Optimization of photoproduction of hydrogen by the bacterium *Rhodobacter capsulatus*: Role of Pyruvate Formate Lyase

Masters Thesis

A Dissertation submitted to the BRAC University in partial fulfillment of the requirement for the Masters of Science degree in Biotechnology

Submitted by

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DECLARATION OF ORIGINALITY OF THE WORK

This is to confirm that the Thesis entitled **"Optimization of photoproduction of hydrogen by the bacterium *Rhodobacter capsulatus*: Role of Pyruvate Formate Lyase"** is submitted in partial fulfilment for the degree of masters in Biotechnology, Department of Mathematics and Natural Sciences, BRAC University, Dhaka, was performed at Laboratoire Chimie et Biologie des Metaux (LCBM), Institute of life sciences research and technologies (iRTSV), French Atomic Energy and Alternative Energies Commission(CEA-CNRS), Grenoble, France during the period of January 2010 to June 2010.

No part of the work has been submitted for another degree or qualification in any other institutes at home or in abroad.

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CERTIFICATION OF ORIGINALITY OF THE WORK

This is to confirm that the Thesis entitled **"Optimization of photoproduction of hydrogen by the bacterium *Rhodobacter capsulatus*: Role of Pyruvate Formate Lyase"**, a requirement for partial fulfilment of the degree of Masters of Science (MS) in Biotechnology under the Department of Mathematics and Natural Science, BRAC University, was carried out in the Laboratoire Chimie et Biologie des Metaux (LCBM), Institute of life sciences research and technologies (iRTSV), French Atomic Energy and Alternative Energies Commission (CEA-CNRS), Grenoble, France during the period of January 2010 to June 2010, under our joint supervision.

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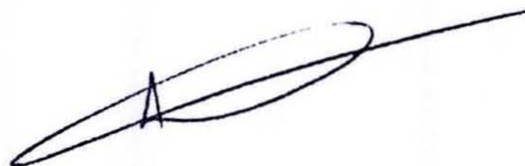

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RESUME

This thesis paper consists of an overview of the project work entitled with "Optimization of hydrogen photoproduction by the bacterium *Rhodobacter capsulatus:* Role of pyruvate Formate Lyase". The laboratory work has been conducted at Laboratoire Chimie et Biologie des Metaux, CEA Grenoble, France, under a joint collaboration between BRAC University and CEA-CNRS. The CEA is the French Atomic Energy Commission. It is a public body established in October 1945 and a leader in research, development and innovation in Europe. The CEA is active in three main fields: Energy, information & health technologies, and defence & national security. In each of these fields, the CEA maintains a cross-disciplinary culture of engineers and researchers, building on the synergies between fundamental and technological research. It is performing its research with the collaborative support of the institute CNRS (The *Centre National de la Recherché Scientifique*) which is a government-funded research organization, under the administrative authority of France's Ministry of research.

This research project has been performed in the fulfilment for the masters program MS in Biotechnology at BRAC University, under the constant supervision of Dr. John Willison, a senior researcher of CEA. The aim of this project is to investigate the factors influencing the production and utilization of hydrogen using anaerobic bacteria, *Rhodobacter Capsulatus.*

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THE AUTHOR

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SUMMARY

The bacterium *Rhodobacter capsulatus* uses solar energy to produce hydrogen, a clean energy from many organic substrates derived from renewable sources. Two types of enzymes can catalyze the reduction of protons to H_2, namely nitrogenase and hydrogenase. Although much progress has been made in the elucidation of gene expression, structure and regulation of these key enzymes, no practical and economically competitive process for the continuous production of biological H_2 (biohydrogen) has, as yet, been put on the market. One of the difficulties is due to the fact that in the photoproduction of H_2, other photofermentation reactions compete with the nitrogenase and thus reduce the efficiency of H_2 production. So, the aim of this project is to enhance hydrogen photoproduction by minimizing the competing photofermentaion reactions. In doing so, we have observed hydrogen production in the presence of lactate or malate as carbon sources and limited concentration of nitrogen. These organic substrates are dissimilated through some pathways and molecular H_2 results from the direct reduction of protons from water. For the complete dissimilation of carbon substrates to H_2 and CO_2, carbon source is depleted from the medium through metabolism. In a step of lactate or malate catabolism, pyruvate is produced by the action of an enzyme pyruvate formate lyase. This produced formate in the metabolic pathway competes with the H_2 by sharing the same metablites, and H_2 production is decreased. For this reason, the gene encoding this enzyme, pyruvate formate lyase, is targeted to switch off by transposon mediated mutagenesis. Here the 2 types of pfl genes B and D were in target and mutagenesis was performed successfully. But at the end, in the biochemical tests for checking formate production from the mutant culture, still it was observed slight production of formate. So from this point of view, the experiment was confusing. But the molecular biology approaches like PCR and hybridization techniques were followed further to check the presence of mutated gene products and the experiments were successful in that regard.

Chapter: 1
Introduction

Hydrogen is a clean and efficient fuel, considered as a potential and more sustainable energy substitute for fossil fuels. It has been predicted that the contribution of hydrogen to global energy consumption will increase dramatically, to approximately 50%, by the end of the 21st century due to the development of efficient end-use technologies, possibly becoming the main final energy carrier. Thus hydrogen will play a strategic role in the pursuit of a low-emission energy source for environmental demand [1, 2]. To this end, it will be necessary for hydrogen to be produced renewably and on a large scale. The global hydrogen production system, initially fossil-fuel based, is shifting progressively toward renewable sources. The following technologies for the conversion of secondary and primary fuels into hydrogen are being investigated extensively: electrolysis, coal gasification, steam methane reforming of natural gas, partial oxidation of fuel oil, solar thermal cracking, biomass gasification and photobiological synthesis [1–5]. Biological hydrogen production stands out as an environmentally harmless process carried out under mild operating conditions with renewable resources. Also, low conversion efficiencies of biological systems can be compensated for, by low energy requirements and reduced initial investment costs.

Biological energy conversions can be categorized into two groups: i) photosynthesis (the process whereby solar energy is fixed to yield energy useful to organisms and industry), and ii) biomass conversion (the product of photosynthesis) into energy. Photosynthesis occurs in plants, algae and photosynthetic bacteria, while biomass conversion reactions often occur in non-photosynthetic microorganisms. Currently, much research on hydrogen production is carried out with laboratory-scale or pilot-scale reactors using photosynthetic microorganisms [3–11].

Among all these photosynthetic microorganisms, photosynthetic non-sulphur (PNS) bacteria drew much attention of the scientists because of high hydrogen yield, hence the study bacteria of this work is photosynthetic non-sulphur bacteria *Rhodobacter capsulatus* which is a genus of *Rhodospirillaceae* family. (Photobacteria subdivision shown in appendix-1)

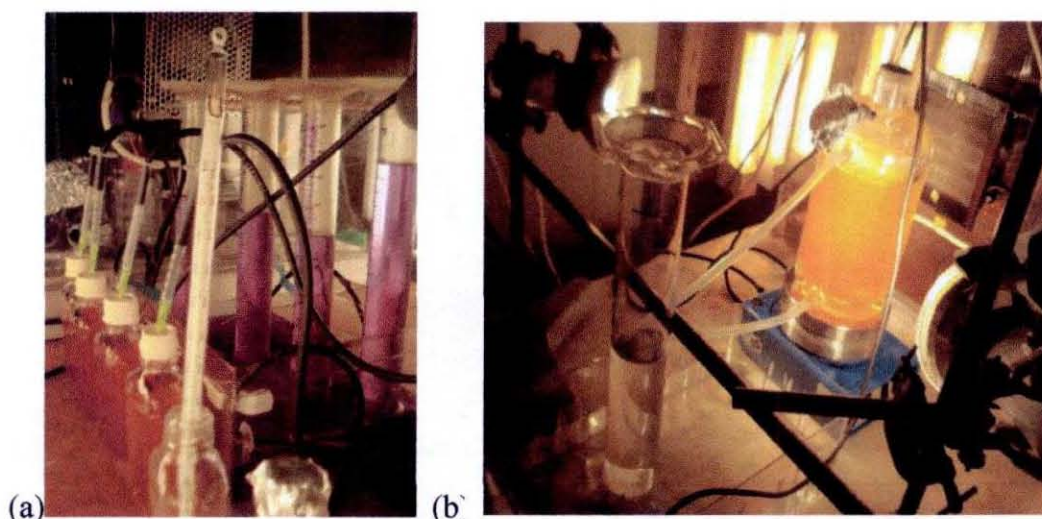


Figure 1: Laboratory scale continuous production of H_2 from PNS bacteria *Rhodobacter capsulatus* in (a) small vessels with mutants and (b) large 1litre volume culture (Photo courtesy: LCBM/iRTSV, CEA-Grenoble, France)

Bacterial photosynthesis is thought to be a relatively old form of photosynthesis. It incorporates the use of either organic or sulfur compounds as electron donors in photosystem I (Fig. 1). Unlike in the case of plant photosynthesis, cyclic photophosphorylation takes place in bacterial photosynthesis, i.e. electrons are repeatedly excited in a cyclic manner, with ATP being generated in each cycle. Photosynthetic bacteria are also capable of reducing electron carriers such as NAD, via a linear reaction similar to the electron transmission which occurs during plant photosynthesis.

CO_2-fixing reactions do not produce energy during bacterial photosynthesis (i.e. equimolar amounts of organic compounds are produced through decomposition of organic compounds), except when sulfur compounds serve as electron carriers. The mechanisms for photosynthetic hydrogen production are summarized in Fig. 2.

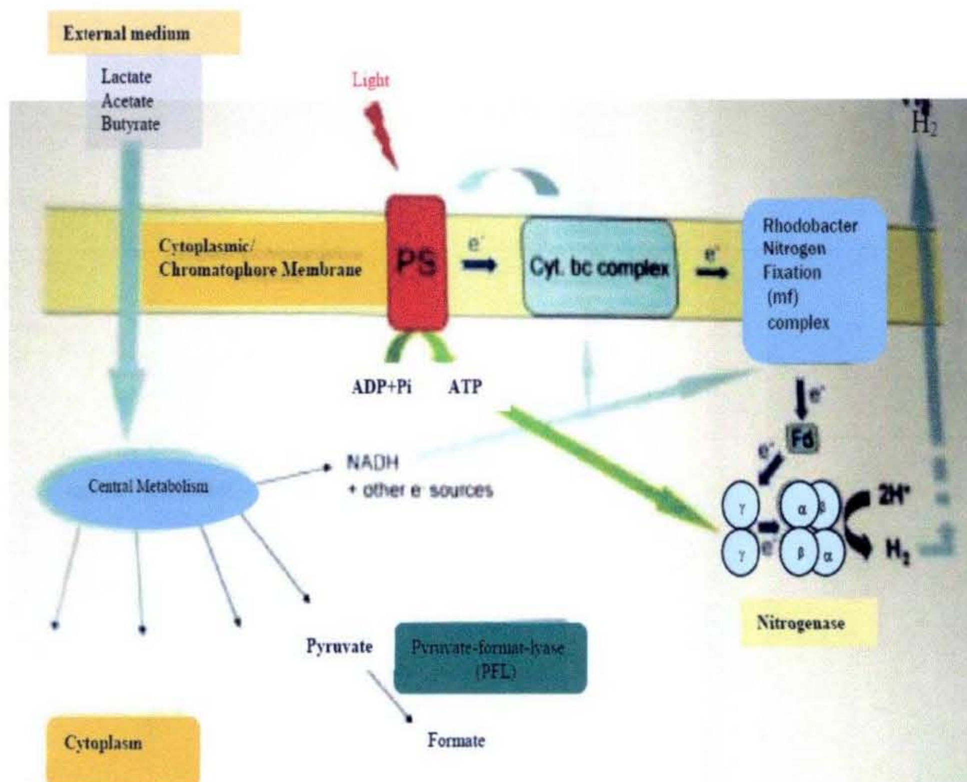
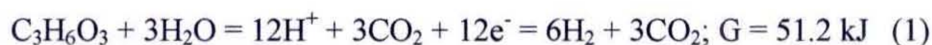


Figure 2: Schematic diagram of H_2 photoproduction by *Rhodobacter capsulatus*
(*Photo courtesy: Dr. John C. Willison, LCBM, CEA, Grenoble, France*)

Upon exposure of ammonia-free media containing photosynthetic bacteria to light, nitrogenase activity is induced, resulting in hydrogen production. Organic substances such as lactic acid (Eq. 1) serve as electron donors in photosynthetic bacteria. In such reactions, G is positive, indicating that the use of solar energy allows photosynthetic bacteria to produce hydrogen through complete decomposition of organic substances. Anaerobes such as *Clostridium* also produce hydrogen, but are incapable of completely utilizing energy or decomposing organic substances.



$$C_3H_6O_3 + 3H_2O = 12H^+ + 3CO_2 + 12e^- = 6H_2 + 3CO_2;\ G = 51.2\ kJ \quad (1)$$

$$C_6H_{12}O_6 + 2H_2O = 4H_2 + 2CH_3COOH + 2CO2;\ G = -184.2\ kJ \quad (2)$$

So according to the equation, 6 mole of hydrogen are expected to be produced per mole of lactate utilized by *rhodobacter*.

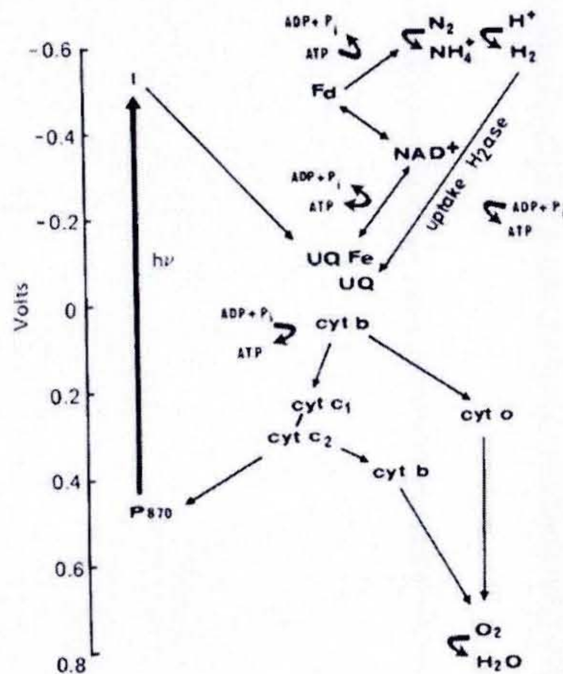


Figure 3: Mechanism for hydrogen production by photosynthetic bacteria [13]

Organic substances are utilized as electron donors by photosynthetic bacteria. The energy required for extracting electrons from these molecules is much lower than that required for the hydrolysis of water. Photo-energy is more often used for nitrogenase activation, i.e. ATP reproduction, than for the decomposition of organic substances. On the other hand, hydrogenases catalyze hydrogen-producing reactions without ATP requirements. Hydrogenase-catalyzed reactions are reversible, and are either biased in favour of hydrogen production or hydrogen uptake. Hydrogenases in *Clostridium* and other bacteria work primarily to produce hydrogen, while hydrogenases in photosynthetic bacteria work toward hydrogen uptake. Hydrogen-producing efficiency is known to be higher in hydrogenase-deplete strains of photosynthetic bacteria.

A key factor in determining the commercial applicability of hydrogen production processes is the rate at which hydrogen is produced. Bacteria have been widely investigated for their rates of hydrogen production. To date, *Rhodobacter capsulatus* has been identified as the bacterium having the highest hydrogen-producing rate [7], with a photoenergy conversion efficiency of 7%, (energy yield by combustion of produced hydrogen/incident solar energy) determined using a solar simulator [7, 8].

Further strain development will potentially elevate the energy conversion efficiency of photosynthetic bacteria to levels comparable to those of solar batteries.

The photoproduction of H_2 by *R. capsulatus* is due to the enzyme nitrogenase, which, in the absence of alternative substrates, is able to reduce protons to H_2 [14, 15]. Anaerobic conditions and high light intensities are required, in addition to an effectively metabolized substrate [16] found that the highest rates of H_2 production were shown by cells grown with DL-lactate or pyruvate as carbon and energy source and glutamate as growth-limiting nitrogen source; lactate was converted to H_2 and CO_2 with a yield of 72% of the theoretical maximum. Other organic acids gave slightly lower rates and yields of H_2 production, while sugars were utilized much less efficiently. The rate of H_2 production could be doubled by growth in nitrogen-limited continuous culture [17], and yields approaching 80% were obtained. However, despite these investigations, little is yet known of the regulatory mechanisms which determine the efficiency of H_2 production from different substrates.

In addition to nitrogenase, *R. capsulata* possesses a membrane-bound hydrogenase, which appears to function exclusively in H_2 uptake, i.e. H_2 oxidation under physiological conditions [18]. This enzyme enables *R. capsulata* to grow autotrophically on a mixture of H_2 and CO_2. Hydrogenase is also synthesized under heterotrophic growth conditions and is present in the highest levels in H_2-evolving cultures, suggesting that its synthesis is induced by H_2 [19]. However, the physiological role of hydrogenase under heterotrophic conditions is not known. In other diazotrophic bacteria, it has been suggested that the role of hydrogenase is to recycle H_2 evolved by nitrogenase, thereby increasing the energetic efficiency of the nitrogen fixation reaction; or, alternatively, to facilitate the protection of nitrogenase against oxygen by allowing H_2 to serve as a substrate for the aerobic respiratory chain [20]. In *R. capsulata*, H_2 can serve as a substrate for the aerobic respiratory chain [21], participate in the photoreduction of CO_2 [22], or act as an electron donor to nitrogenase. However, recycling of H_2 evolved by nitrogenase has been observed only in cells depleted of organic substrates suggesting that H_2 recycling is negligible in cultures actively evolving H_2 [23]. So, it is found that the production rate and the yield of H_2 vary greatly depending on the carbon source used and the experimental, physiological conditions, such as light intensity or pH [15, 21]. On the other hand,

several studies have shown that mutant strains can be isolated and show improved hydrogen producing capabilities compared to the wild-type [24,25].

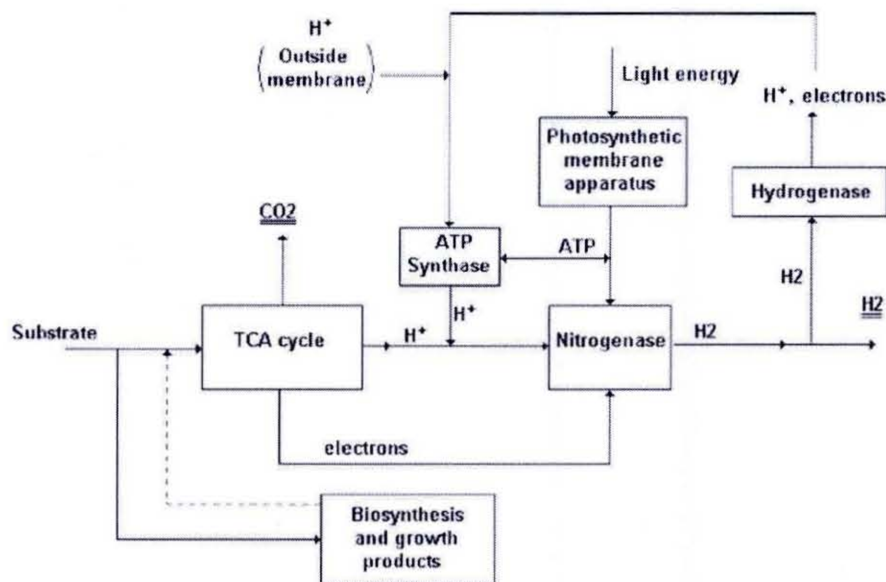


Figure 4: The overall Scheme of H_2 production by Photosynthetic non-sulphur (PNS) bacteria

So, from the above discussion it is obvious that there are several factors that influence the efficiency of hydrogen photoproduction from anoxygenic bacteria. In order to investigate the factors influencing the production and utilization of H_2, scientists have isolated mutants of *R. capsulata* unable to grow photoautotrophically on H_2 and CO_2. Some of these mutants were found to produce increased amounts of H_2 from various organic substrates. The present study emphasized on enhanced H_2 production by purple non-sulphur bacteria *Rhodobacter capsulatus*, by selecting more active strains and mutants optimising the medium and ceasing the components competing with H_2 production. This increased H_2 production was found to be unrelated to specific defects in the enzymes of autotrophic metabolism, and appeared instead to be due to an altered carbon metabolism, which affects the flow of reducing equivalents from organic substrates to nitrogenase.

The metabolic and nutritional versatility of *rhodobacter* was tested by using different carbon and nitrogen sources rather than lactate or malate and ammonium. The growth and H_2 productivity of the mutants were tested on these substrates. These new

substrates like ethanolamine and diols are proven to be very promising substitute of present traditional ones because of their easy availability in biomass. Ethanolamine was tested as sole nitrogen source in producing H_2 by *Rhodobacter capsulatus.* Ammonium salts severely repress the evolution of hydrogen so the possible choice is ethanolamine. It is currently used in the industrial process of fixing carbon dioxide and is discarded as relatively pure waste. So using ethanolamine as nitrogen source is both efficient and renewable system. [26]

Growth experiments were performed with the purple nonsulfur bacterium *Rhodobacter capsulatus* to test its ability to use aliphatic, methyl-substituted, and unsaturated alcohols, as well as di-alcohols, as carbon sources for growth. tested a number of alcohols as growth substrates for this species. Interestingly, we have found that *R. capsulatus* can catabolize a variety of di-alcohols (diols) showing that the nuritiona versatility of this organism is even higher than suspected.Rhodobacter capsulatus strain B10 was previously proven efficient for growth in diols (Panagiotis et al.). So in this experiment, to check growth efficiency of the mutants, B10 was considered as positive control and again greatly expanded the broad carbon nutritional spectrum of this organism. Thus low soluble and high toxic alcohols can be used renewably by *rhodobacter* as a promising end use technology. [27]

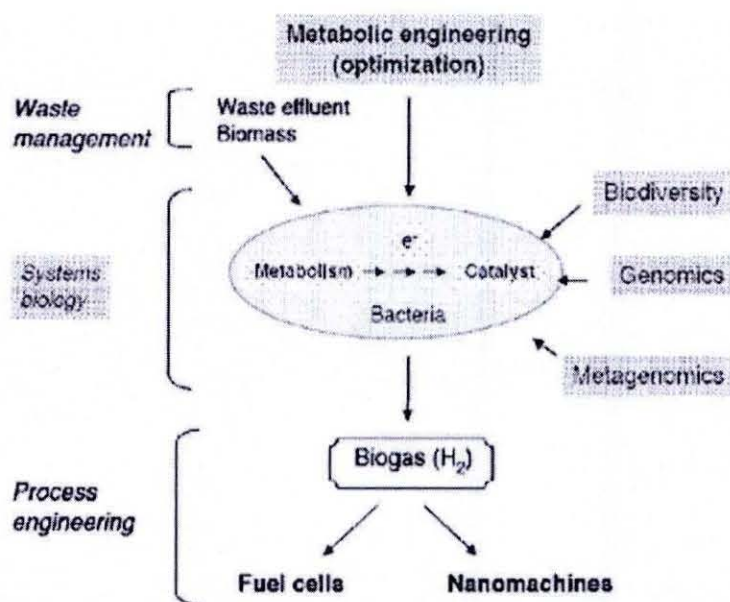


Figure 5: The need for integrated (pluridisciplinary) research in the development of efficient biological systems for hydrogen production and use [47]

This figure indicates how this approach may be integrated into the overall scheme of biological H_2 production, in combination with other approaches, such as genomics and biodiversity studies, as well as the emerging sciences of metagenomics and systems biology. Its application, however, will require a detailed understanding of the metabolic pathways and regulatory circuits involved. [47]

At the end, all the approaches still to be improved as the principal target product competing with H_2 still producing from the culture.

Objectives

General objectives

The general objective of the present study was to optimize hydrogen photoproduction by the photosynthetic bacterium *Rhodobacter capsulatus* to determine the role of pyruvate formate lyase in growth and hydrogen photoproduction in various growth conditions.

Specific objectives

The specific objectives of the present study were:

- To increase growth and yield of hydrogen by inhibiting formate production in the selected strains of *Rhodobacter capsulatus.*
- Insertion of Kanamycin resistance (Km^r) cassette inside the *pyruvate formate lyase gene* by conjugation and transformation in order to deactivate it so that formate production is inhibited
- To determine the efficiency of growth and hydrogen production of the mutant strains in diverse growth conditions

Chapter: 2
Methods and Materials

Photosynthetic bacteria are favorable candidates for biological hydrogen production due to their high conversion efficiency and versatility in the substrates they can utilize. For large-scale hydrogen production, an integrated view of the overall metabolism is necessary in order to interpret results properly and facilitate experimental design. In this study, a summary of the hydrogen production metabolism of the photosynthetic purple non-sulfur (PNS) bacteria will be presented. Practically all hydrogen production by PNS bacteria occurs under a photoheterotrophic mode of metabolism. Yet results show that under certain conditions, alternative modes of metabolism—e.g. fermentation under light deficiency—is also possible and should be considered in experimental design.

Two enzymes are especially critical for hydrogen production. Nitrogenase promotes hydrogen production and uptake hydrogenase consumes hydrogen. Though a wide variety of substrates can be used for growth, only a portion of these is suitable for hydrogen production. The efficiency of a certain substrate depends on factors such as the activity of the TCA cycle, the carbon-to-nitrogen ratio, the reduction-state of that material and the conversion potential of the substrate into alternative metabolites such as PHB. All these individual components of the hydrogen production interact and are subject to strict regulatory controls.

In research targeting large-scale biological hydrogen production, the efficiency of H_2 production is designed to increase by molecular biology tools such as transposon mediated mutagenesis (loss of function mutation) of the genes interrupting efficient H_2 production. So, in this experimental work, *Rhodobacter Capsulatus,* a member of photosynthetic purple non-sulfur (PNS) bacteria has been studied thoroughly by using various biological methods.

2.1 Microbiology methods

2.1.1 Culture of bacteria

2.1.1.1 Preparation of the basic solutions of the culture medium

Super salts (Mixture of mineral salts and vitamins):

4g Mg $SO_4.7H_2O$, 1.5g $CaCl_2.2H_2O$, 0.29g Fe (III) EDTA, 20 mg Thiamine-HCl and 20ml of micro nutrients dissolved in 1 Litre deionised water. (Micro nutrients preparation: 0.3975 g $MnSO_4.H_2O$, 0.7 g H_3BO_3, 0.01 g $CuSO_4.5H_2O$, 0.06 g $ZnSO_4.7H_2O$ and 0.1875 g $Na_2MoO_4.2H_2O$ in the 500 ml deionised H_2O)

Na-lactate, 1M, pH 6.8 (Carbon source for bacterial growth and production of production of hydrogen)

186.6 g sodium lactate (60% w/v) was weighed in a beaker, 800 ml deionised H_2O was added to it and pH was adjusted to 6.8 by 10 M NaOH. The solution was made up to 1L by deionised H_2O.

K-phosphate, 0.64 M, pH 6.8: (KPi buffer)

20 g KH2PO4 and 30 g K2HPO4 dissolved in 500 ml deionised H2O and pH adjusted to 6.8 with 10 M KOH.

$(NH_4)_2$ SO_4, 10% (w/v) (Nitrogen source for pre cultures)

50 g $(NH_4)_2$ SO_4 dissolved in 500 ml deionised H_2O

Na-glutamate, 1 M, pH 7 (Nitrogen source for the production of hydrogen)

14.7 g glutamic acid or 18.7 g sodium hydrogen glutamate. H_2O in the 100ml deionised H_2O. pH was adjusted to 7 with 1 M NaOH.

- All the above solutions are stored in 4° C.

2.1.1.2 Preparation of the culture medium

Table 1: RCV (lactate-ammonium) medium for the pre culture of bacteria

Composition	Volume (ml)
"Super salts" (solution of mineral salts)	50 ml
K-phosphate, 0.64 M, pH 6.8	15 ml
1 M Na-lactate, pH 6.8	30 ml
10% (w/v) $(NH_4)_2 SO_4$	10 ml
Distilled H_2O	Up to 1 L after adding all

→ pH adjusted to 6.8 with 1 N HCl (liquid medium) and autoclaved for 120 min at 121° C.

- For different carbon sources, RCV-C medium was prepared
- For different nitrogen sources, RCV-N medium was prepared
- For different carbon & nitrogen sources, RCV-C-N was prepared.

Table 2: RCV-LG (lactate-glutamate) medium for the production of H2

Composition	Volume (ml)
"Super salts" (solution of mineral salts)	50 ml
K-phosphate, 0.64 M, pH 6.8	15 ml
1 M Na-lactate, pH 6.8	30 ml
1 M Na-glutamate, pH 7	7 ml
Distilled H_2O	Up to 1 L after adding all

→ pH was adjusted to 6.8 with 1 N HCl (liquid medium) and autoclaved for 120 min at 121° C.

2.1.1.3 Preparing pre-cultures of bacteria for hydrogen production

*Rhodobacter*s were preserved inside a special vial (MicroBank) inside the beads at -80° C. 2-3 stock beads were put inside 15 ml of RCV(lactate-ammonia) media. Pre-cultures were grown photosynthetically in the incandescent light at 30-32° C. for 2-3 days.

Strains used: -Wild type *Rhodobacter capsulatus* B10

Mutant *Rhodobacter capsulatus* IR3

2.1.2 Measurements of photo production of hydrogen

2.1.2.1 Preparing culture for hydrogen production

- 2 tubes of 25 ml RCV-C-N media were taken and autoclaved
- 5 ml media was taken out from it to make over head space in the tubes for hydrogen collection
- 600 μl 1 M lactate and 140 μl 1 M glutamate were added to the tubes to make the final concentrations 30mM and 7mM consecutively
- Each tube was treated with argon for 10-15 minutes to make the culture anaerobic
- Sterile rubber stoppers were sealed tightly on the tubes
- 0.5 ml from each pre-cultures of *rhodobacter* (B10 & IR3) were added to the media with syringe.
- Incubated photo synthetically for 16-18 hrs at 28° C
- To exert extra pressure caused by argon, a pin hole was made with sterile needle after 1 hr of incubation.

2.1.2.2 H_2 collection from the bacterial culture

A glass pipette was filled with water and set upside down in half-filled water tub. After ~18 hrs, H_2 was formed as bubbles from the culture and was collected inside the glass pipette at the head space.

Standard H_2 production rate is→ 2 ml/min/L culture

2.1.3 Experiments with GTA: complementation of nitrogen fixation of the Nif- mutants

2.1.3.1 Preparation of the required medium

Table 3: RCV agar medium preparation

Composition	Volume (ml)	Volume (ml)
"Super salts" (solution of mineral salts)	50 ml	50 ml
1 M Na-lactate, pH 6.8	30 ml	30 ml
10% (w/v) $(NH_4)_2 SO_4$	10 ml	------
Agar	15 g	15 g
Distilled H_2O	910 ml	920 ml
K-phosphate, 0.64 M, pH 6.8	15 ml (added separately)	15 ml (added separately)

- No need to adjust pH
- Autoclaved the media components mixture (except KPi) for 20 min at 121° C.
- KPi added separately after autoclaving
- Media poured in the Petri dishes when hand-touch cold
- Solidified for 24 hrs

- For **RCV+Tc^1+Km^{10} plates** → 1 ml Tc^1 (1 mg/ml) & 2 ml Km^5 (5 mg/ml) were added before pouring into Petri dishes

- For **RCV+Km[10] plates** → 2 ml Km[5] (5 mg/ml) was added before pouring into Petri dishes

Table 4: RCV-malate medium preparation

Composition	**Volume (ml)**
"Super salts" (solution of mineral salts)	50 ml
K-phosphate, 0.64 M, pH 6.8	15 ml
10% DL-malate, pH 6.8	40 ml
10% (w/v) $(NH_4)_2SO_4$	10 ml
Distilled H_2O	Up to 1 L after adding all

→ pH was adjusted to 6.8 with 4 N HCl and autoclaved for 120 min at 121° C.

Table 5: YPS medium preparation

Components	**Amount (g or ml)**
Difco yeast extract	3 g
Bacto peptone	3 g
20% (w/v) $MgSO_4.7H_2O$	2.5 ml
7.5% (w/v) $CaCl_2.2H_2O$	4.0 ml
Agar	15 g

- No need to adjust pH
- Autoclaved for 20 min at 121° C.
- Media poured in the Petri dishes when hand-touch cold
- Solidified for 24 hrs
- Preserved in the cold room (4° C.)

2.1.3.2 G-buffer preparation

- 100 mg BSA layered on top of 10 ml d H_2O & let mixed spontaneously
- 0.5 ml BSA added to 10 ml G-buffer
- Mixture filter sterilized

2.1.3.3 GTA crosses: Experiment-1: With RC1 & SB1003

STEP-1:

Preparation of pre culture of *Rhodobacter* SB1003 & RC1

- Prepared from stock beads preserved in -80° C. into sterile 10 ml RCV media
- Both kept in the light room for 24 hrs for photosynthetic growth

Inoculating *Rhodobacter* SB1003 & RC1 cultures for GTA production

- 10 ml YP medium & 10 ml RCV-malate taken in glass tubes kept in the light to be warm for 30 min (as high temperature decrease the half life and growth is fastened)
- 300 µl SB1003 added in the pre warmed YP medium containing in the glass tube for anaerobic culture in the light
-300 µl RC1 added in the pre warmed RCV-malate media in the light
- Both of these cultures were grown photo synthetically for 24 hrs
-300 µl SB1003 added in the 10 ml YP medium containing in the falcon tube and was grown aerobically for 24 hrs

STEP-2:

Bacterial growth was monitored by measuring absorbance at 660 nm ($O.D._{660}$) of the cultures.

STEP-3:

-1000 µl SB1003 (hv) and SB1003 (O_2) each taken in eppendorf tubes and centrifuged for 5min at 6000 rpm.
-The supernatant was taken not touching the bacterial cells and filter sterilized.
-300 µl RC1 centrifuged for 5min at 6000 rpm, supernatant discarded and pellet was re-suspended in 1 ml G-buffer.

-0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from SB1003 (hv) mixed in 15 ml tube →(a)

-0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from SB1003 (O_2) mixed in 15 ml tube →(b)

-0.5 ml RC1 pellet and 0.5 ml YP medium (used as control) mixed in 15 ml tube

-These 3 tubes were incubated for 60 min at 30° C.

STEP-4:

-3 controls were taken: 1. Direct supernatant of SB1003 (hv)

2. Direct supernatant of SB1003 (O_2)
3. Cell pellet of RC1 with YP medium

-For GTA crosses of each type, [SB1003 (hv) × RC1] & [SB1003 (O_2) × RC1], the following combinations were streaked on to RCV plates:

10 μl GTA cross + 90 μl YP medium
20 μl GTA cross + 80 μl YP medium
50 μl GTA cross + 50 μl YP medium
100 μl GTA cross directly

-100 μl of each of the controls were streaked on to the RCV plates

-The 11 plates were grown photo synthetically inside anaerobic jars for 3 days

2.1.3.4 GTA crosses: Experiment-2: with RC1 & SB1003

STEP-1

Making RC1 & SB1003 pre-culture:

- 500 μl SB1003 from old culture in 15 ml YP medium in a falcon tube
- 1 ml RC1 in 17 ml RCV malate in a glass tube
- Photosynthetic growth of the cultures for 24 hrs

Inoculating *Rhodobacter* SB1003 & RC1 cultures for GTA production:

- 3 glass tubes of RCV malate of 17 ml and 3 glass tubes of YP medium of 15 ml pre warmed keeping in the light room for 30 min
- 0.1 ml, 0.2 ml & 0.5 ml of both RC1 and SB1003 pre-cultures inoculated in RCV-malate & YP medium consecutively

- Light incubation for 24 hrs

STEP-2

Bacterial growth was monitored by measuring absorbance at 660 nm (O.D.$_{660}$) of the cultures.

STEP-3

- 1000 μl SB1003 (0.1 ml, 0.2 ml & 0.5 ml) each taken in eppendorf tubes and centrifuged for 5min at 6000 rpm.
- The supernatant was taken not touching the bacterial cells and filter sterilized.
- RC1 culture containing 0.1 ml inocula was taken as the recipient cells
- 300 μl RC1 taken in 3 eppendorf tubes nd centrifuged for 5min at 6000 rpm, supernatant discarded and pellets were re-suspended in 1 ml G-buffer.
- 0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from SB1003 (0.1 ml) mixed in 15 ml tube →(a)
- 0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from SB1003 (0.2 ml) mixed in 15 ml tube →(b)
- 0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from SB1003 (0.5 ml) mixed in 15 ml tube →(c)
- 0.5 ml RC1 pellet and 0.5 ml YP medium (used as control) mixed in 15 ml tube
- These 4 tubes were incubated for 60 min at 30° C.

STEP-4:

- **4 controls were taken:** 1. Direct supernatant of SB1003 (0.1 ml)

  3. Direct supernatant of SB1003 (0.5 ml)

  4. Cell pellet of RC1 with YP medium

- For GTA crosses of each type, [SB1003(0.1 ml)×RC1], [SB1003(0.2 ml)×RC1] & [SB1003(0.5 ml)×RC1], the following combinations were streaked on to RCV plates:

  10 μl GTA cross + 90 μl YP medium

  100 μl GTA cross directly

- 100 μl of each of the controls were streaked on to the RCV plates
- The 9 plates were grown photo synthetically inside anaerobic jars for 3 days

2.1.3.5 GTA crosses: Experiment-3: With Y262 & RC1

STEP-1

Preparing of pre culture of *Rhodobacter* Y262 & RC1:

- 200 μl Y262 from old culture in 17 ml RCV-malate in a glass tube
- ¾ beads of RC1(-80°C.) in 17 ml RCV malate in a glass tube
- Photosynthetic growth of the cultures for 24 hrs

Inoculating *Rhodobacter* Y262 & RC1 cultures for GTA production:

- 200 μl of Y262 in → 20 ml of YP medium in glass tube (hv)
  → 20 ml of YP medium in flask (O_2)
- 3 glass tubes of RCV malate of 17 ml taken
- 0.1 ml, 0.2 ml & 0.5 ml of RC1 pre-culture inoculated in these 3 tubes
- Light incubation for >24 hrs

STEP-2

Bacterial growth was monitored by measuring absorbance at 660 nm ($O.D._{660}$) of the cultures.

STEP-3

- 1000 μl Y262 (O_2) → Centrifuged for 5min at 6000 rpm., then filtered → GTA (A)
  → Filtered directly → GTA (B)

- 1000 μl Y262 (hv) → Centrifuged for 5min at 6000 rpm., then filtered → GTA (C)
  → Filtered directly → GTA (D)

- ×100 dilution of each GTA (10 μl in 1 ml YP medium)

- RC1 culture containing 0.1 ml inocula was taken as the recipient cells while crossing

- 300 μl RC1 taken in 3 eppendorf tubes and centrifuged for 5min at 6000 rpm, supernatant discarded and pellets were re-suspended in 1 ml G-buffer.

- 0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from Y262 (O_2) both direct and centrifuged mixed in 15 ml tube →(a) & (b)

- 0.5 ml RC1 pellet and 0.5 ml supernatant (containing GTA) from Y262 (hv) both direct and mixed in 15 ml tube →(c) & (d)

- 0.5 ml RC1 pellet and 0.5 ml YP medium (used as control) mixed in 15 ml tube

- All of the above tubes were incubated for 60 min at 30° C.

STEP-4:

- **3 controls were taken:** 1. Cell pellet of RC1 with YP medium
  2. Direct supernatant of Y262 (O_2)
  3. Direct supernatant of Y262 (hv)

-100 µl GTA crosses were directly streaked on to RCV-N plates of all the 4 types

(a) [Y262(O_2-Direct)×RC1]

(b) [Y262(O_2-Centrifuged)×RC1]

(c) [Y262(hv-Direct)×RC1]

(d) [Y262(hv-Centrifuged)×RC1]

- The 7 plates were grown photo synthetically and anaerobically inside anaerobic jars for 4-5 days

2.2 Biochemical assays

2.2.1 Metabolite analysis

2.2.1.1 Growth test of Rhodobacter capsulatus with Glycols (diols) as carbon source

Stock solutions preparation (1 M, 10 ml each)

[Following the formula, Density (d) = Molecular weight (M.W.)/ Volume (V)]

1, 3-Propanediol, 98%:

M.W. = 76.09 g/mol, d =1.053 g/cm^3

V= 76.09/1.053 ml=72.05 ml

So 1 M 10 ml solution ≡ 720 µl 1, 3-Propanediol + 9.28 ml dH_2O

1, 2-Propanediol:

M.W. = 76.09 g/mol, d =1.036 g/cm^3

V= 76.09/1.036 ml=73.5 ml

So 1 M 10 ml solution ≡ 735 µl 1, 3-Propanediol + 9.265 ml dH_2O

2, 3-Butanediol:

M.W. = 90.12 g/mol, d =1.003 g/cm^3

V= 90.12/1.003 ml=89.8 ml

So 1 M 10 ml solution ≡ 898 µl 1, 3-Propanediol + 9.102 ml dH_2O

1, 3-Butanediol:

M.W. = 90.12 g/mol, d =1.004 g/cm^3

V= 90.12/1.004 ml=89.7 ml

So 1 M 10 ml solution ≡ 897 µl 1, 3-Propanediol + 9.103 ml dH_2O

Addition of glycols in the media & growth curve of B10:

- 4 tubes of 20 ml RCV-C+NH_4^+ → 0.4 ml of each of the glycols added in the tubes
- 4 tubes of 20 ml RCV-C+ glutamate → 0.4 ml of each of the glycols added in the tubes
- 2 control tubes were prepared inoculating bacteria with NH_4^+ and glutamate but with out the carbon substrates
- Light incubation start time was recorded as T_0

. Growth was monitored by taking absorances at T_{19}, T_{43} and T_{119} (hr)

Growth curve of the mutants:

5 tubes of 20 ml RCV-C+ glutamate → 0.4 ml of each of the glycols added in the tubes

- 0.5 ml of each of the 4 mutants(3 *pfl* B & 1 *pfl* D) in 4 tubes and B10 in one tube inoculated as positive control
- Light incubation
- Growth was monitored by measuring OD at different time intervals

2.2.1.2 Growth test in only 1, 2-Propanediol

5 tubes of 20 ml RCV-C+ glutamate → 0.4 ml of each of the glycols added in the tubes

- 0.1 ml of each of the 4 mutants(3 *pfl* B & 1 *pfl* D) in 4 tubes and B10 in one tube inoculated as positive control
- Light incubation

- Growth was monitored by measuring OD at different time intervals
- Also hydrogen production was tested

(This test was also performed with adding and without adding propane-diol and media was made anaerobic before culture inoculation)

2.2.1.3 Growth and hydrogen production test of *Rhodobacter capsulatus* with Ethanolamine as nitrogen source

Preparation of the precultures

- Precultures were grown in RCV+glutamate media
- Incubated in the light for 2 hrs

Culture with Ethanolamine for growth test and hydrogen production:

- RCV-N (malate) media prepared with 5.8 mM and 11.6 mM ethanolamine
- Before culture inoculation, argon applied to each tube for 15 minutes
- 1 ml of *pfl* B, *pfl* D mutants and B10 as positive control were centrifuged and cell re-suspended in 10 mM KPi buffer
- The resuspended cells were then inoculated inside the tubes with a syringe
- Light incubation
- After 3 hrs, excess argon was released with needles
- Growth was monitored by measuring absorbance and hydrogen collection was done at several hours interval

2.2.2 Enzyme activity assays

Formate dehydrogenase assay (UV method)

For the determination of content of formic acid produced by the mutant culture of *Rhodoacter capsulatus* where the formate producing enzyme pyruvate formate lyase was supposed to be deactivated by the loss-of-function mutation in the gene *pyruvate formate lyase*. So the expected result would be no production of formate (formic acid) by the culture of the mutant.

2.2.2.1 Preparation of the reagents

Phosphate buffer:

2.94 g K_2HPO_4 and 0.286 g KH_2PO_4 were dissolved in 100 ml dH_2O. 20 mg pyrazole was added into it & pH was adjusted to 7.5 (stored at 4°C)

Nicotinamide-adenine dinucleotide, lithium salt, NAD^+-Li:

300 mg NAD^+-Li salt was dissolved in 10 ml dH_2O (stored at 4°C)

Formate dehydorgenase, FDH:

90 mg was dissolved in 0.5 ml dH_2O (stored at 4°C)

Sodium formate, 2mM:

Standard solution was prepared as 2 mM dissolving formic acid in dH_2O (stored at 4°C)

2.2.2.2 For standard curve: With standard solution

In UV visible cuvettes, the following reagents were pipetted first-

Phosohate buffer	0.5 ml
NAD^+-Li salt sol.	0.25 ml

After that, and standard solution of formate were pipetted in the following volumes and absorbance at 340 nm.was measured.

After addition of the enzyme FDH (25 µl), absorbance at 340 nm was measured at each 10 minutes intervals.

2.2.2.3 Formate assay with mutant bacteria: B10 (pRK290::*pflB*::Km^r)

Preparing culture of B10 (pRK290::*pflB*::Km^r) :

- 6×15 ml glass tubes were poured with 12 ml RCV-N media & 84 µl Na-glutamate added into it

- Argon applied to make the media anaerobic

- From colony grown plates derived from the experiment of Y262×B10, 2 growths from each plate were selected and loop full of cells were mixed in the tubes
- Light incubation for 24 hrs

Experiment for the assay:

In UV visible cuvettes, the following reagents were pipetted first-

Phosohate buffer	500 μl
NAD^+-Li salt sol.	250 μl
dH_2O	650 μl
Each sample	100 μl

- After that, absorbance at 340 nm was measured before addition of the enzyme FDH.

- After addition of the enzyme FDH (25 μl), absorbance at 340 nm was measured at each 10 minutes interval.

Calculation: measuring concentration of the formate produced:

Formula: Concentration, $C = \frac{V \times \Delta A}{\varepsilon \times v}$ **mM**

Here, V=1.5 ml, v = 0.1 ml, d= 1 cm, ε = 6.3

So, $\frac{V}{\varepsilon \times v} = 2.38$

So, concentrations of the *pflB* mutants was measured with this formula and put in a table.

2.2.2.4 Formate assay with mutant bacteria: B10 (pRK290::*pflD*::Km^r)

Preparing culture of B10 (pRK290::*pflD*::Km^r) :

- 4×15 ml glass tubes were poured with 12 ml RCV-N media & 84 μl Na-glutamate added into it
- Argon applied to make the media anaerobic

- From colony grown plates derived from the experiment of Y262×B10, 4 colonies were selected and loop full of cells were mixed in the tubes
- Light incubation for 24 hrs

Experiment for the assay:

In UV visible cuvettes, the following reagents were pipetted first-

Phosohate buffer	500 µl
NAD^+-Li salt sol.	250 µl
dH_2O	650 µl
Each sample	100 µl

- After that, absorbance at 340 nm was measured before addition of the enzyme FDH.
- After addition of the enzyme FDH (25 µl), absorbance at 340 nm was measured at each 10 minutes intervals.

Calculation: measuring concentration of the formate produced:

Formula: Concentration, $C = \frac{V \times \Delta A}{\varepsilon \times v}$ **mM**

Here, V=1.5 ml, v = 0.1 ml, d= 1 cm, ε = 6.3

So, $\frac{V}{\varepsilon \times v} = 2.38$

And now, concentrations of the *pflD* mutants was measured

2.3 Molecular Biology approaches

2.3.1 Cloning and mutagenesis

The works done before hand: The *pflB* and *pflD* genes were amplified from genomic DNA of strain RC87 (a mutant of the wild-type strain B10 that has been cured of the endogenous plasmid) using Hot Start Taq polymerase beads (Promega).

The amplified *pfl* genes were cloned into the 4.2 kb low-copy plasmid vector pACYC184 (With the help of 3.9 kb plasmid vector pCR2.1 where *pfl* genes with Km^r cassettes were cloned into using a TA Cloning Kit (Invitrogen) with TOP10F' as host strain)

The kanamycin-resistance cassette was inserted from pUC4-KIXX into the plasmid clone. This plasmid was digested with *Sma*I and the 1.3 kb fragment containing the Km^r gene was purified from a 1% agarose gel.

pACYC184::*pflB* or pACYC184::*pflD* clone was digested with *Pst*I. . Vector and insert DNA were then blunt-end ligated with T4 DNA ligase and transformed into *E. coli* DH5α with selection on LB agar medium containing 10 μg/ml tetracycline and 20 μg/ml kanamycin.

In both *pflD* clones, the Km^r cassette was in the antisense orientation i.e. the Km^r gene was transcribed in the opposite direction to *pflD*. For *pflB*, one clone, B2, was in the antisense orientation and the others were in the sense orientation.

2.3.1.1 Purification of the desired portion of plasmid DNA from the whole plasmid DNA

At first the plasmid DNA was digested with *ECo*RI to get desired gene size:

Buffer (H) 10 μl

ECoRI 20 units

Total DNA 90 μl (10 μg)

- Incubated 1 hr at 30°C.
- After 1 hr, 2 μl of 0.5 M EDTA was added to stop further reaction
- 20 μl of Blue-orange dye was mixed
- Electrophoresis was performed with 1% TAE agarose gel and 0.5× TAE as running buffer. In 4 big wells in the gel, 30 μl of DNA was poured in each of the wells.
- Electrophoresis was performed for 2 hrs at 125V
- The gel was illuminated under UV light, smaller beads at ~2.5 Kb was cut and placed in a pre-weighed sterile eppendorf tubes.

2.3.1.5 Purification of the desired DNA of plasmid from the agarose gel: "Sephaglass Band Prep Kit"

From agarose gel, the DNA portion was purified according to the manual contained in the kit. After electrophoresis, we select the DNA portion at the desired position, so the amount of DNA became less than before. Also after purification with Sephaglass kit, 50% DNA is lost. That is why it is important to determine the amount/concentration of DNA to do further experiments.

"Drop out method" to determine concentration of purified plasmid DNA:

On transparent mini gel tray, 7 spots of 5 μl $EtBr_2$ (2 μg/ml) were spotted at 1 cm distance (3 spotts in 2 rows and 1 spott in middle of the 2 rows). 6 control DNAs were taken which has concentration of- 0 ng/μl, 1 ng/μl, 2 ng/μl, 3 ng/μl, 4 ng/μl & 5 ng/μl and from each of the controls, little was mixed on each of the $EtBr_2$ spotts in rows. The sample DNA was 10 times diluted and 5 μl from there mixed with the spott in middle (to ease the comparison). The tray was exposed under UV light and sample was compared with the controls. The concentration was determined.

Figure 6: Control DNA and sample DNA spots to compare the concentrations

2.3.1.6 Ligation of *pfl* B & *pfl* D containing plasmid DNA with pRK290 plasmid (linearized)

pRK290+*ECo*RI+BAP	2 μl
pfl B/*pfl* D+*ECo*RI (20 ng/μl)	4 μl
Ligation buffer (×10)	1 μl
T4 DNA ligase	1 μl
Bio. Mol. H_2O	2 μl

- Mixed and incubated in the 16°C water bath overnight.

2.3.2 Conjugation and Transformation

2.3.2.1 Conjugation of pRK290-type plasmids into *Rhodobacter capsulatus* Y262

Day 1

1. E.Coli DH5α (pRK290::*pflB*::Km^r) and DH5α (pRK290::*pflD*::Km^r) were inoculated from -20°C. stocks into 3 ml LB+Tc^{10}+Km^{20} medium and grown aerobically overnight at 30° C.
2. E.Coli HB101 (pRK2013) was inoculated from -20°C. stock into 3 ml LB+Km^{20} and grown aerobically overnight at 30° C.
3. 0.5 ml Y262 from previously grown pre-culture in RCV-malate was inoculated into 20 ml RCV-lactate in 100 ml flask and it was grown aerobically overnight at 30° C.

Day 2

4. Growth of *E.Coli* was monitored by measuring OD at 600 nm and growth of *rhodobacter* was monitored by measuring at 660 nm
5. DH5α (pRK290) cultures were diluted 1 in 10 in pre-warmed LB medium and incubated with shaking at 30° C.
6. HB101 (pRK2013) culture weas diluted 1 in 5 in pre-warmed LB medium and incubated with shaking at 30° C.
7. Y262 was diluted 1:1 in pre-warmed RCV-lactate medium and incubated with shaking at 30° C.
8. After 3 hr, each 0.5 ml DH5α (pRK290) was mixed with 0.5 ml HB101 (pRK2013), centrifuged at 6000 rpm for 2 min and resuspended cells in 1 ml LB medium.
9. 10 μl of E.Coli mix was mixed with 10 μl Y262 in the centre of a YPS agar plate and spreaded over a surface area of 1-2 cm^2
10. Incubated the dried plates at 30° C. over night.

Day 3

11. The next day, the growth was streaked in the centre of the plate (mating mixture) onto RCV+Km^{10} plates and RCV+Tc^{1}+Km^{10} plates with 3 sticks
12. Incubated in the light for 3-4 days

2.3.2.2 Preparing pure culture of Y262 (pRK290::*pflB*::Km^{r}) & Y262 (pRK290::*pfD*::Km^{r})

- Individual colonies were found after 4 days and 3 colonies were streaked onto RCV+Km^{10} plates for pure cultrure of Y262.
- Incubated in the light for 4-5 days
- Each of the colonies was the made into stocks inside the beads in the "MicroBank" cryotubes and preserved in the (-80° C.)

2.3.2.3 Plasmid elimination and recombination: From the bacterium *rhodobacter* SB1003 by successive culture:

Inoculating cultures:

- Precultures of SB1003 (pRK290::*pflB*::Km^{r}) and SB1003 (pRK290::*pflD*::Km^{r} were grown on RCV+Km^{10}
- 150 µl of each pre-culture was added into 15 ml YP+Km^{10} medium
- Incubated in the light for 24 hrs

Second inoculation of the cultures:

- - 150 µl of each culture was inoculated into 15 ml YP+Km^{10} medium
- Incubated in the light for 24 hrs

Third inoculation of the culture:

- Same as before

Fourth inoculation of the culture:

- Same inoculation and incubation

- Absorbance of the fourth culture was measured to monitor the bacterial growth

2.3.2.4 Plating bacterial growth in RCV+Km^{10} plates

- **Serial dilutions** of SB1003 (pRK290::*pflB*::Km^{r}) & SB1003 (pRK290::*pflD*::Km^{r}) in RCV-N media (liq.) in the following ways:

100 µl culture
+9.9 ml RCV-N media → 10 ml

100 µl culture
+ 9.9 ml RCV-N media → 10 ml

200 µl culture
+ 9.8 ml RCV-N media

- Then this diluted cultures were spreaded onto 6 plates of RCV+Km^{10} plates
- Cultures were grown photo synthetically in the light for 5-6 days

2.3.2.5 Replica plates with SB1003 (pRK290::*pflB*::Km^{r}) & SB1003 (pRK290::*pflD*::Km^{r}) culture after plasmid elimination

- After 5 days, individual colonies are found in both of the cultures grown in RCV+Km^{10} plates
- Small spots were plotted with toothpick from each of the plates in the following way:

  36 colonies/plate → 4 plates of RCV+Km^{10}

  36 colonies/plate → 4 plates of RCV+Tc^{1}+Km^{10}
- RCV+Km^{10} plates were grown aerobically in the dark at 30°C. for 2 days
- RCV+Tc^{1}+Km^{10} plates were grown anaerobically in the light at 30°C. for 2 days

Observation:

- There was growth in the RCV+Tc^1 plates, so the result was not good. The mutant should be Tc sensitive, so no growth is expected.

2.3.2.6 Experiment of GTA transfer of Kanamycin resistance: With Y262 & B10

Preparing cultures of *Rhodobacter* Y262 & B10 for GTA transfer:

- 1 loop of Y262(pRK290::*pflB*::Km^r) & Y262(pRK290::*pflD*::Km^r) taken from RCV+Tc^1+Km^{10} plasmid purification plates of E.Coli×Y262 in the 10 ml YP medium containing 20 μl Km^r
- ¾ beads of B10(-80°C.) in 15 ml RCV malate in a glass tube
- Photosynthetic growth of the cultures for 48 hrs

GTA transfer of Km^r:

- 1000 μl Y262 (pRK290::*pflB*::Km^r) → Centrifuged for 5min at 6000 rpm., then filtered → prepared GTA (A)
- 1000 μl Y262 (pRK290::*pflD*::Km^r) → Centrifuged for 5min at 6000 rpm., then filtered → prepared GTA (B)
- 300 μl B10 taken in 4 eppendorf tubes and centrifuged for 5min at 6000 rpm.
- Supernatant discarded and pellets were re-suspended in 1 ml G-buffer.
- 0.5 ml B10 pellet and 0.5 ml supernatant (containing GTA) from Y262 (pRK290::*pflB*::Km^r) mixed in 2 tubes of 15 ml and incubated for 1 hr in 30°C→ A1, A2
- 0.5 ml B10 pellet and 0.5 ml supernatant (containing GTA) from Y262 (pRK290::*pflD*::Km^r) mixed in 2 tubes of 15 ml and incubated for 1 hr in 30°C→ B1, B2
- After 1 hr incubation, entire 1 ml mixture of each GTA was inoculated into 9 ml YPS in 4×100 ml sterile flasks
- Incubate with shaking at 30°C for 4 hrs
- Each GTA was transferred to 4 eppendorf tubes & centrifuged at 6,000 rpm for 10 min
- Cells resuspended in 250 μl YP medium
- From each mix, 100 μl were spreaded on RCV+Km^{10} plates
- All of the above plates were incubated photo synthetically for 4-5 days at 30°C

Colony counting in RCV-N plates of Y262×B10:

- After 4-5 days, colonies grown in the plates were counted which prove the successful GTA crosses

- Streaked each colony in RCV+Km^{10} plates like a little circle so that each plate contained 12 colonies.

- Photosynthetic incubation for 2 days

Replica plates of all GTA-Km^r colonies of both *pflB* & *pflD* mutants:

- With toothpick, colonies were first spotted on RCV+Km^{10} plates, then spotted on RCV+Tc^1 plates
- Photosynthetic growth at 30°C. for 4-5 days

Streaking GTA-Km^r B10 colonies onto RCV+Km^{10} plates for pure culture:

- 3 *pfl B* mutant (large+dark brown) colonies → 3 plates
- 3 *pfl B* mutant (small+ pale brown) colonis → 3 plates
- 3 *pfl D* mutant (large) colonies → 3 plates
- Each colony was streaked with 3 sticks so that individual colonies were found
- 4 days required to grow photo synthetically

Preparing B10 pure culture:

- *pfl B* pale colonies were not grown

- *pfl B* dark colonies and *pfl D* colonies were grown and individual colonies were available

- 3 colonies streaked from each plate onto RCV+Km^{10} plates for pure culture

- 6 plates were incubated in the light for 4-5 days

- After 4 days, loop full of cultures were taken from the plates of each colony and prepared stocks for -80°C. in the cryo tube "MicroBank".

2.3.3 Preparation of nucleic acids and plasmid DNA

For performing molecular biology approaches including polymerase chain reaction, we need to extract DNA. Plasmid DNA was used in the cloning protocols.

Extraction of DNA of mutants: B10 (pRK290::*pflB*::Km^r) and B10 (pRK290::*pflD*::Km^r):

2.3.3.1 Extraction procedure-1

Genomic DNA isolation of *rhodobacter* with 'Invitrogen Easy DNA Kit':

- Pure culture of mutant B10 were derived from colonies from the experiment of GTA crosses $Y262 \times B^{10}$ Km^r

- 4 dark and 4 pale colonies of mutant strain B10 (pRK290::*pflB*::Km^r) & 4 colonies from mutant strain B10 (pRK290::*pflD*::Km^r) were taken as sample for DNA extraction and further experiments of molecular biology and biochemistry.

- The extraction procedure was followed exactly according to the manual contained in the kit **'Invitrogen easy DNA Kit'**

Gel electrophoresis with the extracted DNA to check the quality of DNA:

Solution of TBE buffer: (pH 8.3)

0.0089 M Tris borate
0.0089 M Boric acid
0.002 M EDTA

1% agarose gel:

-1 g agarose melted in 100 ml 0.5× TBE buffer, poured 50 ml in the mini gel tray, red dye(for exposing the DNA under the UV light) mixed and solidified for 40 min.

-While running, blue-orange dye was mixed with the DNA samples to trace while gel run.

2.3.3.2 Extraction procedure-2

Preparing cultures for DNA extraction:

- 15 ml RCV-malate in 12 falcon tubes
- The 12 colonies of mutant B10 selected first time for DNA extraction were used again to make the cultures and 0.5 ml from each pre culture was taken
- Incubated in the light for 24 hrs

Growth monitoring of the cultures by measuring OD_{660}:

If the OD is in between 2-3, then growth is sufficient for genomic DNA isolation. For this reason, absorbance at 660 nm was measured to monitor the bacterial growth.

Genomic DNA isolation of *rhodobacter* with 'Invitrogen Easy DNA Kit':

Then DNA isolation was performed by the same kit.

2.3.3.3 Extraction procedure-3

Mini prep of bacterial genomic DNA:

Preparation of the necessary reagents:

CTAB/NaCl solution:

NaCl	4.1 g
CTAB	10 g
dH_2O	upto 100 ml

- 4.1 g NaCl was dissolved in 80 ml dH_2O
- 10 g CTAB dissolved in water by heating and stirring
- Final volume adjusted to 100 ml (10% CTAB in 0.7 M naCl)

5 M NaCl solution (100 ml):

- 29 g NaCl dissolved in 80 ml dH_2O heating and stirring
- Beaker should be sealed to stop evaporation of water
- Volume upto 100 ml

TE buffer preparation:

500mM stock EDTA, pH 8.0 (100 ml):

- 18.61 g EDTA dissolved in 80 ml dH_2O stirring with the aid of solid NaOH to make it soluble in neutral pH
- pH adjusted to 8.0 wit NaOH

10 mM tris-HCl, pH 8.0 (100 ml) :

- 1 ml from 1 M stock Tris base (pH 8.0) was added to 80 ml dH_2O and pH adjusted with HCl

- Volume made upto 100 ml by dH_2O

<u>Finally for $T_{10}E_1$ buffer-</u>

- 0.2 ml 500mM EDTA was added into 100 ml Tris-HCl (pH 8.0)

Preparation of the aerobic pre cultures & anaerobic cultures:

The same cultures were used as precultures for the aerobic culture and directly were used for DNA isolation from the anaerobic cultures-

- 15 ml RCV malate taken in falcon tubes
- 1 ml of old cultures of B10 (sample no 1, 2, 3 & 5) taken and B10 wild type taken as positive control.
- Anaerobic photosynthetic growth for 24 hrs.

Preparation of the aerobic cultures of bigger volume:

- 5 flasks each containing 100 ml YPS media (liq.) and stopped with cotton plugs were autoclaved before.
- 1 ml from each precultures centrifuged for 7 min at 6000 rpm and cells mixed in the YPS media contained in the flasks
- Aerobic incubation overnight at 180 rpm (30°C)

Protocol for genomic DNA preparation of bacteria:

1. From anaerobic cultures, 5 ml centrifuged for 5-10 min at 6,000 rpm
2. Whole of the aerobic cultures centrifuged at 10,000 rpm for 10 min at 10°C. Then each pellet resuspended in 500 µl TE buffer & transferred into 2 eppendorf tubes each containing 250 µl
3. 567 µl TE buffer added to each eppendorf tubes by repeated pipetting
4. 30 µl 10% SDS & 3 µl 20 mg/ml proteinase k mixed and incubated for 1 hr at 37° C
5. 100 µl of 5 M NaCl mixed thoroughly
6. 80 µl CTAB/NaCl solution mixed and incubated for 10 min at 65°C
7. Equal volume of chloroform/isoamyl alcohol mixed and micro centrifuged for 4-5 min at 6-10,000 rpm
8. Supernatant transferred to fresh tubes and equal volume of phenol/chloroform/isoamyl alcohol added and centrifuged for 5 min. Again supernatant transferred to fresh tubes
9. 0.6 volume of isopropanol mixed gently and then centrifuged for 5min

10. Supernatant was removed and pellet (precipitated DNA) rinsed in 1 ml 70 % ethanol (-20°C) and pellet washed.

11. Centrifuged for 5 min, supernatant discarded & dried briefly in lypholizer/heat block

12. Dried pellets were resuspended in 100 μl TE buffer and ready to use further.

RNAse treatment with the isolated DNA samples:

- 2 μl RNAse added in each of the samples
- 30 min incubation at 37°C

Purification of cDNA after RNAse treatment:

1. 100 μl of phenol:$CHCl_3$:Isoamyl alchol (25:24:1) was added into the samples and vortexed for 30 sec.
2. Centrifuged for 2min at 10,000 rpm
3. Upper aqueous phase was removed and transferred to clean tube
4. 100 μl $CHCl_3$ was added and was vortexed for 30 sec.
5. Centrifuged for 2 min at 10,000 rpm
6. Upper aqueous phase was removed and transferred to clean tube
7. The volume was measured and made upto 100 μl with dH_2O
8. 10 μl of 3 M Na-acetate and 275 μl of EtOH (95%) were added
9. Centrifuged for 30 min at 10,000 rpm (4°C.)
10. Pellet rinsed with 500 μl 70% ethanol (-20°C.) and centrifuged for 10 min at 10,000 rpm (4°C.)
11. Supernatant removed and pellet air dried
12. Pellet resuspended in 50 μl dH_2O and preserved at 4°C.

Electrophoresis of both the cultures to test the DNA:

- 1% agarose gel was prepared with 0.5×TEA buffer, stained with red dye and solidified.
- After 45 min of gel run, the gel was photographed with UV exposure
- DNA bands were analyzed from the images

2.3.3.4 Preparation of the cultures for pACYC184::*pflB::Kmr/* pACYC184::*pflD::Km^r* for plasmid DNA purification

- 100 ml LB liquid media taken in a 500 ml flask
- 0.1 ml Km5 (5 mg/ml) and 1 ml Tc1 (1 mg/ml) mixed into it to make the final concentrations of 20 μg/ml and 10 μg/ml culture
- 500 μl from 16% culture of plasmid pACYC184::*pflB::Km^r* and pACYC184::*pflD::Km^r* were mixed in the media
- Incubation at 30°C at 180 rpm overnight.

2.3.3.5 Purification of Plasmid using "QIAGEN Plasmid Midi Kit"

After 24 hrs of incubation, the absorbance of the cultures was in between 2 to 3.
The plasmid DNA was purified following the protocol in the handbook of the "QIAGEN Plasmid Midi Kit (25)"

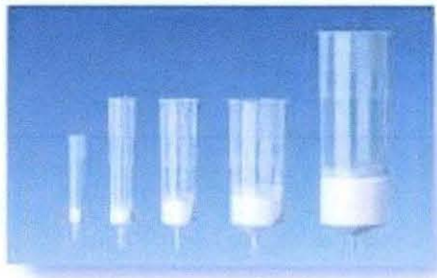


Figure 7: QIAGEN Plasmid Kit Anion Exchange Tips

2.3.1.3 Measuring concentration of plasmid DNA (/ml): (Spectrophotometry method)

Purified plasmid DNA was diluted 100 times in TE buffer and then absorbance was measured at 260 nm. The relation of absorbance and concentration of DNA is:
OD_{260}=1 equivalent to concentration=50 μg/ml

2.3.4 PCR

PCR (Polymerase Chain reaction) was performed to check whether the pfl B and pfl D genes were mutagenised upon insertion of the Km^r cassette into it. The amplified gene products were easier to analyze further by Gel electrophoresis.
Here, the PCR experiments were performed and optimized by changing and taking the following as parameters:

1. Type of DNA taken as template

- Templates directly taken from the bacterial colonies and prepared by boiling at 100°C.
- Extracted DNA from both aerobic and anaerobic cultures
- Extracted DNA with or without RNase treatment

2. Primer

- 16s ribosomal RNA amplification to test if everything is fine with the PCR machine
- Old pfl B and pfl D primers
- New pfl B and pfl D oligos
- Combination of the old and new primers (forward and reverse combinations too)

3. Annealing temperature

4. Wild type B10 as positive control

5. Addition of DMSO (used as an adjuvant that considerably increased amplification efficiency and specificity of PCR)

2.3.4.1 Experiment-1: 16s rRNA amplification

This experiment was not directly related to the amplification of my desired gene but was performed as a control for all the other PCR experiments in order to check the

efficiency of Taq polymerase and all other PCR buffers as well as to be sure the PCR program was performing well.

Sample: Taking cells from the colonies of mutants from plates & extracted DNA

- 3 colonies from 3 plates of *pfl B* mutants and 3 colonies from *pfl D* mutants were selected
- Cells from there dissolved in 100 µl PBS and incubated in 100°C. for 10 min.
- Centrifuged at 12,000 rpm for 7 min
- Supernatants were taken as DNA templates

- 4 samples of DNA extracted before were also taken for the amplification

Master mix preparation:

Table 6: Master mix preparation

Reagent	Volume per 1 sample reaction (µl)
H_2O molecular biology grade	34
Primer 16s-C (50 þ mol/µl)	1
16s-D (50 þ mol/µl)	1
dNTP mix (5 mM each)	2
10× Taq buffer (MgCl2 free)	5
MgCl2, 25 mM	5

- From the master mix, 48 µl equilibrated in each of the PCR tubes
- 2 µl DNA template mixed into it
- 2 beads of paraffin coated Taq polymerase were added then
- PCR was performed according to the following program (program 1)

Thermal cycle: (Program 1)

First step 95°C for 2 minutes (initial denaturation)

Second step 95°C for 1 minute
50°C for 1.5 minute — 29 cycles
72°C for 2 minutes

Third step 72°C for 3 minutes (final extension)

4°C forever

2.3.4.2 Experiment-2: Amplification of *pfl B* and *pfl D* genes

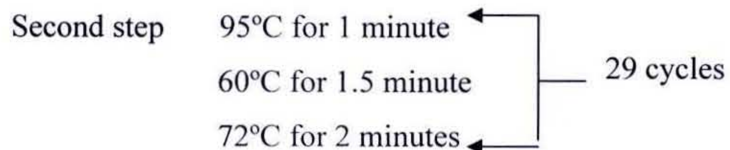
Master mix preparation: for *pfl B*/ *pfl D* gene

Table 7: Master mix preparation

Reagent	Volume per 1 sample reaction (μl)
H_2O molecular biology grade	34
Primer *Pfl B*-F/ *pfl D*-F	1
Pfl B-R/ *pfl D*-R	1
dNTP mix (5 mM each)	2.5
10× Taq buffer (MgCl2 free)	5
MgCl2, 25 mM	5

Thermal cycle: (Program 2)

First step 95°C for 2 minutes (initial denaturation)



Third step 72°C for 3 minutes (final extension)

4°C forever

Optimization of PCR to amplify *pfl* genes:

2.3.4.3 Experiment-3

Addition of DMSO:

- 0%, 2.5% and 5% DMSO added into the PCR tube separately after equilibrating master mix:

- Additional volume was adjusted compensating H_2O

2.3.4.4 Experiment-4

New *pfl D* primers & combinations with old and new primers:

- Stock solutions of 100 þ mol/µl was diluted to 50 þ mol/µl with molecular biology grade H_2O
- Old primer *pfl* D-F/R and new primer *pfl* D-F1/R1
- The combinations used *pfl* D-F1/R1, *pfl* D-F1/R & *pfl* D-F/R1
- PCR was performed with these combinations of primers for both the *pfl* B & *pfl* D mutants as well as wild type B10 as positive control.
- For thermal cycle, program 1 was used where the annealing temperature was 50°C.

2.3.4.5 Experiment-5

Annealing Tm:

- The Tm for pfl D-F1: 57.3°C.(50% GC) and for pfl D-R1: 62.1°C.(54.5% GC)
- So in the program, the annealing temperature was changed from 50°C. to 55°C. for the new primers

Thermal cycle: (Program 3)

First step	95°C for 2 minutes (initial denaturation)	
Second step	95°C for 1 minute 55°C for 1.5 minute 72°C for 2 minutes	29 cycles
Third step	72°C for 3 minutes (final extension) 4°C forever	

2.3.5 Hybridization techniques

2.3.5.1 DNA labelling: with "DIG High Prime DNA Labelling and Detection Kit II (Cat. No. 1 585 614; *Instruction Manual* Version 1, Nov. 2003)

Besides the reagents supplied with the kit, some additional solutions were required to prepare:

Washing Buffer:

- For removal of unbound antibody.

Maleic acid	0.1 M (11.61 g)
NaCl (pH 7.5)	0.15 M (8.76 g)
Tween 20	0.3 % (v/v)

- Preserved in 20°C

Maleic acid buffer:

- For dilution of blocking solutions.

Maleic acid	0.1 M (11.61 g)
NaCl (pH 7.5)	0.15 M (8.76 g)

- pH 7.5 was adjusted with solid NaOH (20°C)

Detection buffer:

- For adjustment of pH to 9.5

Tris-HCl	0.1 M
NaCl	0.1 M

- pH 9.5 was adjusted and preserved at 20°C

Blocking solution dilution:

- For blocking unspecific binding sites on the membrane
- Blocking solution in the kit was 10×
- Diluted to 1× in maleic acid buffer

Antibody solution:

- For binding to the DIG-labelled probe
- Anti-digoxigenin-AP (in the kit) centrifuged for 5 min at 10,000 rpm
- Taken from the surface and 1:10,000 dilution in blocking solution

20× SSC preparation:

NaCl	3 M (175.2 g)
Tri sodium citrate	300 mM (88 g)

- pH was adjusted to 7.

2.3.5.2 The protocol

The protocol was performed according to the manual contained in the kit **"DIG High Prime DNA Labelling and Detection Kit II"** (Cat. No. 1 585 614; *Instruction Manual* Version 1, Nov. 2003)

Labelling DNA:

- *pfl* B (20 ng/μl) & *pfl* D (10 ng/μl) clones were taken
- 100 ng of DNA need to start the labelling
- So, 5 μl from *pfl* B clone and 10 μl *pfl* D clone taken

Then the labelling was done following the manual contained in the kit.

Table 8: Expected yield of DIG-labelled DNA

Template DNA (ng)	Incubation time	Total yield of labelled DNA (ng)	% yield of labelled DNA
100	1 hr	270	15 %
100	20 hr	1500	38 %

Determination of labelling efficiency:

Both template and control DNA (supplied in the kit) were diluted according to the dilution series mentioned in the protocol and 1 μl from each of the dilution was spotted on a nylon membrane. The dilution series is mentioned below:

Table 9: Dilution series to determine labelling efficiency of the probe

Tube	DNA (μl)	From tube #	DNA Dilution Buffer (μl)	Dilution	Final concentration
1		Diluted original			1 ng/μl
2	5	1	495	1:100	10 þg/μl
3	15	2	35	1:3.3	3 þg/μl
4	5	2	45	1:10	1 þg/μl
5	5	3	45	1:10	0.3 þg/μl
6	5	4	45	1:10	0.1 þg/μl
7	5	5	45	1:10	0.03 þg/μl
8	5	6	45	1:10	0.01 þg/μl
9	0	-	50	-	0 þg/μl

Cross linking and washing were performed following the kit manual. At the end, membrane was exposed for 20 min in the UV imager.

Result analysis:

If both the dilutions containing 0.1 þg of DNA were visible in the image, the labelling was considered to reach to the expected labelling efficiency.

2.3.5.3 DNA Fixation

Mutant DNA digestion with restriction enzyme:

- RC 87 was taken as positive control
- All the DNA solutions were diluted to 500 ng/μl

Master mix for DNA digestion:

For mutants		**For control RC87**	
Buffer H (×10)	10 µl	Buffer H (×10)	10 µl
dH_2O	79 µl	dH_2O	64 µl
EcoRI	1 µl	EcoRI	1 µl
Diluted DNA	10 µl (each)	DNA	25 µl

- 2 hr incubation at 37°C
- After 2 hr, 2 µl of 0.5 EDTA was added to each tube
- Then 20 µl of Blue-orange dye was mixed in the tubes

Electrophoresis of the DNA digests:

- 1% agarose gel was used to get DNA bands to hybridize with *pfl* D probe
- 0.7 agarose gel was used to get DNA bands to hybridize with *pfl* B probe

2.3.5.4 Fixation of DNA to the membrane

Preparation of additional reagents:

Denaturation solution preparation:

NaCl	1.5 M (87.75 g)
NaOH	0.5 M (20 g)

- dH_2O upto 1 litre (room temp.)

Neutralisation solution preparation:

NaCl	3 M (175.5 g)
Tris base	0.5 M (60 g)

- dH_2O upto 1 litre (room temp.)

- pH adjusted to 8.0 with 6 N HCl
- Gels were denatured for 2×15 min rotated covering in the denaturisation solution and neutralized for 30 min rotating inside neutralization solution.
- Then electrophoretic transfer of blots were performed in the **BIO-RAD Mini Trans Blot® Electrophoretic Transfer Cell** (Catalog numbers 170-3930, 170-3935)

- After over night transfer of blot, membranes were placed on filter paper rinsed with 10×SSC
- UV cross linked for 5 min without washing.
- After UV cross linking, rinsed briefly in dH_2O & air dried

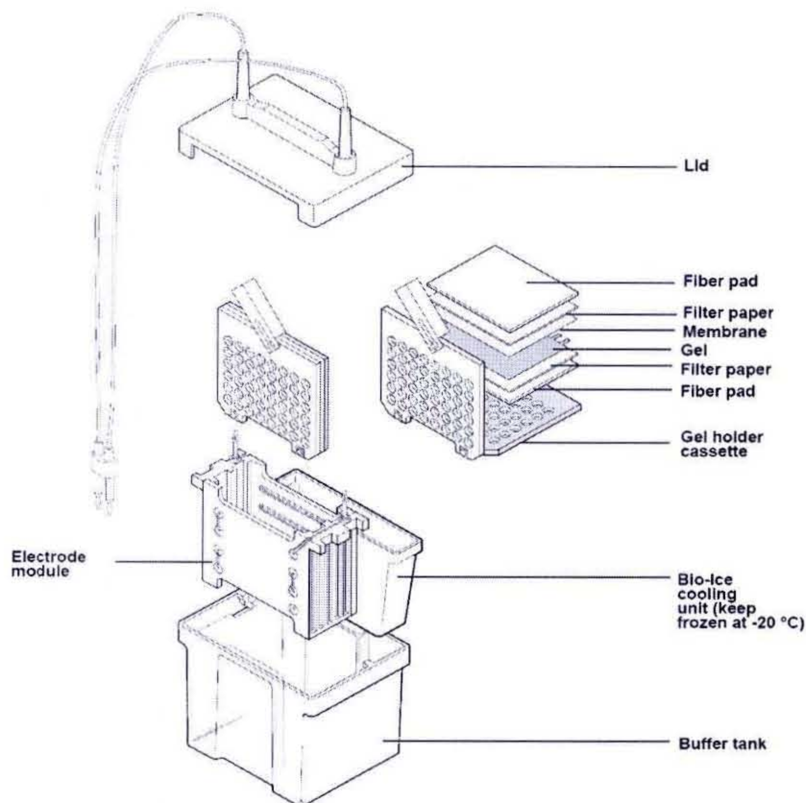


Figure 8: Assembly of parts of the cell and Preparation of the gel sandwich

2.3.5.5 Hybridization

- Calculated hybridization temperature is 55°C

- The steps of - a. Pre-hybridization, b. Hybridization and c. Stringency wash were performed according to the manual of **"DIG High Prime DNA Labelling and Detection Kit II** (Cat. No. 1 585 614; *Instruction Manual* Version 1, Nov. 2003)

Immunological detection:

According to the manual of **"DIG High Prime DNA Labelling and Detection Kit II** (Cat. No. 1 585 614; *Instruction Manual* Version 1, Nov.2003)

Chapter-3

Results and discussion

In this work, the role of pyruvate formate lyase on the growth and metabolism of rhodobacter capsulatus mutant strain involved in the formation of hydrogen was checked by doing various experiments. Though the hydrogen collection with the usual growth medium was not used for the mutants, as it was checked with other mutant strain IR3 prepared and used before [47]. The mutants were instead tested for growth on new carbon and nitrogen sources. The enzyme assay was carried out to check if the target product formate was produced by the strains. All the molecular biology approaches were followed to determine if the transposon was successfully inserted inside the *pfl* gene or not.

3.1 Microbiological methods

3.1.1 Hydrogen collection

Hydrogen collection from the bacteria at different period of incubation is shown in table 10. At first hydrogen photoproduction was performed for 24 hrs and the collected hydrogen showed that, the volume of hydrogen produced by the mutant IR3 was always higher than the wild type B10. The range of hydrogen collected by the old type ranged from 6.5 ml to 30.5 ml while the volume of hydrogfen produced by the mutant IR3 ranges from 9.8 ml to 32.25 ml. Moreover, there was comparatively less increase in the volume of hydrogen collected from 1 day old culture than 2 days old culture.

Table 10: H_2 Collection from bacteria grown for 24 hrs

Total culture time	Total H_2 collected (ml)	
	B10	**IR3**
16 hr	6.5	9.8
18 hr	15.5	20.0
19 hr 15 min	20.7	24.8
21 hr	24.5	27.4
24 hr	30.5	32.25

Hydrogen production (volume) from the same bacterial strain (48-70 hrs) is shown in table 11. From this table we can deduce that, here the increase in hydrogen production per hour is greater than the 1 day old culture. So during the first day the

production rate is slower compared to second or third day. But after 3 days (72 hrs), the production declined gradually after 72 hours (not shown in the table)

Table 11: H_2 Collection from bacteria grown for more than 24 hrs

Total culture time	**Total H_2 collected (ml)**	
	B10	**IR3**
41 hr 30 min	35.35	39.10
45 hr 30 min	40.55	44.0
48 hr 30 min	43.0	47.6
66 hr 30 min	46.1	50.45
70 hr	48.0	64.5

3.1.2 Gene transfer agent mediated crosses

Gene transfer agent (GTA) produced by *Rhodobacter capsulatus* is a virus like element that seem to function solely for mediating gene exchange. It is more like a defective prophage that only carries random pieces of the genome of the producing cell in a process similar to generalized transduction. So to exchange genetic materials in between rhodobacter strains, GTA is being used successfully. [48]

Nif- mutants are unable to fix nitrogen, so to grow it in a nitrogenous media, the nif genes were complemented with the aid of GTA by mediating crosses with wild type *rhodobacter* strains having the Nif gene.

The following experiments successfully proved the efficiency of GTA of donor strains (having Nif genes) by the colony grown from the recipient mutant strains.

3.1.2.1 Experiment-1

Both the donor and recipient strains were checked for growth by measuring their absorbance at 660 nm.

Table 12: Bacterial growth measured by absorbance of the cultures

Culture	**Absorbance $_{660}$**
RC1	3.7
SB1003 (hv)	3.4
SB1003 (O_2)	1.15

It can be observed from the table 12 that both the recipient (RC1) and the donor strain i.e. SB1003 (grown photosynthetically) and SB1003 (grown aerobically) grew well in

the RCV medium (mentioned in the section 2.1.1.2) and were ready for the GTA crosses.

The number of colonies grown on media containing nitrogen shown in table 13 proved that GTA from wild type donor strain SB1003 was successfully transferred into mutant recipient strain RC1.

Here, the SB1003 grown aerobically showed more efficiency than the culture grown anaerobically and photosynthetically.

Table 13: Colony counting in RCV plates of SB1003×RC1

Crosses		**Number of Colonies**
SB1003(hv)×RC1	100 μl GTA cross directly	22
	10 μl GTA cross + 90 μl YP medium	07
	20 μl GTA cross + 80 μl YP medium	04
	50 μl GTA cross + 50 μl YP medium	24
SB1003(O_2)×RC1	100 μl GTA cross directly	104
	10 μl GTA cross + 90 μl YP medium	06
	20 μl GTA cross + 80 μl YP medium	22
	50 μl GTA cross + 50 μl YP medium	47

From the above table 13, it can be inferred that GTA acted efficiently as the concentration was increased. The increase in concentration from 10 μl to 50 μl influenced the growth by increasing the number of colonies from 07 to 47. And when no dilution was made, it produced highest number of colonies for example 104 colonies.

So it can be concluded from the observation that, the dilution of the GTA crosses as well as the YP medium decreases the GTA effiecinecy in a greater amount.

3.1.2.2 Experiment-2: GTA crosses

Both the donor and recipient strains were checked for growth by measuring their absorbance at 660 nm.

Table 14: Bacterial growth measured by absorbance of the cultures

Culture		**$Absorbance_{660}$**
RC1	0.1 ml inocula	3.75
	0.2 ml inocula	3.77
	0.5 ml inocula	3.38

SB1003	0.1 ml inocula	2.30
	0.2 ml inocula	2.39
	0.5 ml inocula	2.59

It can be observed from the table 14 that both the recipient (RC1) and the donor strain i.e. SB1003 (inoculated in different concentrations) grew well in the RCV medium (mentioned in the section 2.1.1.2) and were ready for the GTA crosses.

In the table 15 below, the number of colonies grown on media containing nitrogen proved that GTA from wild type donor strain SB1003 was successfully transferred into mutant recipient strain RC1.
Here, the different volumes of SB1003 were used during the crosses and the data showed more efficiency with the less concentrated recipient strain than the one with more concentration. So it was deduced from the observation that, concentration of the recipient strains should be less than the concentration of the donor strains.

Table 15: Colony counting in RCV plates of SB1003×RC1

Crosses		**Number of Colonies**
SB1003(0.1ml)×RC1	10 µl GTA cross + 90 µl YP medium	183
	100 GTA cross directly	228
SB1003(0.2ml)×RC1	10 µl GTA cross + 90 µl YP medium	149
	100 GTA cross directly	177
SB1003(0.5ml)×RC1	10 µl GTA cross + 90 µl YP medium	33
	100 GTA cross directly	08

It can be inferred also that GTA acted efficiently as the concentration was increased.

3.1.2.3 Experiment-3:

Both the donor and recipient strains were checked for growth by measuring their absorbance at 660 nm.

Table 16: Bacterial growth measured by absorbance of the cultures

Culture		**O.D.$_{660}$**
RC1	0.1 ml inocula	3.82
	0.2 ml inocula	3.22
	0.5 ml inocula	3.46
Y262	Aerobic culture (O_2)	1.39
	Anaerobic culture (hv)	3.71

It can be observed from the table 16 that both the recipient (RC1) and the donor strain i.e. Y262 (grown photosynthetically) and Y262 (grown aerobically) grew well in the RCV medium (mentioned in the section 2.1.1.2) and were ready for the GTA crosses. From the table 17, the number of colonies grown on media containing nitrogen proved that GTA from donor strain Y262 was successfully transferred into recipient strain RC1.

Here, from the number of colonies it was observed that donor strain grown photosynthetically in anaerobic atmosphere showed much higher efficiency than the culture grown aerobically without light even 9 times was observed. Moreover, the GTA used directly proved more efficiency than the GTA centrifuged, even 100 more colonies were observed, for example for centrifuged GTA, total 388 colonies were grown while for GTA directly streaked 486 colonies were grown.

Table 17: Colony counting in RCV-N plates of Y262×RC1

Crosses		**Number of Colonies**
Y262(O_2)× RC1	Direct	52
	Centrifuged	42
Y262(hv)×RC1	Direct	486
	Centrifuged	388

3.2 Biochemical assay

In biochemical assays, growth with new susbstrate were checked as well as formate producing capability was also tested.

3.2.1 Metabolite analysis

To check the versatility of substrate used for growth of *rhodobacter* strains, the mutants were tested for the growth in media containing new carbon and nitrogen sources that are not usually used as the growth substrate by the bacterium *rhodobacter*.

3.2.1.1 Growth test of *Rhodobacter capsulatus* with glycols:

The growth of wild type B10 was monitored at first by using 4 types of diols as carbon source and it was optimized by changing the nnitrgen sources as well. In one set of culture, salts were used as nitrogen sources while in another set of cultures glutamate was used. In control, no carbon source was used.

Table 18: Rhodobacter strain B10 growth measured by absorbance of the cultures grown with different diols

Carbon substrates	**Nitrogen source**	
	NH_4^+	**Glutamate**
	Absorbance $_{660}$ at 19 hours of growth	
RCV+1, 3-Propanediol	0.22	1.02
RCV+1, 2-Propanediol	0.16	1.48
RCV+1, 3-Butanediol	0.18	1.31
RCV+2, 3-Butanediol	0.14	0.94
RCV-C (Control)	0.13	0.98
	Absorbance $_{660}$ at 43 hours of growth	
RCV+1, 3-Propanediol	0.72	1.05
RCV+1, 2-Propanediol	0.18	2.34
RCV+1, 3-Butanediol	0.26	1.13
RCV+2, 3-Butanediol	0.17	1.02
RCV-C (Control)	0.18	1.07
	Absorbance $_{660}$ at 119 hours of growth	
RCV+1, 3-Propanediol	0.20	1.26
RCV+1, 2-Propanediol	0.29	3.43
RCV+1, 3-Butanediol	0.45	1.15
RCV+2, 3-Butanediol	0.21	0.98
RCV-C (Control)	0.26	1.09

The absorbance taken at different time of incubation showed that 1,2-Propanediol showed the highest efficiency to support the bacterial of all other diols. For example, at 119 hours the absorbance was 3.43 with 1,2-porpanediol while the absorbance grown with 1,3-propanediol was observed to be 1.26.

Hence, growth was also visible in control without any carbon source which proved nitrogen alone can support the growth but to a very limited extent. On the other hand, control using glutamate showed ordinary growth like the culture with diols and it is because glutamate can serve both as carbon and nitrogen source. (Table 18)

After checking the higher efficiency of 1,2-Propanediol compared all other diols, it was used as the carbon source while testing the growth of pfl mutants with diols. Glutamate was used as the nitrogen source and B10 served as control. (Table 19 A)

Table 19 A: Mutant bacterial growth measured by absorbance of the cultures Grown with 1, 2-Propanediol (RCV+Glutamate)

Total time	*Pfl* B1	*pfl* B2	*pfl* B3	*pfl* D	B10
16 hr 30 min	0.497	0.595	0.520	0.284	0.381
18 hr	0.641	0.770	0.737	0.575	0.747
20 hr	1.12	1.23	1.21	0.829	1.05
22 hr	1.566	1.679	2.195	1.417	1.936
23 hr 30 min	1.21	1.218	2.16	1.00	1.51
24 hr 30 min	1.15	1.22	2.23	0.926	1.41
25 hr	1.53	1.13	2.61	0.98	1.59

The *pfl* D mutant grew least among the mutants of *pfl* B which proved that *pfl* D gene is associated with 1,2-Propanediol, so the *pfl* D mutant was not much grown in this substrate. (Table 19 A)

Later on, hydrogen producing abibility of the mutants in the media with diol was also checked a well as the growth test with 1,2-propanediol. The table below showed *pfl* D never produced any hydrogen. (Table 19 B)

Table 19 B: Mutant bacterial growth measured by absorbance of the cultures Grown with 1, 2-Propanediol and H_2 productivity (RCV+Glutamate)

Total time	*pfl* B1	H_2	*pfl* B2	H_2	*pfl* B3	H_2	*pfl* D	H_2	B10	H_2
18 hr	0.855	-	1.30	+	1.18	-	0.93	-	1.14	-
20 hr 45	1.11	-	1.54	+	1.42	+	1.13	-	1.31	+
22 hr 30	1.19	+	1.62	+	1.50	+	1.14	-	1.36	+
24 hr 30	1.25	+	1.66	+	1.56	+	1.15	-	1.42	+
26 hr	1.29	+	1.70	+	1.60	+	1.16	-	1.44	+
42 hr	1.42	+	1.70	+	1.62	+	1.21	-	1.50	+

Later on, growth test of each of the mutants was performed by both ways using 1,2-propanediol as carbon source and not using any carbon source. B10 was taken as control. (Table 19 C)

Table 19 C: Experiment with/without adding 1, 2-Propanediol (C) (RCV+Glutamate)

Total time	*pfl* B+C	*pfl* B-C	*pfl* D+C	*pfl* D-C	B10+C	B10-C
24 hr 30 min	0.212	0.252	0.223	0.235	0.208	0.230
26 hr 30 min	0.210	0.244	0.214	0.242	0.213	0.237
28 hr	0.222	0.258	0.232	0.246	0.243	0.267
29 hr 30 min	0.238	0.280	0.261	0.275	0.276	0.299
30 hr 30 min	0.255	0.299	0.270	0.280	0.299	0.307
47 hr	1.40	1.26	1.22	1.26	1.38	1.26
50 hr	1.48	1.29	1.25	1.29	1.44	1.32
52 hr	1.52	1.30	1.26	1.30	1.46	1.32
71 hr	1.66	1.35	1.34	1.37	1.58	1.38

From the data above, it is shown that, growth was more with the culture with out any carbon source but after more than 24 hrs, the opposite incident happened where diol supported growth and absorbace was more than the one with no carbon source. It was again predicted that, at the initial stage, glutamate played well as a carbon source as well as a nitrogen source and when its concentration was declined, diol supported the growth of the bacteria. (Table 19 C)

3.2.1.2 Growth test and hydrogen collection of *rhodobacter* with ethanolamine as N_2 substrate:

Table 20: Monitoring growth with ethanolamine by measuring absorbance (O.D.660)

Bacterial culture	**Concentration of ethanolamine in media**	
	5.8 mM	**11.6 mM**
	O.D. $_{660}$ at 69 hours of growth	
pfl B1	0.8	0.61
pfl B2	1.43	0.38
Pfl D	0.51	0.51
B10	0.65	0.43
	O.D. $_{660}$ at 90 hours of growth	
pfl B1	1.56	1.07
pfl B2	1.17	0.78
Pfl D	1.06	0.90
B10	1.22	0.74
	O.D. $_{660}$ at 116 hours of growth	
pfl B1	2.55	2.17
pfl B2	1.40	1.71
Pfl D	2.39	2.02
B10	2.33	1.29
	O.D. $_{660}$ at 135 hours of growth	
pfl B1	3.6	2.86
pfl B2	1.9	0.38
Pfl D	3.57	0.51
B10	3.45	0.43

The table 20 showed the growth of mutants in different ethanolamine concentrations used as nitrogen source and the compatibility of the *pfl* mutants was compared with B10, wild type strain as control. Good growth was observed with the cultures supplied with 5.8 mM ethanolamine than the more concentrated one. So after comparing with B10, it was proved that *pfl* mutants posses same efficiency for growth with ethanolamine like the wild type. So *pfl* gene does not play any role in it.

Table 21: H_2 collection of the cultures grown with ethanolamine at the given times

Total time	**Volume of H_2 (ml)**			
	5.8 mM ethanolamine			
	pfl B1	*pfl* B2	*pfl* D	B10
69 hr	7.8	---	3.8	10.4
90 hr	4.0	2.2	5.8	7.5
116 hr	3.2	5.8	5.0	7.5
135 hr	---	2.7	---	---
	11.6 mM ethanolamine			
69 hr	0.5	---	0.2	3.8
90 hr	3.4	1.0	2.8	3.8
116 hr	4.3	4.3	5.2	4.6
135 hr	0.8	2.8	2.3	3.4

Table 21 shows, hydrogen collection from the mutants were also similar as the wild type B10 rather B10 grew more than the *pfl* mutants. So it can be inferred that the pyruvatye formate lyase gene does not effect hydrogen production by the photosynthetic bacteria, *rhodobacter capsulatus.*

3.2.2 Enzyme activity assays

Formate dehydrogenase assay

Formate production was checked in the mutant strains by the action of the enzyme formate dehydrogenase (FDH). This enzyme breaks down formate and NADH is produced that increases the optical density as it increases the concentration of the reaction mixture. So to ensure whether formate is present in the culture or not, this assay was performed.

At the beginning, standard solution of formate was used and absorbace was measured and recorded in order to compare the mutant cultures during the assay. It made easy the calculation of the formate produced by the bacteria.

3.2.2.1 Formate dehydrogenase assay with mutant bacteria: B10 (pRK290::*pflB*::Km^r)

At first *pfl* B mutants were tested to check if they were producing formate or not.

When FDH was not added, no reaction was carried out but still the absorbace was recorded to compare the reaction producing formate upon addition of FDH.

Absorbance before addition of the enzyme FDH-

Cuvette no.	Sample	Absorbace $_{340}$
1	A	0.067
2	B	0.057
3	C	0.029
4	D	0.053
5	E	0.279
6	F	0.236

Absorbance after addition of the enzyme FDH (25 µl), absorbance at 340 nm was measured at each 10 minutes interval –

Cuvette no.	0 min.	10 min.	20 min.	30 min.	40 min.
1	0.644	0.969	1.022	1.038	1.039
2	0.824	1.232	1.336	1.324	1.392
3	0.583	0.841	0.869	0.851	0.851
4	0.628	0.876	0.874	0.874	0.900
5	0.353	0.595	0.636	0.648	0.649
6	0.564	0.883	0.927	0.937	0.930

Result: The cultures produced ~ 2 mM formate. As O.D.$_{660}$ 1 = ~2 mM formate produced. This result was unusual and unexpected because mutation of the pyruvate formate lyase gene was expected to be inhibited so that no formate is produced by the mutant *rhodobacter*.

Table 22: Concentrations of the *pflB* mutants

Cuvette no.	Sample	Absorbance $_{340}$	ΔA	C (mM)
1	A	1.039	0.972	2.31
2	B	1.392	1.335	3.177
3	C	0.851	0.822	1.95
4	D	0.900	0.847	2.015
5	E	0.649	0.370	0.88
6	F	0.930	0.694	1.65

In table 22 from the recorded absorbance stated in the former tables, the concentration of formate produced by each culture was calculated by the formula:

Formula: Concentration, $C = \frac{V \times \Delta A}{\varepsilon \times v}$ **mM**

Here, V=1.5 ml, v = 0.1 ml, d= 1 cm, ε = 6.3

$$\text{So,} \quad \frac{V}{\varepsilon \times v} = 2.38$$

3.2.2.2 Formate assay with mutant bacteria: B10 (pRK290::*pflD*::Km^r)

Later on, *pfl* D mutants were tested to check if they were producing formate or not. When FDH was not added, no reaction was carried out but still the absorbace was recorded to compare the reaction producing formate upon addition of FDH.

Absorbance before addition of the enzyme FDH-

Cuvette no.	Sample	Absorbance $_{340}$
1	A	0.141
2	B	0.030
3	C	0.096
4	D	0.0211

Absorbance after addition of the enzyme FDH (25 µl), absorbance at 340 nm was measured at each 10 minutes interval –

Cuvette no.	0 min.	10 min.	20 min.	30 min.	40 min.
1	0.649	1.678	1.817	1.920	1.924
2	0.538	1.038	1.133	1.157	1.165
3	0.567	0.942	1.008	1.026	1.030
4	0.725	1.295	1.392	1.426	1.446

Result: The cultures produced ~ 2 mM formate. As O.D.$_{660}$ 1 = ~2 mM formate produced. This result was unusual and unexpected because mutation of the pyruvate formate lyase gene was expected to be inhibited so that no formate is produced by the mutant *rhodobacter*.

Table 23: Concentrations of the *pflD* mutants

Cuvette no.	Sample	Absorbance $_{340}$	ΔA	C (mM)
1	A	1.039	2.065	4.914
2	B	1.392	1.376	3.27
3	C	0.851	1.06	2.52
4	D	0.900	1.542	3.66

In table 23 from the recorded absorbance stated in the former tables, the concentration of formate produced by each culture was calculated by the formula:

1	A	1.039	2.065	4.914
2	B	1.392	1.376	3.27
3	C	0.851	1.06	2.52
4	D	0.900	1.542	3.66

In table 23 from the recorded absorbance stated in the former tables, the concentration of formate produced by each culture was calculated by the formula:

Formula: Concentration, $C = \frac{V \times \Delta A}{\varepsilon \times v}$ **mM**

Here, V=1.5 ml, v = 0.1 ml, d= 1 cm, ε = 6.3

So, $\frac{V}{\varepsilon \times v} = 2.38$

3.3 Molecular Biology approaches

3.3.1 Cloning and mutagenesis

In the drop out method, the exact concentration of the sample was determined by comparing with standard plasmid DNA of known concentrations. In the following image, it was inferred that, the concentration of the purified plasmid DNA was 2 ng/µl

"Drop out method" to determine concentration of purified plasmid DNA:

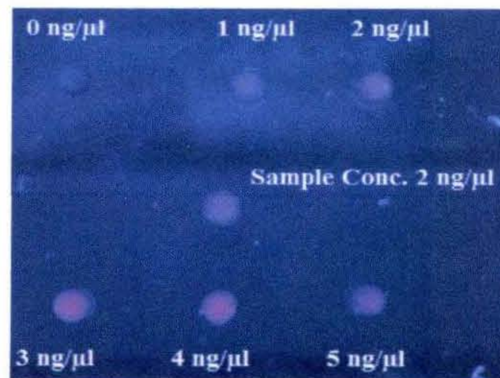


Figure 9: Control DNA and sample DNA spots exposed under UV

3.3.2 Conjugation and Transformation

Conjugation of pRK290-type plasmids into *Rhodobacter capsulatus* Y262:

Table 24: Bacterial growth measured by absorbance of the cultures

E.Coli Culture	**O.D.$_{600}$**
DH5α (pRK290::*pflB*::Km^r)	7.48
DH5α (pRK290::*pflD*::Km^r)	3.52
HB101	6.46
Rhodobacter Culture	**O.D.$_{660}$**
RC1	0.43

Plasmid elimination and recombination: From the bacterium *rhodobacter* SB1003 by successive culture:

The last culture after three successive cultures was measured for absorbance to check the growth only.

Table 25: Bacterial growth measured by absorbance of the cultures

Culture	**Absorbance$_{660}$**
SB1003 (pRK290::*pflB*::Km^r)	1.56
SB1003 (pRK290::*pflD*::Km^r)	2.78

Experiment of GTA transfer of Kanamycin resistance: With Y262 & B10: Colony counting in RCV-N plates of Y262×B10:

Crosses		Number of Colonies
Y262 (pRK290::*pflB*::Km^r)×B10	Plate1-A1	4
	Plate2-A1	6
	Plate3-A1	9
	Plate4-A1	3
Y262 (pRK290::*pflB*::Km^r) ×B10	Plate1-A2	11
	Plate2- A2	2
	Plate3- A2	9
	Plate4- A2	5
Y262 (pRK290::*pflD*::Km^r)×B10	Plate1-B1	4
	Plate2-B1	3
	Plate3-B1	5
	Plate4-B1	5
Y262 (pRK290::*pflD*::Km^r) ×B10	Plate1-B2	6
	Plate2- B2	6
	Plate3- B2	4
	Plate4- B2	7

The above table shows the colonies produced by the GTA mediated crosses in between Y262 with Kmr cassette in the pfl genes and with B10. The mutant was successfully grown which porved the efficiency of the gta complementation of the mutants. *Pfl* B and *pfl* D mutants showed the same effects.

3.3.4 Preparation of nucleic acids and plasmid DNA

Extraction of DNA of mutants: B10 (pRK290::*pflB*::Km^r) and B10 (pRK290::*pflD*::Km^r):

Extraction procedure-1:

After the DNA extraction of the B10 mutants, electrophoresis was performed and image was analysed to check for the availability of the DNA bands.

Image analysis of gel electrophoresis:

After electrophoresis, DNA bands were visible for only the following samples:

Sample # 1 (*pfl* B)
Sample # 3 (*pfl* B)
Sample # 5 (*pfl* D)

So it was understood that DNA only from these three samples were isolated successfully.

Extraction procedure-2:

Table 26: Growth monitoring of the cultures by measuring OD_{660}

Sample	OD_{660}
1	4.37
2	4.11
3	4.14
4	4.23
5	4.04
6	3.94
7	3.84
8	3.36
9	4.10
10	3.96
11	4.27
12	3.23

The data from the above table showed that, the cultures were in good growth to be used in the extraction procedure. So, all of the above samples were taken for DNA extraction.

Image analysis of gel electrophoresis:

After DNA extraction of the mutant bacteria, electrophoresis was carried out and DNA band was visible only in one sample:

Sample # 2 (*pfl* B)

In total four mutants gave good DNA bands and they were from sample 1,2,3 and 5. Among these, the first three were *pfl* B mutans and the fourth was *pfl* D mutant.
So, later on, all the molecular biology works and biochemical tests were performed on these four mutants.

Extraction procedure-3:

Mini prep of bacterial genomic DNA:

Image analysis of gel electrophoresis:

(A)

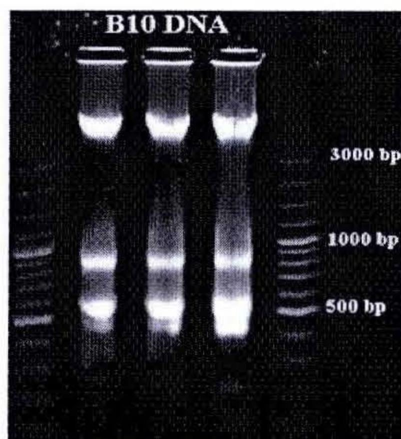


Figure 10: Wild type B10 DNA isolated from aerobic culture (as positive control) (Before RNAse treatment)

In the image A, DNA bands were visible approximately at 10,000 bp position which proved that DNA was isolated from the B10 cultures successfully.

(B)

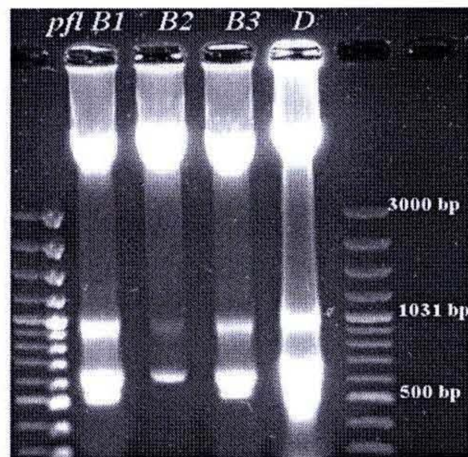


Figure 11: DNA of mutants isolated from anaerobic cultures
(After RNAse treatment)

In the image B, DNA bands were visible approximately at 10,000 bp position which proved that DNA was isolated from the pyruvate formate lyase mutants successfully.

3.3.5 PCR

3.3.5.1 Experiment-1: 16s rRNA amplification:

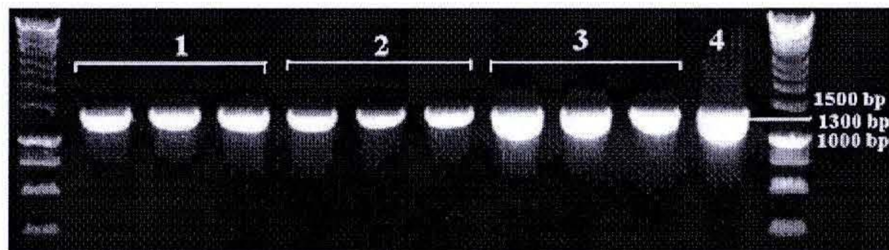


Figure 12: 16s rRNA amplification of the mutants (First 6 templates from colonies and last 4 from extracted DNA)

In the image, 1 = pfl B mutants from colonies from plates
2 = pfl D mutants from colonies from plates
3 = pfl B mutants from extracted DNA
4 = pfl D mutant from extracted DNA

This amplification was done as a control test before doing all other amplifications with the mutant genes. Amplified bands visible at 1300 bp position proved successful amplification of the 16 rRNA.

3.3.5.2 Experiment-2: Amplification of *pfl B* and *pfl D* genes

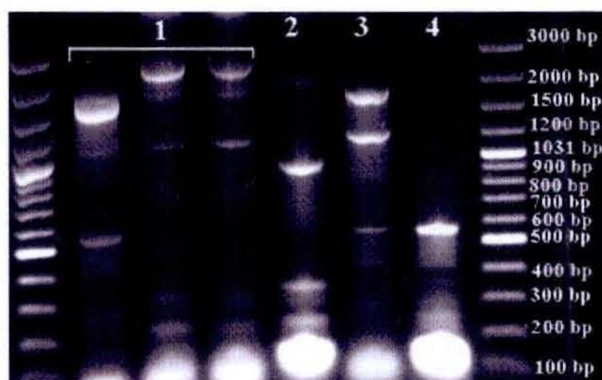


Figure 13: Amplification of *pfl* B and *pfl* D genes of the mutants and wild type B10

In the image, 1 = *pfl* B mutants
2 = *pfl* D mutant
B10 = Amplified by *pfl* B primer
B10 = amplified by *pfl* D primer

The *pfl* B gene with Km^r cassette was expected to amplify gene of ~2600 bp and *pfl* D with Km^r cassette was expected to amplify gene of ~2270 bp (the length of Km^r cassette is 1400 bp). So, amplified bands at 2600 bp position for *pfl* B mutants and at 2270 bp position for *pfl* D mutants proved successful amplification of *pfl* genes inserted with Km^r cassette. That Km^r cassette was inserted in the *pfl* genes was proved in such amplification reactions.

Optimization of PCR to amplify *pfl* genes:

3.3.5.3 Experiment-3:

Addition of DMSO:

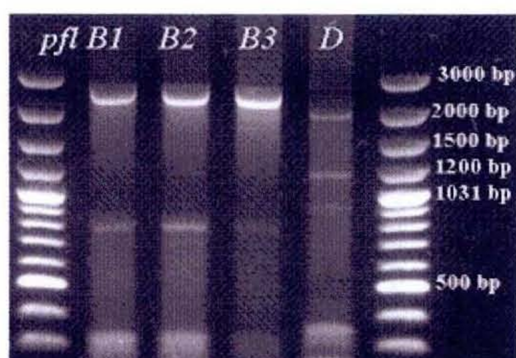


Figure 14: Specific amplification for addition of DMSO in the PCR reaction mixture

Amplified bands at 2600 bp position for *pfl* B mutants and at 2270 bp position for *pfl*

D mutants proved successful amplification of *pfl* genes inserted with Km^r cassette. That Km^r cassette was inserted in the *pfl* genes was proved in such amplification reactions.

3.3.5.4 Experiment-4:

New *pfl D* primers & combinations with old and new primers:

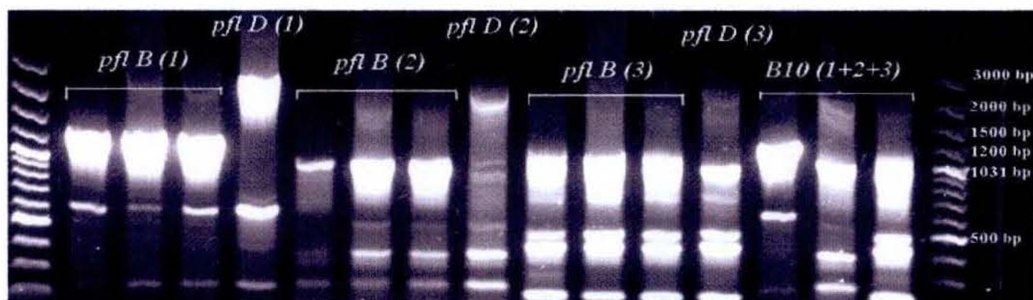


Figure 15: 4 mutants amplified with old and new *pfl* D primers

In the image, Combination # 1 → *pfl* D F1/R1
Combination # 2 → *pfl* D F1/R
Combination # 3 → *pfl* D F/R1

Here, Old primers are *pfl* D F/R
New primers are *pfl* D F1/R1

The combinations of old and new *pfl* D primers were used in this PCR to check whether there was any problem with the oligos or not. If the primers got any problem, whether the forward or the reverse got this defect was also interpreted from this image. It was showed that the *pfl* D forward primer basically got the problem with the efficiency to amplify the *pfl* D genes.

3.3.5.5 Experiment-5:

Annealing Tm: (from 50°C to 55°C)

Figure 16: B10 amplified with new pfl D primers with changed annealing temperature

New primers were at first amplified at 50°C annealing temperature and later on it was optimized by using annealing temperature of 55°C. At this temp., it was found that the amplification of *pfl* D gene with new primers was better.

Here the B10 is a wild type so amplified DNA bands were visible at the position of 870 bp.

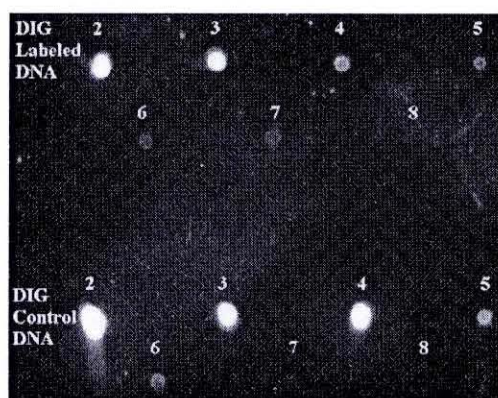
3.3.6 Hybridization techniques

Determination of labeling efficiency:

After labeling DNA with the kit, the labelling efficiency should be measured as labeled probe with specific concentration is required for a hybridization reaction. Efficient labelling and time duration of the labeling reaction effect the amount of DNA labeled.

Result analysis:

Both the dilutions that is DIG labelled probe and DIG control DNA containing 0.1 þg (dilution number 6) of DNA were visible in the image, so the labeling was considered to reach to the expected labeling efficiency. (According to the manual)



Here,
Dilution # 2 → 10 þg/µl
Dilution # 3 → 3 þg/µl
Dilution # 4 → 1 þg/µl
Dilution # 5 → 0.3 þg/µl
Dilution # 6 → 0.1 þg/µl
Dilution # 7 → 0.03 þg/µl
Dilution # 8 → 0.01 þg/µl

Figure 17: Determination of labeling efficiency of DIG labeled DNA probe

Hybridization:

Blot image:

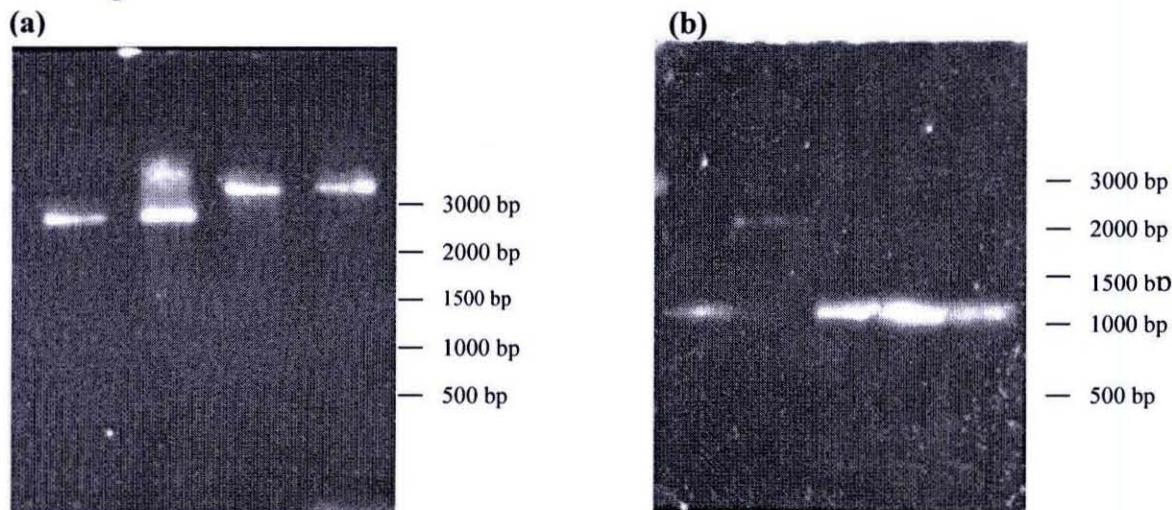


Figure 18: Blot Image [(a) with *pfl* B probe, (b) with *pfl* D probe]

The above images were taken after hybridization of the mutant *pfl* genes. Both the *pfl* B and *pfl* D mutants were used to carry out this hybridization technique. Image (a) was done with *pfl* B probe and image (b) with *pfl* D probe. With EcoRI digestion, the expected length of the mutated *pfl* B gene with Km^r cassette was above ~4100 bp (*pfl* B gene→ 2760 bp and Km^r cassette → 1300 bp) and for *pfl* D gene, it was ~2170 bp (*pfl* D gene → 870 bp and Km^r cassette → 1300 bp). From the image, the DNA bands were observed at the appropriate position of the expected size and so it was proved that *pfl* gene was inserted successfully with Km^r cassette.

Discussion

Microbiology Methods

Hydrogen production by *R. capsulatus* and other photosynthetic non-sulfur bacteria occurs under illumination in the presence of an inert, anaerobic atmosphere (such as argon), from the breakdown of organic substrates such as malate and lactate. The culture medium should be under a nitrogen limitation (i.e. a high C/N ratio), which forces the bacteria to 'dump' the excess energy and reducing power through the production of hydrogen. Several individual components make up the overall production system and these may conveniently be grouped as: (i) the enzyme systems, (ii) the carbon flow—specifically the TCA cycle and (iii) the photosynthetic

membrane apparatus. These groups are interconnected within the hydrogen production scheme by means of the exchange of electrons, protons and ATP.

Mode of Metabolism

It can be inferred from the preceding description that for the PNS bacteria, hydrogen production of any significance occurs under a photoheterotrophic growth mode, which is also the preferred growth mode for these microorganisms. Yet, PNS bacteria are capable of several alternative metabolic modes such as aerobic/anaerobic respiration, fermentation and photoautotrophy. Normally, photobioreactor conditions have to be carefully adjusted such that the photoheterotrophicmode prevails. However, conditions favoring the alternative modes are sometimes unavoidable. For instance, if light availability is poor in deep regions of the reactor, or if the experiment is carried out under natural sunlight, the bacteria may switch to a fermentative type of metabolism. This probability is more pronounced in the latter case, and one of our experiments provides evidence for substrate consumption in the dark periods, possibly indicating a fermentative metabolism.

Hydrogen photo production and collection

The formation of molecular hydrogen results from the direct reduction of protons from water. The photo evolution of hydrogen can be preceded under an atmosphere of 100% H_2. But photo evolution of H_2 occurs in the absence of N_2 and of high concentrations of ammonium ions, under conditions in which ATP from photophosphorylation and reducing equivalents from organic substrates are produced in excess [41-43]. In growing cultures of *R. capsulata*, the highest rates of H_2 production (130 μl hr^{-1} ml $culture^{-1}$) were obtained with DL-lactate with carbon source. That is why, the H_2 producing media were produced with 1 M Na-lactate. More over, glutamate was used as growth limiting N_2 source but the ratio of the concentrations of glutamate (or NH_4^+ salts as 7mM) and lactate (30 mM) was lower than 1.0 as at higher ratios, net production of NH_4^+ from glutamate occurred resulting in the inhibition of nitrogenase [23].

Increased light intensity resulted in an increased nitrogenase synthesis and as a result increased H_2 photo evolution. The rate of H_2 production was found to be proportional to light intensity upto 12,000 lux [44]. Argon was applied through the media before

to light intensity upto 12,000 lux [44]. Argon was applied through the media before culture inoculation in order to make the environment anaerobic as well as to get rid of excess N_2.

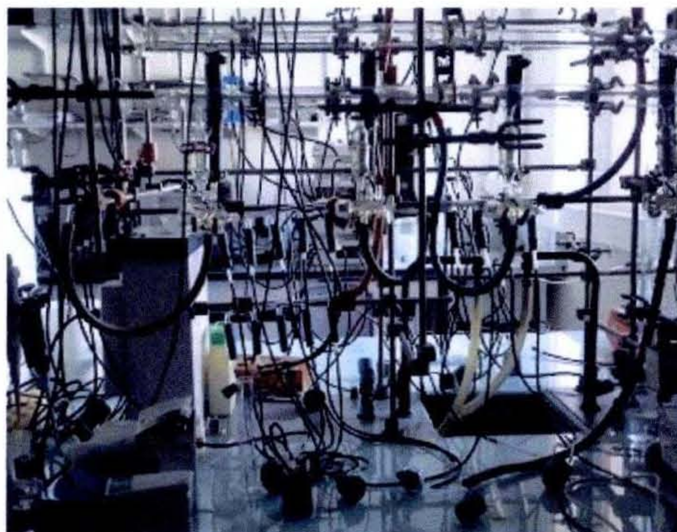


Figure 19: Argon column (courtesy: LCBM, CEA, Grenoble)

Also for the small culture, glass tubes instead of plastic ones were used to ensure anoxygenic bacterial growth. Special type of tefflon pipes were used to collect H_2 from the culture as through rubber pipe, some H_2 may diffuse away. Temperature in the light room was always kept in between 30-34°C which is optimum for good production of H_2. In winter, additional incandescent lights are added inside the room and in summer, cooler was used to lower the room temperature.

Experiments with GTA mediated crosses

In such experiments, sometimes it took a long time for production mediated with GTA or found no colonies at all. The possible reasons could be: donor strain did not make GTA; problem could be with recipient strains, or with media (YP or RCV-N). Sometimes anaerobic jar might fail to create an anaerobic atmosphere. G-buffer could also cause problem or the BSA which was used into it. To overcome it, we used different recipient cells, tried less concentrated YP medium (1 g/L or 2 g/L). Molecular biology grade BSA was another solution. Moreover, anaerobic jar was tested for efficiency by growing B10 by successive dilution. GTA was prepared by filtering a donor culture through 0.45-µm membrane filter, since removing cells by low-speed centrifugation results in a 50% loss in gene transfer activity compared to

purified further to remove inhibitory substances that work against the effectiveness of the GTA. Such inhibitory substances are peptone and yeast extract, so G-buffer could be used for dilution of the crosses instead of YP medium. Moreover, the donor cell concentration should be more than recipient cells as at higher recipient cell concentrations, O_2 becomes limiting and a gradual decrease in transferants is observed. [44].

Biochemical assay

Biochemical assays are laboratory methods for measuring enzymatic activity. They are vital for the study of enzyme kinetics and enzyme inhibition.

Photosynthetic bacteria produce H_2 under anaerobic conditions, in the absence of nitrogen gas, with illumination and with stressful concentrations of nitrogen sources. Photo heterotrophic bacteria, such as *R. capsulatus*, can grow anaerobically to produce H_2 either from reduced substrates such as organic acids or from reduced compounds. These bacteria use enzyme nitrogenase to catalyze nitrogen fixation for reduction of molecular nitrogen to ammonia. Nitrogenase can evolve H_2 simultaneously with nitrogen reduction. Stressful concentrations of nitrogen are therefore required for H_2 evolution. Total hydrogen production is limited due to several metabolic events occurring in cells such as production of poly-3-hydroxybutyrate or consumption of H_2 by hydrogenase uptake. Membrane-bound uptake hydrogenase decreases H_2 production efficiency by catalyzing conversion of molecular H_2 to electrons and protons. Inactivation of uptake hydrogenase has resulted in total increase in H_2 production.

Formate dehydrogenase assay

It was supposed not to produce any formate by the mutants like the wild type but formate was produced slightly. So to get rid of confusion, molecular biology approaches like PCR and Hybridizations were performed to be sure of the mutation. But primarily it was assumed that, the formate was being produced by pyruvate formate lyase activating enzyme which is encoded by the gene *pfl* A and lies beside the *pfl* B gene in the genome.

Formate dehydrogenase

The reduction of CO_2, to formate is an essential process in both the catabolism and the anabolism of many strict anaerobes.

Growth test with Diols

pfl D gene is associated with 1,2-propanediol metabolism but *pfl* D mutant grew in the media containing 1,2-propanediol. But the data showed that, the absorbance monitored after 72 hrs was the least among all other mutants. It was observed that serial dilutions of diol-grown *R. capsulatus* cells made from mid-exponential phase cultures grew rapidly while transfers from stationary phase cultures often failed to grow (Panagiotis E. Pantazopoulous and Micheal T. Mdigan) (Primary alcohols and di-alcohols as growth substrates for the purple non-sulfur bacterium *Rhodobacter capsulatus*)

Genomic DNA extraction

There was a trouble with the genomic DNA extraction in spite of several trials. In case of using kit for such procedures, too old reagent mixtures or reagents with precipitation should be avoided. Also culture was prepared for successful DNA extraction which was aerobic culture with bigger volume. In the procedure with aerobic cultures, CTAB / NaCl used was first heated and then pipetted carefully to maintain the exact volume otherwise it could affect the DNA precipitation.

PCR

In amplifying *pfl* genes, faint bands were observed in the electrophoresis images at the beginning. PCR was optimized in various ways. Like DMSO was used which made increased specificity during annealing the primers. There was more trouble with amplifying *pfl* D genes and after purchasing new oligos, the amplification was much better. The annealing temperature in the PCR program was changed to increase the specificity and good amplification. The template DNA was from extracted DNA of the mutants and after RNAse treatment, the PCR was more specific.

Hybridization techniques

The concentration of the labelled probe should be determined properly otherwise the membrane is not hybridized well. After DNA digestions, 0.7% agarose gel was used

to get bigger fragments and well separated. While transferring blot to the membranes, sandwich was made with good pressure and before that, rolling pen/pipette on the membrane placed on the gel. This small work made unsuccessful transfer to successful ones.

Conclusion

Considering the energy security and the global environment, there is a pressing need to develop non-polluting and renewable energy source. Hydrogen is a clean energy source, producing water as its only by-product when it burns. Compared to other gaseous fuels like methane, hydrogen is harmless to humans and the environment. Phototrophic bacteria are indicated in the current literature as the most promising microbial system for the biological production of hydrogen. This is mainly because of their: (1) higher theoretical conversion yields, (2) lack of O_2-evolving activity which causes O_2 inactivation problems of the catalyst in different biological systems or of separation of O_2 from H_2, (3) the ability to use wide spectral light energy and can withstand high light intensities, (4) the ability to consume organic substrates derived from wastes in association with wastewater treatment, (5) the H_2 porduction catalysed by nitrogenase can proceed under an atmosphere of 100% nitrogen gas, (6) the great metabolic versatility of photosynthetic bacteria enable them to remain functional under many different environmental conditions; and (7) genetic techniques are rapidly being extended to photosynthetic bacteria which now can be transformed by exogenous plasmids . These types of Purple bacteria are able to produce molecular hydrogen (H_2) catalyzed by nitrogenase under nitrogen limiting conditions. Phototrophic bacteria, especially *Rhodobacter capsulatus* are very active nitrogen fixers and H_2 photoproduction by *R. capsulatus* might reach as high as 200 $\mu l\ h^{-1}\ mg^{-1}$ proteins. There are several ways to enhance H_2 production by purple bacteria, such as selecting more active strains and mutants optimising the medium, temperature and pH, analyzing the competition between H_2 production and storage product accumulation, and using a two-stage chemostat culture.

In this project work, hydrogen production is observed in the presence of lactate or malate as carbon sources and limited concentration of nitrogen. These organic

substrates are dissimilated through some pathways and molecular H_2 results from the direct reduction of protons from water. For the complete dissimilation of carbon substrates to H_2 and CO_2, carbon source is depleted from the medium through metabolism. In a step of lactate or malate catabolism, pyruvate is produced by the action of an enzyme pyruvate formate lyase (*pfl*). This produced formate in the metabolic pathway competes with the H_2 by sharing the same metablites, and H_2 production is decreased. For this reason, the gene encoding this enzyme, pyruvate formate lyase, is targeted to switch off by transposon mediated mutagenesis. Here the 2 types of *pfl* genes B and D were in target and mutagenesis was performed successfully. But at the end, during the biochemical tests for checking formate production from the mutant culture, it was observed that still slight production of formate occurs. Molecular biology approaches like PCR and hybridization techniques were followed further to check the presence of mutated gene products and the experiments were successful in that regard.

The metabolic versatility of *rhodobacter* was tested by using different carbon and nitrogen sources rather than lactate or malate and ammonium salts. These new substrates like ethanolamine and diols are proven to be much promising substitute for present traditional ones and they should be influenced to use in future because of their presence in biomass wastes, higher efficiency in *rhodobacter* growth and hydrogen productivity.

In conclusion, metabolic engineering and molecular biology tools are considered as promising approaches for the improvement of biological hydrogen production by microorganisms like *Rhodobacter capsulatus*, particularly as regard the redirection and optimization of the flow of reducing equivalents to the H_2-production related enzymes are concerned. So, all the approaches need to exclude the formation of formate by the hydrogen producing culture. Further researches in this field will lead to development of an efficient microbial biotechnology methods for higher production of hydrogen- "a potential fuel for future".

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Appendix-1

Division 1: Gracilicutes

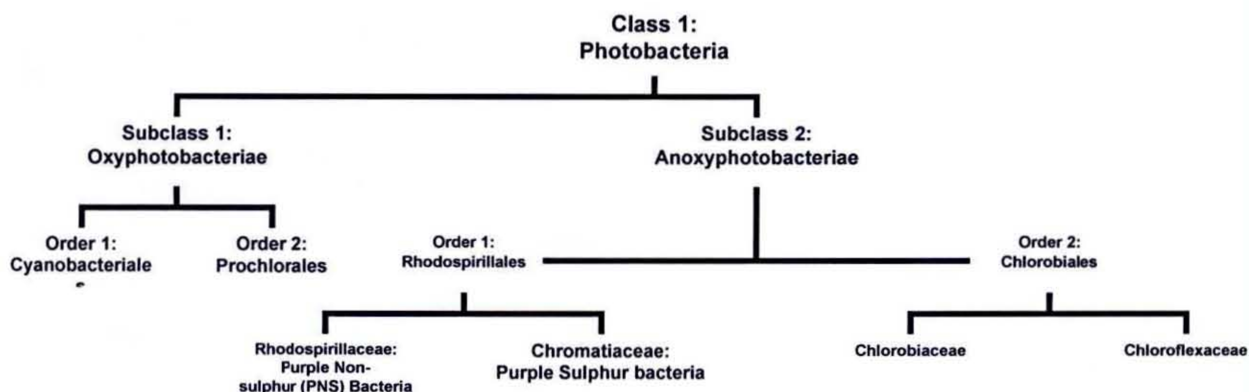

Figure: Subdivision of kingdom "Procaryotae" Murray (1968) as proposed by Gibbons and Murray (1978)