocket. The residues 300 – 349 are the most variable, most involved, and most likely to change the enzyme's binding capabilities when mutated and therefore were selected as the mutable region of the LadA gene. Note that some synthesized mutants will contain a mutation of the catalytic residue, His311, and may turn up positive in this binding assay. In future work, these false positives will be eliminated through a separate reaction assay.

3.2 Docking Analysis and Rational Protein Design

We proceeded to computationally predict the most suitable LadA mutant that binds to polyethylene. The residue interactions were analyzed to determine the ones that interacted with multiple alkane chains. These recurrently interacting residues were then marked as residues that need to be modified in order to be able to accommodate polyethylene. The region marked for mutagenesis was a fifty residue region in IS4. The sequence of the region is shown below in figure 7.

310	320	330	340	350
KYWSLEGHLA	SKYSSNDYIG	HYGGGTGYDL	SISVGEIINN	MSKLDGKWFK

Figure 7. The fifty residue LadA region marked for mutation.

This region was previously mentioned to be the key region in the interactions between LadA and its substrates. This congruity between docking and literature affirmed the validity of our analysis and narrowed down the region to be mutated.

The three residues that seem to have the most antagonistic interactions with the very long alkane chains were Isoleucine 337, Asparagine 340, and Tryptophan 348 (highlighted in figure 8) are targeted as residues that specifically needed to be modified. Because there is no accurate way to computationally model the protein folding of a large protein such as LadA, to preserve the original protein folding as much as possible, the number of modified residues was kept to a

minimum. Though Histidine 311 displayed possible hindrances to the binding of the very long chain alkanes, its catalytic properties excluded it from being chosen as a mutable residue as to minimize the effects on protein function.

310	320	330	340	350
KYWSLEGHLA	HYGGGTGYDL	SKYSSNDYIG	SISVGEIINN	MSKLDGKWFK

Figure 8. Highlighted are the three residues specifically designated for mutation

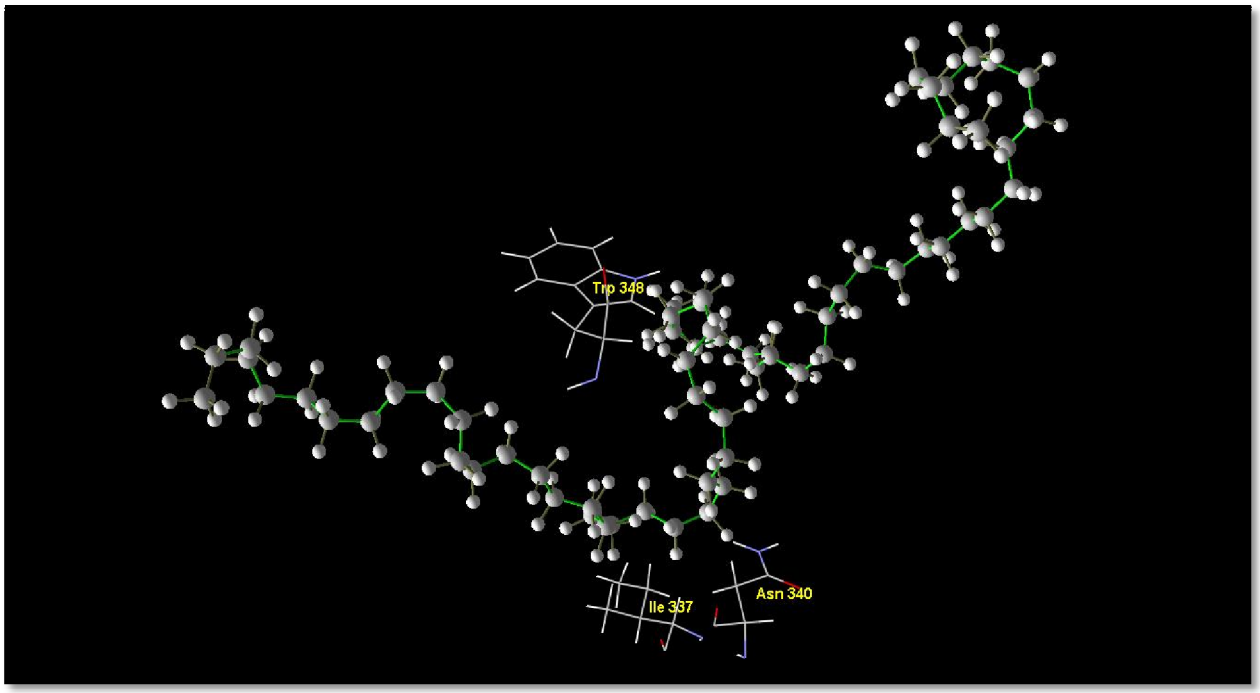


Figure 7 A three dimensional view of the three residues (Asparagine 340, Isoleucine 337, Tryptophan 348) designated for mutation in relation to the bound polyethylene molecule.

In judging suitable replacements, two main criteria were considered: polarity and structure. Due to the non-polarity of polyethylene, the first and foremost consideration was polarity of residues. Polar residues were replaced with non-polar ones. However, in the case of replacing non-polar residues, the hydropathy index value of the replacement residue was kept similar to that of the original residue to minimize effect on protein function. The other important consideration in the selection of alternate residues was shape and structure. When replacing a polar residue with a non-polar one, the specific non-polar residue was chosen based on its similarity to the

structure of the original polar residue to minimize the impact on protein folding. We assumed that non-polar repulsive interactions with the alkane chain were due to steric conflicts and not polarity conflicts. Consequently, the non-polar residues were replaced with smaller non-polar residues less likely to cause steric conflicts.

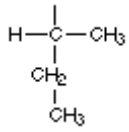
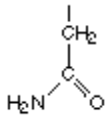
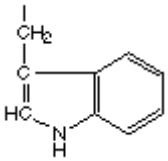
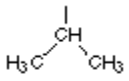
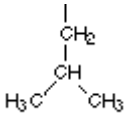
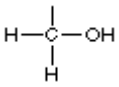
Original Residues		***Isoleucine***	***Asparagine***	***Tryptophan***
	Polarity	*Non-polar*	Polar	Non-polar
	Hydropathy Index [19]	4.5	-3.5	-0.9
	Structure			
Mutated Residues		***Valine***	***Leucine***	***Serine***
	Polarity	Non-polar	Non-polar	Polar
	Hydropathy Index [19]	4.2	3.8	-0.8
	Structure			

Figure 8 Comparison between the original residues and the replacement residues

Isoleucine 337 is one of the most non-polar amino acids and thus should have no polarity clashes with the alkane chain. However, the computational data show that the residue does conflict with the alkane chain. Because isoleucine 338 does not have similar problems, the repulsion interactions of isoleucine 337 with the alkane chain can be attributed to steric conflicts.

In order to solve the steric conflict, isoleucine 337 was modified into valine. Though valine is similarly non-polar, its smaller size makes it less likely to cause steric conflicts

Asparagine 340 was mutated into leucine. Asparagine is polar and will clash with the non-polar alkane chain. Leucine on the other hand is non-polar. The structure of leucine also bears a strong resemblance to that of asparagine.

Tryptophan 348 was replaced by serine. Unlike isoleucine, tryptophan is only slightly non-polar. It was decided that this did not present any polarity conflicts with the alkane chains. The primary problem with tryptophan was its large size leads to steric conflicts with the alkane chains. Because of this, tryptophan was mutated into a serine, which has a similar hydropathy index but is much smaller.

Figure 9: Highlighted residues from figure 8 after mutation.

After determining the best-suited replacements for the four marked residues, the changes were made using the PyMOL[20] mutagenesis wizard. For each mutation, a rotamers had to be individually considered and selected in order to minimize the steric incompatibility of the new residues with the rest of the LadA enzyme.

Once the mutagenesis was complete, the modified LadA enzyme was computationally bound, using the same conditions as the previous runs, to the 63 carbon chain to see if there was a greater binding affinity. The resulting rerank score (the unitless value that Molegro [18] uses to judge binding affinity) was -65.2628. This was almost a six-fold increase in binding affinity from that of the wild-type LadA to the 63 carbon chain; the original binding affinity was only -11.13.

While these results are certainly promising, the problem of unintended changes to protein folding and to enzyme function must be kept in mind. The number of residues modified and the shape of the modified residues were all taken into account in order to minimize the impact of these variables. However, there is no efficient and precise method to predict how the modifica-

tions of these select residues will affect the folding and the function of the LadA enzyme. Once we experimentally isolate the mutants that bind well to polyethylene, we will compare those sequences with the computationally predicted sequence above.

4 EXPERIMENTAL

A library of LadA monooxygenase mutant genes will be synthesized and screened for the desired mutant through a phage display assay with one mutant varying from the others by random mutations within its subregion of IS4. Each mutant will be spliced into a phage vector T7select10-3b (Novagen) and amplified through the host *E. coli* BLT5403 [21]. The produced phages will express their respective LadA mutant on their protein coat without its coenzyme FMN which will allow binding but inhibit catalysis and is essential for the assay, allowing for the identification of mutants that bind well to the non-native substrate as mentioned in the introduction. The library of phages will then be introduced to micronized polyethylene powder immobilized on ELISA plates [21]. The polyethylene powder will be eluted afterwards to identify which phages (and by extension, LadA mutants) exhibit strong binding.

We are currently extracting the wild-type LadA monooxygenase gene from the bacteria *Geobacillus thermodenitrificans* (ATCC 29492). We isolated the megaplasmid pWL1071 on which we performed PCR to extract the LadA gene. This wild-type gene will be spliced into the Novagen T7select10-3b vector and assayed for binding of LadA to one of its native substrate, octadecane. This initial run with the wildtype enzyme provides a baseline indication of a positive assay result and strong binding affinity for comparison to future assays of the mutant enzymes.

DNA Extraction method *Geobacillus thermodenitrificans* was acquired from ATCC (29492). The LadA monooxygenase wild-type gene is found on the megaplasmid pWL1071, which is

highly conserved (99% similarity) among strains of this species [22]. The bacteria were incubated in nutrient broth and nutrient agar at 60 °C for 24 to 72 hours and the plasmid was extracted using the Promega DNA purification kit. The gene was amplified from pWL1071 through PCR, using forward and reverse primers TCTTATAGATCCTCCCCCTT (GC%: 45, T_m: 52 °C) and ACGTGGGAACCCTAAATCC (GC%: 53, T_m: 55 °C) and stored at -20°C. Phusion high-fidelity DNA polymerase, HF buffer, and dNTPs used in PCR were obtained from Finnzymes.

Substrate preparation LadA natural substrate, octadecane, was acquired from Sigma-Aldrich (catalog# O652-2G). Low-molecular weight polyethylene samples were gratuitously provided by Micro Powders Inc: MPP-230VF (1000 g/mol, broad range), MPP-480VF (~1000 g/mol), MPP-308VF (1000 g/mol narrow range), MPP-620VF (~1500 g/mol), and MPP-635VF (~2000 g/mol). In our experiment, we chose to use MPP-230VF. Both octadecane and polyethylene will be immobilized on ELISA plates for exposure to phages and elution.

Mutant Library Synthesis Mutants will be synthesized through the combination of two different approaches: random mutations in the fifty residue subregion of IS4 and synthesis of mutant library containing all predicted mutants using a high-throughput oglionucleotide synthesizer [23]. Our approach differs from that used in most directed evolution experiments where error-prone PCR is employed for site-directed mutagenesis [24].

Cloning LadA wildtype and mutant genes, amplification, panning, and sequencing The wild-type and mutant genes will be prepared with 5'-AATT and 5'-AGCT sticky ends and spliced directionally into the *EcoRI/HindIII* cloning region of the phage vector. The inserts will then be packaged and amplified in *E. coli* BLT5403 and the synthesized phages will be exposed to immobilized polyethylene in ELISA plates [21]. Bound phages will be eluted and sequenced to identify the mutant that best binds to polyethylene. The binding phages will serve as the popu-

lation for the subsequent round of mutant library synthesis, culminating after multiple repetitions of the process in one or few mutants that bind significantly better to polyethylene than its original substrate. These phages will be transformed and amplified in *E. coli* BLT5403. Splicing, packaging, panning, elution, sequencing, and amplification constitute one round. Subsequent rounds will be done with the sequenced genes of the previous round. A total of five rounds will be conducted to ensure binding is strong and to enrich the phage population that exhibit this characteristic.

5 FUTURE WORK

Multiple rounds of the directed evolution/phage display assay will be performed, with the strongest-binding mutants of each round serving as the parent of a new pool of mutants used for the next round of screening. The positive mutants of each round will be ran through a separate binding affinity assay to quantitatively compare the energy of binding to that of wild-type LadA with its original substrates. These mutants will then be screened for mutations of the catalytic residues, discarding such mutants which will likely no longer retain the ability to perform catalysis. Finally, the desired LadA will be removed from the protein coat of its corresponding bacteriophage and introduced to polyethylene in the presence of FMN to ensure the preservation of its hydroxylation capability.

The gene of the well-binding LadA mutant will be synthesized and spliced into *E. coli* after codon optimization and investigations will be conducted as to what factors or stressors will induce high-levels of LadA transcription.

Ultimately, we will create a system capable of producing LadA, an alcohol dehydrogenase (such as AlcA, which is not membrane bound, from the *Pseudomona putida* chromosomal

DNA [14]), an aldehyde dehydrogenase(such as AldA , also from *P. putida* [14]), and an enzyme system such as the human fatty acid synthase (or its multiple components found in bacteria). This *in vitro* system will have each step coordinated in a bioreactor in such a way that the products of catalysis at each stage can be directed to the compartment for the next stage.

6 CONCLUSION

Our computational results and *in silico* mutagenesis have identified the key residues preventing the binding of polyethylene to LadA, suggesting that mutations of these residues may allow for a mutant that can hydroxylate polyethylene chains, accomplishing the first and critical step in the biodegradation of polyethylene. An effective method of isolating such a mutant has been devised in the form of directed evolution targeted at a subset of IS4, the key region other than the active site pocket that interacts with LadA substrates, and phage display assay to detect binding. This project comprises the first, and most critical stage in a system by which polyethylene can be safely and efficiently (in terms of energy investment required) degraded *in vitro*, providing a feasible solution to the environmental threat posed by the prevalent polymer.

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