Production and Engineering of Hydrogenase as a Biocatalyst for Hydrogen Fuel

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Objectives

1) *Genetically manipulate indigenous hydrogenase biosynthetic pathway in Escherichia coli;*
2) *Heterologously produce the regulatory hydrogenase (RH) from Ralstonia eutropha using synthetic biology approaches;*
3) *Improve catalytic activities of RH using gene-shuffling and random mutagenesis;*
4) *Produce stable hydrogenase as biocatalyst for hydrogen fuel cell.*

Technical Barriers

1) *Heterologous production of hydrogenase;*
2) *Engineering and production of stable hydrogenase;*
3) *Application of hydrogenase in hydrogen fuel cell;*
4) *Hydrogen production in microbiological systems.*

Abstract

Hydrogenases are fascinating redox proteins, showing tremendous promise in hydrogen fuel as a bioelectrocatalyst. They play critical roles in both biohydrogen production and hydrogen oxidation. In particular, the recent establishment of the comparability of the oxidative activity of the active site of [NiFe] hydrogenase being comparable to that of a platinum fuel cell catalyst marks a significant milestone for the potential application of hydrogenase in fuel cells to replace platinum. However, the ability of producing hydrogenase in heterologous expression hosts and the sensitivity of hydrogenases to oxygen and carbon monoxide, etc. have seriously limited the potential application of hydrogenases in hydrogen fuel cells, as well as their potential application in hydrogen production.

This research project integrates knowledge of structural biology, molecular biology, and principles of metabolic engineering to produce and engineer a stable hydrogenase as a bioelectrocatalyst for hydrogen fuel. In particular, the proposed work tackles issues of "expensive noble metal catalysts (e.g. platinum) and their limited reserves threatening the long-term sustainability of a hydrogen economy"; it also provides essential knowledge for "design artificial materials and enzyme catalyst" for "efficient and cost-effective technologies" for a clean and sustainable energy future.

There are three key aspects for the proposed research. First, based on our current molecular and genetic knowledge of hydrogenase maturation mechanisms and pathways, we engineer hydrogenase maturation pathways and Escherichia coli strains for the production of hydrogenases from *Ralstonia eutropha*. It presents the first example of producing hydrogenase in the conventional expression host using modern metabolic engineering principles and tools. Also, for the high production yield of hydrogenase, protein degradation pathways are altered to prevent hydrogenase degradation. This part of the proposed work will provide a frame work for the design of hydrogenase production pathways for desirable bioengineering purposes. The results of this part research will be significantly beneficial to research in the areas of enzyme fuel cells, bioelectrocatalyst production, and biohydrogen production as well as basic research in microbial hydrogen production.

The second aspect of the proposed research focuses on improving electrochemical and catalytic properties of the stable hydrogenase. With guidance from current knowledge of [NiFe] hydrogenase structure information, this part of the proposed research applies protein engineering approaches to generate hydrogenase mutants and mutant libraries. The research will use high-throughput methods to screen for hydrogenase with desirable enzymatic traits and will provide comprehensive examination of the effects of different mutations on electrochemical and catalytic properties of the hydrogenase. This part of the research will potentially result in new hydrogenase mutants with improved catalytic properties and will greatly enrich our knowledge of the catalytic mechanism of hydrogenase.

The third aspect of the proposed research uses current fermentation and genetic strategies to optimize the production yield of the selected hydrogenase mutants. We will balance metabolic burdens and change growth and induction conditions for maximum production of the engineered hydrogenase in genetically engineered *E. coli* strains. This presents a good example of the application of modern fermentation technologies in bioelectrocatalyst production.

Overall, the proposed research integrates techniques and knowledge of multiple scientific disciplines to address technical issues and basic scientific questions, which are critical to hydrogen economy. In particular, the results of the proposed work will advance our current technologies of hydrogenase as a bioelectrocatalyst in hydrogen fuel. The resulting materials can be readily used for the research of enzyme fuel cells and photobiological hydrogen production as well as basic research for hydrogenases and biohydrogen production in the Hawaii Natural Energy Institute at UH Manoa and other researchers in the scientific community. The proposed work not only carries significant intellectual merit, but also casts broader impacts on renewable energy research in the private sector and the community.

Progress Report and Future Directions

Funding for the project arrived in April, 2006. Since then, we have made efforts to reach goals of the proposed research. To express heterologus hydrogenase, we have engineered a hydrogenase-null *E. coli* strains (GW1234). Crude extract resulted from the strain GW1234, which harbor *hoxBC* and *hyp* genes of *Ralstonia eutropha*, displayed up-take hydrogenases activity (Figure 1). SDS-PAGE and western-analysis revealed unbalanced expression level of HoxB and HoxC proteins (Figure 2). Our current efforts have been made to balance the expression level of HoxB and HoxC proteins for the increased expression yield of RH

hydrogenase. To increase the protein production of HoxB and HoxC protein, both *hoxB* and *hoxC* genes have been engineered with strep tag and cloned into pCDFDuet vector. Currently, efforts have been made to optimize induction conditions (temperature and inducer concentration) and to purify HoxB and HoxC proteins. In addition, efforts have been made to construct synthetic operons carrying different accessory genes to understand which accessory genes in *E. coli*, if any, are essential for the production of active RH hydrogenase. Because of the size of these accessory genes (about 10 kb), constructions of synthetic accessory gene operons had slowed down our progress in this project. At this point, synthetic operons, which carry different accessory genes from *R. eutropha*, have been cloned into pCOLADuet. In the near future, pCDFDuet carrying strep-tagged *hoxB* and *HoxC* and pCOLADuet vector harboring different accessory genes from *R. eutropha* will be co-transformed into GW1234 strains for understanding the importance of each accessory gene in the production of RH in *E. coli*. At the same time, to facilitate the heterologous expression of hydrogenases from *R. eutropha*, a cosmid library has been constructed using pWEB vector and DNA of pHG1 megaplasmid. The average insert size of the libraries is approximately 36 kb genes. Clones of the cosmid library will be randomly co-transformed with the hydrogenase-null strain harboring HoxBC proteins. Individual clone, which enhance the production level of hydrogenase in the strain GW1234, will be subject to sequencing analysis for identification of any potential gene with ability to enhance RH production.

To improve the catalytic activity, we started working on mutagenesis of hoxC-strep using the QuikChange Sited directed Mutagenesis Kit (Stratagene). Our efforts have been made to change residues in the regions of element 3 and 4 of the gene. We have finished replacement of Ala101, Leu09, and Phe11 with His, and Pro113 into Leu. Currently, we are in the process of confirming these mutations using sequencing analysis. Once mutations are confirmed, up-take hydrogenase activities of these mutants will be tested in the presence or absence of oxygen. At the same time, we also start generating mutants using error prone PCR and gene-shuffling approaches. In the next a few months, focus will be on mutant screening for this part of proposed work.

Several protease genes, including *rpoH*, *lon*, and *ompT*, have been deleted from the hydrogenase-null strain GW1234. This protease deficient strain is used to express RH hydrogenase. Induction condition (temperature, inducer concentration, and media) and culture volume have been used to improve the production yield of the wild-type and mutant protein of HoxB and HoxC. Finally, this project currently supported one postdoctoral researcher (Dr. Dongping Lu), one graduate student (Ms. Juanita Mathews) and one undergraduate student (Ms. Shoko Kono). Dr. Lu recently received his Ph.D. in Molecular Science and Bioengineering at UH Manoa. His expertise centers in molecular biology and protein biochemistry. Ms Mathews is in her third year of her Ph. D. program in Molecular Science and Bioengineering. Her research interests are metabolic pathway engineering and biohydrogen production. Ms. Kono is a junior in Global Environmental Science and her research interests focus on bioenergy production and global warming.

Publications and patent

1. J. Mathews and G. Y. Wang. Characterization of biohydrogen production in engineered *Escherichia coli* strains. Poster presentation for the 30th Symposium on Biotechnology for Fuels and Chemicals, New Orleans, LA, May 4 -7, 2008.

2. J. Mathews and G. Y. Wang. Potential and perspective of metabolically engineered microbial hosts for hydrogen fuel. Invited talk for International Workshop on Biohydrogen Production Technology, India Institute of Technology, Kharagpur, India, February 07-09, 2008.
3. J. Mathew and G. Y. Wang. Potential of metabolically engineered *Escherichia coli* strains in production of biohydrogen and hydrogenase. Invited speaker for the Pacific Rim Summit on Industrial Biotechnology & Bioenergy, Honolulu, Hawaii, November 14-16, 2007.
4. J. Mathews and G. Y. Wang. Metabolic pathway engineering for hydrogen fuel. International Journal of Hydrogen Energy (in review).
5. G. Y. Wang and Q. Z. Li (2007) Metabolically engineered *Escherichia coli* strain for the production of hydrogen and hydrogenase (PCT/US2007/002778-32-787)

Figures

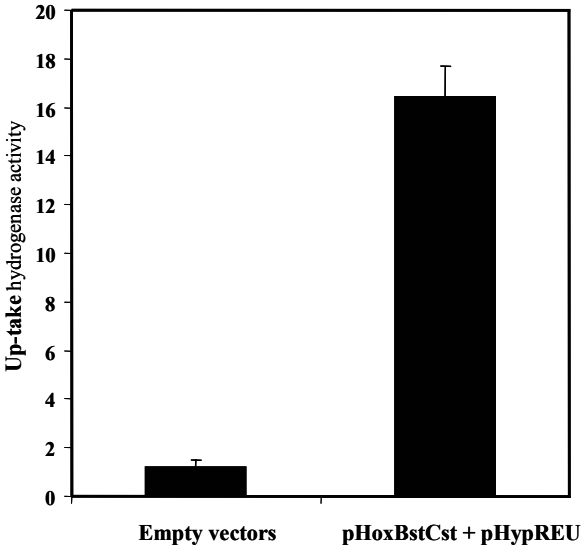

Figure 1. Uptake hydrogenase activiey of heterologously expressed HoxBC proteins in the engineered *E. coli* strains GW1234. Uptake hydrogenase activity was determined using the reduction of methyl viologen. *E. coli* cells were induced for 3 hours at OD 0.6 and 26 °C.

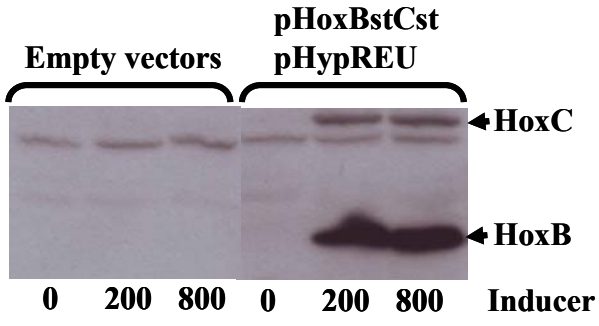

Figure 2. Western blot analysis of heterolously expressed HoxB and HoxC in the hydrogenase-null strain GW1234. GW1234 strain cells containing empty vectors or pHoxBstCst and pHypREU were induced with anhydrotetracycline as inducer at 3 concentrations (pg/L) for 3 hours at OD0.8 and 28 °C.