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Design, construction and introduction of artificial DNA encoding a FeFe hydrogenase into unicellular cyanobacterial cells

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Abstract

Everyday we are stepping towards technological advancements and developmental process. In every sector from small to big scale attributes there is always need of energy to come out with the desired result. The higher consumption and need of energy has created a raising demand of energy globally causing the rise in prices, limited supply and higher emission of greenhouse gases. To overcome all these problems and to show a small effort to promote the green interest of all the scientists globally this project involves in making an efficient construct to make a better cyanobacterial strain (*Synechocystis sp.* PCC 6803) which can produce bio-hydrogen efficiently to be used directly as a biofuel. The engineered Synechocystis sp. PCC 6803 will have a highly efficient [FeFe]-hydrogenase encoded by hydA from *Clostridium acetobuatylicum* along with three other genes hydE, hydF, hydG crucial for its maturation. This heterologous expression of an efficient FeFe-hydrogenase will replace the active [NiFe]-hydrogenase encoded by the hox operon.

The project involves the isolation of the functional gene hydA along with its maturation genes hydE, hydF and hydG from *Clostridium acetobuatylicum* and put in one operon. This operon was driven by ptrc2O promoter regulated by lacI and under the selection of antibiotic resistance Chloramphenicol both present in a second operon driven by pJ23101 promoter. The replicon is taken from pSB1AC3 which is the only part of the "backbone" of the final construct. And the whole construct will be put onto the hox genes of *Synechocystis sp.* PCC 6803 which will act as the integration sites. The assembly of the parts were done using one-step isothermal recombination and overlap extension method.

Abbreviations

Ori	Origin of replication
bp	base pairs
dH2O	deionized sterilized water.
DNA	deoxyribonucleic acid
dsDNA	double stranded DNA
hyd	hydrogenase
dNTPs	deoxyribonucleotides
kb	kilo base pairs
LB	lysogeny broth (Luria-Bertani medium)
PCR	polymerase chain reaction
U	units of enzyme
X-gal	bromo-chloro-indolyl-galactopyranoside
IPTG	Isopropyl β-D-1-thiogalactopyranoside
TAE	Tris base, acetic acid and EDTA
PCC	Pasteur culture collection
e^-	electron
Cm	Chloramphenicol

Objective

The main objective of this master's thesis is to construct an artificial DNA that encodes for highly efficient [FeFe]-hydrogenase encoded by hydA from *Clostridium acetobuatylicum* along with its three maturation genes hydE, hydF, hydG and introduce them into *Synechocystis sp*. PCC6803 by homologous recombination to replace their native [NiFe]-hydrogenase system. This heterologous recombination has been shown to produce hydrogen (H_2) with rate 500 times that of the native [NiFe]-hydrogenase system in the unicellular cyanobacteria, *Synechocystis* (Ducat, Sachdeva, & Silver, 2011).

Introduction

Biofuel

Biofuels, also known as agro-fuels are the renewable fuels mainly produced from biomass or metabolic byproducts like food or organic wastes. They are mostly biodegradable and non-toxic in nature. Biofuel can be produced from any carbon source using the photosynthetic plant for production where energy is derived from the biological carbon fixation (http://www.alternative-energy-news.info/technology/biofuels/). Biofuel is the best solution as an alternative fuel against the conventional fossil fuels which have limited avaibility and also to reduce the emission of greenhouse gases. The interest of global scientific community has increased highly on the matter of biofuel because of the drawbacks of fossil fuels like limited availability, raise in price, and environmental pollution (http://biofuel.org.uk/).

Currently two major sources of biofuels are in use. In the first method fermentation is carried out where ethanol is produced from the sugar crops or starch like sugarcanes, soybeans, corns etc. The second method involves green algae and plants like jatropha which are grown naturally to produce oil and can be used directly as fuel for diesel engines. The major producers and users of the biofuel in the present day context are Asia, Europe and America. United States of America uses soybean and corn to produce ethanol while Brazil and Asian countries use sugarcane to produce ethanol. Similarly Europe uses sugar beet and wheat to produce ethanol and in India they use palm oil and oil from jatropha plant as fuel (http://biofuel.org.uk/).

Synthetic biology for biofuels

Use of synthetic biology enables to choose various properties of different genes freely from the DNA library and assemble them in an expressible combination to make a novel biological system or organism with the desired properties which will finally lead to the desired product. The use of synthetic biology makes it possible to design, engineer and implement new biological system which might not exist in the nature or re-design the existing ones to make them more useful and efficient. Synthetic biology these days has been used much frequently and efficiently in the field of pharmaceuticals for drug production, design of biosensors, bioremediation and biofuel production.

In the field of biofuel production, synthetic biology has become very promising by creating the possibilities of construction of efficient novel pathways for different biofuels like ethanol,

butanol, hydrogen, etc. In my project I am using synthetic biology to design and make a novel construct for photo production of hydrogen using cyanobacteria.

Hydrogen as fuel

H_2 is one of the major potential renewable future bio-fuel to replace fossil fuel and switch off the global warming. It is the only fuel known so far with almost zero emission and hence known as the cleanest fuel available till date. It is the most abundant element in the Earth's environment and exists in nature as compounds in combination with other elements. To use up the H_2 as fuel it must exist as free Hydrogen (H_2) (Sarma, Brar, Le Bihan, & Buelna, 2012).

When used as fuel H_2 works as energy carrier and the energy is delivered as heat when it is combusted. The specially designed engine system converts the chemical energy of H_2 to mechanical energy by burning H_2 in an internal combustion engine or by reacting H_2 with O_2 in a fuel cell to run electric motors. The use of H_2 fuel has already been done in space rockets, as well as automobiles (Melis & Happe, 2001).

Bio-hydrogen

Biohydrogen production is among one of the most challenging areas in biotechnology for the scientists and researchers who are working for the alternative fuel to contribute to the global energy crisis. Biohydrogen is one of the promising renewable source of energy which can be produced from renewable feedstocks like biomass or biowastes by biochemical or biophotolytic process and hence known as clean and environmently friendly fuel (Allakhverdiev et al., 2009).

The microorganisms involved in biohydrogen production use this biomass as a source of electrons and convert the energy into production of molecular H_2 by one of the following biological processes.

- Biophotolysis
- Indirect biophotolysis
- Photo-fermentation
- Dark fermentation

Phototrophic organisms like green algae and cyanobacteria can efficiently produce biohydrogen via biophotolytic reactions. The biophotolytic reaction involves a photosynthesis process where water molecules are split into protons, electrons and O_2 molecules when light is received. The

protons are then reduced by combining with electrons with the help of hydrogenase enzyme to from molecular H_2.

Photosynthesis reaction:

$2H_2O \rightarrow 4H^+ + 4e^- + O_2$

Hydrogen production reaction:

$4H^+ + 4e^- \rightarrow 2H_2 + 2e^-$

The fermentation of the biomass substrate is accompanied by inoculation of the microbial strain which can efficiently convert biomass to molecular hydrogen driven by pyruvate metabolism. This process causes the release of stored energy through fermentation of endogenous carbohydrates and as a result protons are reduced by hydrogenase enzyme to form molecular H_2 (Angermayr, Hellingwerf, Lindblad, & de Mattos, 2009).

Hydrogenases

Hydrogenases are the class of metalloproteins that catalyses the reversible oxidation of molecular hydrogen (H_2) (Berto et al., 2011).

$2H^+ + 2e^- \rightleftharpoons H_2$

The hydrogenases catalyze this redox reaction with an equilibrium constant dependent on the reducing potential of the electrons which are carried along by their redox partner. (Ducat et al., 2011) Reportedly two main classes of hydrogenases are found in wide variety of organisms depending upon the metallic cluster found in their catalytic sites. Namely they are: [NiFe] hydrogenase and [FeFe] hydrogenase. Although the redox machinery of both hydrogenases catalyses the same reaction, still there is distinct difference between both hydrogenases on their structural basis, metallic cluster found in their active site and the electron carriers involved in redox chemistry (Ducat et al., 2011).

[NiFe] hydrogenase

[NiFe] hydrogenases are found in archea and eubacteria including cyanobacteria (Berto et al., 2011). The x-ray crystallography shows that [NiFe]-hydrogenase is heterodimer in structure with active site residing in the large subunit. The active site contains Nikel and Iron ion which has carbon monoxide and cyanide molecules serving as ligands bound to the Iron ion. Various iron-sulfur clusters are present in the small subunit of enzyme which serve as electron transfer

cofactors (Horch, Lauterbach, Lenz, Hildebrandt, & Zebger, 2012).

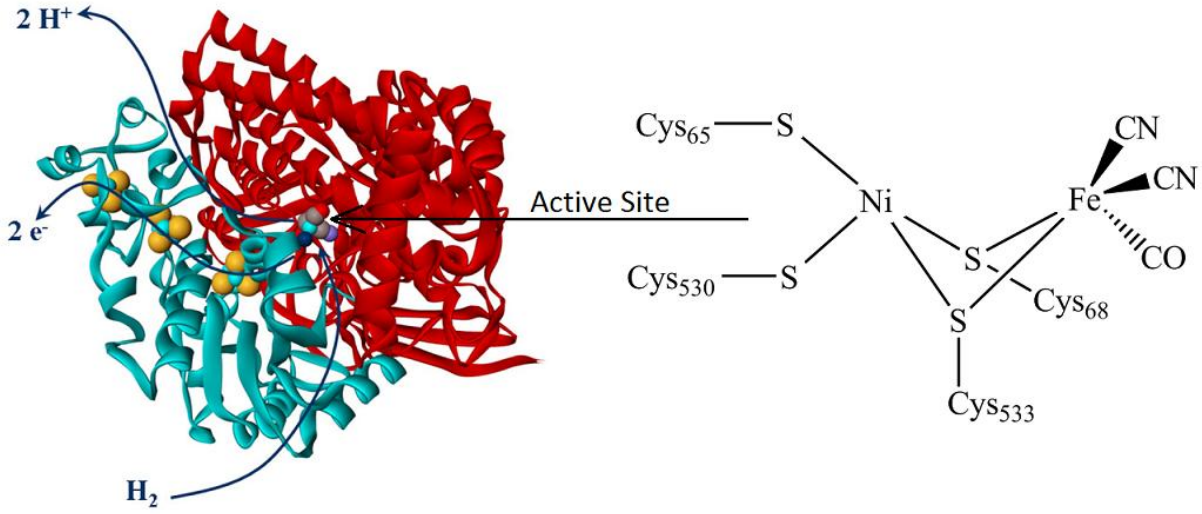

Figure 1. X-ray crystallographic structure and active site of [Ni-Fe] hydrogenage (Ludwig, Cracknell, Vincent, Armstrong, & Lenz, 2009)

[FeFe] hydrogenase

[FeFe]-hydrogenase are found only in eukaryotice microbes and a few prokaryotes but are not present in any of the cyanobactera investigated till today's date (Ducat et al., 2011). The active site contains two iron ions each coordinated by a terminal carbonyl and a terminal cyanide molecules serving as ligands. Various accessory iron-sulfur clusters are present in the catalytically active site working as cofactors. These iron-sulfur clusters are thought to play an important role in efficient electron transfer to and from the H-cluster (Schwab et al., 2006).

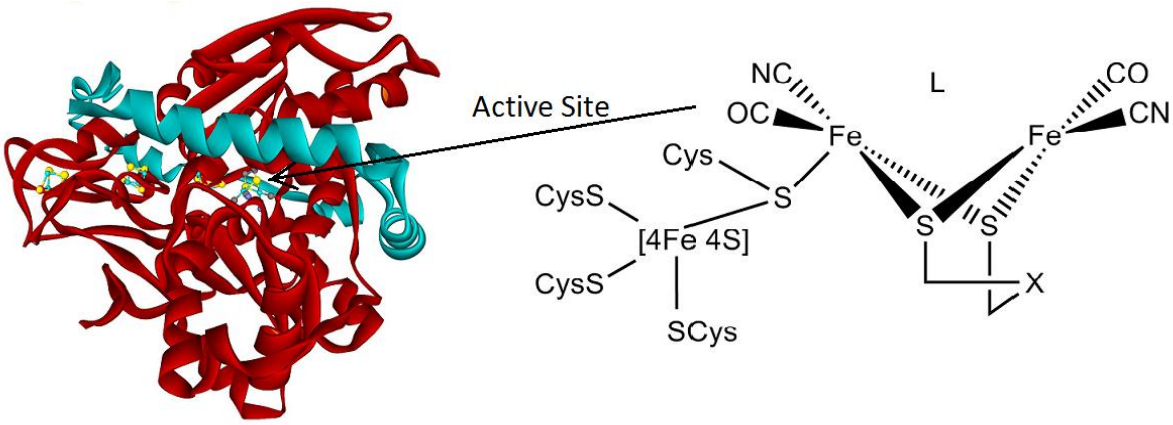

Figure 2. X-ray crystallographic structure and active site of [Fe-Fe] hydrogenage (Ludwig et al., 2009)

[FeFe]-hydrogenase are coupled with ferredoxin protein that can bear electrons with low reduction potential of about -420 mV which is closer to the value of H_2/H^+ pair. [NiFe]-hydrogenases which is coupled to electron carrying partner NADH has reducing potential +320mV which states that [FeFe]-hydrogenase thermodynamically favors the hydrogen production over the previous one. In a research when [FeFe]-hydrogenase encoded by HydA from *Clostridium acetobutylicum* was heterogously expressed in non-nitrogen fixing cyanobacteria *Synechococcus elongatus* sp. 7942 then the rate of hydrogen gas produced was more than that produced by the native [NiFe]-hydrogenases. This explains the higher efficiency of [FeFe]-hydrogenase over [NiFe]-hydrogenases when expressed heterologously in cyanobacterial system (Ducat et al., 2011).

Cyanobacteria

Cyanobacteria are aquatic and photosynthetic micro-organism. Hence they live in water and are capable of preparing their own food. Cyanobacteria have a bluish pigment called phycocynin and in Greek cyano means blue. Because of this property of their color they are also known as blue green algae. Apart from being classified as one of the most largest and important group of bacteria on the earth, they are also considered as the oldest known fossils for being existing in the earth for more than 3.5 billion years (Parmar, Singh, Pandey, Gnansounou, & Madamwar, 2011).

The natural habitat of cyanobacteria can be anywhere from terrestrial to aquatic systems. They can be found in fresh as well as marine water bodies, damp rocks, soils and even in sinks and drains. They can exist as unicellular or in colonial forms which includes filaments, sheets or hollow balls and are often surrounded by a gelatinous or mucilaginous sheath. Cyanobacteria also play an important role in nourishing plants and help them in their growth and develpment by fixing atmospheric free nitrogen into the absorbable organic form such as nitrate or ammonia. Because nitrogen fixation cannot occur in presense of oxygen, cyanobacteria have a special kind of cell with anerobic environment to favor nitrogen fixation. These type of specialized cells are called heterocyst. Hence cyanobacteria are among the very few organisms which can perform nitrogen fixation (Risser, Wong, & Meeks, 2012).

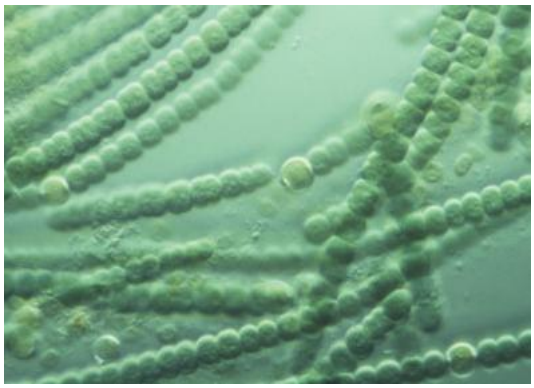

Figure 3. Filamentous cyanobacteria

Figure 4. Rod shaped Cyanobacteria

Synechocystis sp. PCC 6803

Synechocystis is a modular cyanobacterial strain which is capable of both phototrophic and heterotrophic growth. Phototrophic growth is accompanied by oxygenic photosynthesis during sunlight and heterotrophic growth is accompanied via glycosis and oxidative phosphorylation in dark conditions. The strain was discovered in 1968 from a fresh water lake. It was then isolated and put in the Pasteur culture collection (PCC). It is one of the model organism used by scientists globally. It can survive in wide range of environmental conditions, can integrate any foreign DNA into its genome via homologous recombination and hence favors spontaneous transformation (http://synechocystis.asu.edu/).

One step isothermal assembly of overlapping dsDNA (Gibson DNA assembly method)

Because of the same fundamental chemical structure of DNA in all the living organisms, the invention of molecular cloning techniquess made it possible to isolate different genes from different organisms and put them together to work in a single system. The classical cloning techniques make the use of DNA fragments of interest, restriction enzymes to cut the combining ends and the enzyme ligase to join them together to form a new combination which may or may not exist in nature.

Gibson's DNA assembly method makes the use of cloning method in a different approach which can favor the recombination of multiple DNA fragments in just one isothermal reaction. The number of DNA fragments to be combined can be more than a dozen and the combined DNA fragments can be much larger as 583 kb. The process makes the use of the specific primers which will eventually lead to the PCR products having overlapping sequences between every

two DNA fragments adjacent to each other. The recombination is obtained when the equimolar amount of each DNA fragment is incubated at 50 °C for 1 hour with the Gibson master mixture which contains isothermal buffer, T5 exonuclease, Taq DNA ligase and Phusion DNA polymerase (Gibson et al., 2009).

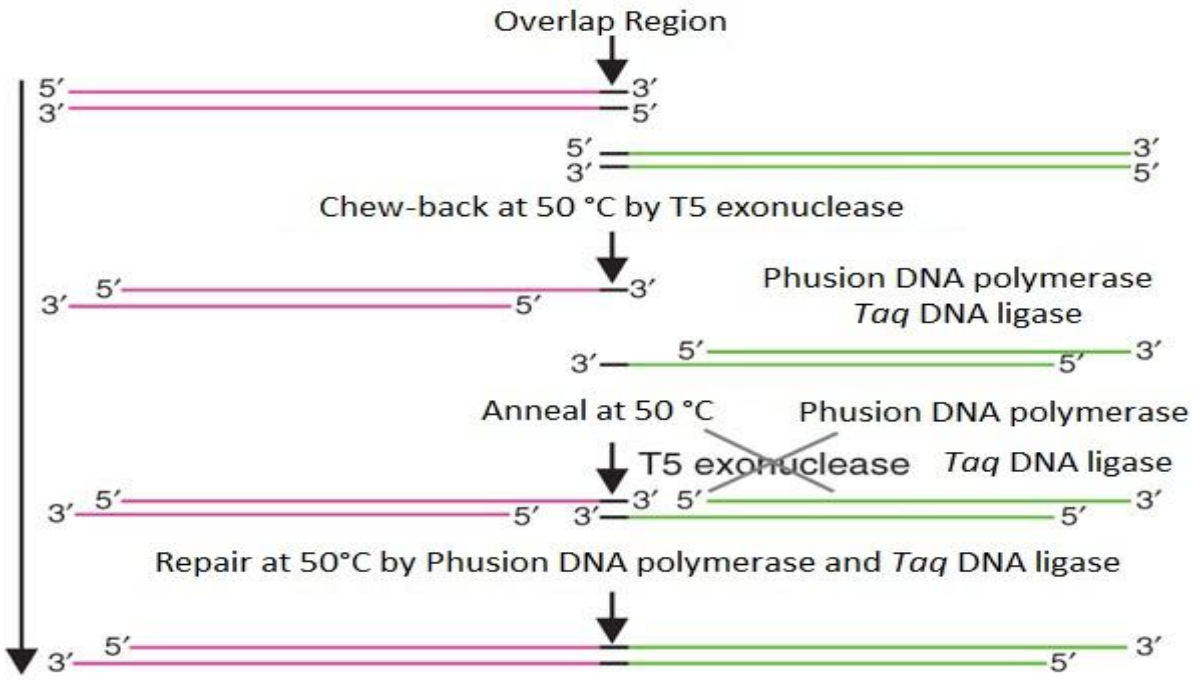

Figure 5. One step isothermal assembly of overlapping dsDNA (Gibson et al., 2009)

When incubated at 50 °C, the T5 exonuclease chews back and removes the nucleotides from 5' end region of the double stranded DNA. This heat liable enzyme is then inactivated on further incubation. Then the two adjacent DNA molecules sharing the complementary terminal sequence overlaps at 3' region anneal together. Phusion DNA polymerase then comes into the action by adding the nucleotides and filling the gap and Taq DNA ligase seals the gap to form a complete recombined DNA molecule. The whole process takes 1 hour of time regardless the number of fragments to be combined and saves a lot of time that would have otherwise consumed in classical ligation methods. The Gibson assembled products can be directly used for transformation reactions depending upon the nature of product (Gibson et al., 2009).

Project Plan

Construct design

The design of the construct was done using J5 software by a senior researcher, Thosten Heidorn working on the same project. The final construct will look like as follows:

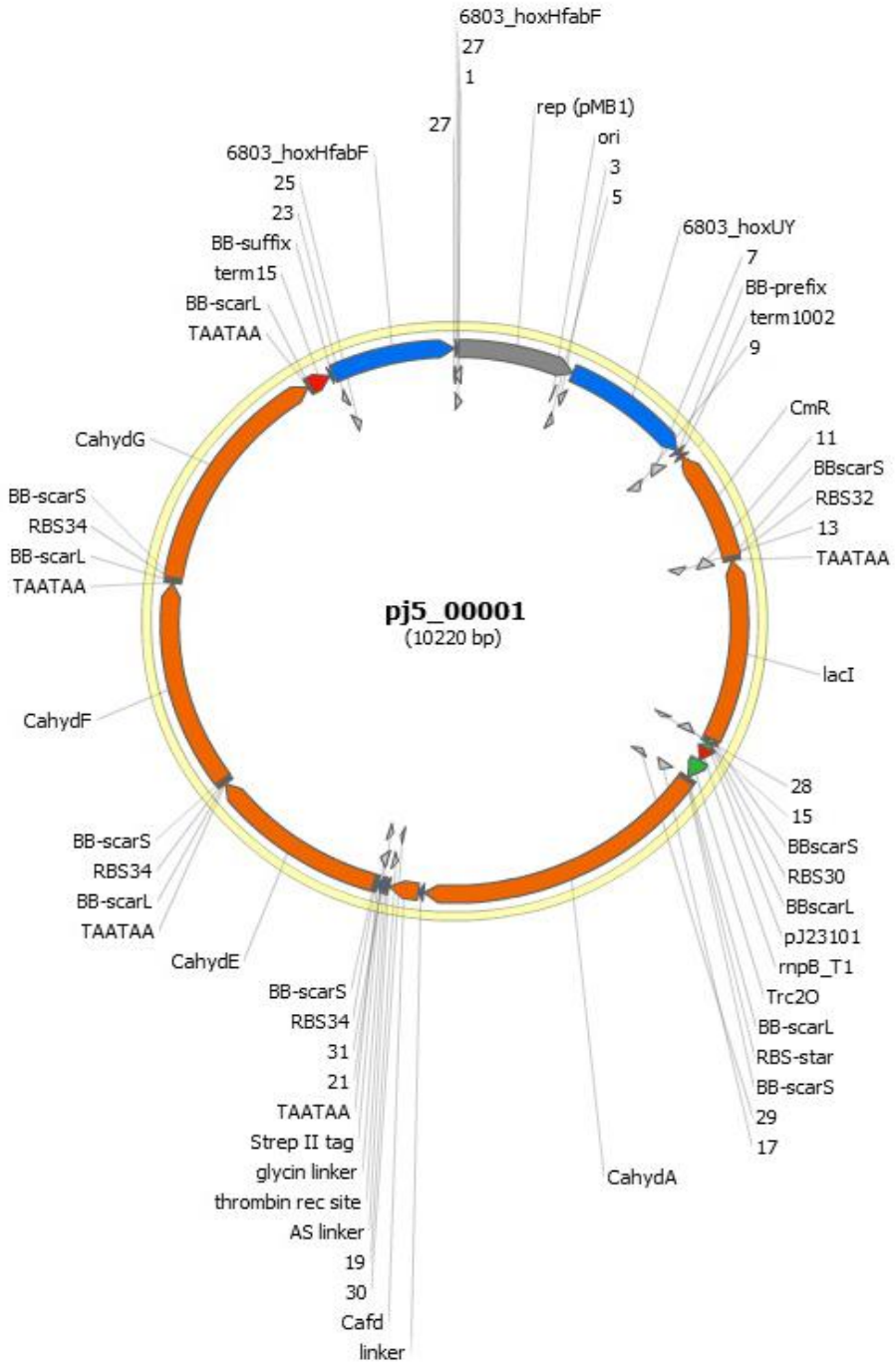

Figure 6. Final Construct

The final construct will consist of two flanking regions hoxUY and hoxHfab native to the *Syenchosists sp. 6803* and everything in between these two regions will be heterologously inserted in the hoxUY and hoxHfab of *Synechosists* to replace the native NiFe hydrogenase system. An origin of replication, Ori for replication of the plasmid which gives the high copy number is obtained from pSBIAC3 plasmid and is present in between two flanking regions in the construct.

The construct consists of a Chloramphenicol antibiotic cassette which will provide the criteria for selection, a lacI repressor for the IPTG inducible promoter, a CahydA gene which codes for hydrogenase enzyme important for hydrogen gas production, a streptavidin tag for the purification of hydrogenase enzyme and CahydE, CahydF, CahydG genes for proper folding and maturation of hydrogenase enzyme.

Construction Strategy

Gibson's assembly seems to be simple and very straight forward since a dozen of DNA can be combined in a single isothermal reaction. But previous trials in our lab suggested that the lesser the number and approximate similar in size favors the Gibson process better. In my case I had nine DNA fragments to be put together shown in table 1 with their fragment number and respective size. We planned to put all of them together in two installments. First we planned to combine the smaller fragments roughly of the same size to form four larger fragments by Gibson's method and then combine those four larger fragments in the final assembly step to form the whole construct.

Table 1. showing the genes, their sizes and templates

Fragment No.	Name of the gene	Size(bp)
1	ORI	709
2	hoxUY	811
3	CmR	732
4	LacI	1134
5	Promoter	335
6	CahydA	1954
7	StrepII tag	109
8	CahydEFG	3981
9	hoxHfabF	770

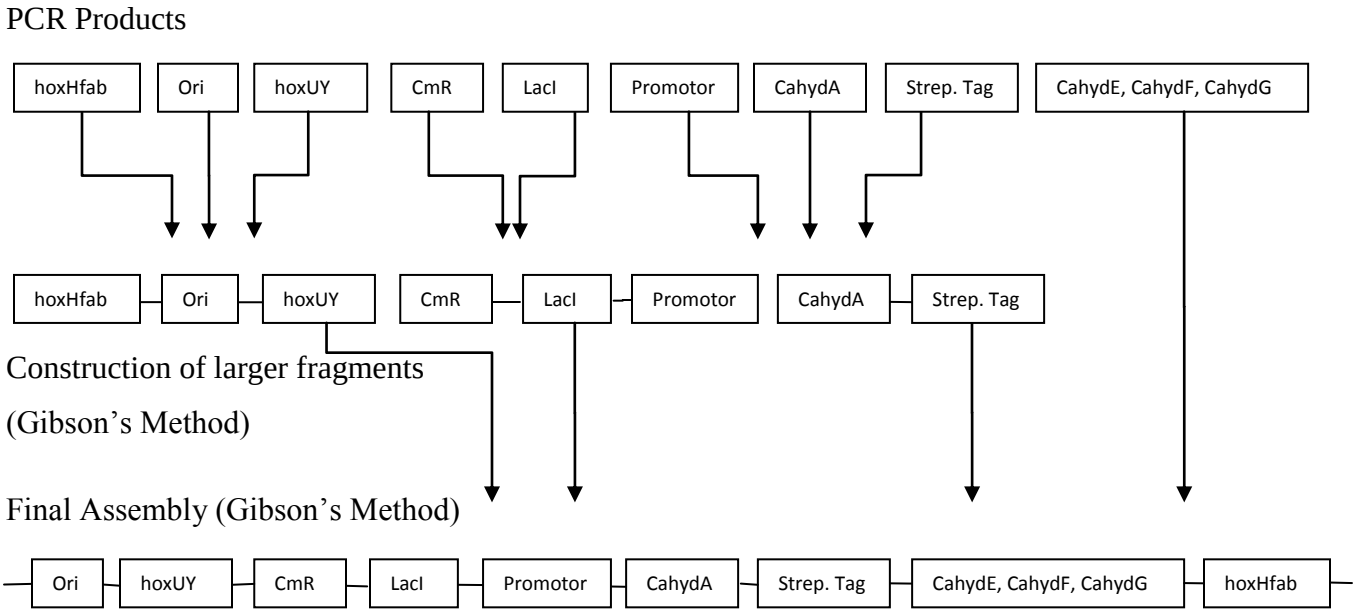

Figure 7. Construction strategy of artificial DNA encoding a green algal hydrogenase using Gibson's Method

Materials and Methods

1. Chemicals used

Finnzymes Phusion® Hot Start II, High-Fidelity DNA Polymerase was used for all the PCR amplification reactions. Finnzymes Phusion® DNA Polymerase was used for all the Gibson assembly and Overlap extention reaction. T5 exonuclease and Taq DNA ligase from Thermo Scientific was used for chewing DNA from 5' end and sealing the nick respectively in Gibson's assembly method. dNTP mix containing equimolar concentration of all four dNTs were obtained from Finnzymes. Dream Taq DNA Polymerase obtained from Fermentas was used for colony PCR. DNA primers (oligonucleotides) for all PCR amplifications and sequencing the DNA samples were purchased from Eurofins MWG operon. The purification of PCR amplified product and DNA extraction from gel was done using GeneJET^{TM} PCR purification Kit and GeneJET^{TM} gel extraction kit respectively. The plasmid prepration from the culture was done using GeneJET^{TM} plasmid mini prep kit.

2. Organisms used

Escherichia coli strain DH5α was used to make competent cells which was frequently used for transformation. *Synechosystis sp. 6803* was used to amplify the hoxUY and hoxHfabF (flanking regions of the whole construct) which will be used to transform the whole construct in it by heterologous recombination.

3. Vectors used

pBluescript II KS^{-} vector

pBluescript II KS^{-} is a commercially available phagmid with 2961 bp in length. As shown in figure 8 it consist of intergenic region of phage F1, replicon pMB1 for the replication of phagemid, the bla(AmpR) gene which codes for beta-lactamase and confers resistance to ampicillin and confers resistance to ampicillin which helps in selection of recombinant *Escherichia coli*. A multiple cloning site within the lacZ gene which codes for the N-terminal fragment of beta-galactosidase. The successful insertion of a DNA causes the disruption of the coding region of lacZ gene thus resulting insertion inactivation of lacZ gene which allows the blue/white screening of the recombinant phagemids. The recombinant *Escherichia coli* colonies will appear white in colour while the non recombinant will have the blue colonies. (http://www.fermentas.com/en/support/technical-reference/phage-plasmid-dna/pbluescriptII).

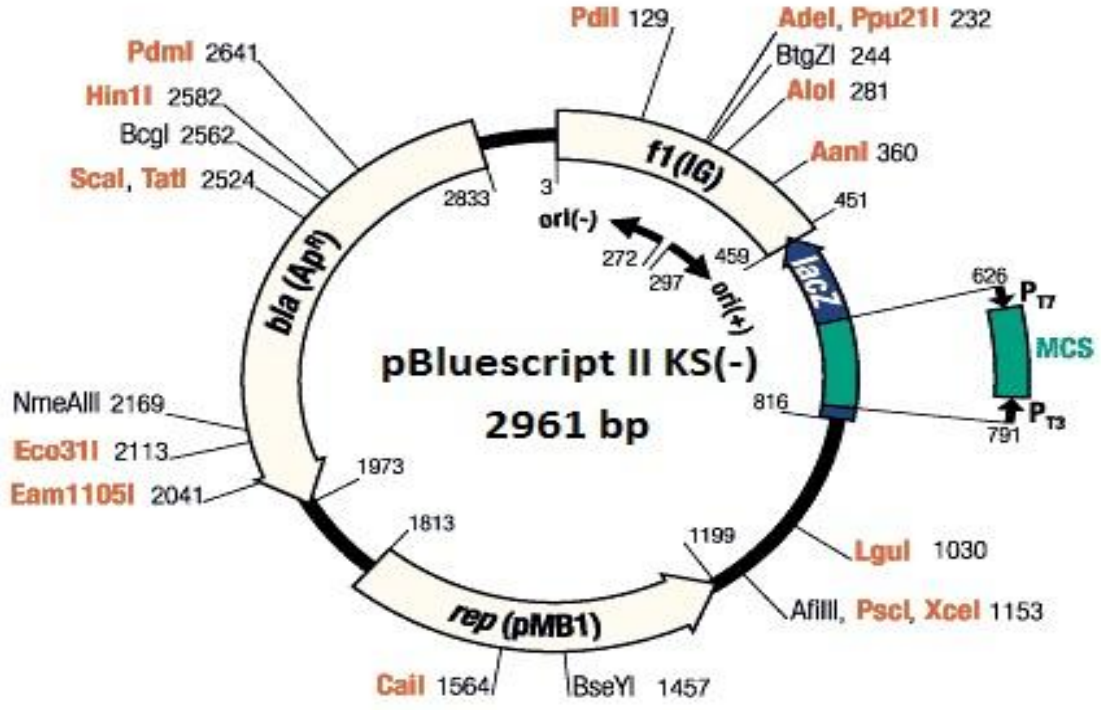

Figure 8. pBluescript vector Reference : http://www.fermentas.com/en/support/technical-reference/phage-plasmid-dna/pbluescriptII

pJet vector

It is also commercially available vector with 2974 bp in length and is a high copy number plasmid. The vector map in the figure 8 shows it consists of replicon pMB1 for replication of plasmid, the bla(AmpR) gene coding for beta-lactamase and confers resistance to ampicillin which helps in selection of recombinant *Escherichia coli*. The vector also consists of a modified Eco47IR gene which includes a multiple cloning site and codes for Eco47I restriction endonuclease which is lethal to all *E. coli.* strains and are not protected by cognate methylation. So all the successful cloning at the multiple cloning sites causes insertion inactivation of Eco47IR gene inhibiting the expression of lethal Eco47I restriction endonuclease. Because of this only those cells which have recombinant plasmid will survive causing the positive selection. It further consists of T7 and P_{lacUV5} promoters which are responsible for invitro transcription of the inserted gene and expression of eco47IR gene respectively. It also consists of multiple cloning site at position 328-422 where the cloning of desired insert at desired site can be performed and is also used for mapping and screening purposes of the insert/product (http://www.fermentas.com/en/support/technical-reference/phage-plasmid-dna/pjet12).

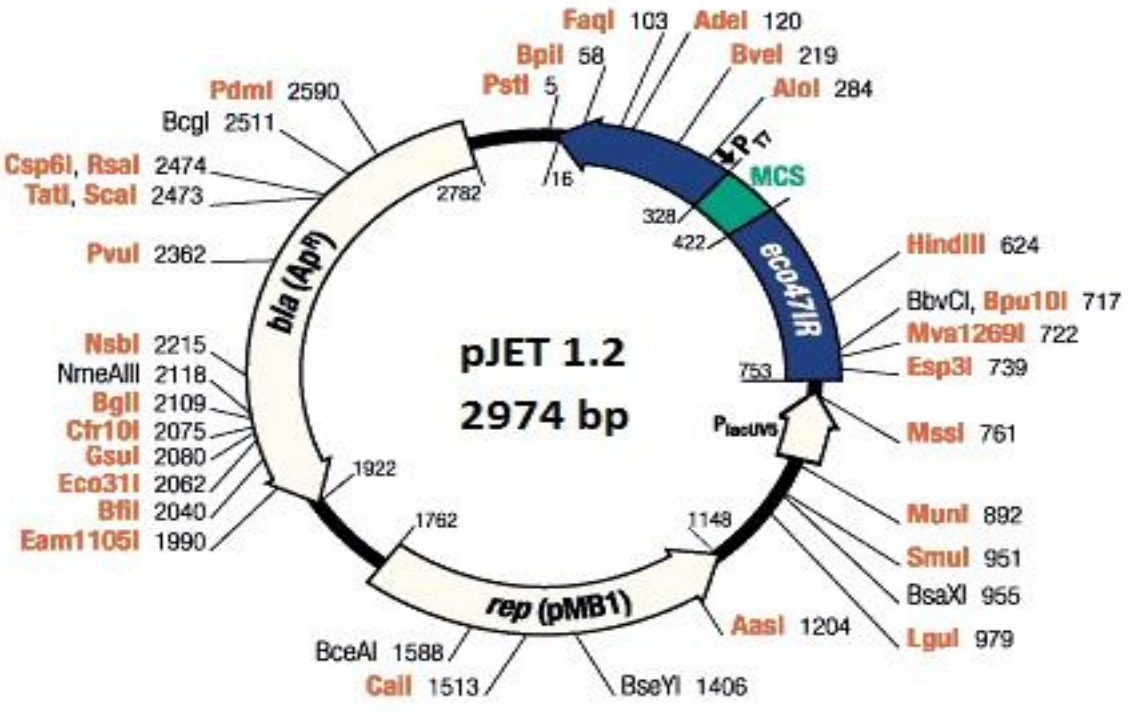

Fig 9. pJET 1.2 vector (Reference: http://www.fermentas.com/en/support/technical-reference/phage-plasmid-dna/pjet12)

4. DNA amplification

The DNA fragments were amplified using a pair of forward and reverse primers as mentioned in table 3. Two flanking regions in the final construct, hoxUY, hoxHfab and origin of replication (Ori) were amplified using genomic DNA of *Syenchosists sp.* 6803 as template. All the rest of the genes that codes for chloramphenicol antibiotic cassette, lacI, promoter, streptavidin tag, CahydA, CahydE, CahydF, CahydG, were obtained by amplifying directly which were present as inserts in pSB1A3 vectors supplied by the company. The set of the primer pair that were used to amplify DNA were designed in such a way that they included a 40 bp tail on their 5' ends to create an overlap region between the adjacent DNA to be assembled with the use of Gibson's assembly method.

PCR protocol

Table 1. General PCR protocol - content

Components	Volume
5x Phusion HF Buffer	10 µl
10mM dNTPs	1 µl
Template DNA (4 ng/µl)	2.5 µl
Forward primer (10 µm)	2.5 µl
Reverse primer (10 µm)	2.5 µl
Phusion® Hot Start II DNA Polymerase (2 U/µl)	0.5 µl
dH_2O	31 µl
Total	50 µl

PCR conditions

Table 2. General PCR protocol for DNA amplification - conditions

Steps	Temperature	Time	Number of Cycles
Initial denaturation	98 °C	30 Seconds	1
Denaturation	98 °C	10 Seconds	35
Annealing	Depending on Tm of Primers	30 Seconds	35
Extension	72 °C	30seconds/kb	35
Final Extension	72 °C	10 minutes	1
Holding	4°C	∞	

Primers used

Table 3. Primers used for DNA amplification

Primer	Sequence	Use
11_01_01	GGCACTGTGGTTATCAAAGGCACCCG TAGAAAAGATCAAAGGATCTTCTTGA GA	Forward primer for Ori of pSBIAC3 vector

11_01_03	GGAATGGGAACCCATGTGAGCAAAAG GCCAGCA	Reverse primer for Ori of pSBIAC3 vector
11_01_05	GCCTTTTGCTCACATGGGTTCCCATTC CCACCCTTTGC	Forward primer for flanking region hoxUY in Synechosystis 6803 sp.
11_01_07	GGGTTCGTTTTTTCTCTAGAAGCGGCC GCGAATTCGAGCCATTCGTCCATATC AAGGAAGGACATA	Reverse primer for flanking region hoxUY in Synechosystis 6803 sp.
11_01_09	GCCGCTTCTAGAGAAAAAACGAACCC CGCCGAAGCGGGGTTACGCCCCGCCC TGCCAC	Forward primer for CmR (Chloramphenicol resistant gene)
11_01_11	GCGGGCAGTAATAATCACACAGGAAA GTACTAGATGGAGAAAAAAATCACTG GATATACCACCGTTG	Reverse primer for CmR (Chloramphenicol resistant gene)
11_01_13	CCATCTAGTACTTTCCTGTGTGATTAT TACTGCCCGCTTTCCAGTCGG	Forward primer for LacI repressor.
11_01_15	AGAGGAGAAATACTAGATGGTGAATG TGAAACCAGTAACGTTATACGATG	Reverse primer for LacI repressor.
11_01_17	AGAGTAGTGGAGGTTACTAGATGAAA ACTATTATCTTAAATGGAAATGAAGT GC	Forward primer for CahydA gene (Gene coding hydrogenease)
11_01_19	GGAACTAAAGAAGCGCTAGCTTCTTG CACGGGGG	Reverse primer for CahydA gene (Gene coding hydrogenease)
11_01_21	CGCAATTTGAGAAATCCTAATAAAAA GAGGAGAAATACTAGATGGATAATAT CATC	Forward primer for CahydEFG gene (maturation genes for hydrogenase)
11_01_23	CAACTAAGGCAGGCTGCAGCGGCCGC TACTA	Reverse primer for CahydEFG gene (maturation genes for hydrogenase)
11_01_25	GCGGCCGCTGCAGCCTGCCTTAGTTGT TCTACCCATGCAGCGGGAC	Forward primer for flanking region hoxHfabF in Synechosystis 6803 sp.
11_01_27	CCTTTGATCTTTTCTACGGGTGCCTTT	Reverse primer for flanking region

	GATAACCACAGTGCCCAGC	hoxHfabF in Synechosystis 6803 sp.
11_01_28	GTTACTGGTTTCACATTCACCATCTAG T	Forward primer for Promoter pJ23101
11_01_29	CCATTTAAGATAATAGTTTTCATCTAG TAACCTCCA	Reverse primer for Promoter pJ23101
11_01_30	GTGCAAGAAGCTAGCGCTT	Forward primer for StrepII tag
11_01_31	CCATCTAGTATTTCTCCTCTTTTTATTA GGATTT	Reverse primer for StrepII tag

5. Agarose gel electrophoresis

The amplified amplicons after the PCR reactions were checked for the correct size by running it on 1% Agarose TAE (Tris-Acetate EDTA) gel against standard DNA ladder at 70 volts for about 30-45 minutes depending upon the size of the amplicons and then visualized under the UV illuminator fitted with a camera to take a picture. DNA being negatively charged travels towards the positive terminal of the apparatus which causes the separation according to it sizes.

6. Restriction Digestion

To confirm the amplified fragments of DNA that we had was the correct one after we further checked for the successiveity of PCR by performing restriction digestion method for cutting amplicons by using restriction enzymes and checked on 1% Agarose TAE (Tris-Acetate EDTA) gel against standard DNA ladder at 70 volts for about 30-45 minutes depending upon the size of the amplicons. The choice of the different restriction enzymes for different fragments was done using computer software “Gentle” (Put picture of restriction digestion in the result section)

7. Purification

Dpn1 digestion

One important thing is to get rid of the bacterial DNA (template) from the PCR mixture before carrying out any cloning experiment. To accomplish this, PCR mixtures were subjected to Dpn1 digestion. This enzyme recognizes the methylated bacterial DNA and cleaves them in the specific sequences which were then removed in further purification step.

PCR purification

After methalyted bacterial DNA was digested using Dpn1 digestion method, further purification

was employed using GeneJET^{TM} PCR purification Kit to get rid of all the proteins and other unwanted chemicals in the PCR mixture and pure amplified DNA was extracted.

Gel Extraction

In some cases like in case of DNA that codes for Streptavidin TagII there were several unspecific bands seen after checking it on 1% TAE agarose gel. In such conditions the gel was cut at their respective position by visualizing it under blue box and purified using GeneJET^{TM} gel extraction Kit. The elutes were obtained after the purification was performed and then taken forward for the measurement of DNA concentration with the help of nanodrop using elution buffer as blank.

8. Constructs assembly

After amplifying all the required fragments the assembling of the DNA fragments together was done using Gibson's assembly (1 step isothermal DNA assembly method). The final construction was done in two installments ie. for assembling total of nine fragments together first we assembled small number of fragments together ie. 2 or 3 in number to form a larger fragment and then the final assembly was performed to make the final construct. For this to achieve the equimolar (3×10^{-4} fragments) amount of each DNA fragments having overlap sequences were put in a microfuge tube and then concentrated in the concentrator. Then 20 µl of Gibson master mix was added to the concentrated DNA mixture and incubated at 50°C for 1 hour to favor the assembly process. The product was finally checked in 1% TAE agarose gel to check the success of the assembly reaction.

Gibson master mix components

Table 4. Gibson master mix components

Component	Volume
5x Isothermal buffer	15 µl
T5 exonuclease (0.2 u/µl)	1.5 µl
Taq DNA ligase (40 u/µl)	7.5 µl
Phusion DNA polymerase (2u/µl)	1 µl
dH_2O	75 µl
Total volume (5 Reactions)	100 µl

5x Isothermal buffer content

Table 5. Components in 5x Isothermal buffer

Component	Amount
PEG-8000	1.5 gram
1M Tris-HCl pH7.5	3 ml
2M MgCl2	150 µl
1M DTT	300 µl
100 mM dGTP	60 µl
100 mM dATP	60 µl
100 mM dTTP	60 µl
100 mM dCTP	60 µl
100 mM NAD	30 µl
Autoclaved dH_20	Add up to final volume 6 ml
Total volume	6 ml

9. Overlap Extension

Small DNA fragments like the gene coding for StrepII tag (109 bp) might get digested completely by T5 exonuclease when assembled using Gibson's assembly. So overlap extension method was applied to combine it with other DNA fragment to have an efficient assembly. In this kind of assembly method equimolar concentration of the both the fragments were used without need of any primers since the overlap region themselves act as primer for the extension of overlapped regions.

Table 6. Overlap extension master mix and reaction conditions.

Component	Volume
Autoclaved dH_2O	Add to make final volume 50 µl
5x Phusion HF Buffer	10 µl
10 mM dNTPs (200 µM each)	1 µl
DNA Templete 1	150 ng
DNA Templete 2	(Equimolar amount of wt to DNA Template 1) ng

Phusion DNA Polymerase (0.02 u/µl)	0.5 µl
Total volume	50 µl

10. Preparation of competent cells

DH5α strains of *E. coli* were used for preparing competent cells. The DH5α strains were streaked out and cultured in a test tube in 5 ml LB broth. They were then grown over-night at 37°C and 250 rpm shaker. Next day the 5ml of cultured DH5α strain was inoculated in a 2 liter sterile conical flask containing 400 ml of LB medium. Then it was left to grow at 30°C at 125 rpm on a shaker. The OD measurement was carried out at a regular interval of about 1 hour at 600nm of light wavelength. When the OD measurement was found to be around 0.35 then the culture was removed from the shaker and the provided standard protocol was followed for preparing the competent cells.

Protocol for preparing competent cells

The culture was transferred into the pre-chilled sterile polypropylene tubes and left on ice for 5 to 10 minutes. The cells were then centrifuged for 7 minutes at 1600g (3000 rpm) at 4°C. The supernatant was discarded and re-suspended in 10 ml ice-cold CCMB 80 buffer. Then the cells were centrifuged at 5 minutes at 1100g (2500 rpm) at 4°C. The supernatant was discarded and the pellets were re-suspended in 10 ml ice-cold CCMB 80 buffer. The re-suspended cells were kept on ice for 30 minutes. Then the cells were centrifuged for 5 minutes again at 1100g at 4°C and the supernatant was discarded. The cells were then re-suspended in 2 ml ice cold CCMB 80 buffer solution and the tubes were let on ice in the fridge for overnight. The next day the cells were then transferred to pre-chilled sterile poly propylene tubes (250 µl aliquots) and were frozen immediately at -80°C. The competency of the cells was checked by following standard transformation protocol using Red Fluorescent Plasmid (RFP) and the transformation efficiency was found to be 7.8×10^6.

11. Transformation

The assembled constructs were transformed using DH5α competent cells which were prepared as described above. The preserved competent cells were thawed on ice and approximately 5 ng of the Gibson mixture was mixed with 100 µl of competent cells in a 1.5 ml eppendorf tube and were suspended by mixing gently by pipetting up and down. Then the mixture was incubated on

ice for 30 minutes. Then heat shock was given for one minute at 42°C which plays an important role for uptake of foreign DNA. Then the mixture was again incubated on ice for 5 minutes. 950 µl of fresh media was added to the transformation mixture and was incubated at 37 °C for 1 hour. Finally the transformation mix was plated in 3 different plates with Chloramphenicol as antibiotic used for selection taking 3 different volumes (1µl, 10µl, 100µl). Then the plates were incubated overnight at 37 °C.

12. Plasmid preparation

A single colony was picked from the streaked plate with the help of a pipette tip and was inoculated in 5ml of LB containing Chloramphenicol as selection antibiotic in a 20 ml culture tube. It was then incubated for 16 hours (overnight) at 37 °C while shaking at 250 rpm. Next morning the cells were harvested by centrifuging the culture at 8000 rpm (6800 g) for 2 minutes at room temperature. The supernatant was discarded and the pellet was further used for plasmid preparation using Plasmid mini prep kit from Fermentas. The pellets were re-suspended in 250 µl of resuspension solution containing RNase A and the bacteria were re-suspended completely by vortexing and pipetting up and down. Then 250 µl of lysis solution was added and mixed thoroughly. 350 µl neutralization solution was added and the cells were centrifuged at 12000 rpm for 5 minutes to pellet down the unwanted cells debris and the chromosomal DNA. The clear supernatant was pooled out by pipetting and added to the GeneJET spin column which was then centrifuged for 1 minute. The flow through was discarded and the column was washed using 500 µl of wash solution (diluted with ethanol) by centrifuging for 1 minutes at 12000 rpm. The flow through was discarded and then the plasmid DNA was eluted with 50 µl of provided elution buffer.

13. Screening

<u>Colony screening PCR:</u>

The selected positive colonies that were obtained after the transformation were applied for colony screening PCR to check whether the results were positive. The PCR system consisted of Dream Taq DNA Polymerase and its buffer which were obtained from Fermentas.

Table 7. Colony screening PCR protocol

Components	Volume
10x Dream Taq Buffer	2 µl
dNTP Mix (10mM)	0.4 µl
Template DNA	1 µl
Forward primer (10 µm)	1 µl
Reverse primer (10 µm)	1 µl
Dream Taq DNA polymerase	0.1 µl
dH_2O	14.5 µl
Total volume	20 µl

Table 8. Colony screening PCR conditions:

Steps	Temperature	Time	Number of Cycles
Initial denaturation	95 °C	3 minutes	1
Denaturation	95 °C	30 Seconds	35
Annealing	Tm - 5°C	30 Seconds	35
Extension	72 °C	1minute/kb	35
Final Extension	72 °C	15 minutes	1
Holding	4°C	∞	

Sequencing

After confirming the positive results from colony PCR, the positive colonies were cultivated in LB using ampicillin. The plasmids were extracted using GeneJET^{TM} plasmid mini prep kit. The results were further confirmed for their positivity by cutting the plasmid using the specific restriction endonucleases and once they were confirmed the plasmid samples were further sent for sequencing to Macrogen.

Table 9. Sequencing primers used:

Primers	Sequences	Use
Seq_A_1	GCTGGCTGAACTGATTCAATGC	For sequencing larger fragment A
Seq_A_2	CGCTCTGCTAATCCTGTTACC	
Seq_A_3	CCTATGGAAAAACGCCAGCAACG	
Seq_A_4	GCAAGATATGGCCATTACGGTGG	
Seq_A_5R	CCTCAAAATCCTCTCATTACTTCC	
Seq_B_1	CCATATCACCAGCTCACC	For sequencing larger fragment B
Seq_B_2	GCTGTCTTCGGTATCGTCGTATCC	
Seq_B_3	GCAGGCAGCTTCCACAGCAATGG	
Seq_B_4R	GCTCAATGTACCTATAACCAGACC	
Seq_C_1	GCACACGGACAAAGATATTACC	For sequencing larger fragment C
Seq_C_2	CGATCGATCTAAATGTGTATTATGC	
Seq_C_3	GCTTAGCCCAAAATTATCATCC	
Seq_C_4	CCTGTCCCGGTGGATGTATCAATGG	
Seq_C_5R	CCATTCCATCTTCTACTTTGG	

Results

Gene isolation

All the nine fragments of DNA that constitutes the final constructs were amplified using the primes with around 40 bp overlap to favor the 1 step isothermal DNA assembly method. Those nine fragment with the name of their genes and their sizes are mentioned in the table: 10. The amplicons were confirmed to be the correct ones by performing agarose gel electrophoresis in 1% TAE gel using standard 1 kb ladder to compare sizes.

Table 10. showing the genes, their sizes and templates

Fragment No.	Size(bp)	Name of the gene	Template
1	709	ORI	pSBIAC3
2	811	hoxUY	Genomic DNA
3	732	CmR	pSBIAC3
4	1134	LacI	pSBIAC3
5	335	Promoter	Direct Synthesis
6	1954	CahydA	pSBIAC3
7	109	StrepII tag	Direct Synthesis
8	3981	CahydEFG	pSBIAC3
9	770	hoxHfabF	Genomic DNA

Restriction Digestion

All the nine amplified fragments were then further checked for their accuracy by digesting them using restriction endonucleases and then checking them in 1% TAE gel. The restriction digestion enzymes used, the number of fragment produced and the size of fragments they formed after digestion is shown in table: 11.

Table 11. showing the genes, restriction enzymes used, number of digested fragments and their sizes

Fragment No.	Size(bp)	Name of the gene	Restriction Enzymes	Fragment sizes	Number of Fragments
1	709	ORI			
2	811	hoxUY	ECORI	34 & 777	2
3	732	CmR	StyI	180 & 552	2
4	1134	LacI	BSSHII	343 & 991	2
5	335	Promoter	VspI	102 & 233	2
6	1954	CahydA	HindIII	664 & 1290	2
7	109	StrepII tag	NheI	10 & 99	2
8	3981	CahydEFG	StyI	138, 395, 751 & 2697	4
9	770	hoxHfabF	ECORI	239 & 531	2

Overlap extension

Because of the small size of fragment no. 7 (106 bp) that codes for strepII tag, the direct employment of Gibson's assembly method (one step isothermal recombination) might result in complete degradation of this fragment by T5 exonuclease. So I employed overlap extension to combine fragment no.6 and 7 together.

1 step isothermal recombination (Construction of larger fragments)

As per suggestion of the senior researchers and lab members working on the 1step isothermal recombination method, I was suggested that the assembly method works best when the numbers of fragments to be recombined are less and roughly of the same size. So I used maximum of three different adjacent amplicons to combine them together and form larger fragments roughly of the same size in first round of 1 step isothermal DNA assembly method. During the assembly 3×10^{-4} moles of each fragments mixed with Gibson master mix before 1hour of incubation at 50ºC. The assembled product were then amplified using a pair of forward and reverse primers and checked in 1% TAE gel shown in figure: 10. The table: 12 shows the larger fragments formed, their constituent small fragments and their final size after recombination.

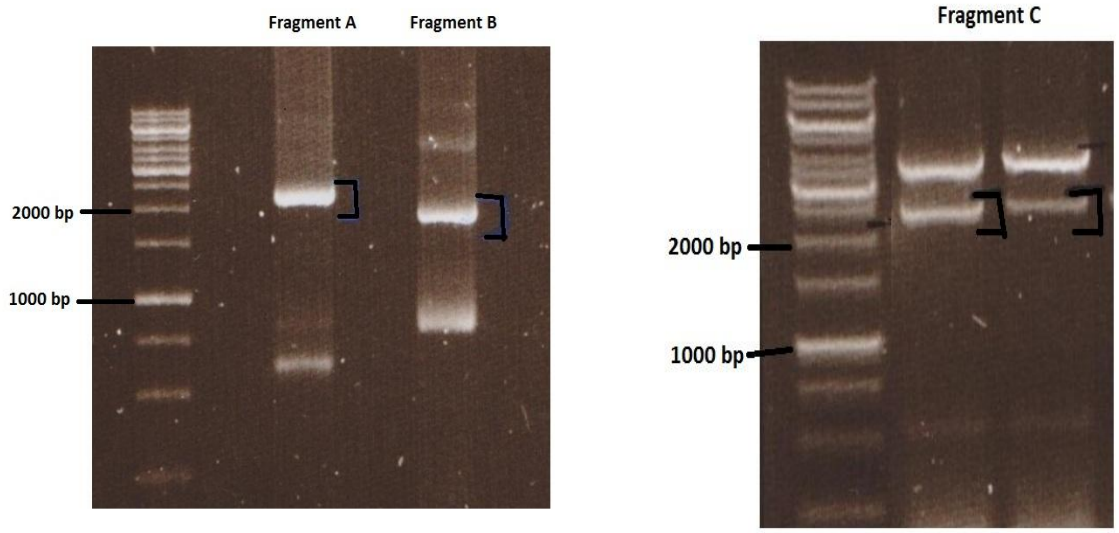

Figure 10. Construction of larger fragments using 1 step isothermal recombination

Table 12. showing Gibson assembled product, their constituent genes and their sizes

Larger fragments	Constituent Smaller fragments	Final Size after recombination
A	hoxHfabF, Ori & hoxUY	2220
B	CmR & LacI	1829
C	Promotor, CahydA & StrepII tag	2326

Cloning larger fragments into vectors

After combing the small DNA fragments together to form the larger fragments and confirming them for their size they were further taken to be cloned into a vector. Firstly the fragments were amplified to get larger amount of product and then they were purified by gel extraction method. Fragments A and C were cloned into pJet vector whereas fragment B was cloned into pBluescript all using blunt end cloning method. Fragment D which consists of genes HydEFG was already present in pSB1C3 plasmid. After the ligation reaction, the DNA ligation mixture was mixed with 100 µl of DH5α competent cells to favor transformation following the standard protocol and was plated in LB plates with ampicillin as selection antibiotic. The plates were then incubated overnight at 37 ºC.

Colony screening PCR

After transformation a number of colonies were seen in the growth plates. The obtained positive colonies were then taken forward for colony screening PCR. Out of total screening there were 8 positive colonies for fragment A, 16 for fragment B and 2 for fragment C shown in figure: 11, 12 and 13. The positive colonies were then cultured in LB and plasmid mini-preparation was carried out to prepare the DNA samples and were send further for DNA sequencing.

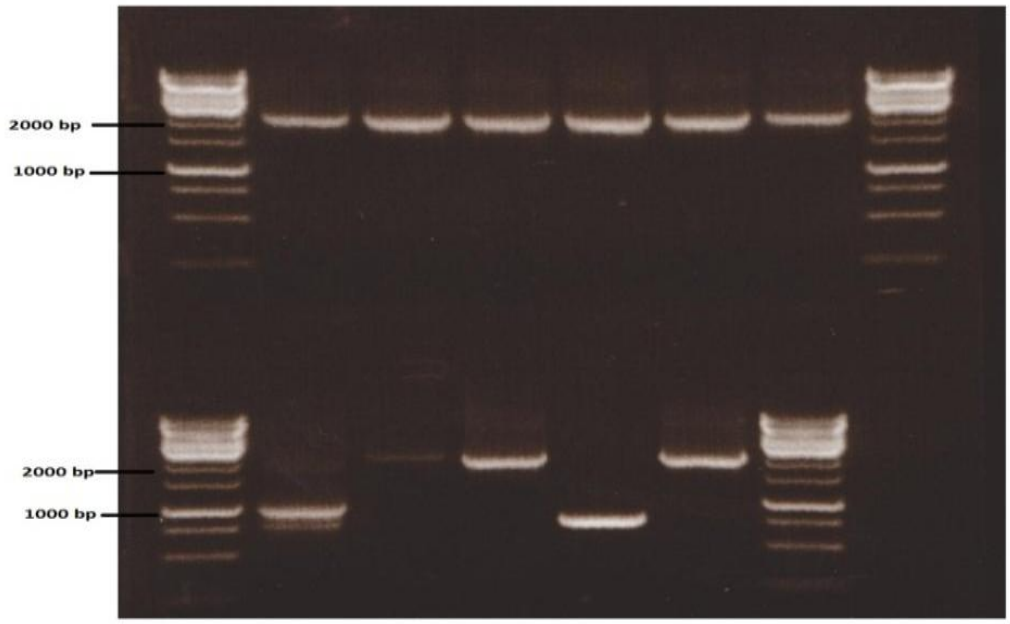

Figure 11. Colony screening PCR for fragment A

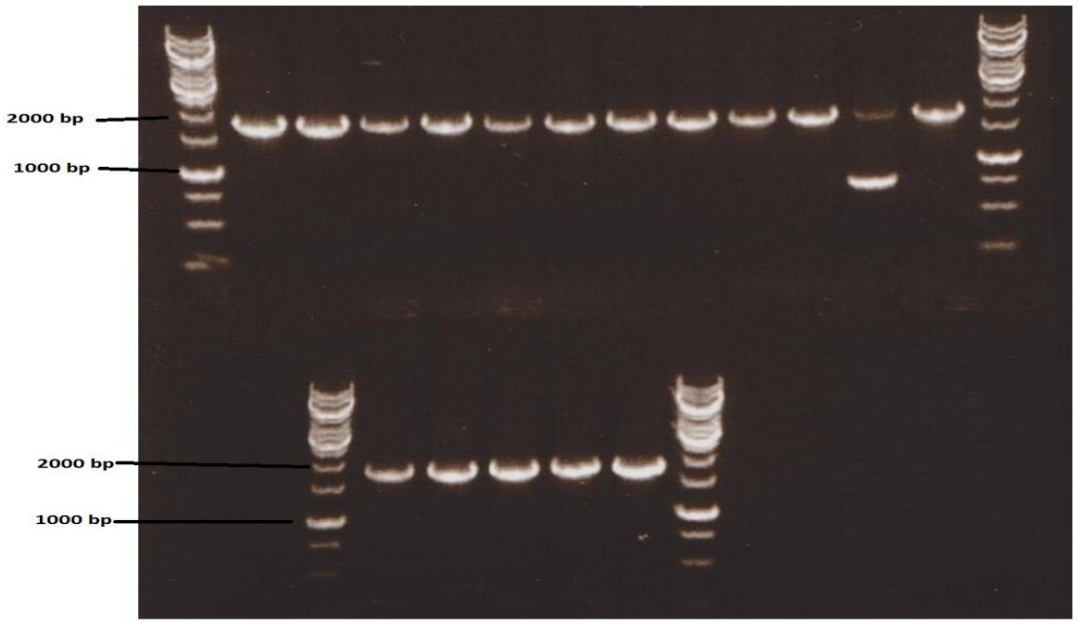

Figure 12. Colony screening PCR for fragment B

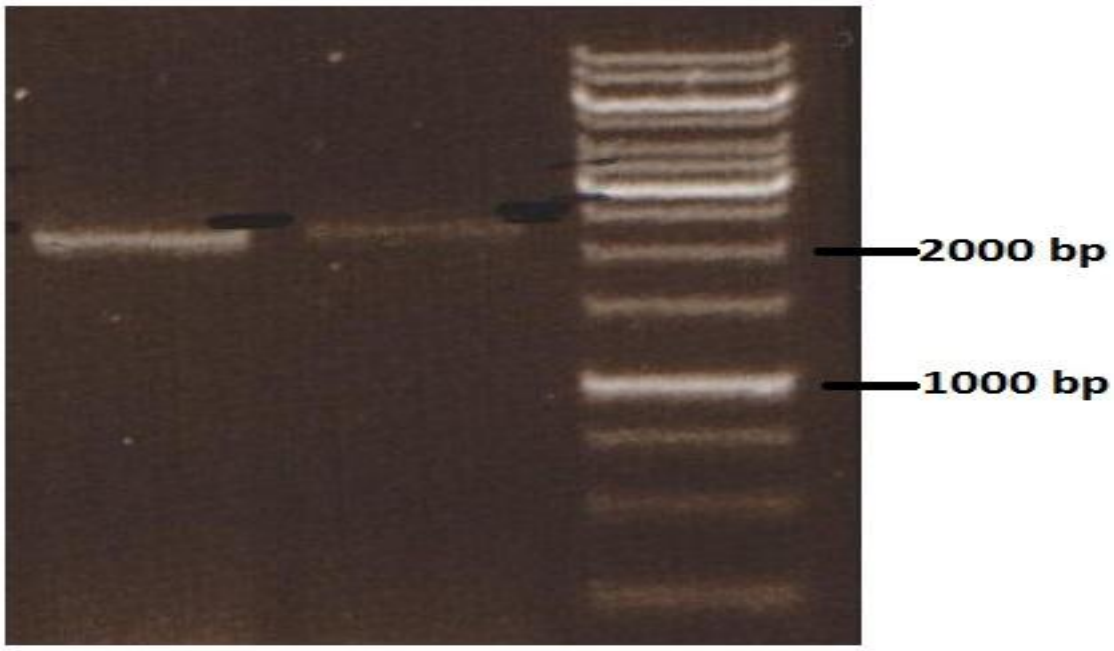

Figure 13. Colony screening PCR for fragment C

Sequencing to check the assembled fragments

To check if the cloning experiments was done successfully and that there was no mutation between the nucleotides the mini-preparation samples after screening of the colonies were sent for sequencing. All the fragments were found to be assembled correctly.

1 step isothermal recombination to form final construct

All the larger fragments A, B, C and D were amplified, purified using gel extraction method and then 3×10^{-4} moles of each fragments were mixed with Gibson master mix followed by 1 hour of incubation at 50ºC to favor 1 step isothermal recombination. A negative control was introduced which contained the same amount of different DNA fragments but dH_2O was added to it instead of Gibson master mix. The Gibson mix was then directly used to transform into DH5α cells and plated in LB with Chloramphenicol as selection antibiotic. I tried it many times but till date I haven't been able to find any colonies in any of those experiments.

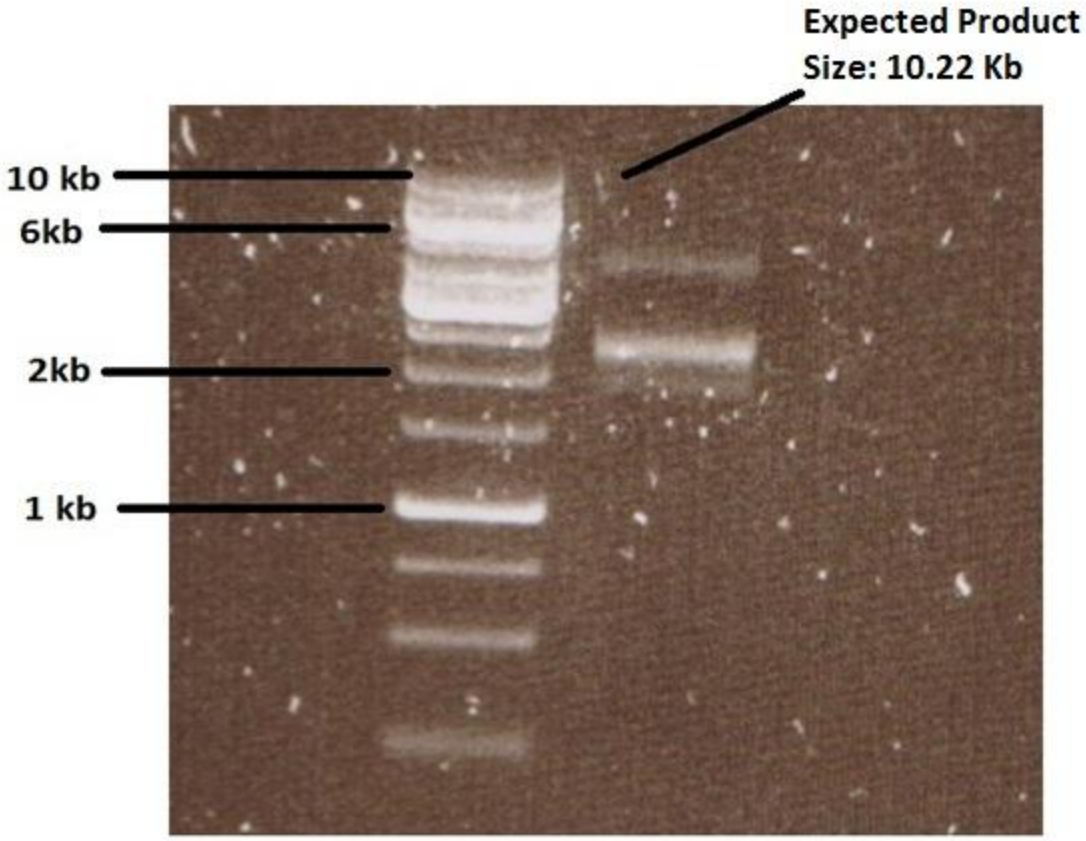

Figure 14. 1 step isothermal recombination to form final construct

Discussion

Starting with the amplification of all the small nine fragments as mentioned in table 10, the amplicons of the correct size were amplified which were confirmed on the basis of their size by checking the amplified product and also their restriction digestion product under 1% TAE gel. Those fragments were taken further for 1 step isothermal DNA assembly to construct larger fragments roughly of the same size which were named as A, B and C as mentioned on table: 12. These fragments were finally achieved and were checked for their size by running under 1% TAE gel as shown in figure 10, which finally were confirmed by sequencing. I tried several times to make my final construct using Gibson's assembly (1 step isothermal DNA assembly) method and transformed the product into competent cells and plated them in appropriate LB medium with Chloramphenicol as selection antibiotic. But always there was something fishy about it and the result didn't turn out be as expected. I never found any DNA band on the gel for the expected final product of 10.22 Kb or colonies growing in the plates which opens up the possibilities among one of the several reasons.

May be the DNA fragments have formed coils and secondary structure inhibiting the proper assembly, may be the self-replication part or the antibiotic cassette didn't function leading no growth.

Gibson's assembly method (1 step isothermal DNA assembly) is considerably new method in comparison to other ligation and cloning methods. It seems very straight forward, easy and saves quite a lot of time. Keeping that in mind I have been using this method in my project to make my construct. Till date I was able to combine the different pieces of DNA to form the larger fragment of around 2.5 kb but I was not able to combine the fragments more that this size and nor I was able to make a circular plasmid with this method. So it might be necessary to well characterize and optimize the conditions at which the Gibson's assembly method favors.

The construct was desined by a senior researcher Thorsten Heidorn who was working on the same project before. There might be quite a lot of information missing that could have been passed on to me with a face to face meeting. Some of the thing like the concept of the design, their past learnings, what didnt work and what was his critical view about the project was just limited to a bunch of the files that I was provided.

The construct which I am working on will be a circular plasmid as shown in figure: 6 will be inserted heterologously into the *Synechocystis sp*. PCC 6803. But because I have not been able to get the final construct till date I haven´t got the opportunity to check whether it works in the foreign system. So still the prediction of the higher efficiency of hydrogen gas production by the synthetic system is questionable.

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