Developing enzyme and biomimetic catalysts for upgrading heavy crudes via biological hydrogenation and hydrodesulfurization.

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Summary:
The recovery and conversion of heavy oils is limited due to the high viscosity of these crudes and their high heteroatom content. Conventional technology relies on thermochemical hydrogenation and hydrodesulfurization to address these problems and is energy intensive due to the high operating temperature and pressure. This project was initiated to explore biological catalysts for adding hydrogen to the heavy oil molecules. Biological enzymes are efficient at hydrogen splitting at very mild conditions such as room temperature and pressure, however, they are very specific in terms of the substrates they hydrogenate. The goal of the project was to investigate how the specificity of these enzymes can be altered to develop catalysts for oil upgrading. Three approaches were used. First was to perform chemical modification of the enzyme surface to improve binding of other non-natural substrates. Second approach was to expose the deeply buried catalytic active site of the enzyme by removal of protein scaffolding to enable better interaction with other substrates. The third approach was based on molecular biology to develop genetically engineered systems for enabling targeted structural changes in the enzyme. The first approach was found to be limited in success due to the non-specificity of the chemical modification and inability to target the region near the active site or the

site of substrate binding. The second approach produced a smaller catalyst capable of catalyzing hydrogen splitting, however, further experimentation is needed to address reproducibility and stability issues. The third approach which targeted cloning of hydrogenase in alternate hosts demonstrated progress, although further work is necessary to complete the cloning process. The complex nature of the hydrogenase enzyme structure-function relationship and role of various ligands in the protein require significant more research to better understand the enzyme and to enable success in strategies in developing catalysts with broader specificity as that required for crude upgrading.

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Problem description: The processing of heavy oils is currently plagued by two major problems, one involving presence of asphaltenes, which increases oil viscosity and secondly, the heteroatom content which poses corrosion and complex refining problems. Technology to process heavy crudes in order to reduce viscosity and heteroatom content under mild conditions is therefore necessary. Biological processing has the unique advantage of low temperature and pressure operation and high specificity. Addition of hydrogen to aromatic and heteroatom molecules via a biological route can be a potentially attractive alternative to upgrade heavy crudes. In nature, hydrogen is added to simple compounds such as sulfate and oxygen as well as complex compounds such as aldehydes, aryl sulfonates [1] and biological mediators using hydrogenase enzymes [2]. The enzymes existing naturally have been evolved to convert compounds which are encountered in nature and not designed to attack hydrocarbons. In order to make the biological enzymes work with compounds in crude oil, it is necessary to create favourable binding interactions between the oil substrates and enzymes. Further, to address the broad substrate specificity of compounds in oil, it is necessary to make the biocatalyst binding sites flexible to accommodate the variety of compounds.

Experimental approach: Hydrogenation biocatalysts can potentially be developed for upgrading crude oils via chemical and structural modification of hydrogenase enzymes. These enzymes typically have a transition-state metal (Fe, Ni, Mo, Co) at the active site responsible for hydrogen activation. These same metals are also the catalytic sites of chemical hydrogenation and hydrodesulfurization catalysts. Further, the structure of some of the enzyme metal active sites are also similar to those found to be efficient hydrodesulfurization (HDS) catalysts, e.g., a Co-Fe-Ni cubane structure [3]. Simplistically, hydrogen addition to a substrate takes place via three steps, first is the hydrogen activation step followed by the transfer of the active species (e.g. hydride) to the substrate's hydrogenation site and lastly addition of the active H species to the substrate. Our approach was targeted to the last step and to try to improve the enzyme-substrate interaction without altering the enzyme's intrinsic hydrogen activation mechanism.

To achieve this goal, three different methods were used. In order to catalytically hydrogenate a substrate, it is first necessary to enable binding of the substrate to the catalyst. Each of the methods were designed to develop a biocatalyst suitable for binding substrates present in oils such as unsaturated and sulfur containing hydrocarbons. The three methods consisted of 1. chemical modification of hydrogenase, 2. protein digestion to obtain catalytic active center, 3. cloning of hydrogenase to develop a genetic

modification system. The report below is divided into three sections based on the experimental details and results for each of these three approaches.

Section 1. Chemical modification of hydrogenases:

Experimental methods and techniques:

In the approach to chemically modify hydrogenases, the difficulty of coupling the enzyme with the hydrophobic substrates in oil was addressed by modifying the surface of the enzyme to make it more hydrophobic and less hydrophilic. Reducing the polarity or increasing the hydrophobicity of the enzyme's substrate docking site was expected to enable improved binding with the substrate. A survey conducted by Karlin [4] suggested that changes in the labile group coordinated to the Fe of Ni, Fe_xS_y cluster in hydrogenase enzymes can impact substrate binding. Previous work at ORNL with hydrogenase enzymes has shown up to 10-fold increase in activity of the enzyme in toluene solvent by chemical attachment of hydrophobic entities such as polyethylene glycol (PEG). Attachment with alkyl and benzyl groups was conducted using established techniques to alter the hydrophobicity of the docking site in the vicinity of the outermost iron-sulfur cluster (Figure 2).

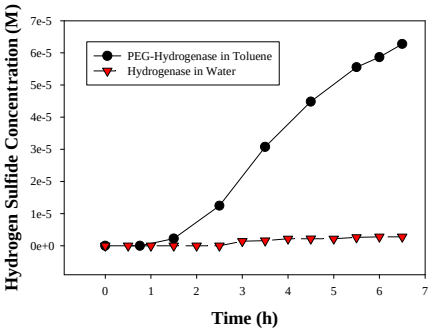


Figure 1. Activity of modified hydrogenase for hydrogenation of sulfur in toluene.

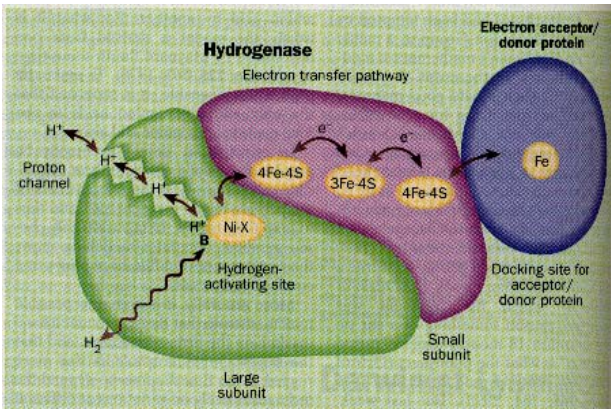


Figure 2. The relative positions of various metal ligands in hydrogenase from *D. gigas*.

Assessment of hydrogenation activity:

Assessment of enzyme activity was conducted via standard hydrogen uptake assay (De Lacey and et. al., 2000) to determine activity of enzyme after modification. To assess activity with diffferent substrates, model compounds such as dibenzothiophene and polyaromatic hydrocarbons were used. The assay was conducted with two enzymes, hydrogenase from *Desulfovibrio gigas* and *Pyrococcus furiosis* and their modified derivatives. The modification was done by two methods: attachment of polyethylene glycol and alkyl groups such as ethyl and propyl groups at lysine residues on the enzyme. The enzyme assay to determine substrate-enzyme interaction was done in 10-20 % acetonitrile solvent with balance being Tris buffer. The activity of the enzyme in organic-

aqueous mixtures was assessed using standard assays and found to be about 50% in the 20% acetonitrile The assays were conducted in sealed glass containers using hydrogen in headspace at 30^{o}C. The concentration of the substrates was measured over a 2-24 hour period. The conversion of the substrates was assessed by HPLC.

The results showed that the interaction between the substrate and the modified enzymes was not effective enough for hydrogenation of these substrates to occur. Thelimitation of this approach was soon realized. The residues where the chemical modification takes place is not resulting in changing the binding site of the enzyme. It was observed that even if residues close to the active site were available, the ability to engineer the active site binding for interaction with multiple substrates would be difficult. What was therefore, needed was a catalyst which would be able to activate hydrogen and hydrogenate substrates via the reducing potential of the active hydrogen or a hydride ion. Such an approach was believed to be more successful in developing a catalyst for oil application. Thus, the attention was focussed on this approach, as described in the next section.

Section 2. Protein digestion to isolate catalytic active site

The active site of hydrogenase enzyme (E.C. 1.12) consisting of Ni-Fe (or Fe-Fe) center is situated deep within the enzyme. In the following work, we have explored with the idea of isolating the Ni-Fe active center-peptide complex from the hydrogenase enzyme from the sulfate reducing bacteria, *Desulfovibrio gigas* and to demonstrate the capability of this complex to function as a catalyst in hydrogenation reactions. A partially purified enzyme was digested with a non-specific protease, proteinase K (Sigma, lyophilized powder) and the peptide containing the active center was purified by ultrafiltration and size exclusion chromatography. The details of the isolation procedure are given in Figure 3.

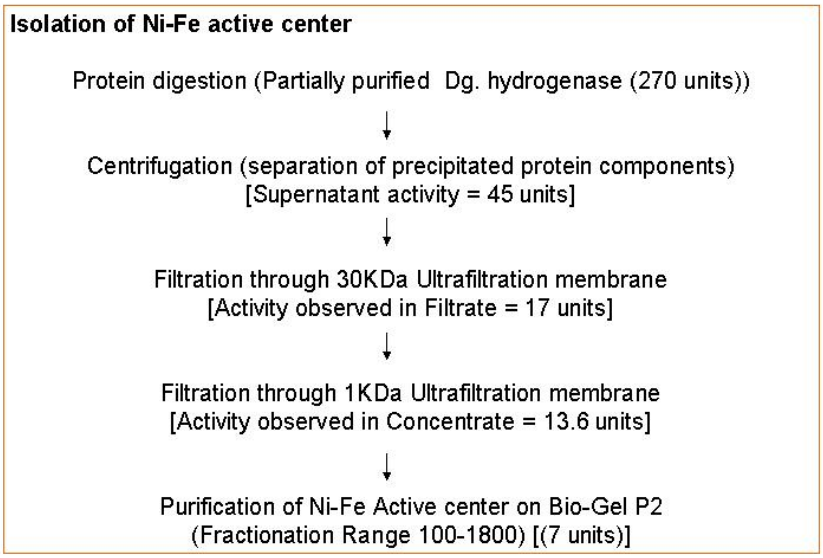


Figure 3: Protocol for isolation of Ni-Fe active center-peptide complex.

Active center characterization:

The concentrate obtained from the 1 kDa membrane was analyzed by ICP and found to contain 3.27 mg/L nickel and 11.6 mg/L iron. Further, based on the ultrafiltration results, the molecular weight of the complex is expected to be between 1211 and 30,000. The lower estimate is based on complete digestion by the protease enzyme (Proteinase K) used for digestion. The structure of the complex based on complete digestion is given in Figure 4. The biochemical formula for the complex given in Figure 2 is Ni-X-Fe](I-S_{Cys})(T- S_{cys}-V-G- S_{cys})(V-G- S_{Cys})$(CY)_2$(SO), where X = S or O; Y = O or N.

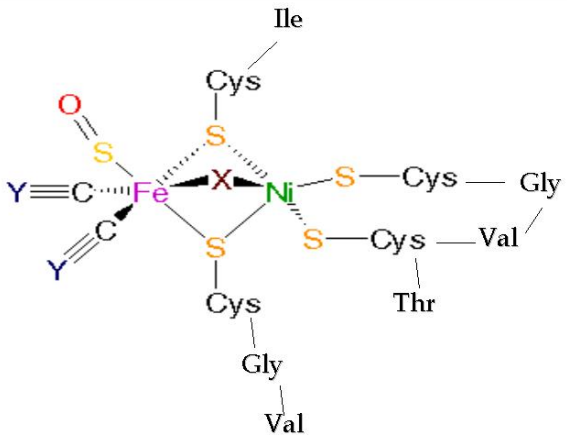


Figure 4: Sturcture of Ni-Fe active center-peptide complex based on complete digestion of the hydrogenase enzyme.

Activity assessment:

The active center-peptide complex was found to be able to reduce substrates such as benzyl viologen, methyl viologen and sulfur. The activity for measurement of benzyl and methyl viologen activity is as follows.

Hydrogen uptake assay: (using benzyl/methyl viologen)

· 2 mL (hydrogen saturated) 20 mM Tris buffer pH 7.6, in a spectrophotometer cuvette + 1 mM benzyl viologen + 2 mM sodium dithionite

· 10 mL solution of Ni-Fe active center in Tris buffer activated by hydrogen bubbling for 10 minutes (corresponds to 0.03 units of activity as defined for *D. gigas* hydrogenase)

· Absorbance measurement at 604 nm

Hydrogen uptake assay:

· 2 mL, 20 mM Tris buffer, pH 7.6 (deaerated with nitrogen), in a spectrophotometer cuvette + 1 mM benzyl viologen + 30 mM sodium dithionite

· 10 mL solution of Ni-Fe active center in Tris buffer (corresponds to 0.03 units of activity as defined for *D. gigas* hydrogenase)

Sulfur reduction activity:(Conversion of sulfur to hydrogen sulfide)

- 5 mL 20 mM Tris buffer, pH 7.6 + 1 mM sodium dithionite + 1 mM benzyl viologen + 0.1 g sublimed sulfur
- Saturate with hydrogen (bubble for 5 min) 5 psi head pressure
- Incubation tempeparure: 30°C and 60°C with shaking at 175 rpm
- Incubation Time: 48 hours.

Activity results:

Hydrogen uptake assay

Activity with Benzyl viologen at 30^{o}C: 1.36 units/mL

Activity with methyl viologen at 30^{o}C and 10% acetonitrile : 0.19 units/mL

Hydrogen uptake assay

No hydrogen was produced by the standard hydrogenase assay.

Sulfur reduction activity

30°C: H_2S produced : 2.5 ± 0.15 % (headspace concentration)

60°C: H_2S produced : 1.8 ± 0.20 %

Control, 30°C : H_2S produced : 0.00 %

Control, 60°C : H_2S produced : 0.00 %

The complex also showed superior thermo-stability as compared to the parent enzyme. The results are shown in Figure 5.

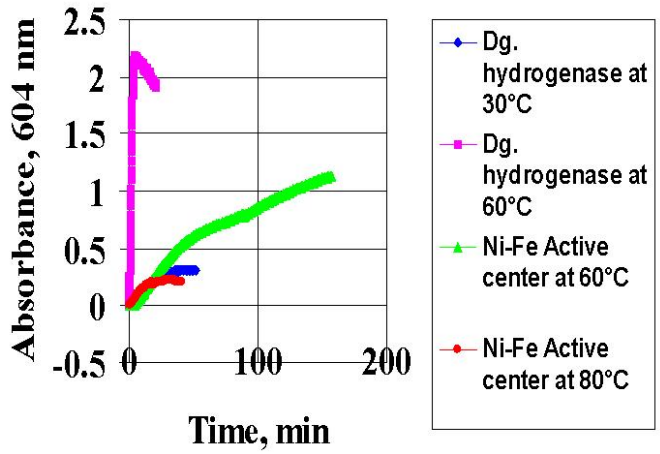


Figure 5. Thermostability of Ni-Fe active center complex at various temperatures as compared to the hydrogenase enzyme. The complex was found to be stable at 60°C for more than three hours.

Effect of solvent on activity:

The Ni-Fe active center-peptide complex was also found to retain significant activity in the presence of 10% solvent (ethanol and acetonitrile). Almost 100% activity was observed in 10% acetonitrile, while about 50% activity was observed in 10% ethanol. This suggests potential for use of the complex in organic phase biocatalysis.

In conclusion, a Ni-Fe active center-peptide was isolated from *D. gigas* hydrogenase after protein digestion. The isolated Ni-Fe center-peptide demonstrated hydrogen uptake activity via benzyl viologen, methyl viologen and sulfur reduction, but no hydrogen evolution activity. The hydrogen uptake activity was observed up to 90°C, with a maximum at 80°C. The isolated peptide also showed higher thermostability then the native enzyme at 60°C. These results demonstrate that the Ni-Fe active center-peptide complex can serve as a superior catalyst for carrying out hydrogenation reactions, compared with any enzymes or other catalysts available currently.

Further work on development of the Ni-Fe active site catalyst was pursued by trying to obtain a larger amount of the catalyst. However, this proved to be difficult. The yeild of the active site from the digestion of the hydrogenase enzyme was not sufficient to conduct more detailed analysis such as X-ray diffraction or improved metal and protein analysis. Additional work in this are is necessary to identify and reproduce the conditions to isolate the active site from the hydrogenase. The problem is obtaining sufficient enzyme from the cells. Due to the small amounts of enzyme expressed in most of the bacteria producing hydrogenase, this becomes a limiting step in pursuing further work. Secondly, significant time and effort was spent in obtaining even nanogram quantities of the catalyst, thus these problems need to be addressed prior to developing a program to investigate the various conditions for digestion and characterization of the catalyst.

Experiments in collaboration with ChevronTexaco:

In addition to the three approaches discussed in this report, work was also conducted in collaboration with ChevronTexaco to assess a fourth alternative. In this alternative, a combined chemical + biological catalysis was investigated for achieving mild conditions for hydrogenation. Studies were conducted using hydrogenase enzyme and a HDS catalyst together to study the synergy of the two catalysts. Tyically, high temperatures and pressures are required for HDS catalysts to perform the hydrogenation reactions. Since hydrogenase enzymes carry out hydrogen activation at mild conditions, the idea was to see if the hydrogen activated by hydrogenase can be transferred to the HDS catalyst which can then bind with the substrate to perform hydrogenation. Initial work was carried out in aqueous-organic mixtures with the two catalysts. However, no conversion was obtained for the substrate, dibenzothiophene used in the study. The HDS catalyst was used in pellet as well as powder form, however, no hydrogenation was observed. The HDS catalysts used in the study were provided by ChevronTexaco. The configuration and interfacing of the two catalysts is thought to be critical in development of an active formulation for the reaction to occur. Additionally, use of 100% solvent was thought necessary for the catalyst to work in conjunction. However, the scope of this project could not be widened to include such parametric testing. Further work targeted at using chemically-modified enzymes active in organic solvents and doing focussed studies along with structural analysis of the formulations can potentially yield positive results with this approach.

Section 3. Expressing hydrogenase in *E. coli*

In this approach, the goal was to express the hydrogenase from P. furiosis in other hosts such as E. coli to enable a genetic system to alter the structure of the enzyme. This approach would also allow production of sufficient quantities of the hydrogenase enzyme for further work along the lines of the results in Section 2. Expression of hydrogenase in E. coli was conducted by Dr. Mike Adams at the University of Georgia, Athens via a subcontract.

Results from University of Georgia, Athens

Overall Goal:
To obtain an active recombinant form of the soluble hydrogenase 1 (HYD1) from the hyperthermophilic archaeon, *Pyrococcus furiosus,* using the bacterium *E. coli* as the host. The long term goal was to ultimately use the results to design a "minimal" soluble hydrogenase with tailored catalytic activity, oxygen sensitivity and electron donor specificity.

The rationales for recombinant expression within *E. coli* are 1) the *hyp* genes are highly conserved based on homology searches, 2) *Pfu* alpha subunit is 28% identical / 44% similar to several E. coli large subunits and 3) the established genetic system for manipulation of hydrogenases

Background:
It is the enzyme hydrogenase that is responsible for the metabolism of molecular hydrogen and hydrogen evolution in nature. The general enzymatic functions of hydrogenases are to catalyze the oxidation of molecular hydrogen or its production from protons and electrons (1). This process is a reversible reaction as expressed by:

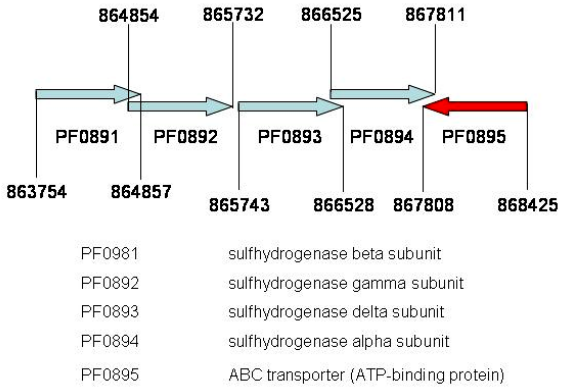
$$H2 \leftrightarrow 2H+ + 2e-$$

There are two major classes in which these enzymes are classified based on their active site cofactor: Ni-Fe and Fe-only hydrogenases (1). An active Fe center with CO and CN coordination is an unusual characteristic shared between these two evolutionarily unrelated types of hydrogenases. The operon being studied within *Pyrococcus furiosus* contains a Ni-Fe center as its active site. So far, there has been no efficient way to recombinantly express Hydrogenase I for industrial use or on a large scale.

Specific Aim 1: To aerobicially express a recombinant form of the entire Hydrogenase I operon from *Pyrococcus furiosus* using the bacterium *E. coli* as the host.

Operon Structure:

Hydrogenase 1 Operon = 4057bp



The cytoplasmic Hydrogenase I has been successfully PCRed as a multiple open reading frame (mORF) giving rise to the entire Hydrogenase I operon. The PCR method has been partially optimized for the amplification of Hydrogenase I mORF. The mORF originally contained a 5' BamHI restriction site and a 3' NotI restriction site for cloning purposes. The 5' end primer was redesigned to incorporate a Sma1 site in place of the BamH1 because PF0892 contains 2 BamH1 sites. With the new primers, the mORF was again successfully PCRed. Using Structural Genomics effort at UGA protocol for cloning, I used pET24dBamH1 as the vector for further cloning. With all attempts yielding no positive transformations, I switched vectors and protocol to the StrataPrep PCR-Script Amp Cloning Kit (cat#: 211190) from Stratagene based on conversations with experienced lab members with previous experience with my situation/ experiment.

Following the protocol from the kit, the PCR product was gel purified and polished using *Pf* polymerase to rid the product of the overhang *Taq* polymerase creates during amplification. From there, it was blunt ended cloned into Srf I-digested pPCR-Script Ampt SK(+) cloning vector. A blue-white selection screen was additionally used, along with ampicillin antibiotic resistance when transforming the vector in XL Gold Kan ultracompotent cells. After many attempts, there was no successful transformation of the plasmid containing the insert into any host strain of *E. coli*.

The focus therefore shifted to the large subunit (PF0894) with the aim of achieving metal insertion within the active site and catalytic activity.

The experimental scheme is as follows:
1. His-tagged aerobic expression with aerobic / anaerobic purification
2. Aerobic-anaerobic switch expression of PF0894 with anaerobic purification
3. Development of anaerobic expression system

1. His-tagged Aerobic Expression with Aerobic / Anaerobic Purification

A his-tagged PF0894 was obtained by aerobic expressed but the purified protein was devoid of proper metal incorporation in the active site; it contained only 0.27 zinc atoms per monomer of protein. This left two questions to be answered:

1. Did the metals fall out during aerobic purification?
2. Is PF0894 being processed in *P. furiosus*?

Thus, the expression of the alpha subunit was repeated using aerobic and anaerobic purification techniques. PF0894 was ligated into pET24Bam and transformed into *E. coli* strain JM109(DE3)pRIL instead of BL21(DE3)pRIL as the Structural Genomics effort had previously done because of the lower hydrogenase evolution activities had been reported for BL21(DE3) strain. The recombinant protein was expressed in 1 liter cultures, lyzed, heat treated (80°C for 30 minutes) and passed through a Ni-NTA affinity and a size exclusion column.

From these experiments, it was found that the his-tagged alpha subunit was heat stable at 80°C for 30 minutes. The following table details the results from the aerobic / anaerobic purification attempts from aerobically grown *E. coli* cells.

Sample	Expected Mass* (Da)	Observed Mass* (Da)	Expected Metal Content (per monomer)	Observed Metal Content (per monomer)
Aerobically Purified	49223	49223	1 Fe 1 Ni	0.07 Fe 0.22 Zn
Anaerobically Purified	49223	49223	1 Fe 1 Ni	0.07 Fe 0.25 Zn

* All Proteins were aerobically expressed with a 6 histidine tag for purification purposes

The purified protein was devoid of metal insertion and there was no processing of carboxy-terminus in the *Pfu* cytosolic Ni-Fe hydrogenase large subunit. This was to be expected due to the oxygen-sensitive nature of the enzyme and enzyme's maturation assistance from the *hyp* genes from *E. coli*. Since the culture was aerobically induced, the native *E. coli* hydrogenases would not have been turned on along with the *hyp* genes needed for the maturation of the recombinant protein.

Is there processing of carboxy-terminus of the *Pfu* cytosolic Ni-Fe hydrogenase large subunit as in other homologous Ni-Fe hydrogenases? It has been reported that a must for C-terminal processing is the incorporation of the nickel into the active site (Theodoratou *et al.*, 2005).

Native Processing of Alpha Subunit of HYD 1

D. gigas sequence **TVHSYDPCIACGVH VIDPESNQVHKFRI**
E. coli HYD1 sequence **TLHSFDPCLACSTH VLGDDGSELISVQVR**

P. furiosus PF0894 (Predicted) **VVRAYDPCISCSVH VVRL**

P. furiosus PF0894 **VVRAYDPCISCSVHV VRL**

The underlined amino acids represent the highly conserved c-terminal nickel binding motif found within all Ni-Fe hydrogenases. The amino acids highlighted in red represent

the conserved cleavage site of the alpha subunit from Ni-Fe hydrogenases between a highly conserved histidine residue and a semi-conserved valine residue.

Result: It was experimentally determined that the *Pfu* cytosolic Ni-Fe hydrogenase large subunit from Hydrogenase 1 (HYD1) was c-terminally processed, but the oligopeptide cleaved was 3 amino acids in length instead of the predicted 4 amino acids.

Predicted total cleaved peptide molecular weight: 500.54
Experimental cleaved peptide molecular weight: 399.01
Difference = 101.53

Experimental cleaved peptide molecular weight is 2.45 Da too small.

Amino Acid Molecular Weights as a peptide:
Valine (99.08)--Valine (99.08)--Arginine (156.18)--Lysine (146.2) = 500.54
Valine (99.08)--Arginine (156.18)--Lysine (146.2) = 401.46

Conclusion: The native PF0894 subunit is processed by an endopeptidase as in other Ni-Fe hydrogenases. Previous publications report c-terminal processing only after the required nickel has been inserted in the active site (Theodoratou *et al.*, 2005). If the *hyp* genes are necessary for metal insertion, then the next logical step would be to induce these proteins under their particular conditions. Under anaerobic conditions the *hyp* genes would be transcriptionally turned on and could act upon the recombinant protein and aid in metal insertion.

Specific Aim 2. To Carry Out an Aerobic-Anaerobic Switch For Expression of Active Recombinant Hydrogenase Obtained Using an Anaerobic Purification Procedure

For this goal, both cytosolic large subunits (PF0894 and PF1332) from *Pfu* Ni-Fe hydrogenases were ligated into pET24Bam and transformed into *E. coli* strain JM109(DE3)pRIL. In both cases, the recombinant protein was expressed in 1.2 liters of rich media (LB) at 37°C to OD_{600} of 0.6. At this point, the culture is induced with 0.4M IPTG for a one hour (shaking at 100 rpm or static culture at 37°C). After one hour, the cultures are slowly poured into Wheaton bottles until volume reached 4 centimeters from the top to minimize aerobic conditions. Then, the cultures were briefly agroned/ de-gassed to remove any excess oxygen. 0.2-0.5% glucose and 30mM formate were added to induce the native *E. coli* hydrogenase-3 operon. This operon is the most active of the hydrogenases within *E. coli and* is easily detected by a hydrogen evolution assay. The culture was allowed to turn anaerobic over a 4.5 hour period. Cells were harvested and purified anaerobically. Anaerobic purification is defined as lysis in the presence of 2mM DT, heat treatment (80°C for 30 minutes), passage through a Ni-NTA affinity column performed in an anaerobic chamber (non reducing, but in an anaerobic environment) and then passage down a size exclusion column with 2mM DT and DTT.

Result: Expression of both subunits was successful, yet both yielded no cytoplasmic hydrogenase activity.

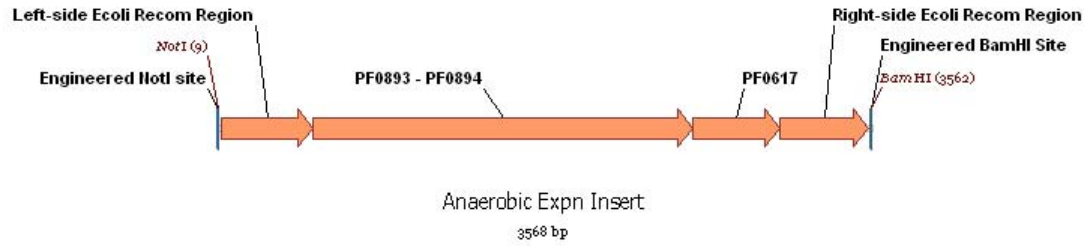
Specific Aim 3. To design an anaerobic expression system for *P. furiosus* hydrogenase

Plasmid expression of PF0893-PF0894 (small and large subunit of HYD1) / entire *Pyrococcus furiosus* HYD1 operon (PF0891-PF0894) behind *E. coli hya* promoter

Placement of recombinant insert on a plasmid should be the first step. Once on the plasmid, strains can be tested to optimize the growth and expression of the recombinant proteins along with their maturation. Once a particular strain has been found to be suitable, the recombinant proteins will be inserted into the strains chromosome using double recombination (Hamilton *et al*, 1889; Link *et al*., 1997). The two recombinant inserts consist of:

Left-side homologous region with E. coli (includes *hya* promoter)
PF0893 (small subunit of HYD1)
PF0894 (large subunit of HYD1)
PF0617 (hycI homolog – endopeptidase)
Right-side homologous region with E. coli

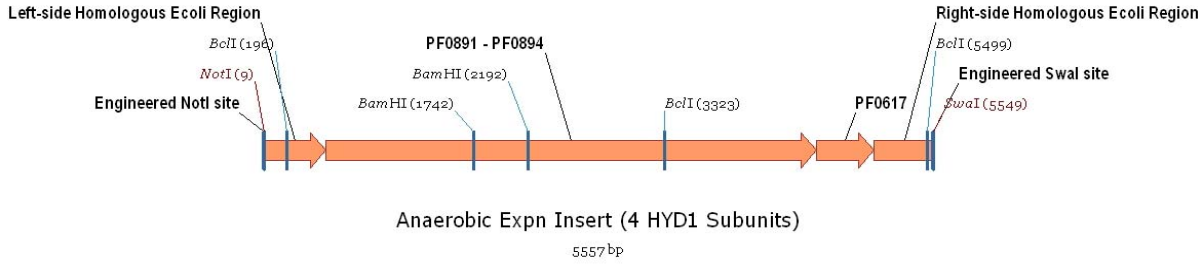
NOTE: The left side *E. coli* Recombinant Region contains the *hya* promoter. Engineered restriction site at 5' side (NotI) and at the 3' side is an engineered BamHI site.



Or

Left-side homologous region with E. coli (includes *hya* promoter)
PF0891 (Beta subunit of HYD1)
PF0892 (Gamma subunit of HYD1)
PF0893 (small subunit of HYD1)
PF0894 (large subunit of HYD1)
PF0617 (hycI homolog – endopeptidase)
Right-side homologous region with E. coli

NOTE: The left side *E. coli* Recombinant Region contains the *hya* promoter. Engineered restriction site at the 5' side (NotI) and an engineered 3' SwaI restriction site.



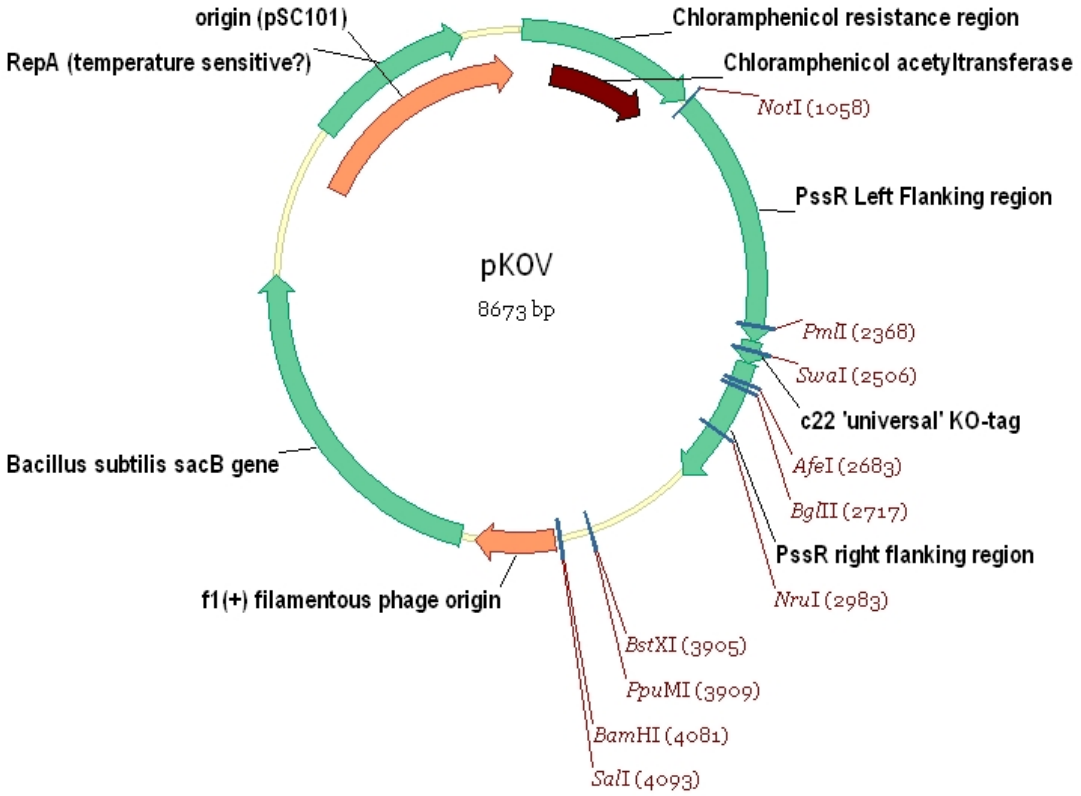
We chose to use the *hya* promoter for expression based on the more resistant nature of *hya* operon under slightly aerobic conditions (still transcriptionally active), a wider range for the pH optimum (6 to 8), the ability to enhance growth by the addition of formate and maybe glucose, and its ability to express during E. coli's stationary phase.

The expression of PF0893-PF0894 should not be under a constitutive promoter (eg. Em7) because a) the expression should be controlled b) we have already shown that metal insertion and processing does not occur aerobically, and thus, would be a wasted effort to have it expressed when the native hydrogenases and *hyp* genes are not active.

Recombinant expression will be accomplished in the absence of the pRIL plasmid. The purpose of the plasmid is to compensate for the bacterial rare codons that some archaeal transcripts require. The absence of the pRIL plasmid might be viewed as a detriment to expression, but in my case, quite the opposite. The restriction on expression might reduce the local concentration of hydrogenase transcripts requiring the assistance of the *hyp* genes along.

Should the host strain be wild-type or a mutant strain defective in specific hydrogenases? The benefit of using a hydrogenase mutant for plasmid/ chromosomal recombinant expression is that under anaerobic conditions the *hyp* genes would be turned on, which in the absence of the native hydrogenases, would be in a higher concentration to act upon the recombinant proteins.

The construct will be done without a his-tag to prevent additional obstacles during



folding and metal insertion.

Plasmid: pKOV as described in Link *et al*., 1997.

Progress:
To date we have completed all of the initial PCR products needed to start cross-over PCR experiments. The next step will be to attempt to use the initial PCR fragments as templates to create a fused PCR product of the two template PCR fragments.

Summary of hydrogenase cloning work:
1. Alpha subunit of hydrogenases I has been successfully aerobically expressed in monomeric form.
   Alpha subunit is heat stable (80°C for 30 min).
   Appears to be expressed devoid of metals.
   Reconstitution attempts are currently being attempted

2. Aerobic-anaerobic switch expression with anaerobic purification of alpha subunit from HYD 1 yielded pure protein devoid of metal insertion

3. Anaerobic expression system is currently being developed

Project conclusions

The goal of this project was to investigate development of biological catalysts for hydrogenation and hydrodesulfurization. Three approaches were followed to achive this goal. The first approach was based on chemical modificationof enzymes to change the active site for enabling binding of oil substrates. However, the limitations of this approach in addressing the broad specificity needs for a oil hydrogenating catalyst were identified early in the study, leading to alternate improved ideas for catalyst development. In the second approach, the target was to determine the feasibility of isolating the active site from the hydrogenase enzyme. The results from this approach show promise, however, various parameters affect the activity , stability and yield of the active site. Thus, significant work is needed to develop such methodologies further. The third approach was based on developing a genetic system for expression of hydrogenase enzyme, which would then allow molecular approaches to alter and design the specificity of the enzymes for oil upgrading via hydrogenation or hydrodesulfurization.

Note: Argonne National Laboratory was a third partner in the project, however, no contribution was received from them to the report.

Impact to the Industry

Biological hydrogenation technology can result in substantial energy as well as economic savings to the petroleum industry due to the mild/moderate operating conditions, minimization of hydrogen usage due to controlled specificity of catalyst and reduction in material of construction cost. This technology can potentially use the available refinery equipment (e.g., hydrogenation reactors) enabling retrofitting of existing infrastructure. A field/pipeline or downhole application may be feasible if viscosity reduction under mild conditions is realized.

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