ABSTRACT

Title of Document: USING ENZYMATIC COMBINATIONS TO REDUCE ASPHALTENE AGGREGATION

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Team BACTERIA's research aims to determine an optimal mixture of enzymes produced by fungi that would effectively reduce asphaltene aggregation in heavy crude oil, also known as bitumen. One of the biggest challenges associated with handling heavy crude oil is the asphaltene aggregation, which leads to a spontaneous flocculation that causes clogging of the pipelines. The key to impede the flocculation is preventing the formation of the asphaltene nanoaggregation by reducing the polycyclic aromatic hydrocarbons (PAHs) within the asphaltene. Conventional methods of asphaltene de-flocculation utilize chemicals that are both energy-intensive and expensive, while a biological method can improve the sustainability of heavy crude oil refinement. In this study, extensive experiments were conducted to determine whether the enzymes laccase and chloroperoxidase reduced flocculation by oxygenation, thereby reducing PAHs and increasing the oxygen-containing functional groups. A combination of these enzymes was also tested to determine whether the combination of enzymes would be more effective at degrading asphaltene than the individual enzymes. Enzymatic treatment of asphaltene demonstrated a significant reduction in flocculation when compared to untreated asphaltene, but the combination of laccase and chloroperoxidase did not exhibit

a significant reduction in flocculation when compared to the individual enzymes. Based on the results of the flocculation tests and FTIR analysis, the team provided for the first time an example mechanism of the chemical pathways of such enzyme-mediated asphaltene degradation. This research, therefore, offers possibly the first comprehensive and systematic investigation of the technique of enzyme-mediated asphaltene oxygenation and degradation.

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By

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Table of Contents

List of Figures

Chapter 1: Introduction

Energy Demand

Oil is a critical, nonrenewable resource[17] required to meet increasing global energy demand. The International Energy Agency predicts that the global demand for energy supplies will increase by 47% by the year 2035 (Stringham, 2012). Additionally, current fossil fuel reserves are projected to be depleted within the next 100 years (Shafiee & Tofal, 2009). There is a high demand for light crude oil, which has low viscosity, low density, free-flowing properties at room temperature, and a low volume of asphaltene (Leon & Kumar, 2005). Light crude oil is prominently used in transportation, production of plastic and synthetics, and manufacturing of food (Miller, 2014). The continuous use of light crude oil has led to its depletion, necessitating the extraction of heavy crude oil as a substitute (Castañeda, Muñoz, & Ancheyta, 2014).

Heavy crude oil, also known as bitumen[4], is known for its high density, viscosity, and asphaltene content. Asphaltenes, commonly referred to as the "cholesterol" of pipelines, are heterogeneous chemical compounds that contribute to many of the difficulties associated with using heavy crude oil. A greater input of energy is required to produce petroleum products from heavy crude oil than from light crude oil (Martinez-Palou et al., 2011). Even though there is seven times heavier crude oil present in oil sands than light crude oil present in the world's oil reserves, heavy crude oil is still a largely untapped resource for global energy use, primarily due to the challenges present in recovering heavy crude oil (Leon & Kumar, 2005). One of the central challenges of handling and using the heavy crude oil is preventing the flocculation of the asphaltene present in the crude oil. This flocculation is caused by the multi-scale aggregation of the

asphaltene molecules forming nano-aggregates, which results in the invariable clogging and damaging of pipelines and other installations transporting the crude oil. Estimates indicate that developing a sustainable method to lower the asphaltene aggregation in heavy crude oil could facilitate access to an estimated 2.4 trillion barrels of heavy crude oil, which could power the world for nearly 70 years at current usage rates (Miller, 2014).

Preparation of Heavy Crude Oil for Transportation

In Canada, where heavy crude oil is abundant, 700,000 barrels of synthetic crude oil are obtained from bitumen or tar sands and transported to refineries in Canada and the United States (Hart, 2014). Current pipeline transportation methods involve a pressure drop to minimize the pump power required to move oil over long pipeline distances (Hart, 2014). Although pipeline transportation is the primary way to move crude oil to refineries, many problems in efficiency of flow arise over time due to factors such as viscosity and asphaltene aggregation (Hart, 2014).

Several methods to reduce flocculation in light and medium crude oil pipelines exist, but their application to heavy crude oil is more difficult. Crude oil dilution and heat tracing represent the most common methods used. Dilution involves the addition of a lighter solvent at a predetermined ratio of solvent to heavy crude oil. This method can reduce viscosity but presents other problems such as increased asphaltene deposition. Another method is using heat to conserve the elevated temperature (<373.15 K) at which oil is produced at the wellhead through insulation of pipelines. External heating is used at pumping stations through direct-fired heaters and burying the pipeline creates insulation to conserve heat (Martinez-Palou et al., 2011).

Pipeline Transportation

After crude oil is extracted, it must be transported to oil refineries. Every year, about 346 billion gallons of crude oil are transported throughout the United States via pipelines (Etkin, 2009). Pipelining is the easiest and most conventional method of transporting lighter crude oil. The transportation of heavy crude oil via pipelines is hindered by the low mobility and flowability of heavy crude oil (Martínez-Palou et al., 2011). Asphaltene deposition and high viscosity are two factors that can cause reduction in the flow of heavy crude oil due to the eventual narrowing of flow passage within the pipeline (Martinez-Palou et al, 2011). Asphaltene aggregation also stabilizes water-in-oil emulsions, which cause pipeline corrosion as water reacts adversely with the iron pipelines, which is very damaging to the transportation process (Martinez-Palou et al, 2011). This reaction can increase the risk of pipeline rupture and oil spills, which threatens the health and safety of both humans and the environment. Between 1998 and 2007, approximately 3.2 million gallons of oil were spilled from pipelines every year, and the U.S. spent over $2.1 billion on crude oil spill cleanup each year (Etkin, 2009).

One method used to improve pipeline transportation of crude oil is dilution, which is described in the previous section (Martinez-Palou et al, 2011). While dilution is the most commonly used technique and can be used for large volumes of crude oil, some problems can occur when diluting light natural gas or light crude oil with heavy crude oil (Hart, 2012). Naphtha or light crude oils seem to effectively dilute the crude oil, but with these compounds, more asphaltene flocculation was observed (Martinez-Palou et al, 2011). The diluents may increase the tendency for asphaltene to aggregate, causing more flocculation and deposition within the pipeline, ultimately hindering the flow of the oil.

Additionally, the light hydrocarbons normally used for dilution can be expensive and less readily available for large volumes of heavy crude oil (Hart, 2012).

Another method used to improve the flow is by heating the pipeline, which causes a reduction in viscosity to lower the resistance of the oil to flow (Hart, 2012). The heavy crude oil must be preheated before transporting it through a heated pipeline (Hart, 2012). While effective, this heating process uses a large amount of energy and is costly, especially over long distances (Hart, 2012). Heating can also exacerbate problems in other areas of the pipeline, such as corrosion (Hart, 2012).

Heavy crude oil can also be emulsified in water and stabilized with surfactants for pipeline transportation (Hart, 2012). The oil disperses as droplets in the water and forms a stable oil-in-water emulsion that has a reduced viscosity (Hart, 2012). Dispersing machines, mixing with rotor-stator, colloid mills, high-pressure homogenizers applying high shearing stresses, and emulsification by membrane and ultrasonic waves are used to produce the oil droplets (Hart, 2012). Once the droplets form, the surfactants prevent phase separation to stabilize the emulsion (Hart, 2012). Once transported, the emulsion is separated using techniques such as thermal demulsification, freeze-thaw method, and pH modification (Hart, 2012). The main disadvantages of using this technique are the cost, the ability of the surfactant to stabilize the emulsion during transport, and the need for an additional process to separate the emulsion after transport (Hart, 2012).

Oil Recovery

One method of oil recovery that has received much attention in recent years is microbial enhanced oil recovery (MEOR). This method takes advantage of the ability of various bacterial and fungal species to reduce the viscosity of oil through the production

of enzymes and surfactants (Ding et al., 2014). In comparison to chemical treatments, a biological process consumes significantly less energy and also has a lower negative impact on the environment (Ding et al., 2014). Current literature suggests MEOR can be used to alter the chemical structure of bitumen to make the oil lighter and less viscous, therefore increasing its value and making it easier to refine and transport (Suthar, Hingurao, Desai, & Nerurkar, 2009). For this reason, Team BACTERIA chose to study the abilities of two microbially produced enzymes to degrade asphaltene and reduce the viscosity of bitumen.

Asphaltene

Many of the challenges in transporting crude oil can be attributed to the asphaltene component of crude oil (Leon & Kumar, 2005). As previously stated, asphaltenes are heterogeneous compounds, meaning they do not have a single uniform structure. However, all asphaltene molecules contain a polycyclic aromatic hydrocarbons (PAHs) complex, as well as heteroatoms such as nitrogen, oxygen, and sulfur atoms (Leon & Kumar, 2005). Asphaltenes are the heaviest and most polar fractions found in oil (Leon & Kumar, 2005). These chemical properties contribute to the viscous nature of oil, and pose major problems in oil transportation as they increase the cost to refine and transport oil (Lavania, Cheema, Sarma, Mandal, & Lal, 2011).

The PAH is the site where intermolecular attraction occurs, causing the aggregation[1] or clustering of asphaltene (Mullins, 2010). The nanoaggregates can then further cluster together into aggregates of suspended particles, which results in high viscosity of oil and clogged pipelines (Mullins, 2010). An example of a asphaltene structure is shown in the leftmost diagram of Figure 1. Asphaltene aggregation stabilizes

emulsions of oil and water by undergoing van der Waals interactions, keeping the molecules close together and causing increased viscosity (Leon & Kumar, 2005). The structure and density of the asphaltene in heavy crude oils cause most of the inefficiencies in extracting, separating, and upgrading[28] heavy crude oil (Leon & Kumar, 2005).

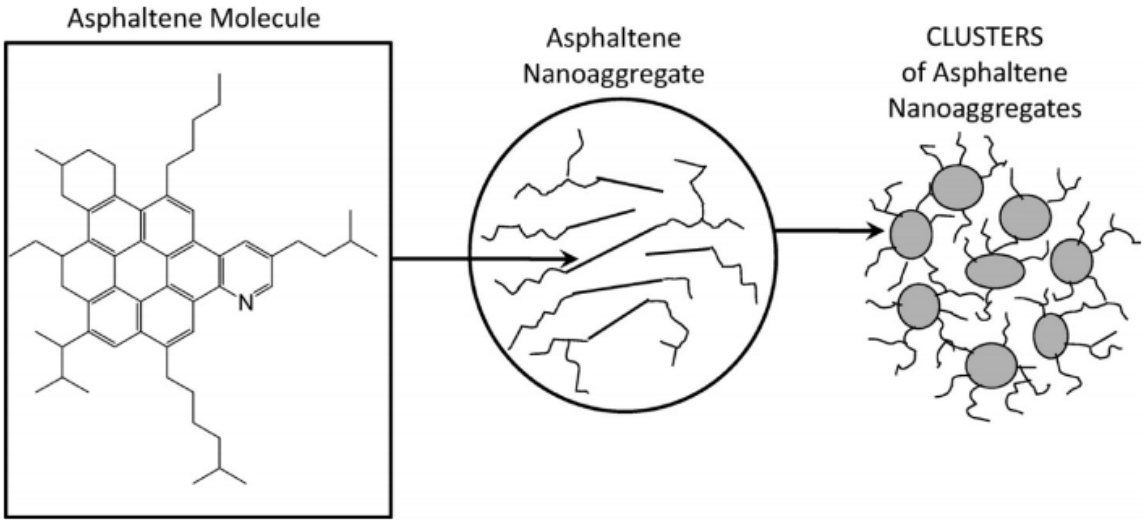

Figure 1: The Yen-Mullins Model of asphaltene structure. This figure has been adapted with permission from O.C. Mullins. Mullins, O.C. (2010). The Modified Yen Model. *Energy & Fuels*, *24*(4), 2179-2207.

Flocculation

Asphaltene flocculation[8] in heavy crude oil causes many issues during production, processing, storage, and transportation (Demirbas, 2016). Flocculation causes asphaltenes to aggregate and interact with surfaces, such as the inner surface of the oil pipelines (Savvidis, Fenistein, Barré, & Béhar, 2001). The onset of asphaltene aggregation leads to the formation of larger clusters, called flocs. This results in the deposition of crude oil, which clogs the pipelines and can eventually cause ruptures;

asphaltene has thus been dubbed the “cholesterol” of the pipeline (Mullins, 2010). Asphaltene deposition causes blockage in the wells of oil reservoirs and pipelines, which significantly decreases the efficiency of transportation (Savvidis, Fenistein, Barré, & Béhar, 2001). The deposition of asphaltene aggregates can lead to other detrimental problems such as reservoir plugging and wettability reversal (Demirbas, 2016). Asphaltene deposition typically follows asphaltene flocculation, which is why it is crucial to understand how to reduce flocculation and thereby reduce asphaltene deposition. Variables such as temperature, pressure, and shear rate of flow can lead to greater chances of blockage due to precipitation and deposition (Demirbas, 2016). Current methods of de-precipitation have shown a slowed re-precipitation of asphaltene with the use of deflocculants (Demirbas, 2016).

Currently, the Colloid Stability Index is used to predict the stability of asphaltene in oil by determining the contents of asphaltene fractions and their structural characteristics (Petrova, 2013). The index is defined as the ratios of aromatic hydrocarbons and resins to paraffinic-naphthenic hydrocarbons and asphaltene (Petrova, 2013). If this ratio is low, the asphaltene particles may form aggregates, which clump together, causing asphaltene deposition (Petrova, 2013).

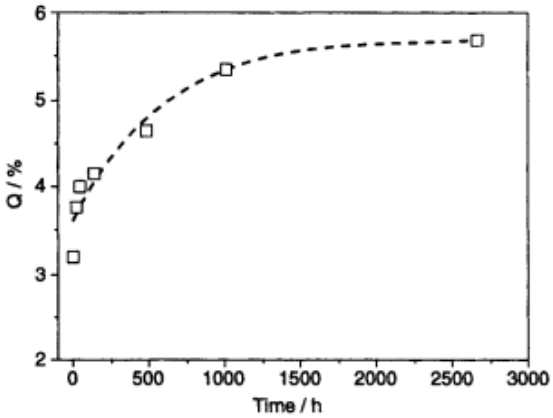

Figure 2: General example of an asphaltene flocculation curve. This figure has been adapted with permission from Savvidis, Fenistein, Barri & Emmanuel Bihar. Savvidis, T. G., Fenistein, D., Barré, L., & Béhar, E. (2001). Aggregated structure of flocculated asphaltenes. *AIChE Journal*, *47*(1), 206–211.

Figure 2 illustrates a general example of a flocculation curve. The general pattern among flocculation curves is a rapid increase in precipitation with time before reaching the maximum precipitation of asphaltene. The curve allows for the interpretation of flocculation rates (or amount of precipitate formed over a time interval) with variables such as solvent concentration and enzyme concentration **(**Savvidis, Fenistein, Barri & Emmanuel Bihar, 2001).

Research Objective

The current methods for preparing heavy crude oil for pipeline transportation have raised environmental concerns, calling for the development of an alternative biological approach to reducing asphaltene flocculation. However, treating heavy crude oil with enzymes produced by bacteria or fungi to reduce asphaltene flocculation has yet to be implemented. Utilizing enzymes to disrupt asphaltene aggregation has the potential to reduce the detrimental impact of oil transportation on the environment by preventing the deposition of heavy crude oil, thus reducing the likelihood of pipeline bursts. As the supply of lighter crude oil declines, it is imperative to improve upon the current methods of heavy crude oil refinement to decrease the environmental hazards that currently exist.

Team BACTERIA's research sought to define a mixture of enzymes that would effectively reduce asphaltene flocculation enough to improve the pipeline transportation

of heavy crude oil. Individual enzymes have already been identified in previous literature from bacteria and fungi to breakdown components of asphaltene through the processes of biodesulfurization, biodenitrogenation, and oxidation, but there are currently no studies on using mixtures of enzymes in oil refinement or specifically to treat asphaltenes (Vazquez-Duhalt, Torres, Valderrama, Le Borgne, 2002). These methods will allow researchers to continue studies that seek to recover oil from alternate sources.

Our research aimed to determine whether a combination of two enzymes – laccase and chloroperoxidase– would degrade asphaltene more effectively than the individual enzymes. The team tested the hypothesis that the combination of laccase and chloroperoxidase will be more effective at degrading asphaltene than each of the enzymes individually, such that it decreases asphaltene flocculation. By studying how a mixture of enzymes affects asphaltene structure, the team set the stage for the implementation of enzymatic mixtures in heavy crude oil extraction and refinement. Such experiments provide proof of concept for a biological alternative to current standard refinement processes. Furthermore, this research carried out a detailed FTIR analysis and thereby provided a comprehensive analysis of the potential chemical pathways for enzyme-mediated asphaltene degradation.

Based on the literature, Team BACTERIA hypothesized the following:

1. The enzyme treatments will reduce asphaltene aggregation through an enzyme-catalyzed mechanism.
2. A mixture of enzymes will degrade asphaltene more effectively compared to singular enzymes.
3. The enzyme treatments will reduce asphaltene aggregation by disrupting PAHs

through oxygenation. Therefore, after treatment, the asphaltene sample should have more functional groups containing oxygen and fewer aromatic rings.

Chapter 2: Literature Review

Several microbes are known to degrade hydrocarbons and have applications in bioremediation and MEOR. MEOR is a relatively new method that is more environmentally friendly compared to traditional methods of oil recovery(Vazquez-Duhalt, Torres, Valderrama, Le Borgne, 2002). Various bacterial and fungal species have been found to produce enzymes that alter the structure of compounds in oil, but little is known about the mechanisms behind this activity. Understanding how enzymes perform in non-aqueous environments, such as bitumen, is necessary for the advancement of of biotechnology in the oil industry, yet so far, research has focused on only using enzymes in aqueous solutions of oil and water (Vazquez-Duhalt, Torres, Valderrama, Le Borgne, 2002). Enzymes, such as oxidases and oxygenases, have been used in previous heavy crude oil research as a means of degrading large oil fractions such as asphaltene and resins[20]. However, the effect of an enzyme mixture on asphaltene flocculation and analysis of the chemical pathways through which enzyme-mediated asphaltene degradation occurs has not been researched.

Enzymes and Asphaltene Degradation

Heavy crude oil is not easily recovered due to its high volume of resin-asphaltene structures, which are not easily degraded by microbes due to their complex structures (Kopytov, Filatov, & Altunina, 2014). Microbial interactions with resins and asphaltene were observed when oxidation reactions occurred due to enzymatic activity, and these reactions were then used as an indicator for microbial activity (Kopytov, Filatov, & Altunina, 2014). Kopytov found that resins are easier to oxidize than asphaltene and experience more detachment of peripheral paraffin[18] structures, even though asphaltenes

contain a greater amount of paraffin structures than resins (Kopytov, Filatov, & Altunina, 2014). Additionally, mixed bacterial strains are able to degrade more of the complex components in the asphaltene structure than individual bacteria (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012).

Experimenting with microbes, such as fungi, adds confounding variables due to the presence of exterior components and complex interior structure, making it more difficult to observe precise changes in asphaltene structure. While fungi contain the necessary enzymes for treatment of asphaltene fractions, precise controls and low energy requirements make experimentation using purified enzymes more favorable (Vazquez-Duhalt, Torres, Valderrama, Le Borgne, 2002). Using fungi also requires extensive control of its enzymatic cascades[4], increasing the amount of variables during experimentation. In comparison, the use of purified enzymes allows for a more straightforward management of stabilization properties during experimentation (Ayala, Verdin, & Vazquez-Duhalt, 2007). It is easier to control purified enzymes individually and isolate the variables pertaining to the enzymatic reaction (Ayala, Verdin, & Vazquez-Duhalt, 2007).

Enzymes

The two purified enzymes used to investigate asphaltene degradation in this study were laccase and chloroperoxidase. While both the enzymes have been used in previous research on oil degradation, the impact of the combination of these enzymes has not been tested. Experimentation using individual enzymes provided a basis for testing subsequent enzymatic combinations and their effect on asphaltene. The enzymes were screened among many others including cytochromes C and P450, aldehyde dehydrogenase, and

various lignolytic enzymes, to evaluate each of their abilities to break down PAHs, and were also screened for optimal enzymatic activity and regulatory factors, such as pH, temperature, salinity. This evaluation was used in the determination of the optimal measures and conditions for a mixture of enzymes. The enzymes selected for experimentation have similar optimal pH, temperature, and salinity requirements to ensure that the enzymes in combination can work in their optimal conditions. Ultimately, the other enzymes were ruled out due to their inability to greatly disrupt PAHs or for having significantly different optimal pH, temperature, and salinity levels. Laccase and chloroperoxidase, described below, have the potential to reduce asphaltene flocculation and thereby improve oil transportation.

Laccase. Laccases are oxidases found in over 60 strains of fungi, including Pleurotus ostreatus, Trametes versicolor, and Agaricus bisporus (Mayolo-Deloisa, Machín-Ramírez, Rito-Palomares, & Trejo-Hernández, 2011). These enzymes catalyze one-electron substrate oxidation while coupling to four-electron reduction of molecular oxygen, O2, specifically with compounds containing phenols, polyphenols, and anilines (Kunamneni et al., 2008). Laccase stores the four electrons from the oxidation reaction of the substrate to reduce molecular oxygen to two molecules of water by having its four copper atoms bound to three redox sites (Kunamneni et al., 2008). The mechanism for laccase-catalyzed oxidation of phenols is shown in Figure 3.

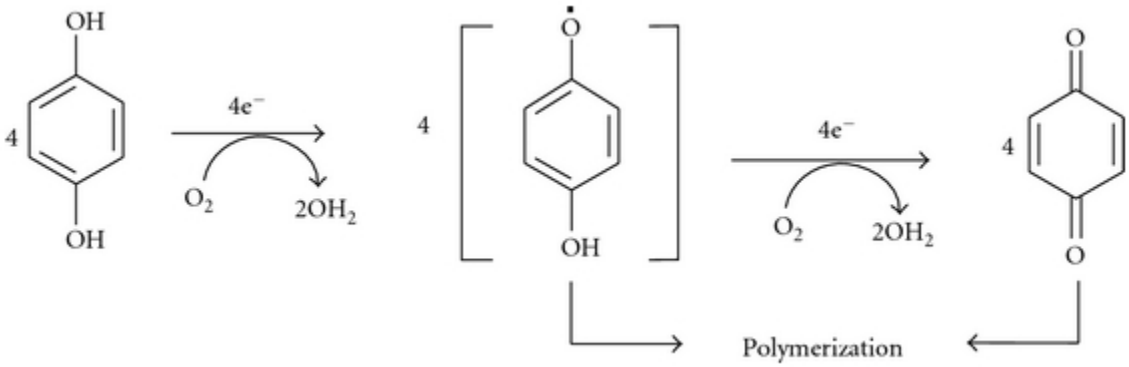

Figure 3: General mechanism for laccase-catalyzed oxidation of phenols. This figure has been adapted with permission from Karigar and Rao. Karigar, C. S., & Rao, S. S. (2011). Role of Microbial Enzymes in the Bioremediation of Pollutants: A Review. Enzyme Research, 2011, 1–11.

Laccases are viable enzymes to test in this study because of their low substrate specificity, which allows them to oxidize a greater variety of substrates without steric hindrance [23] (Mayolo-Deloisa, Machín-Ramírez, Rito-Palomares, & Trejo-Hernández, 2011). Additionally, they do not require reaction initiators such as hydrogen peroxide (H_2O_2), since the electron acceptor is oxygen (Mayolo-Deloisa, Machín-Ramírez, Rito-Palomares, & Trejo-Hernández, 2011). Optimal conditions include a pH of 4 and temperatures ranging from 50 to 70°C (Baldrian, 2006). According to a study by Mayolo-Deloisa et. al. in 2011, laccase from *Agaricus bisporus*, or the White Button Mushroom, was able to oxidize several varieties of PAHs, such as anthracene, benzo[a]pyrene, and benzo[a]anthracene, which indicates its potential use for degrading the larger aromatic complexes of asphaltene in bitumen to smaller and simpler molecules. Additionally, if other enzymes, such as chloroperoxidase, create more of these phenolic,

polyphenolic, and anilinic compounds, combinations of these enzymes with laccase could potentially be used to further enhance asphaltene degradation (Kunamneni et al., 2008).

Chloroperoxidase. Chloroperoxidase is an enzyme isolated from the fungal species Caldariomyces fumago (Ayala, Hernandez-Lopez, Perezgasga & Vazquez-Duhalt, 2012). In crude oil applications, chloroperoxidase catalyzes the transformation of asphaltene using hydrogen peroxide as an electron acceptor (Ayala, Hernandez-Lopez, Perezgasga & Vazquez-Duhalt, 2012). According to Ayala et al., previous research has been done to test the ability of chloroperoxidase to transform asphaltene fractions. The fluorescence analysis of the enzymatic activity indicates changes in the molecular structure of the asphaltene fractions after transformation (Ayala, Hernandez-Lopez, Perezgasga & Vazquez-Duhalt, 2012). Specific changes included an increase of alcohol, ketone, halogenated alkyl chain, and aldehyde functional groups, as well as a decrease in general aromatic ring structures in the asphaltene fractions (Ayala, Hernandez-Lopez, Perezgasga & Vazquez-Duhalt, 2012; Hernández-López, Ayala & Vazquez-Duhalt, 2015). Chloroperoxidase contains low enzyme specificity, optimal enzymatic activity at 60°C and a pH of 3.0, increasing in stability as more substrate is added (Ayala, Hernandez-Lopez, Perezgasga & Vazquez-Duhalt, 2012; Liu & Wang, 2007). In addition, chloroperoxidase can be chemically modified to increase activity and stability by adding phthalic anhydride, maleic anhydride or citraconic anhydride (Liu & Wang, 2007). Based on this analysis, chloroperoxidase is a viable enzyme to be studied in combination with laccase.

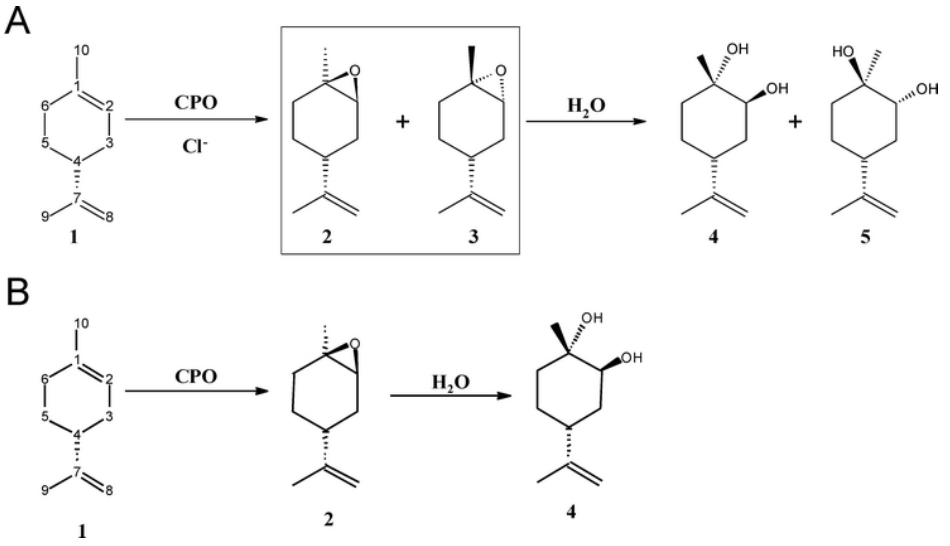

Figure 4: General reaction for chloroperoxidase in catalyzing the formation and opening of an epoxide ring in the presence of water. This figure has been adapted with permission from Águila et. al. Águila, S., Vazquez-Duhalt, R., Tinoco, R., Rivera, M., Pecchi, G., & Alderete, J. B. (2008). Stereoselective oxidation of R-( )-limonene by chloroperoxidase from Caldariomyces fumago. *Green Chemistry Green Chem., 10*(6), 647.

Based on the mechanisms of the individual enzymes, we have proposed a generic mechanism to illustrate the pathway through which a combination of the enzymes, chloroperoxidase and laccase, will affect the asphaltene model, shown below. The structure used for the mechanism is based on the continental model of asphaltene, which is the most descriptive of Canadian asphaltene. However, the continental model is not a definitive model, as asphaltene molecules are highly heterogeneous (Harbi, Benyounes, & Khodja, 2017).

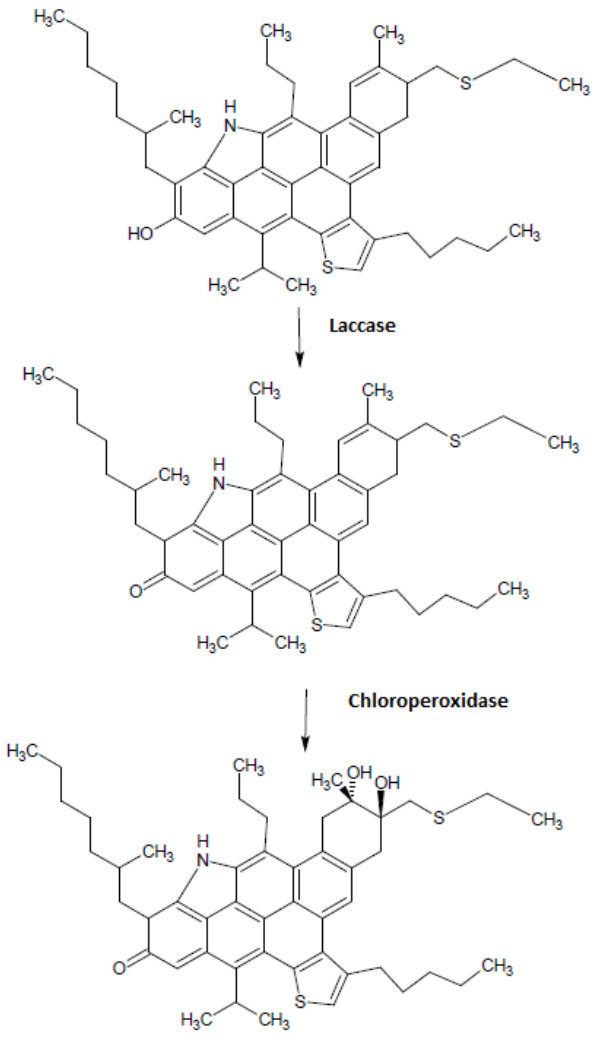

Figure 5: Proposed mechanism for combination of enzymes. This mechanism was devised from an adaptation of a figure obtained with permission from Gharbi, Benyounes, and Khodja. Gharbi, K., Benyounes, K., & Khodja, M. (2017). Removal and prevention of asphaltene deposition during oil production: A literature review. *Journal of Petroleum Science and Engineering, 158*, 351-360.

Laccase is expected to target phenols and perform an oxidation reaction to convert the phenol into a ketone, while also producing a water as a byproduct. Chloroperoxidase

would likely use this water byproduct as a reagent to oxidize one of the double bonds in an aromatic ring to add two more alcohol groups. The final product pictured in Figure 5 is the predicted result after the addition of laccase and chloroperoxidase. The enzymatically treated asphaltenes are predicted to have disrupted *pi*-conjugation in the internal ring complex, allowing for less aggregation to occur by minimizing the site of intermolecular attraction. The disrupted *pi*-conjugation is the result of the addition of hydroxyl groups by chloroperoxidase and the formation of ketones by laccase.

Analytical Techniques

Qualitative analysis. According to the 2013 study by Petrova et. al., observing the behavior of asphaltene fractions in the flocculation and precipitation process involves using ultraviolet-visible spectroscopy (UV-Vis) with automatic recording, IR-Fourier spectroscopy, mass spectroscopy with matrix-activated laser-induced desorption/ionization, and dielectric analysis. Moving forward, only IR-Fourier spectroscopy was used in experimentation due to the technique's high sensitivity and team's access to instrumentation.

Changes in the microscopic asphaltene structure can be detected through the use of Fourier Transform Infrared Spectroscopy (FTIR) (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012). FTIR is a qualitative technique that can be used to determine the chemical structure of asphaltene before and after treatment with enzymes. An interference light beam from the infrared source is directed to an interferometer, and is split by a beam splitter (Faix, O., 1992). A fixed mirror and a moving mirror each reflect half of the light beam, and the light beam halves combine to form a reconstructed beam (Faix, O., 1992). This reconstructed beam is the interference wave, which is analyzed by

a computer, and an infrared spectrum is constructed (Faix, O., 1992). A solvent is not required for analyzing asphaltene with an FTIR instrument (Wilt, Welch, & Rankin, 1998). The advantages of utilizing FTIR include high sensitivity, quick and effective analysis, and the production of qualitative data (Faix, O., 1992). The downside to this technique is that FTIR may pick up byproducts produced by the enzymes as a result of breaking down asphaltene between the 700 – 1,500 cm^{-1} region (Lavania, Cheema, Sarma, Mandal, & Lal, 2011).

A study completed in 2012 by Tavassoli et al., compared an original sample of asphaltene without treatment to a sample of asphaltene after two months of degradation from bacterial species. There was a complete elimination of carbon triple bonds, which were seen at a peak of 3412 cm^{-1} (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012). There were also decreases in peaks at 1600 cm^{-1} and 1300-1450 cm^{-1}, indicating that the bacteria degraded aromatic or conjugated alkane and amine components (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012). The bacteria sampled by Tavassoli failed to eliminate the aldehyde compounds as they had a stronger peak after the treatment than in the original sample (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012). Anticipated results of FTIR analysis for laccase and chloroperoxidase would show larger peaks in oxygen functional groups and few to no peaks in carbon double and triple bonds because in all of the reaction mechanisms of the two enzymes, the enzymes attack double and triple bonds in the asphaltene sample and oxygenate them (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012).

Quantitative analysis. Gel Permeation Chromatography (GPC) analysis is a form of quantitative measurements that can be used to identify any change in the

molecular weight of asphaltenes before and after treatment (Ali, El-Gendy, Moustafa, Roushdy, & Hashem, 2012). Due to the heavy composition of asphaltenes, the use of GPC can be limited in its capacity to measure these large molecules. Other forms of mass measurement of asphaltenes involve the process of extraction using chloroform, hexane, and methanol (Oudot, 1984; Lavania, Cheema, Sarma, Mandal, & Lal, 2011). Since asphaltenes are hexane insoluble, filtration can be used to separate it from the other fractions of oil and the resulting asphaltene can be measured gravimetrically, since a change in molecular weight is an indicator of structural change in asphaltene (Oudot, 1984; Lavania, Cheema, Sarma, Mandal, & Lal, 2011).

Nuclear magnetic resonance (NMR) spectroscopy can be a successful technique to characterize and identify the chemical constituents of crude oil (Silva et al., 2011). NMR predicts the total content of hydrogen and its distribution among the functional groups that are present in the sample (Silva et al., 2011). This technique can obtain information about the structural characteristics of the sample and estimate the molecular weight of the crude oil fractions (Silva et al., 2011). FTIR analysis can provide ample information about the effects of enzymatic treatments on asphaltene degradation and can construct a potential degradation pathway Therefore, GPC and NMR were not utilized in Team BACTERIA's experimentation. However, future research on the enzymatic treatment of heavy crude oil could include the use of these analytical methods for an even more comprehensive analysis of the degradation.

There is great potential for biological treatment of oil to reduce environmental risk. Enzymes produced by various microbes can aid in asphaltene de-flocculation, which can potentially improve pipeline transportation of oil, while simultaneously granting the

ability to tap into largely underutilized heavy crude oil sites. The inherent properties of laccase and chloroperoxidase allow them to catalyze oxygenation reactions at PAH sites. Oxygenating the asphaltene lowers its tendency to form nanoaggregates, thereby reducing the risk of pipeline clogs and ruptures.

Chapter 3: Methodology

Research Objective

The following research examined whether a mixture of two enzymes can reduce PAHs within asphaltenes more effectively than individual enzymes to prevent asphaltene aggregation. To analyze the physical changes in enzymatically-treated asphaltene, the rate of flocculation of asphaltene samples was observed. Fourier Transform Infrared Spectroscopy (FTIR) was used to detect and understand the molecular changes in asphaltene and determining whether the enzymes oxygenated the sample, which would indicate a reduction in PAHs. The experimental design consisted of three phases: benchmark testing of the untreated asphaltene compounds, flocculation testing with the individual enzymes, and flocculation testing with the combinations of enzymes.

Benchmark Phase

It was necessary to observe the behavior of the pure asphaltene to affirm whether the enzymes affected asphaltene flocculation. The team observed changes in amount of asphaltene precipitate collected over time with varying concentrations of *n*-heptane. The asphaltene molecules were examined after the flocculation tests using FTIR to determine the molecular structure of asphaltene after precipitation. These studies served as the standard for comparison with the flocculation of asphaltene samples treated with individual enzymes and the combination of enzymes.

Preparation of asphaltene. The pure asphaltene sample was obtained from the Centre for Oil Sands Innovation Laboratory, University of Alberta. The original asphaltene mixture was extracted from Athabasca bitumen, which was obtained from a Nexen plant (Mehranfar, Gaikwad, Das, Mitra, & Thundat, 2014). The sample was

purified to obtain 100% pure asphaltene rock. 10 mL of HPLC grade toluene was added to 0.100 g of crushed asphaltene to dissolve the solid.

Flocculation test with untreated asphaltene. To precipitate asphaltene, *n*-heptane was added to the solution of asphaltene and toluene and incubated for 24 hours at room temperature to allow for maximum precipitation. Three trials were conducted for each volume of *n*-heptane, and volumes ranged from 100 mL to 900 mL. After the incubation period, vacuum filtration was performed on the asphaltene solution and the remaining asphaltene precipitate was isolated and weighed. The baseline flocculation curve was developed from the data produced from this experiment.

Individual Enzymes Phase

Preparation of Individual Enzymes.

Laccase. Laccase was supplied in a powder form from *Agaricus bisporus* and stored in a -20°C freezer before experimentation. 3 mg of the enzyme were added to 13.2 mL of 3 mM phosphate-buffered saline (PBS). 2 mL of the enzyme-buffer solution were added to 0.100 g of asphaltene dissolved in 10 mL toluene in a 20 mL glass vial. The vial was then equipped with a stir bar and incubated at room temperature (25°C) for 30 minutes on a stir plate.

Chloroperoxidase. Chloroperoxidase was supplied from *Caldariomyces fumago*, suspended in 0.1 M sodium phosphate pH 4.0. Chloroperoxidase was stored in a 4°C refrigerator before experimentation. For treatment of chloroperoxidase with asphaltene, 0.100 g asphaltene was dissolved in 10 mL of toluene. 0.0025 mL of the chloroperoxidase was added to 1.997 mL of 3 mM buffer. The enzyme solution was

added to the asphaltene-toluene solution, which was agitated via mechanical stimulation for 30 minutes.

Flocculation tests. Asphaltene solutions were prepared and treated with each enzyme individually: laccase and chloroperoxidase. Technical grade *n*-heptane was added to the asphaltene/enzyme mixture at specified volumes ranging from 100 mL - 900 mL to allow for comparison with the flocculation curve of the control group. To precipitate the asphaltene, the enzyme-treated asphaltene and *n*-heptane mixture was incubated for 24 hours at room temperature to allow for maximum precipitation. Three trials were conducted for each volume of *n*-heptane, and after the incubation period, the asphaltene precipitate was isolated via vacuum filtration. Once the asphaltene precipitate had air-dried, the sample was weighed using a scale.

Enzyme Combination Phase

Asphaltene solutions were prepared and treated with a 1:1 combination of laccase and chloroperoxidase. The enzymes were prepared as previously mentioned and 1 mL from the laccase solution was added to 1 mL of the chloroperoxidase solution for a final enzyme solution volume of 2 mL. The enzyme mixture was added to 0.100 g of asphaltene dissolved in 10 mL of toluene. The reaction vial was equipped with a stir bar and allowed to incubate for 30 minutes on a stir plate at a low setting. The *n*-heptane was added to the samples to reprecipitate the asphaltene. The volume of *n*-heptane used ranged from 100 mL - 900 mL to parallel the control and individual enzyme flocculation curves. The samples were incubated for 24 hours. The asphaltene precipitate was then isolated by vacuum filtration and weighed. After the asphaltene precipitate was air-dried, the sample was weighed.

Fourier Transform Infrared Spectroscopy (FTIR)

After the asphaltene fractions were extracted from the bitumen before and after treatment, FTIR analysis was used to analyze specific bonds that were degraded due to the enzymes (Faix, O., 1992). FTIR analysis can describe the functional groups and constituents of hydrocarbons (Faix, O., 1992). Peaks in the FTIR spectra were measured and compared to post-treatment analysis, where a decrease in certain key markers of asphaltene was a strong indicator of enzymatic degradation in the context of this experimentation (Faix, O., 1992).

The peaks depict different bonds that correlate to specific functional groups (Faix, O., 1992). The related software detects and analyzes the peaks found on the FTIR spectra. The FTIR instrument used was the Thermo Nicolet NEXUS 670 FTIR, and OMNIC Esp software provided the spectra graph and analytical tools to understand the peaks and tailings from the spectrum. The FTIR spectra is shown from a 4,000-400 cm^{-1} and stored as electronic files.

Yoon et al., 2009, indicated presence of aromatic rings in asphaltene samples' FTIR spectrum with peaks at 810 cm^{-1} and 755 cm^{-1} with a tail at 3,052 cm^{-1}. There were also bands that indicated O-H and N-H stretching in multiple hydrogen bonded components in the asphaltene (Yoon et al., 2009).

Chapter 4: Results

The data gathered from the flocculation tests and FTIR analysis was used to evaluate the first hypothesis and the proposed chemical pathway of an enzyme-catalyzed mechanism. The weight percentages of asphaltene recovered from the control group and the enzymatic treatments were used to construct a flocculation curve to test the the second hypothesis: a mixture of enzymes would degrade asphaltene more effectively than would singular enzymes. FTIR analysis of the samples was used to test the third hypothesis: more functional groups containing oxygen would appear in asphaltene after enzymatic treatment. Lastly, the data from flocculation curves was analyzed with ANOVA to determine statistical significance, and FTIR peaks were compared to reported literature values.

Asphaltene Precipitation with *n*-heptane

The flocculation curves were constructed from the weight percent of asphaltene recovered as a function of *n*-heptane volume used for each trial of each treatment.

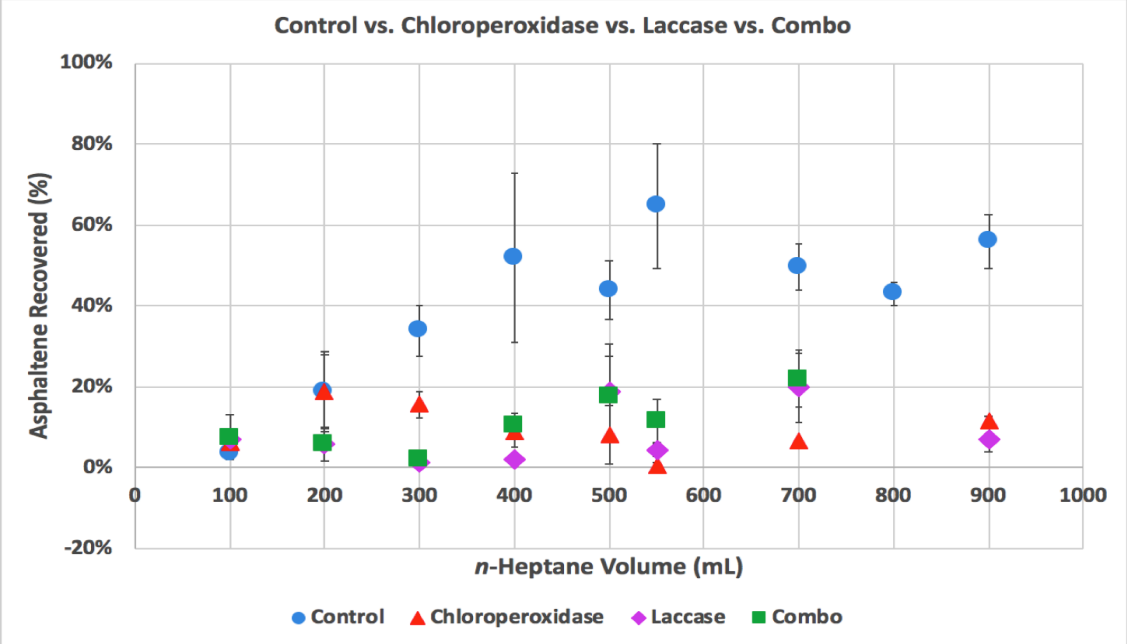

Figure 6: Weight percentage of asphaltene recovered after incubation in *n*-heptane with the addition of no enzyme, laccase, chloroperoxidase, and a combination of chloroperoxidase and laccase.

The percent asphaltene recovered in Figure 6 represents asphaltene precipitated out of the mixture. The addition of chloroperoxidase and laccase to the mixture of asphaltene and toluene reduced the percentage of asphaltene recovered. The percent asphaltene recovered was nearly identical for both enzymes at 100 mL of *n*-heptane added. Chloroperoxidase diverged from the flocculation curve of the control at 300 mL of *n*-heptane added. With increasing volumes of *n*-heptane, the percent asphaltene recovery also decreased to nearly 1% with 550 mL *n*-heptane, compared to a peak at 18.91% with 200 mL of n-heptane. Beyond 550 mL *n*-heptane, the percent asphaltene recovery increased again.

The flocculation curve of laccase decreased as the volume of *n*-heptane increased from 100 mL to 400 mL. However, for volumes 500 mL and above, the amount of asphaltene recovered oscillated around a 12.5% average. The combination of both enzymes initially resulted in a decrease in average weight percent of asphaltene recovered between *n*-heptane volumes of 100 mL and 300 mL. At volumes of 400 mL and above, the amount of asphaltene recovered as the result of the enzymatic treatment oscillated around a 15.4% average.

ANOVA Statistical Analysis

ANOVA statistical analysis was performed on the asphaltene recovery data. In this case, the factor is enzymes and fours levels are no enzymes, chloroperoxidase, laccase, and mixture of chloroperoxidase and laccase enzymes. Using SAS (see

Appendix D), the F statistic is calculated to be 15.35, indicating the mean square for treatments is much higher than mean square error, casting considerable doubt on $H_0: \mu_1 = \mu_2 = \mu_3 = \mu_4$. The calculated F statistic is higher than the F critical value $F_{3,28} = 2.95$with significance level α of 0.05. The p-value is calculated to be less than 0.0001. Using a significance level α of 0.05, the null hypothesis is rejected because 15.35 > 2.95 and < 0.0001 is less than 0.05. Therefore, mean asphaltene recovery does appear to depend on the treatment of enzymes used. Tukey's method was also used in order to find out which treatment mechanisms differ significantly from one another. From the SAS results (see Appendix D), the no enzyme treatment appears to significantly differ from the chloroperoxidase, laccase, and mixture of chloroperoxidase and laccase enzymes treatments.

FTIR Results

Several FTIR spectra were acquired for this analysis. The FTIR spectra of toluene, PBS, individual enzymes, and pure asphaltene from Oil Sands Innovation Laboratory were used as controls. The FTIR spectra of asphaltene resulting from the enzymatic treatments were compared to the FTIR spectrum of pure asphaltene.

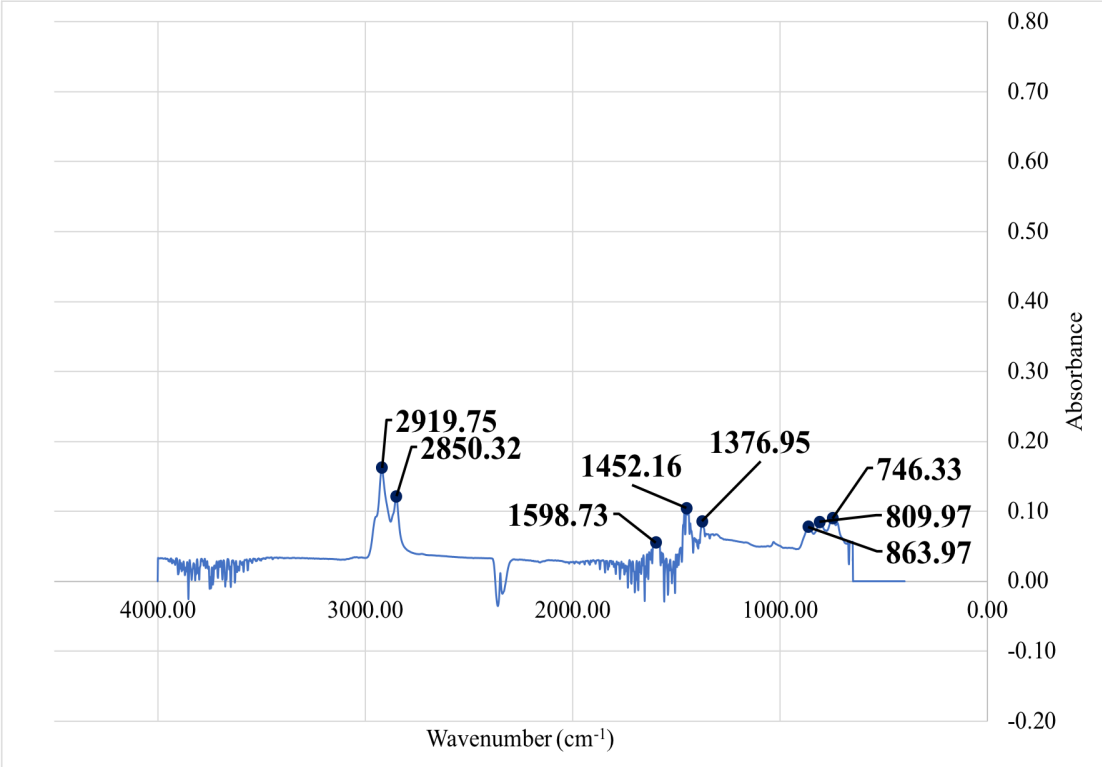

Figure 7: FTIR spectrum of pure asphaltene control.

The FTIR analysis of pure asphaltene sample from the Athabasca oil sands in Figure 7 shows the presence of asymmetric carbon-hydrogen bonds within a carbonyl group, indicated at absorbances of 0.153 and 0.120 at 2919.75 cm^{-1} and 2852 cm^{-1}, respectively (Wilt, B. K., Welch, W. T., & Rankin, J. G. 1998). Another significant peak is located at 1600 cm^{-1} with an absorbance of 0.05, indicating a C=C aromatic ring. CH_2 and CH_3 bending is indicated by peaks between 1300-1400 cm^{-1} with absorbances at 0.1 and 0.08 (Wilt, B. K., Welch, W. T., & Rankin, J. G., 1998). Finally, the triplet with peaks between 746 to 863 cm^{-1} and absorbances of approximately 0.08 is indicative of aromatic CH out-of-plane vibrations.

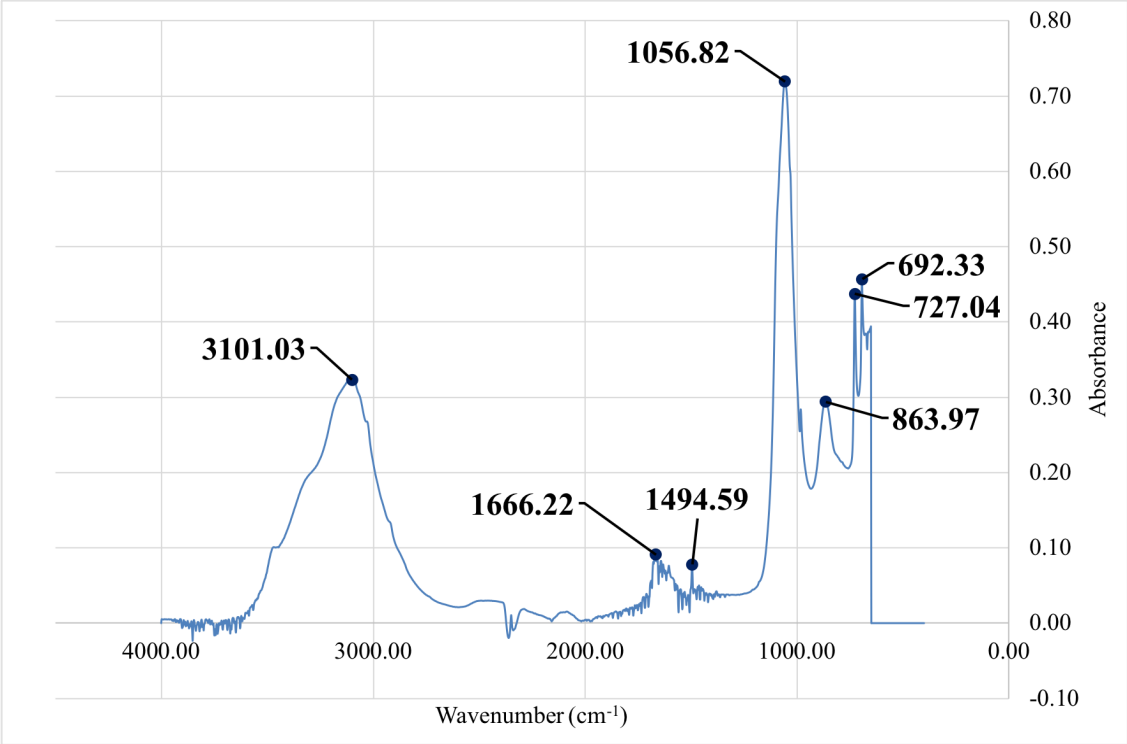

Figure 8: FTIR spectrum of toluene.

The FTIR spectrum of toluene in Figure 8 shows a significant peak at 3108 cm^{-1}, with an absorbance of 0.3, which is indicative of C-H stretch in a carbonyl aromatic ring (Asemani, M., & Rabbani, A. R., 2015). The peaks at 1664 cm^{-1} and 1494 cm^{-1} with absorbances of 0.1 correlate to the literature value associated with C=C stretches in an aromatic ring (Wilt, B. K., Welch, W. T., & Rankin, J. G., 1998). The significant peaks between 730 cm^{-1} and 1000 cm^{-1} with absorbances of 0.4 and 0.7 are indicative of in-plane and out-of-plane C-H bending.

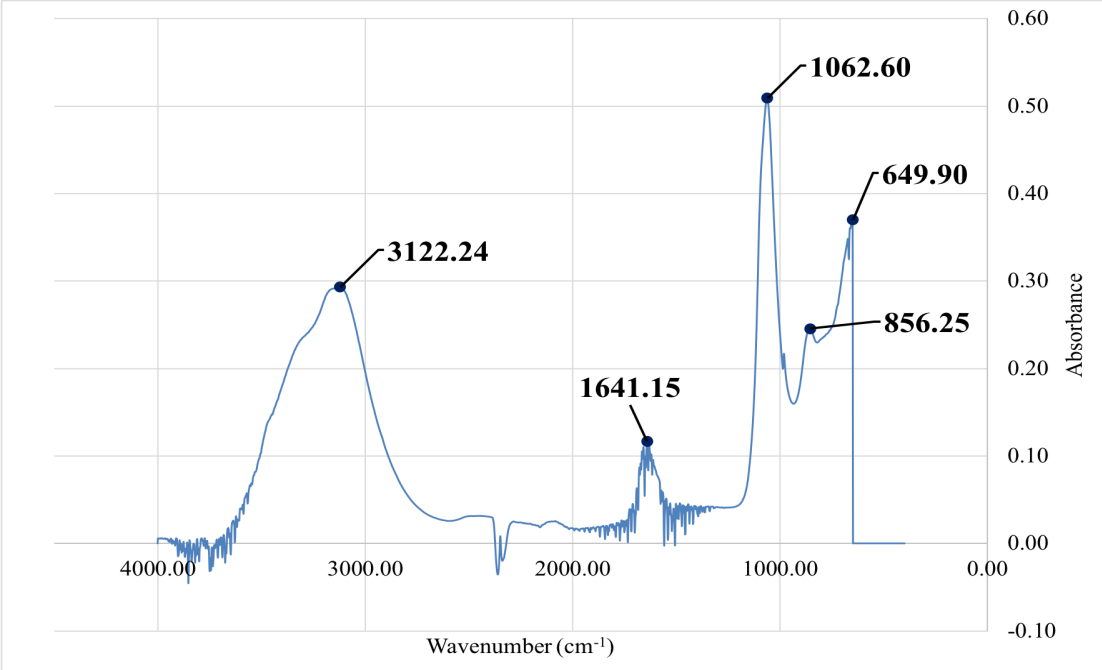

Figure 9: FTIR spectrum of chloroperoxidase enzyme solution, 1 vol% PBS solution.

The FTIR spectrum of chloroperoxidase, n-heptane, and PBS solution in Figure 9 shows a significant peak at 3130 cm^{-1} with an absorbance of 0.30, which is indicative of C-H bonding in aromatic compounds (Asemani, M., & Rabbani, A. R., 2015). The peak at 1648 cm^{-1}, with an absorbance of 0.10, corresponds to C=C bonding in aromatic rings. The peaks between 650 cm^{-1} to 1060 cm^{-1} indicate S=O bonds in sulfoxides.

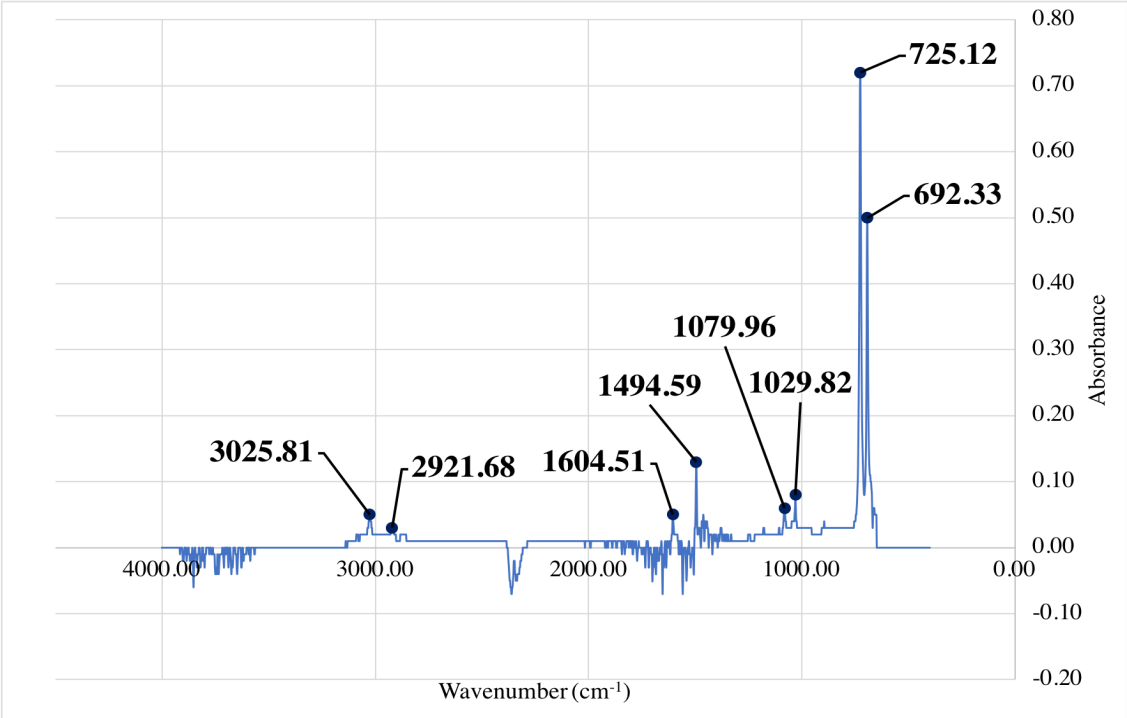

Figure 10: FTIR spectrum of chloroperoxidase/asphaltene reaction mixture, composed of chloroperoxidase enzyme, 1 vol% PBS solution, 0.100 g of asphaltene, and toluene solution.

In Figure 10, the peaks at 3000 cm^{-1}, corresponding to C-H bonding in aromatics, are reduced from 0.15 and 0.17 absorbance in asphaltene to 0.01 absorbance in the presence of chloroperoxidase (Fedorak, P. M., Semple, K. M., Vazquez-Duhalt, R., & Westlake, D. W., 1993). The C=C bonding present at 1600 cm^{-1} in asphaltene is reduced from 0.05 absorbance to 0.02 absorbance in the presence of chloroperoxidase (Wilt, B. K., Welch, W. T., & Rankin, J. G, 1998). After chloroperoxidase application, a peak remains at 1494 cm^{-1} with a slight reduction in absorbance from the asphaltene absorbance in this region, which is also consistent with the absorbance of toluene in this region.

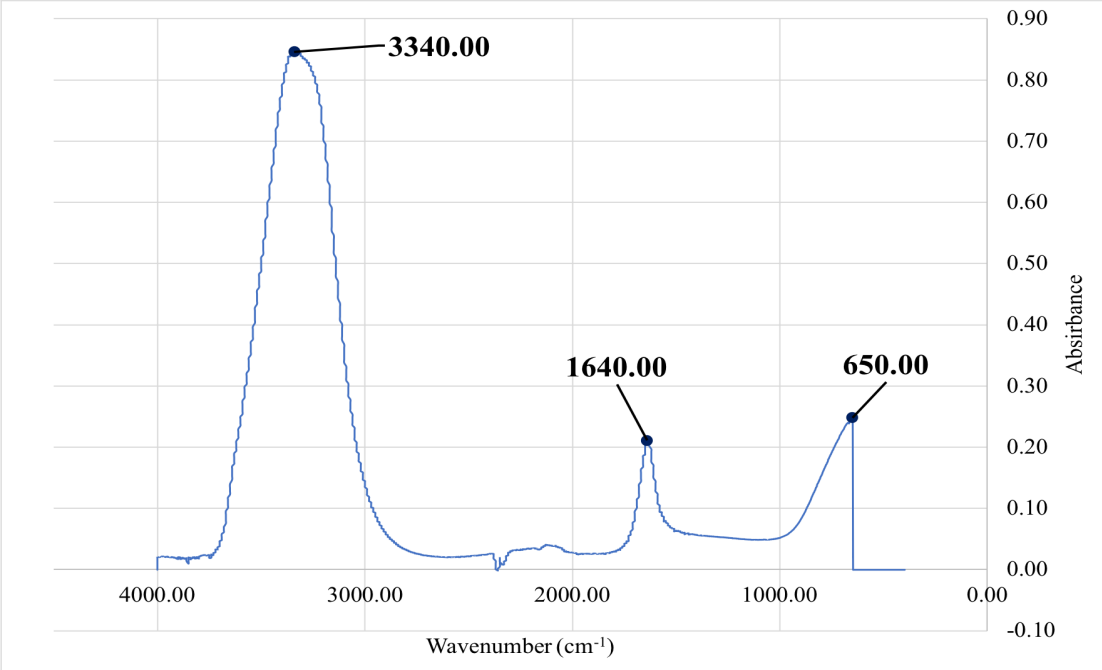

Figure 11: FTIR spectrum of laccase enzyme solution, 1 vol% PBS solution.

Peaks of significance in the FTIR spectrum shown in Figure 11 include a broad peak at 3317 cm^{-1} with an absorbance at 0.82, indicative of a C-H of aromatics, C-H of phenols, or the presence of N-H shifting the peak more downfield. The peak at 1602 cm^{-1} with an absorbance of 0.2 is likely indicative of C=C stretches in aromatics, shifted slightly downfield due to the presence of nitrogen (Wilt, B. K., Welch, W. T., & Rankin, J. G, 1998).

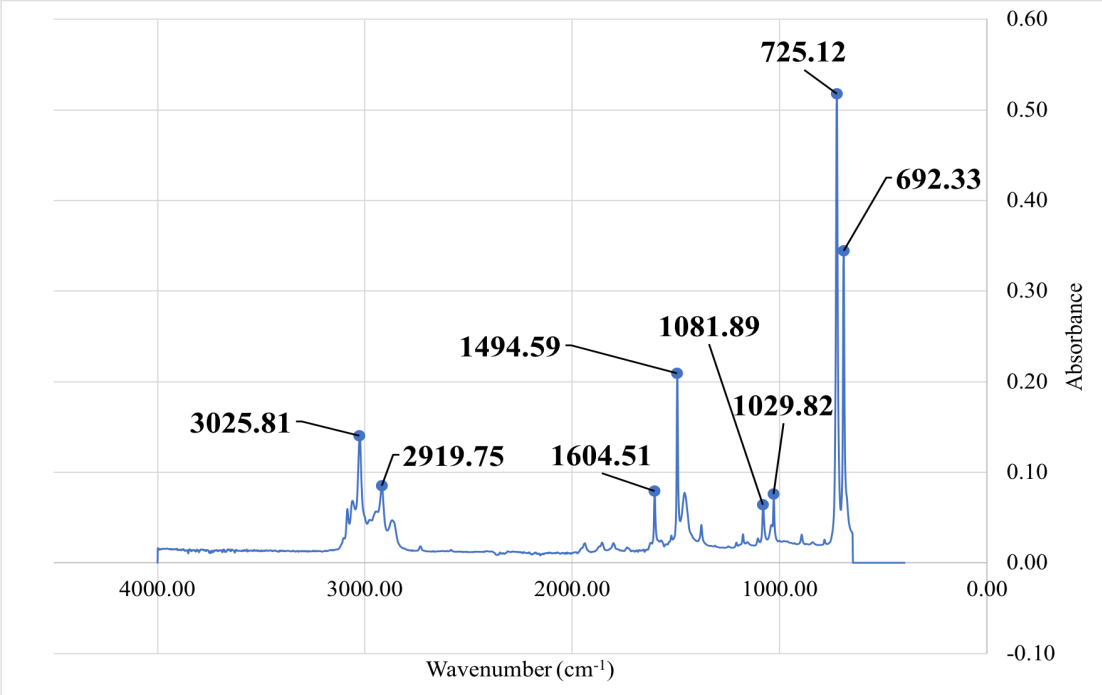

Figure 12: FTIR spectrum of laccase/asphaltene reaction mixture, composed of laccase enzyme, 1 vol% PBS solution, 0.100 g of asphaltene, and toluene solution.

The absorbance in the region of 3000 cm^{-1} shows a reduction in absorbance of C-H bonding in aromatics, a decrease from the asphaltene sample (Fedorak, P. M., Semple, K. M., Vazquez-Duhalt, R., & Westlake, D. W., 1993). There is still a peak present around 1500 cm^{-1} with an absorbance of 0.2, which was also present in C=C stretching of the laccase enzyme, as well as carbonyl stretching in toluene (Wilt, B. K., Welch, W. T., & Rankin, J. G, 1998). The peak at 725 cm^{-1} is indicative of out-of-plane C-H bending, which was also present in the toluene sample.

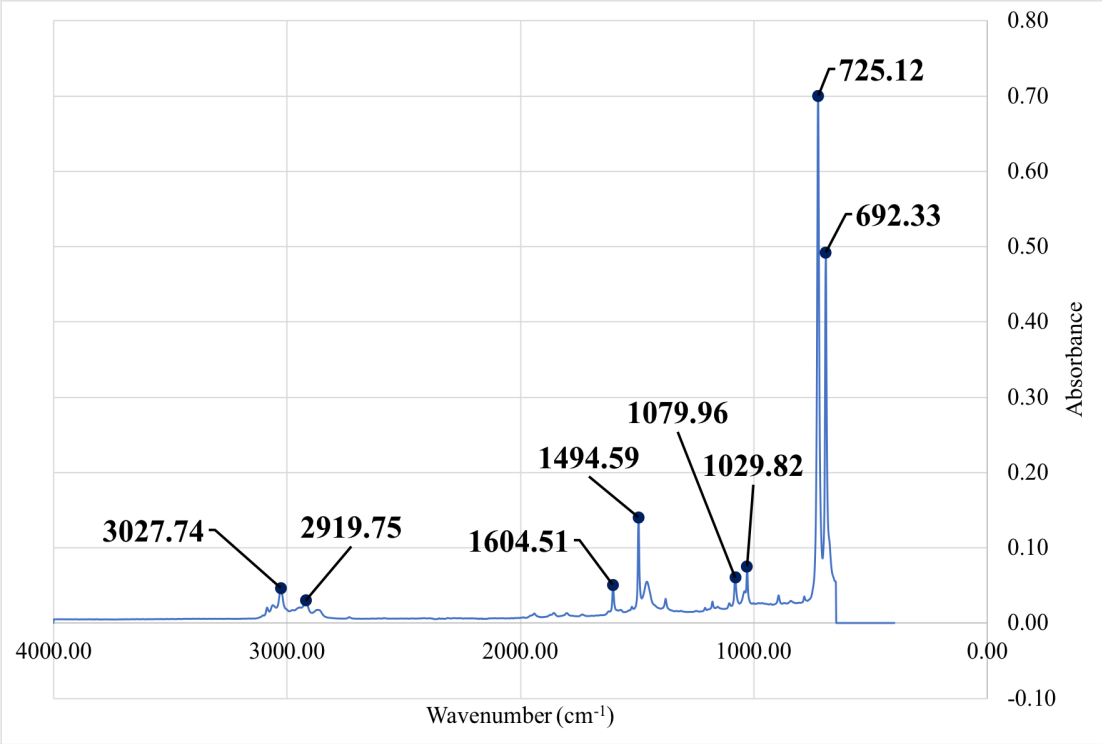

Figure 13. FTIR spectrum of laccase and chloroperoxidase/asphaltene reaction mixture, composed of 1:1 laccase to chloroperoxidase enzymes, 1 vol% PBS solution, 0.100 g of asphaltene, and toluene solution.

Each of the significant peaks associated with carbonyl aromatics are reduced in this FTIR spectrum when asphaltene is in the presence of both enzymes. The reduction of the C-H bending associated with aromatics at 3000 cm^{-1} is reduced to 0.04 absorbance (Asemani, M., & Rabbani, A. R., 2015). The C=C aromatic ring bonding is reduced to an absorbance of 0.05, decreased from the asphaltene sample. The peaks associate with C=C stretches in aromatic ring or C-H stretching in toluene at 1500 and 725 cm^{-1} are still present, indicative of toluene still being present in mixture.

Chapter 5: Discussion

Analysis of Flocculation Curves

Based on ANOVA F-statistical analysis, enzymatic treatment of asphaltene demonstrated a significant reduction in flocculation when compared to pure asphaltene ($p<0.0001$). Chloroperoxidase-treated asphaltene exhibited a strong decrease in flocculation. The addition of laccase exhibited similar decreases in flocculation, but not as drastic (see Fig 12). The combination of laccase and chloroperoxidase did not exhibit a significant reduction of flocculation in comparison to the single enzyme trials, indicating that the combination of enzymes did not produce a synergistic effect on asphaltene flocculation. Furthermore, the use of a combination reaction mixture did not reduce asphaltene flocculation in a nonlinear manner when compared to the single enzymes. This is not consistent with part one of the proposed hypothesis; however, FTIR analysis of the enzymatically treated asphaltene provided further insight into the mechanisms by which these enzymes degraded asphaltene.

Analysis of FTIR

FTIR analysis of the asphaltene treated with laccase showed a 1.3-fold decrease in the presence of aromatic groups which corresponded to the peaks at 2919 and 3025 nm, shown in Figure 14. Analysis of samples containing chloroperoxidase, as well as the combination of the enzymes, showed a 4-fold decrease in the presence of aromatic groups as seen in peaks at 2920 and 3020 nm.

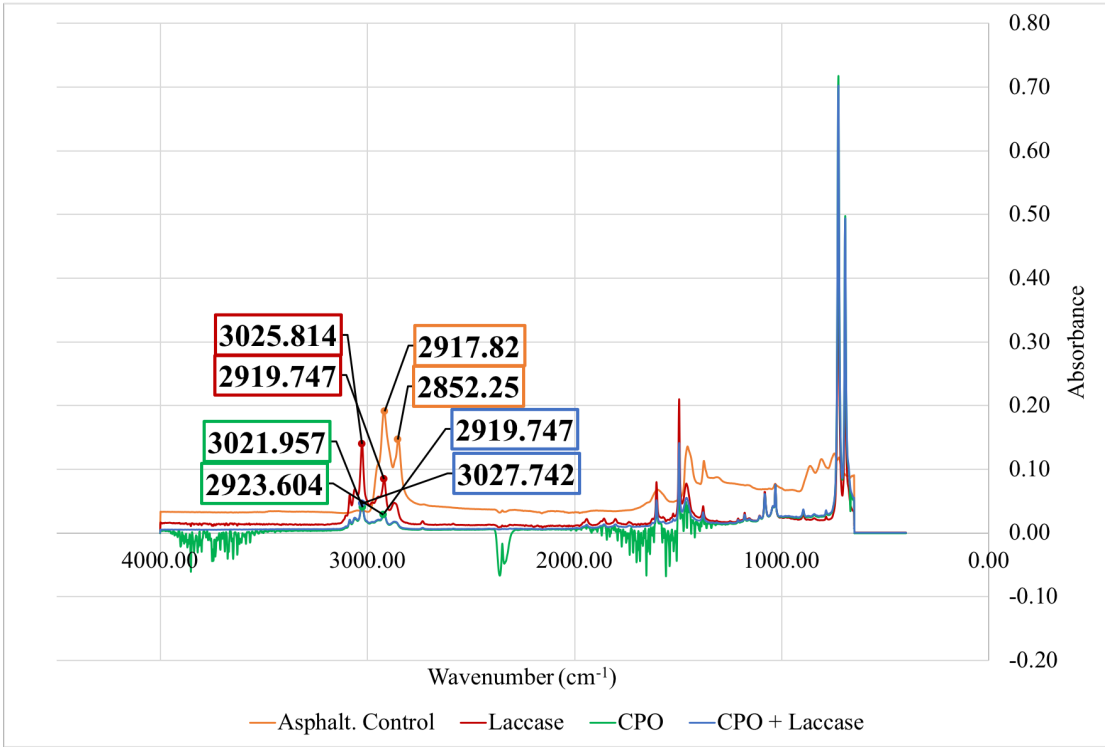

Figure 14. FTIR spectra of pure asphaltene and asphaltene samples treated with laccase, chloroperoxidase, and a combination of both enzymes.

The decrease in aromatic groups is consistent with the mechanism of these two enzymes. In the proposed mechanism, the oxidation of phenol groups by lacasse results in fewer CH bonds in aromatic groups. Similarly, the formation and opening of epoxides by chloroperoxidase reduces the pi bonds in aromatic groups. The proposed mechanism indicates that a combination of these reactions will result in the formation of ketones and alcoholic groups, which can explain the shift seen in the aromatic peaks from the original FTIR analysis of asphaltene.

The difference in aromatic peak intensity between lacasse and chloroperoxidase can be explained by the composition of the Canadian asphaltene. Canadian asphaltene contains 2.60 wt% oxygen, and based on the known structure of asphaltene, these oxygens are part of the alcohol substrate with which lacasse reacts (Gharbi, Benyounes,

& Khodja, 2017). Additionally, Canadian asphaltene contains 83.60 wt% carbon (Gharbi, Benyounes, & Khodja, 2017). Many of these carbon atoms are found in aromatic groups that provide double bonds necessary to form epoxide rings in the chloroperoxidase reaction (Gharbi, Benyounes, & Khodja, 2017). FTIR analysis of the combination of these two enzymes showed similar results to the FTIR analysis obtained solely from the chloroperoxidase trial, which is attributed to the aforementioned availability of the specific substrate for each enzyme.

The peaks in the chloroperoxidase-asphaltene reaction mixture spectrum are significantly diminished compared to those of the asphaltene and toluene controls spectra. These smaller peaks represent the disruption of sites with significant pi-conjugation when chloroperoxidase and laccase are added to the reaction mixtures. This indicates that the chloroperoxidase enzyme is breaking down the asphaltene. Treatment with chloroperoxidase enzyme resulted in a 4-fold decrease in aromatic groups in the asphaltene samples. Similar results were found in the spectra of the enzyme combinations and asphaltene reaction mixture (see fig. 2). However, the spectrum of the laccase-asphaltene reaction mixture only demonstrates a 1.3-fold decrease in aromatic groups (see fig. 2). Since the combination of enzymes produced the same reduction in aromatic peaks as the chloroperoxidase, it can be concluded that the effect of laccase on asphaltene is not sizable enough to justify the use of the combination to degrade asphaltene. The combination of enzymes does not produce a considerably greater degradation of asphaltene than the single enzymes. Thus, the use of single enzymes to degrade asphaltene structures is demonstrably more efficient than the use of a combination.

Confounding Variables

The complex structure of asphaltene is a major confounding variable in the experimentation. Asphaltene is a family of compounds that does not possess a single, uniform structure (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012). Because of this, there is not a single definitive model of asphaltene molecules (Tavassoli, Mousavi, Shojaosadati, & Salehizadeh, 2012). Each asphaltene molecule contains a common base structure, but can vary in functional group placement and size of carbon chains and aromatic ring structures. The highly variable nature of asphaltene composition makes generalizing the results of our enzymatic treatment of asphaltene to the treatment of any asphaltene challenging. We tested each volume of *n*-heptane in triplicate to ensure that the variability of asphaltene did not drastically affect asphaltene recovery, and the standard error values for each trial were consistently low (see Appendix B). Still, the reproducibility of these experiments is crucial to the validity of our results.

Chapter 6: Conclusion

Asphaltene flocculation causes a multitude of issues in pipelines that transport both crude and refined oil products, and can lead to disastrous environmental consequences when the flow of oil is impeded or interrupted by pipeline blockages or ruptures. Current methods for dealing with this issue use costly machinery and chemical processes, which bring about negative externalities to the oil transportation process. This research has shown that enzymatic treatment of asphaltene can significantly reduce asphaltene flocculation due to the ability of the enzymes to alter the chemical structure of asphaltene.

Before embarking on lab experimentation, the team developed the following hypotheses:

1. The enzyme treatments will reduce asphaltene aggregation through an enzyme-catalyzed mechanism.
2. A mixture of enzymes will decrease asphaltene aggregation more effectively compared to singular enzymes.
3. The enzymes treatments will reduce asphaltene aggregation by disrupting PAHs through oxygenation. Therefore, after treatment, the asphaltene sample should have more functional groups containing oxygen and fewer less aromatic rings.

Based on the results of the flocculation tests and FTIR analysis of asphaltene samples and statistical analysis of these results, we concluded that a mixture of laccase and chloroperoxidase does not more significantly degrade asphaltene than does singular enzymes. While chloroperoxidase greatly contributed to the reduction of asphaltene flocculation and presence of aromatic groups, the addition of laccase did not significantly

reduce asphaltene flocculation or the presence of aromatic groups in recovered asphaltene. Therefore, the application of a mixture of laccase and chloroperoxidase in a 1:1 ratio to asphaltene is no more efficient than the application of solely chloroperoxidase to asphaltene. The FTIR spectra of enzymatically-treated asphaltene samples and untreated asphaltene samples demonstrate that laccase and chloroperoxidase oxidized phenols and aromatic rings in asphaltene, which confirms our second hypothesis that more functional groups containing oxygen would be present in the recovered, enzymatically-treated asphaltene. Furthermore, the FTIR analysis are consistent with the proposed mechanism (see fig. 5). Our analysis of the FTIR spectra characterizes the chemical pathway through which enzyme-mediated asphaltene degradation occurs. Such analysis has not been conducted previously and offers scientists a basis for further exploration of the aforementioned chemical pathway. Finally, since we did not apply our enzyme treatments to heavy crude oil, we cannot conclude that the decreased asphaltene flocculation as a result of enzyme treatment would translate to reduced asphaltene aggregation in heavy crude oil. However, since we observed a significant reduction in the flocculation of enzymatically-treated pure asphaltene, and asphaltene should behave the same way in heavy crude oil, we can infer that the degradation of asphaltenic compounds would manifest as decreased asphaltene aggregation in heavy crude oil. Furthermore, since the literature indicates that asphaltene aggregation is the primary contributor to the blockage of heavy crude oil flow in pipelines, we can infer that the enzymatic treatment of heavy crude oil would decrease asphaltene aggregation, thereby improving the flow of heavy crude oil through pipelines and potentially reducing the risk of pipeline bursts.

In the future, exploring the addition of other classes of enzymes, such as oxygenases, would be valuable in determining an optimal enzymatic mixture for the reduction of asphaltene flocculation. Testing combinations of enzymes at varying ratios would also be a worthwhile endeavor, as our experimentation only tested laccase and chloroperoxidase in a 1:1 ratio. Since chloroperoxidase reduced asphaltene flocculation more than laccase, a mixture that contains more chloroperoxidase than laccase may be more effective. Additionally, a study that tested the kinetics of the reaction would help to refine the proposed mechanism. The current methodology utilizes a batch process where both enzymes are added at once. Alternatively, the enzymes could be added in series to observe how it may affect which functional groups are targeted first and therefore reduce flocculation.

Our research endeavored to characterize the chemical pathway of enzyme-mediated asphaltene degradation and determine the chemical composition of enzymatically-treated asphaltene. While implementing an enzymatic treatment on heavy crude oil on a larger scale was beyond the scope of this project, we hope that our research lays the foundation to make this possible and improve pipeline transportation in the future.

Appendix A: Statistical Analysis

ANOVA and Tukey's Procedure were performed using SAS.

The ANOVA Procedure

Class Level Information		
Class	**Levels**	**Values**
Treatment	**4**	A (no enzyme) B (CPO) C (Laccase) D (CPO+Laccase)

Number of Observations Read	32
Number of Observations Used	32

Source	**DF**	**Sum of Squares**	**Mean Square**	**F Value**	**Pr > F**
Model	3	0.6271	0.2090	15.35	<.0001
Error	28	0.3813	0.0136		
Corrected Total	31	1.008			

R-Square	**Coeff Var**	**Root MSE**	**Recovery Mean**
0.6219	63.7307	0.1167	0.1831

Tukey's Studentized Range (HSD) Test for Recovery.

Note: This test controls the Type I experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	28
Error Mean Square	0.0136
Critical Value of Studentized Range	3.8612

Comparisons significant at the 0.05 level are indicated by ***.				
Treatment Comparison	**Difference Between Means**	**Simultaneous 95% Confidence Limits**		
A - D	0.2957	0.1351	0.4562	***
A - B	0.3107	0.1559	0.4655	***
A - C	0.3237	0.1689	0.4785	***
D - A	-0.2957	-0.4562	-0.1351	***
D - B	0.0150	-0.1499	0.1799	
D - C	0.0280	-0.1369	0.1929	
B - A	-3.0107	-0.4655	-0.1559	***
B - D	-0.0150	-0.1799	0.1499	
B - C	0.0130	-0.1463	0.1723	
C - A	-0.3237	-0.4785	-0.1689	***
C - D	-0.0280	-0.1929	0.1369	
C - B	-0.01230	-0.1723	0.1463	

Appendix B: Raw Data

Overall Summary and Comparison							
***n*-heptane Volume**	**Control**	**Chloroperoxidase**	**Standard Error**	**Laccase**	**Standard Error**	**Chloroperoxidase + Laccase**	**Standard Error**
100	3.67%	6.25%	2.52%	7.09%	0.0164	7.52%	0.0555
200	18.67%	18.91%	9.86%	5.74%	0.0095	5.96%	0.0407
300	34.00%	15.73%	3.25%	1.26%	0.0090	2.24%	0.0124
400	52.00%	8.75%	3.75%	1.98%	0.0080	10.33%	0.0318
500	44.00%	8.06%	7.21%	18.97%	0.1154	17.82%	0.0976
550	64.70%	0.59%	0.59%	4.41%	0.0150	11.58%	0.0533
700	49.70%	6.69%	0.74%	19.81%	0.0867	22.05%	0.0698
800	43.00%	-	-	-	-	-	-
900	56.00%	11.56%	1.17%	6.90%	0.0309	-	-

Chloroperoxidase Summary				
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
100 mL	Trial 1:	0.11	0.005	4.55%
	Trial 2:	0.125	0.014	11.20%
	Trial 3:	0.1	0.003	3.00%
			Average:	**6.25%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
200 mL	Trial 1:	0.113	0.002	1.77%
	Trial 2:	0.103	0.037	35.92%
	Trial 3:	0.105	0.02	19.05%
			Average:	**18.91%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
300 mL	Trial 1:	0.1034	0.013	12.57%
	Trial 2:	0.108	0.024	22.22%
	Trial 3:	0.105	0.013	12.38%
			Average:	**15.73%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
400 mL	Trial 1:	0.1174	0.004	3.41%
	Trial 2:	0.1021	0.007	6.86%
	Trial 3:	0.1063	0.017	15.99%
			Average:	**8.75%**

		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
500 mL	Trial 1:	0.1114	0.025	22.44%
	Trial 2:	0.1153	0.002	1.73%
	Trial 3:	0.1039	0	0.00%
			Average:	**8.06%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
550 mL	Trial 1:	0.1135	0.002	1.76%
	Trial 2:	0.1102	0	0.00%
	Trial 3:	0.1062	0	0.00%
			Average:	**0.59%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
700 mL	Trial 1:	0.098	0.008	8.16%
	Trial 2:	0.116	0.007	6.03%
	Trial 3:	0.102	0.006	5.88%
			Average:	**6.69%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
900 mL	Trial 1:	0.1077	0.012	11.14%
	Trial 2:	0.1017	0.014	13.77%
	Trial 3:	0.1022	0.01	9.78%
			Average:	**11.56%**

Chloroperoxidase and Laccase 1:1 Summary				
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
100 mL	Trial 1:	0.116	0.002	1.72%
	Trial 2:	0.102	0.019	18.63%
	Trial 3:	0.135	0.003	2.22%
			Average:	**7.52%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
200 mL	Trial 1:	0.107	0.015	14.02%
	Trial 2:	0.109	0.001	0.92%
	Trial 3:	0.102	0.003	2.94%
			Average:	**5.96%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered

300 mL	Trial 1:	0.106	0.005	4.72%
	Trial 2:	0.100	0.001	1.00%
	Trial 3:	0.100	0.001	1.00%
			Average:	**2.24%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
400 mL	Trial 1:	0.100	0.014	14.00%
	Trial 2:	0.100	0.013	13.00%
	Trial 3:	0.100	0.004	4.00%
			Average:	**10.33%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
500 mL	Trial 1:	0.1237	0.008	6.47%
	Trial 2:	0.1047	0.039	37.25%
	Trial 3:	0.1027	0.01	9.74%
			Average:	**17.82%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
550 mL	Trial 1:	0.1263	0.028	22.17%
	Trial 2:	0.1352	0.01	7.40%
	Trial 3:	0.1158	0.006	5.18%
			Average:	**11.58%**
		Asphaltene Mass (g)	Asphaltene Recovered (g)	% Recovered
700 mL	Trial 1:	0.105	0.0118	11.24%
	Trial 2:	0.104	0.0365	35.10%
	Trial 3:	0.103	0.0204	19.81%
			Average:	**22.05%**

Appendix C: Glossary

All citations are IUPAC Compendium of Chemical Terminology: Gold Book, unless specified

Nič, M., Jirát, J., Košata, B., Jenkins, A., & McNaught, A. (Eds.). (2009). *IUPAC Compendium of Chemical Terminology: Gold Book* (2.1.0 ed.). Research Triangle Park, NC: IUPAC.

1. Aggregates: material or structure formed from a loosely compacted mass of fragments or particles

2. Asphaltene: A heterogeneous compound that contains polycyclic aromatic hydrocarbon rings (PAHs) and heteroatoms such as nitrogen, oxygen, or sulfur atom. They are characterized as being soluble in toluene and insoluble in *n*-alkanes. Asphaltene aggregates are the heaviest and most polar fractions found in oil (Leon & Kumar, 2005; Mullins, 2010)

3. Beer-Lambert Law: A linear relationship between absorbance and concentration of a species. The general Beer-Lambert Law is written as $A= \varepsilon * l * c$. The constants as represented in this equation are A (measured absorbance), ε (molar absorptivity), l=path length, and c (concentration)

4. Bitumen: A black viscous mixture of hydrocarbons composed of aromatics and asphaltene fractions

5. Enzymatic Cascades: A process similar to a signalling pathway in which a series of activation reactions involving enzymes are performed

6. Catalytic Hydrogenation: A process in which molecular hydrogen (H_2) is added, usually in the presence of a catalyst, in order to saturate organic compounds (Leon &

Kumar, 2005)

7. Denaturation: A process in which enzymatic folding structures are altered due to exposure to chemical or physical factors, causing the enzymes to become biologically inactive

8. Flocculation: The process by which individual particles of a substance aggregate into masses or clumps

9. Fossil Fuel: Hydrocarbons - primarily coal, petroleum oil, and natural gas - formed from the
remains of dead plants and animals

10. Greenhouse Gases: Gases that absorb infrared radiation and radiate heat. Examples of greenhouse gases include carbon dioxide (CO_2), sulfur dioxide (SO_2), and nitrogen oxides (Nabavi-Pelesaraei et al., 2014)

11. Hydrophobic: The inability of a compound to mix or dissolve with liquid water

12. *In situ*: In its original position

13. Intermolecular interactions: Weak forces of attraction and/or repulsion that occur between atoms, molecules, and ions

14. Lyophilized: A process in which substances are freeze dried

15. Metalloprotein enzyme: An enzyme that, in the active state, contains one or more metal ions which are essential for its biological function

16. Naphthenic acid: Monocarboxylic acids derived from naphthenes, or cycloalkanes and their alkyl derivatives

17. Nonrenewable Resource: A resource that does not renew itself at a sufficient rate for sustainable extraction in human time

18. Peripheral Paraffins: Saturated hydrocarbons

19. Renewable Resource: A resource that is naturally replenished in human time

20. Resins: Highly viscous substances that typically contain prepolymers

21. Rheological: The study of flow and deformation of materials under applied forces

22. Shear Stress: Force acting tangentially to a surface divided by the area of the surface

23. Steric Hindrance: The prevention of intermolecular and intramolecular interactions due to crowding of substituents

24. Thermal Cracking: A process in which hydrocarbons are subjected to high temperature to break molecular bonds so that smaller hydrocarbons can be extracted

25. Unit of Chloroperoxidase Activity: The amount of enzyme that converts 1 μmol of monochlorodimedone to dichlorodimedone per minute at pH 2.75 and 25 °C in the presence of KCl and H_2O_2

26. Unit of Dioxygenase Activity: The amount of enzyme that oxidizes 1.0 μmole of protocatechuate to 3-carboxy-cis,cis-muconate per min at pH 7.5 at 37 °C

27. Unit of Laccase Activity: The amount of enzyme that converts 1 μmol of catechol per minute at pH 5.0 and 25 °C

28. Upgrading: A process in which heavy oil is improved or raised to a higher standard of oil, light oil, in order to make the crude oil more stable (Zhang, 2014)

29. Viscosity: For a laminar flow of a fluid, the ratio of the shear stress to the velocity gradient perpendicular to the plane of shear

30. Volatile Organic Compounds (VOCs): organic molecules that have a high vapor pressure at room temperature

31. Zero Discharge Policy: A policy implemented by industrial organizations in order to prevent release of any toxic or harmful chemicals and materials into the environment (Quagraine, Peterson, & Headley, 2005)

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