# DETECTION AND AMPLIFICATION OF PUTATIVE *HYDROGENASE 3* OPERON IN *CITROBACTER* SP. L17

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I would like to dedicate this thesis to my beloved people; my father, my mother, my sisters and brothers for their endless love, prayers and encouragement. Also not forgetting my father, mother and sisters in-law.

My special dedication goes to my husband who always cheering me up and for his patience, sacrifices that he has made for me.

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# ABSTRACT

Our ultimate dependence on fossil fuels will lead to dramatic depletion of these limited resources. Some developed countries have started to use renewable energy, which efficiently utilizes physical or environmental resources. Hydrogen has been expected to be the energy carrier of the future to replace fossil fuels. Hydrogen can currently be produced using many methods, most of which use fossil fuels. Biological hydrogen production is gaining wider interest now due to the cleaner production process. Hydrogenase is among the main enzymes involved in bacterial hydrogen production. Among the four types of hydrogenases reported, hydrogenase 3 is considered as the enzyme responsible for hydrogen production only. The others are involved in hydrogen production and utilization. In this research, *Citrobacter* sp. strain L17 was used. 15 genes in the putative hydrogenase 3 operon of this particular bacterial strain have been successfully detected and amplified. All the clustered genes have been predicted to be coded by 13,456 nucleotides. It has been predicted to be translated into 15 different proteins of 4,485 amino acids. EXPASY TRANSLATE was used to predict the amino acid sequence of the proteins coded by the hydrogenase 3 operon. The results of this study will guide future research of biohydrogen generation by *Citrobacter* sp. L17, in terms of maximizing biohydrogen yield via metabolic engineering.

# ABSTRAK

Kebergantungan mutlak kita kepada bahan api fosil akan membawa kepada pengurangan dramatik sumber-sumber yang terhad.Beberapa negara maju telah mula menggunakan tenaga boleh diperbaharui, yang menggunakan sumber fizikal atau alam sekitar dengan efisien.Hidrogen telah dijangkakan menjadi pembawa tenaga masa depan bagi menggantikan bahan api fosil. Hidrogen kini boleh dihasilkan menggunakan banyak kaedah, yang kebanyakannya menggunakan bahan api fosil.Kini, pengeluaran hidrogen biologi semakin menarik minat ramai kerana proses pengeluaran yang lebih bersih.Enzim hydrogenase adalah antara enzim utama yang terlibat dalam pengeluaran hidrogen oleh bakteria.Antara empat jenis hydrogenase yang dilaporkan, hydrogenase 3 dianggap sebagai enzim yang bertanggungjawab untuk pengeluaran hidrogen sahaja.Hidrogenase jenis lain terlibat dalam kedua-dua pengeluaran hidrogen dan penggunaannya. Dalam kajian ini, *Citrobacter* sp. strain L17 telah digunakan.15 gen dalam operon yang dianggap hydrogenase 3 bagi strain bakteria tertentu ini telah berjaya dikesan dan dibesarkan.Kesemua gen yang dikelompokkan telah diramal untuk dikodkan oleh 13,456nukleotida.Ianya telah diramal untuk diterjemahkan kepada 15 protein yang berbeza daripada 4485 asid amino.EXPASY TRANSLATE telah digunakan untuk meramal jujukan asid amino yang dikodkan oleh operon hydrogenase 3.Keputusan kajian ini akan membimbing penyelidikan masa depan bagi penjanaan biohidrogen oleh *Citrobacter* sp. L17 dari segi memaksimumkan hasil biohidrogen melalui kejuruteraan metabolik.

# TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| °C | - | Degree Centigrade Celsius |
| CN | - | Cyanide |
| CO | - | Carbon monoxide |
| ETBR | - | ethedium bromide |
| Fe-S | - | Iron-Sulfur |
| FHL | - | Formate Hydrogen Lyase |
| HYD-1,2,3,4 | - | Hydrogenase 1,2,3,4 |
| KDa | - | Kilodalton |
| mg | - | Milligram |
| mg/L | - | Milligram/Liter |
| min | - | Minute |
| Ml | - | Milliliter |
| mM | - | Millimol |
| NA | - | Nutrient Agar |
| NiFe | - | Nickel-Iron |
| PCR | - | Polymerase Chain Reaction |
| w/v | - | weight/volume |
| μL | - | Microliter |

# LIST OF APPENDICES

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of Study

Energy has been playing a main role in global prosperity, but the excessive reliance on fossil fuels as the basic source of energy can lead to global warming, environmental issues and health complications (Levin*et al.*, 2004). Over the past few years, further considerations have been taken towards hydrogen gas and its powerful applications in transport and electrical generation. This is basically due to hydrogen being an unpolluting gas, renewable energy source, have high ratio of energy product and does not lead to greenhouse gas effect (Agrawal *et al.*, 2008).

Generally, hydrogen gas can be produced by several ways such as electrolysis of water, thermocatalytic reformation of organic materials and biological processes (termed biohydrogen) (Mielenz *et al.*, 2007). There are two main mechanisms to produce biohydrogen: sunlight microbial photosynthetic mechanism, which utilizes water or organic substrate; and dark fermentations by heterotrophic bacteria using starch, sugar and other substrates. Biohydrogen production mechanisms are primarily based on hydrogen producing enzyme, hydrogenase (Hallenbeck *et al.*, 2002).

Hydrogenase is an essential enzyme that can oxidise molecular hydrogen ($H_2$) reversibly in various microorganisms. These microorganisms have four kinds of hydrogenases. *E. coli* carries many hydrogenases operons, for example *hyaABCDEF* which encodes for hydrogenase 1(hyd-1), *hybABCDEFG* encodes for hydrogenase 2 (hyd-2) (Richard et al., 1999), *hycABCDEFGHI* encodes for hydrogenase 3(hyd-3) (Bagramyan *et al*., 2003), and *hyfABCDEFGHIR* encodes for hydrogenase 4 (hyd-4) (Andrews *etal.,* 1996). Hyd-1 and hyd-2 are hydrogen consuming only, hyd-4 has investigated to be inactive, and hyd-3 has both activity of hydrogen production and consumption (Maeda *et al.,* 2008).

Hydrogenase 3 (hyd-3) is a [NiFe]-type of hydrogenase which has hydrogen synthesis activity (Rossmann *et al*., 1994). hycABCDEFGHI operon encodes the seven subunits of hyd-3 (Sauter *et al*., 1992). hycB and hycF genes encode small subunits thought to have role in transferring the electron within the FHL complex, hycE genes encodes the hydrogenase large subunit that contains the Ni–Fe center while hycG encodes the hydrogenase small subunit, and the hycC and hycD genes encode polytopic membrane proteins. The hycH gene encodes a hydrophilic protein, which probably to include a portion of the hyd-3 complex. hycA encodes a repressor (anti-activator) of the hyc operon, and hycI encodes a protease that is essential for maturation of the hyd-3 large subunit (Dragal *et al*., 1998; Magalon *etal.,* 2000, Bagramyan *et al*., 2002).

## 1.2 Problem Statement

Hydrogen production pathway in *Citrobacter*sp. L17 is not understood yet. In addition, most studies have focused on understanding the biochemical and molecular aspects of hydrogenase 1 and 2. This study was performed to characterize the operon encoding hyd-3 structural components that is responsible for biohydrogen uptake and production by extracting the *Citrobacter*sp. L17 genome, amplifying the operon

encoding hydrogenase 3 structural components and finally sequencing the *hyd-3* subunit genes and accessory proteins.

## 1.3 Objective of Study

The objectives of this study are:

1. To extract the genomic DNA of *Citrobacter* sp. L17.
2. To amplify the genes coding for hyd-3 enzyme subunits and accessory genes by PCR.
3. To sequence the amplified genes and to verify the identity of hyd-3 by using bioinformatics analysis.

## 1.4 Scope of Study

This study was conducted to characterize genes for hyd-3 operon *Citrobacter* sp. strain L17. The whole genome of this particular bacterial strain has been extracted. The gene coding the hyd-3 operon has been amplified using polymerase chain reaction (PCR). Finally the sequencing of these genes has been performed, followed by bioinformatics analysis.

## 1.5 Significance of Study

Hydrogenase is an important enzyme which has hydrogen synthesis activity (Rossmann *etal.,* 1994). Hydrogen considers as one of the most powerful and unpolluted carrier of energy (Thompson *et al.,* 2008). By utilizing the fuel cell, hydrogen can be improved to electricity, which is recently applied in developed countries (Nakada *et al.,* 1999). This study will focus on molecular characterization of the subunits and accessory genes of hyd-3 in *Citrobacter* sp. L17. And the knowledge will be used in the knock-out study to understand biohydrogen production by *Citrobacter* sp. L17.

## REFERENCES


Agrawal, P., Hema, R., & Kumar, S. M. 2012. Experimental Investigation onBiological Hydrogen Production Using Different Biomass. *Jurnal Teknologi, 47*(1), 13–24

Akella, A. K., Saini, R. P., & Sharma, M. P. 2009. Social, Economical and Environmental Impacts of Renewable Energy Systems. *Renewable Energy, 34*(2), 390-396.

Bagramyan, K., & Trchounian, A. 2003. Structural and Functional Features of Formate Hydrogen Lyase, An enzyme of Mixed-acid Fermentation from *Escherichia coli*. *Biochemistry (Moscow), 68*(11), 1159-1170.

Carere, C. R., Sparling, R., Cicek, N., & Levin, D. B. 2008. Third Generation Biofuels via Direct Cellulose Fermentation. *International Journal of Molecular Science, 9*(7), 1342-1360.

Casalot, L., & Rousset, M. 2001. Maturation of the [NiFe] Hydrogenases. *Trends In Microbiology, 9*(5), 228-237.

Cassidy, D. P., Hirl, P. J., & Belia, E. 2008. Methane Production from Ethanol Co-products in Anaerobic SBRs. *Water Science Technology, 58*(4), 789–793.

Das, D., & Veziroğlu, T. N. (2001). Hydrogen Production by Biological Processes: A survey of Literature. *International Journal of Hydrogen Energy, 26*(1), 13-28.

Demirbas, Ayhan. 2009. Biohydrogen: For Future Engine Fuel Demands.*Springer*:London

Demirbas, Ayhan. 2000. Recent Advances in Biomass Conversion Technologies. *Energy Education Science Technology*, 6, 19-41.

Forzi, L., & Sawers, R. G. 2007. Maturation of [NiFe]-hydrogenases in *Escherichiacoli*. *Biometals, 20*(3-4), 565-578.

Garibyan, L. andAvashia, N. 2013. Polymerase Chain Reaction. *Journal of Investigative Dermatology* 133.

Hallenbeck, P. C., & Benemen, J. R. 2002. Biological Hydrogen Production. *International journal of Hydrogen Energy*, 1359–1365.

Kim, D. H., & Kim, M. S. 2011. Hydrogenases for Biological Hydrogen Production. *Bioresource Technology, 102*(18), 8423-8431.

King, P. W., & Przybyla, A. E. 1999. Response of *Hya* Expression to External pH in *Escherichia coli*. *Journal of Bacteriology, 181*(17), 5250-5256.

Leach, M. R., & Zamble, D. B. 2007. Metallocenter Assembly of The Hydrogenase Enzymes. *Current Opinion in Chemical Biology, 11*(2), 159-165.

Liu, X., Ren, N., Song, F., Yang, C., & Wang, A. 2008. Recent Advances in 8Fermentative Biohydrogen Production. *Progress in Natural Science, 18*(3), 253-258.

Levin, D. B., Pitt, L., & Love, M. 2004. Biohydrogen Production: Prospects and Limitations to Practical Application.*International Journal of Hydrogen Energy*,*29*(2), 173-185.

Lubitz, W., Ogata, H., Rüdiger, O., & Reijerse, E. 2014. Hydrogenases. *Chemical reviews, 114*(8), 4081.

Lutz, S., Böhm, R., Beier, A., & Böck, A. 1990. Characterization of Divergent NtrA-dependent Promoters in The Anaerobically Expressed Gene Cluster Coding for Hydrogenase 3 Components of *Escherichia coli*. *Molecular Microbiology*, 4(1), 13-20.

Maeda, T., Sanchez-Torres, V., & Wood, T. K. 2007. *Escherichia coli* Hydrogenase 3 is a Reversible Enzyme Possessing Hydrogen Uptake and Synthesis Activities. *Applied Microbiology and Biotechnology, 76*(5), 1035-1042.

Maeda, T., Sanchez-Torres, V., & Wood, T. K. 2008. Protein Engineering of Hydrogenase 3 to Enhance Hydrogen Pproduction. *Applied Microbiology and Biotechnology*, *79*(1), 77-86.

Magalon, A., & Böck, A. (2000). Analysis of the *HypC-HycE* Complex, a Key Intermediate in the Assembly of the Metal Center of the *Escherichia coli* Hydrogenase 3.*Journal of Biological Chemistry*, *275*(28), 21114-21120.

Mielenz, J.R., Klasson, K.Th, Adney, W.S., McMillan, J.D (Eds.) 2007. Biotechnology for Fuels and Chemicals. Denver, Colorado: Humana Press

Meher kotay, S., & Das, D. 2008. Biohydrogen as a Renewable Energy Resource-Prospects and Potentials. *International Journal of Hydrogen Energy, 33(1).*

Meuer, J., Kuettner, H. C., Zhang, J. K., Hedderich, R., & Metcalf, W. W. 2002. Genetic Analysis of the Archaeon *Methanosarcina Barkeri* Fusaro Reveals a Central role for Ech Hydrogenase and Ferredoxinin Methanogenesis and Carbon Fixation. *Proceedings of the National Academy of Sciences, 99*(8), 5632-5637.

Nath, K., & Das, D. 2004. Improvement of Fermentative Hydrogen Production: Various Approaches. *Applied Microbiology and Biotechnology, 65*(5), 520-529.

Nakada, E., Nishikata, S., Asada, Y., & Miyake, J. 1999. Photosynthetic Bacterial Hydrogen Production Combined with a Fuel Cell. *International Journal of Hydrogen Energy*, *24*(11), 1053-1057.

Richard, D. J., Sawers, G., Sargent, F., McWalter, L., & Boxer, D. H. 1999. Transcriptional Regulation in Response to Oxygen and Nitrate of the Operons encoding the [NiFe] Hydrogenases 1 and 2 of *Escherichia coli*. *Microbiology, 145*(10), 2903-2912.

Rossmann, R., Maier, T., Lottspeich, F., & Böck, A. 1995. Characterisation of a Protease from *Escherichia coli*I in Hydrogenase Maturation. *European Journal of Biochemistry, 227*(1-2), 545-550.

Rychlik, W. J. S. W., Spencer, W. J., & Rhoads, R. E. 1990. Optimization of the Annealing Temperature for DNA Amplification In-vitro.*Nucleic Acids Research*, 18(21), 6409-6412.

Sauter, M., Böhm, R., & Böck, A. 1992. Mutational Analysis of the Operon (*hyc*) Determining Hydrogenase 3 Formation in *Escherichia coli*. *Molecular Microbiology, 6*(11), 1523-1532.

Schlensog, V., Lutz, S., & Böck, A. 1994. Purification and DNA-binding Properties of FHLA, the Transcriptional Activator of the Formate Hydrogenlyase System from *Escherichia coli*. *Journal of Biological Chemistry, 269*(30), 19590-1959.

Thompson, L. J., Gray, V. M., Kalala, B., Lindsay, D., Reynolds, K., & von Holy, A. 2008. Biohydrogen Production by *Enterobacter cloacae* and *Citrobacter freundii* in Carrier Induced Granules. *Biotechnology Letters*, *30*(2), 271-274.

Van Ginkel, S. W., & Logan, B. 2005. Increased Biological Hydrogen Production with Reduced Organic Loading. *Water Res, 39*(16), 3819-3826.

Vignais, P. M., Billoud, B., & Meyer, J. 2001.Classification and Phylogeny of Hydrogenases1. *FEMS Microbiology Reviews, 25*(4), 455-501.

Vignais, P., & Colbeau, A. 2004. Molecular Biology of Microbial Hydrogenases. *Current Issues in Molecular Biology, 6*(2), 159-188.

Vignais, P. M., & Billoud, B. 2007. Occurrence, Classification, and Biological Function of Hydrogenases: an overview. *Chemical Reviews, 107*(10), 4206-4272.

Vignais, P. M. 2008. Hydrogenases and $H^+$-reduction in Primary Energy Conservation, *Bioenergetics,*45, 223-252.

Weiland, P. 2010. Biogas Production: Current State and Perspectives. *Applied Microbiology and Biotechnology, 85*(4), 849-860.