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Metabolic Engineering to Improve Biohydrogen Production by *Rhodobacter capsulatus* JP91

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Metabolic Engineering to Improve Biohydrogen Production by *Rhodobacter capsulatus* JP91

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RÉSUMÉ

La demande pour l'énergie augmente de jour en jour, ce qui se traduit par une attention mondiale à l'égard d'autres carburants respectueux de l'environnement parce que les combustibles fossiles nuisent à l'environnement. La production biologique d'hydrogène est une méthode alternative pour la production d'hydrogène, grâce à laquelle elle est produite dans des conditions douces, respectueux de l'environnement. La production photo-biologique d'hydrogène par les bactéries photosynthétiques pourpres non sulfureuses est un processus prometteur dans lequel les bactéries peuvent capturer de l'énergie lumineuse pour conduire la production d'H_2 avec leur système de nitrogénase. Cependant, certaines voies métaboliques, tel que la fixation du CO_2 et la biosynthèse du PHB, rivalisent avec la nitrogénase pour les électrons. Récemment, l'génie la métabolique a été appliqué pour améliorer le taux et le rendement de production d'H_2.

Le but de la présente étude était d'améliorer le rendement de la production d'H_2 pendant la Photosynthèse par *Rhodobacter capsulatus* JP91 en utilisant des approches d'ingénierie métabolique. Notre hypothèse était que l'inactivation de PHB synthase (*phbC*) arrêterait la biosynthèse de PHB et dirigerait plus de flux des électrons dérivés du substrat vers la nitrogénase pour catalyser la production d'H_2. Dans des études antérieures, des mutants dans la PHB synthase ont été développés dans d'autres bactéries photosynthétiques, y compris *R. sphearoides* et *Rhodopseudomonas palustris* mais pas *Rhodobacter capsulatus*. Dans cette étude, nous avons développé une nouvelle souche *R. capsulatus* RS15 doublement mutée (*hup*$^-$, *phbC*$^-$) qui était dérivée de *R. capsulatus* JP91 (*hup*$^-$). Nos résultats montrent que la nouvelle souche, *R. capsulatus* RS15 pourrait croître dans différentes sources de carbone et d'azote. Notre mutant a une longue phase de décalage. Cependant, une foi démarrée, lorsqu'elle atteignait la phase logarithmique, sa croissance était similaire à ce de la souche parentale.

À partir des résultats de la production d'hydrogène, la souche RS15 nouvellement développée est capable de convertir l'acétate, le lactate et le glucose en hydrogène l'acétate semble d'être la meilleure source de carbone pour la production de H_2 par RS15 mais pas pour sa croissance.

Surtout, *R. capsulatus* RS15 (*hup*$^-$, *phbC*$^-$) semble d'être un candidat prometteur pour le système hybride à deux niveaux puisque les principaux effluents de fermentation sombre sont l'acétate et le butyrate. Donc, ce processus peut potentiellement rendre la technologie de production de bio hydrogène possible à l'échelle industrielle.

Mots-clés : production de bio hydrogène, génie métabolique, bactérie violette sans soufre *R. capsulatus* JP91, *R. capsulatus* RS15, photofermentation, polyhydroxy butyrate, et PHB synthase

ABSTRACT

Energy demand is increasing day by day, resulting in global attention towards alternative eco-friendly fuels. Biohydrogen is considered the most promising energy carrier to replace conventional fossil fuels because its production and combustion is not harmful to the environment. Biological hydrogen production is an alternative method for hydrogen production, by which hydrogen is produced under mild conditions, making it environmentally friendly. Photo- biological hydrogen production by purple non-sulfur photosynthetic bacteria is a promising process in which bacteria can capture light energy to drive H_2 production with their N_2ase system. Some metabolic pathways, such as CO_2 fixation and PHB biosynthesis, compete with nitrogenase for electrons (reducing equivalents). Recently, in attempts to improve the yield of H_2, metabolic engineering has been applied to increase electron flow to N_2ase.

The purpose of the present study was to improve the yield of photosynthetic H_2 production by *Rhodobacter capsulatus* JP91 using a metabolic engineering approach. The hypothesis was that inactivation of PHB synthase (*phbC*) would block PHB biosynthesis, thus directing more electron flux towards nitrogenase to catalyze increased H_2 production.

In previous studies, PHB synthase mutants were developed in other photosynthetic bacteria, including *R. sphearoides* and *Rhodopseudomonas palustris* but not *Rhodobacter capsulatus*.

In this study, a new doubly mutated *R. capsulatus* strain RS15 (*hup*$^-$, *phbC*$^-$), a derivative of *R. capsulatus* JP91 (*hup*$^-$) was created. The results show that the newly created strain, *R. capsulatus* RS15, could grow in different carbon and nitrogen sources. This mutant has a long lag phase. However, once it reached log phase, growth was similar to the parental strain. From hydrogen production studies, the new developed strain RS15 is able to convert acetate, lactate, and glucose to hydrogen.

Acetate was shown to be the best carbon source for H_2 production by RS15 but not for its growth. Overall, *R. capsulatus* RS15 *(hup⁻, phbC⁻)* seems to be a promising candidate for use in two - stage hybrid systems since the main dark fermentation effluents are acetate and butyrate. Such a process could potentially make biohydrogen production technology feasible on an industrial scale.

Keywords: biohydrogen production, metabolic engineering, purple none sulfur bacteria, *R. capsulatus* JP91, *R. capsulatus* RS15, photofermentation, polyhydroxy butyrate, and PHB synthase

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ABBREVIATIONS

Acetyl-CoA: acetyl coenzyme A

ATP: adenosine triphosphate

ADP: adenosine diphosphate

Ap: ampicillin

CBB: calvin-Benson-Bassham cycle

CO_2: carbon dioxide

CH_4: methane

C/N: carbon to nitrogen ratio

DraT: dinitrogenase reductase ADP-ribosyltransferase

DNA: deoxyribonucleic acid

DNS: dinitrosalicylic acid

e^-: electron

Fe: iron

Fd: ferredoxin

GC: gas chromatography

Gm: gentamycin

GHG: greenhouse gases

GlnK: signal transduction protein, belongs to PII group

GlnB: signal transduction protein, belongs to PII group

h: hour

H_2: hydrogen

Hup: uptake hydrogenase

H_2ase: hydrogenase

Km: kanamycin

ISL: isocitrate lyase

LH1: light harvesting complex1

LH2: light harvesting complex 2

MFC: microbial fuel cell

MEC: microbial electric cell

μM: micromolar

mM: millimolar

M: molar

mg: milligram

μg: microgram

ml: milliliter

μl: microliter

NAD`: nicotinamide-adenine dinucleotide (oxidised)

NADH: nicotinamide-adenine dinucleotide (reduced)

NADP: nicotinamide-adenine dinucleotide phosphate

NFOR: nADH: ferredoxin oxidoreductase

N_2ase: nitrogenase

NH_3: ammonia

NifA: transcriptional activator of *nif* genes

nm: nanometer

OD: optical density

PHA: poly-hydroxyalkanoate

PHB: poly-hydroxybutyrate

phbA: ß-ketothiolase

phbB: acetoacetyle CoA dehydrogenase

phbC: PHB synthase

PCR: polymerase chain reaction

PNSB: purple non-sulfur bacteria

PSB: purple sulfur bacteria

PS1: photosystem1

PS2: photosystem 2

R. capsulatus: *Rhodobacter capsulatus*

R. rubrum: *Rhodospirillum rubrum*

R. palustris: Rhodopseudomonas palustris

R. sphaeroides: Rhodobacter sphaeroides

SO_2: sulfur dioxide

TCA: tri carboxylic acid

Tet: tetracycline

VFA: volatile fatty acid

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Introduction

1. Hydrogen is one of the best alternative fuels:

In the last few decades, economic growth has been strongly dependent on fossil fuels as a source of energy resulting in the depletion of fossil fuels along with increasing greenhouse gases emissions (GHG) into the air including; CO_2, CH_4, NO_2, and SO_2 and other toxic pollutants (Chandrasekhar et al, 2015). The increasing concentrations of GHG in the atmosphere has many negative effects including; climate change, receding of glaciers, rise in sea levels, loss of biodiversity, health problems, and others (Singh and Nigam, 2014). These concerns have focused global attention towards alternative, renewable, sustainable, efficient, and cost-effective ecofriendly energy sources with fewer emissions to replace fossil fuels. Biofuel production processes have been extensively investigated. As a result of these efforts, commercially successful biofuels such as; biodiesel, bioethanol, and biomethane have been developed. Among alternative energy sources, hydrogen is a very promising energy carrier that could play a significant role in the reduction of greenhouse gas emission because hydrogen (H_2) only produces water when burned with oxygen, so it does not have any harmful impact on the environment. It has the highest energy per unit weight, 141.90 MJ/Kg, and the lowest CO_2 emission compared to other fuels. Hydrogen can be converted efficiently to electricity by fuel cells. In recent years, hydrogen has been used for hydrogen-fueled transit buses, ships and submarines, and chemical and petrochemical applications (Miyake et al, 1999; Chandrasekhar et al, 2015; Reith et al, 2003; Akkermana et al, 2002; Hallenbeck, 2009; Singh and Nigam, 2014; Sarma et al, 2016). Even though hydrogen is the most abundant element on the planet it does not exist in any significant quantities in elemental form. Unfortunately, at present hydrogen is obtained from energy-exhaustive, expensive, nonrenewable, and environmentally unfriendly sources.

Nowadays, approximately 98% of hydrogen is produced from fossil fuels through steam reforming of natural gas or methane (Kalinci et al, 2009; Chandrasekhar et al, 2015). A variety of process technologies can be used, including; chemical, biological, electrolytic, photolytic and thermochemical. Each technology is in a different stage of development, and each offers unique opportunities and benefits. Steam methane reforming is the most common and least expensive way to produce hydrogen at present and hydrogen can also be extracted from oil, gasoline, and methanol through reforming. Coal can also be reformed to produce hydrogen, through gasification. H_2 can be produced from biomass either though gasification, like coal, or through pyrolysis. These processes have one or more of these disadvantages, such as, being expensive, inefficient, and greater resource use and emissions. Also, electrolysis, which is the process of producing H_2 by using electricity to split water into H_2 and O_2, is a promising long- term method of producing hydrogen. This process is a cost-effective way to produce small amounts of pure H_2; however, it is expensive on a large scale (Dunn, 2002; Momirlan and Veziroglu, 2002). The cost of producing hydrogen via current electrolytic processes is largely dependent on the cost of electricity, the efficiencies of the systems, and the capital costs of the systems. Hydrogen produced via electrolysis can result in zero GHG emissions in air, depending on the source of the electricity used. When electrolysis is from renewable generated electrical power (wind, solar, etc.), that would be seen as potentially economical for large scale use (Saliba-Silva et al, 2009).

Therefore, there is a need to find a cost-effective hydrogen production process that can be applied on a large scale. In the long term, H_2 would preferably be produced from renewable sources such as: electrolysis, photobiological H_2 production, biomass gasification, etc. At the present, hydrogen production from biomass and waste through biological processes has been has been the subject of much research due to its sustainable nature (Venkata Mohan et al, 2013; Barretoa et al, 2003).

1.1 Biohydrogen:

Biological H_2 production, catalyzed by microorganisms, has been investigated for many decades. Investigations have facilitated better understanding of basic principles behind the phenomenon, and have created the possibility for large-scale production of biohydrogen as a renewable source of energy. Despite the production price, biological hydrogen production would combine the advantage of ecological production with clean combustion and other goals, such as waste water treatment and carbon dioxide reduction (Akkermana et al, 2002; Asada and Miyake, 1999).

The chemical reaction of hydrogen production is represented in Eq. (1).

$$2H \rightarrow 2e^{-} + H_2 \quad (1)$$

1.2 Hydrogen production microorganisms:

Several organisms can produce H_2 in nature including; the archaea, anaerobic and facultative anaerobic bacteria, cyanobacteria, and lower eukaryotes (*i.e.*, green algae and protists) Heterotrophs are the most common H_2 producing biocatalysts in fermentative process, and dark fermentative bacteria, operating in the absence of oxygen, do not need light as an energy source. These bacteria are obligate anaerobes, and can be further classified based on their sensitivity to O_2 and their growth temperature **(**Chandrasekhar et al, 2015).

1.3 Biohydrogen production enzymes:

Specific enzymes are required for all biological processes, and for biological hydrogen production the presence of one of three enzymes is required: iron hydrogenases, nickel-iron hydrogenases, or nitrogenases, for the reduction of protons or the oxidation of hydrogen (Seifert et al, 2012).

1.3.1 Nitrogenase:

Nitrogenase is the enzyme that carries out N_2 fixation by converting nitrogen to ammonia, a form that can be used by most organisms. This enzyme is found only in some prokaryotes, including;

purple non-sulfur (PNS) bacteria, green sulfur bacteria, some cyanobacteria, etc. N_2ase is the key enzyme involved in photo- fermentative hydrogen production by the PNSB. There are three genetically different types of nitrogenase consisting of two dissociable components: - reductase (Fe protein) and dinitrogenase (either Mo-Fe protein, or V-Fe protein, or Fe-Fe protein) and their reactions are shown in equations 2-4. Mo-nitrogenase is the most commonly found and is the most efficient nitrogenase for converting N_2 to NH_3. The main task of the reductase is the delivery of electrons of high reductive potential to nitrogenase.

Mo - nitrogenase: $N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$ (2)

V - nitrogenase: $N_2 + 12H^+ + 12e^- + 24ATP \rightarrow 2NH_3 + 3H_2 + 24ADP + 24Pi$ (3)

Fe -nitrogenase: $N_2 + 24H^+ + 24e^- + 48ATP \rightarrow 2NH_3 + 9H_2 + 48ADP + 48Pi$ (4)

The use of the nitrogenase pathway for hydrogen production is great deal of energy in the form of ATP because the electron transfer steps within the enzyme are highly energy consuming processes. Substantial energy is needed to break the stable triple bond in the dinitrogen molecule with 16 ATP molecules required per one molecule of nitrogen fixed as shown in equitation (1). Specifically, electron transfer from nitrogenase reductase to dinitrogenase is accompanied by the hydrolysis of 2ATP. Therefore, 4 ATP are required by nitrogenase to produce one hydrogen in the absence of molecular nitrogen, equation (4) (Keskin and Hallenbeck, 2012; Seifert et al, 2012; Adessi and De Philippis, 2012).

$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4Pi$ (5)

Under non-nitrogen-fixing conditions, hydrogen production is associated with the function of hydrogenase. The presence of hydrogenase has been found to be a common feature of the photosynthetic bacteria (Liang et al, 2009). Nitrogenase activities are inhibited by the presence of O_2, as it destroys enzyme activity, or by NH_3, since it represses the synthesis of the enzyme; however, nitrogenase's activities are restored after the removal or consumption of ammonia

(Keskin and Hallenbeck, 2012; Seifert et al, 2012). The regulation of nitrogenase has been studied in *Rhodobacter capsulatus,* which only contains Mo and Fe nitrogenases. In *R. capsulatus*, regulation has been demonstrated to consist of a three-level control mechanism, but this regulation may not be found in all PNSB strains due to the presence or the absence of three isoenzymes (Adessi and De Philippis, 2012).

1.3.2 Hydrogenases:

Hydrogenases are enzymes that catalyze the oxidation of hydrogen and the reduction of protons to hydrogen as shown in equation (6) (Appel and Schulz, 1998).

$$H_2 \xleftrightarrow{H_2ase} 2H^+ + 2e^- \qquad (6)$$

Hydrogenases consist of distinct groups of metalloproteins that have been classified to different classes based on the metals contained in the active center (fig 1) (Liang et al, 2009).

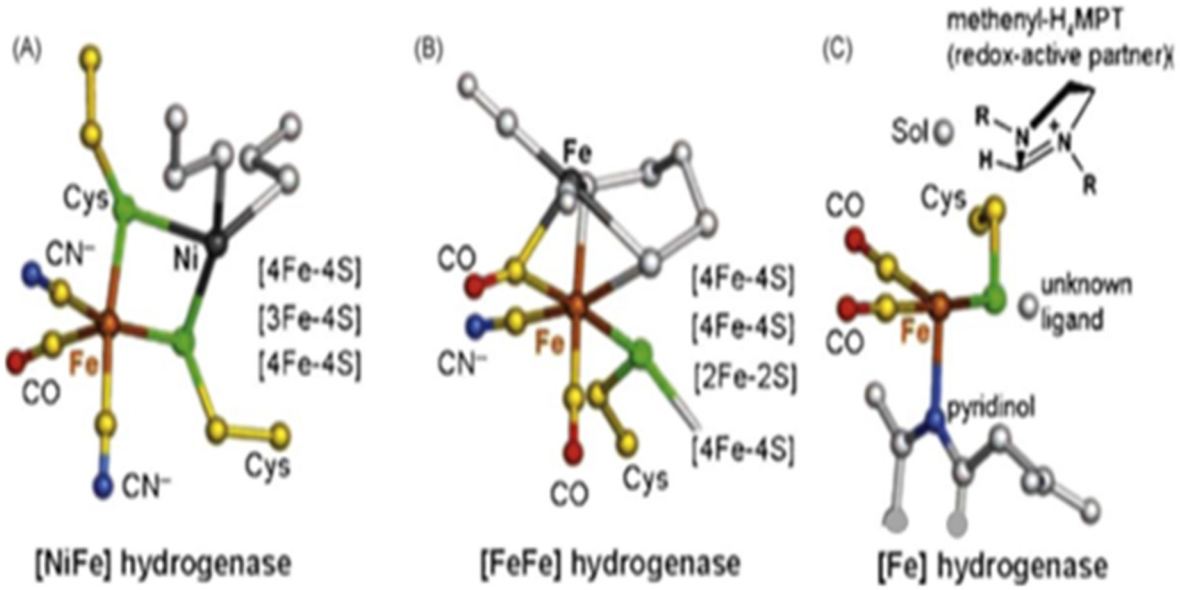


Figure 1. Structure of the three classes of hydrogenases. [NiFe]-hydrogenases and [FeFe]-hydrogenases have some features in common in their structures. In their active sites, each enzyme has two metals (either an iron and a nickel atom or two iron atoms), and both have CN^- ligand and a few Fe-S clusters that are buried in the protein. [Fe]-hydrogenase has an only one iron atom in its active site. All the three hydrogenases have some similarities in their structure since an iron atom linked to a CO group at the active sites (adapted from Allakhverdiev et al, 2010, used with permission).

1- FeFe hydrogenases have been found in anaerobic prokaryotic microorganisms, such as clostridia and sulfate reducers, and in eukaryotic microorganisms (Kim et al, 2011). They are responsible for reversible reduction of protons to H_2 under anaerobic conditions as shown in equitation (6) (Adessi and De Philippis, 2012; Eroğlu and Melis, 2011).

2- Fe hydrogenases use H_2 to reduce CO_2 to methane using H_2, and it only was found in Archaea. Also, the activities of Fe hydrogenase are rapidly lost under aerobic phototrophic conditions which would make the isolation and the characterization very difficult (kim et al, 2011; Rousset and Liebgott, 2014).

3- NiFe hydrogenases carry out hydrogen oxidation. The [NiFe]-hydrogenases are the most studied and the most frequently found in photosynthetic bacteria (Adessi and De Philippis, 2012). The synthesis of these enzymes occurs under anaerobic conditions, and so is usually negatively regulated by O_2. NiFe hydrogenases are divided into four groups:

1-Uptake hydrogenases: involved in anaerobic respiration. Active uptake hydrogenases are undesirable in H_2 production processes as they affect the H_2 production rate: inactivation or inhibiting these enzymes usually leads to enhanced hydrogen production (Adessi and De Philippis, 2012). This can be done using genetic manipulation to inactivate uptake H_2ase, or it can be inhibited chemically, since these hydrogenases are nickel enzymes, by limiting the amount of nickel available. Thus, the presence of ethylendiaminotetraacetic acid (EDTA) inhibits hydrogenase activity due to its Ni chelating abilities (Keskin and Hallenbeck, 2011).

Also, a study reported that EDTA boosted H_2 photoproduction by (I) inhibiting the biosynthesis of Hup hydrogenase and (II) mobilization of iron, therefore biosynthesis of nitrogenase complex will be active (Kern et al, 1992).

2- Cytoplasmic H_2 sensors: Regulatory enzymes, able to activate the cascade regulating the

respiratory hydrogenases in the presence of H_2.

3- Bidirectional heteromultimeric cytoplasmic (reversible H_2ase): Enzymes able to bind NAD and NADP and to work in both directions, either to generate reduced nucleotides, or to dispose of excess electrons.

4- H_2 evolving, energy-conserving, membrane-associated hydrogenases: These multimeric enzymes seem to combine the anaerobic oxidation of one-carbon-atom organic compounds to H_2 production (Adessi and De Philippis, 2012). The most well-known of these hydrogenases are the NiFe hydrogenase in cyanobacteria and photosynthetic bacteria and FeFe hydrogenase in obligate anaerobic fermentative bacteria and green algae. Both are sensitive to various chemical agents. FeFe and NiFe hydrogenases are involved primarily in H_2 production and consumption, respectively. Studies have revealed that the hydrogen consuming activity of hydrogenase is greater than its hydrogen producing activity (Keskin and Hallenbeck, 2012).

1.4 Biohydrogen production methods:

Biological hydrogen production methods are classified into one of the following processes:

i. Biophotolysis of water using algae/cyanobacteria.

ii. Photodecomposition (photofermentation) of organic compounds using photosynthetic bacteria.

iii. Dark fermentative hydrogen production using anaerobic or facultative anaerobic bacteria

iv. Bioelectrohydrogenesis or microbial fuel cell (MFC).

v. Hybrid system (Saratale et al, 2013).

Each process has advantages and disadvantages when compared with the other available methods (table 1) (Chandrasekhar et al, 2015).

System	Input	Organism	By-product	Advantage	Drawback
Biophotolysis	Light, water	Green algae or cyanobacteria	–	Inexhaustible solar energy source Nearly zero substrate cost	Low production rate Light dependent Light saturation at low sunlight intensity Low conversion efficiency from light Process easily inhibited by O_2 Product contains CO_2 or O_2
Photo-fermentation	Light, a variety of organic compound	Photosynthetic bacteria	CO_2	High production yield Complete substrate conversion Ability to use a wide range of substrate including VFAs	Low production rate Light dependent Light saturation at low sunlight intensity Low conversion efficiency from light Sensitive to fixed nitrogen Product contains CO_2
Dark fermentation	Carbohydrates	Fermentative bacteria	VFAs, solvent, CO_2	High production rate Simple reactor design Light independent Many substrate usable	Low yield Generation of waste effluent Product contains CO_2
Microbial electrolysis cell	Electricity, a variety of organic compound	Exoelectrogens	CO_2	High production yield Complete substrate conversion Light independent	Low current density Extra electricity required Methanogenesis problem
Hybrid system	Organic compound, light or electricity	Fermentative bacteria plus photosynthetic bacteria or exoelectrogens	CO_2	High production yield	More complicated reactor design

Table 1. The advantages, disadvantages, and by-products of biohydrogen production methods (adapted from Ding et al, 2016, used with permission).

1.4.1 Biophotolysis (Water-Splitting Photosynthesis):

In recent years, the production of hydrogen from water and sunlight by biological catalysts has been the subject of several studies. (Miyaboto et al, 1979). Oxygenic photosynthetic microorganisms such as green microalgae (e.g., *Scenedesmus obliquus*, *Chlamydomonas reinhardtii*, *Chlorella*, and *Scenedesmus*, among others) and cyanobacteria (e.g., *Anabaena variabilis*, *Nostoc punctiforme*, and *Synechocystis* sp, among others) use this process which only require water and sunlight to produce H_2 as shown in equation 7:

$$2H_2O + \text{light energy} \rightarrow 2H_2 + O_2 \quad (7)$$

A (FeFe)-hydrogenase in green algae drives the evolution of H_2, whereas nitrogenase is

responsible for this process in heterocystous cyanobacteria (fig 2). Even though this approach is attractive, it still suffers from major challenges that may require years of research and development to overcome. Biophotolysis is further divided into direct and indirect processes:

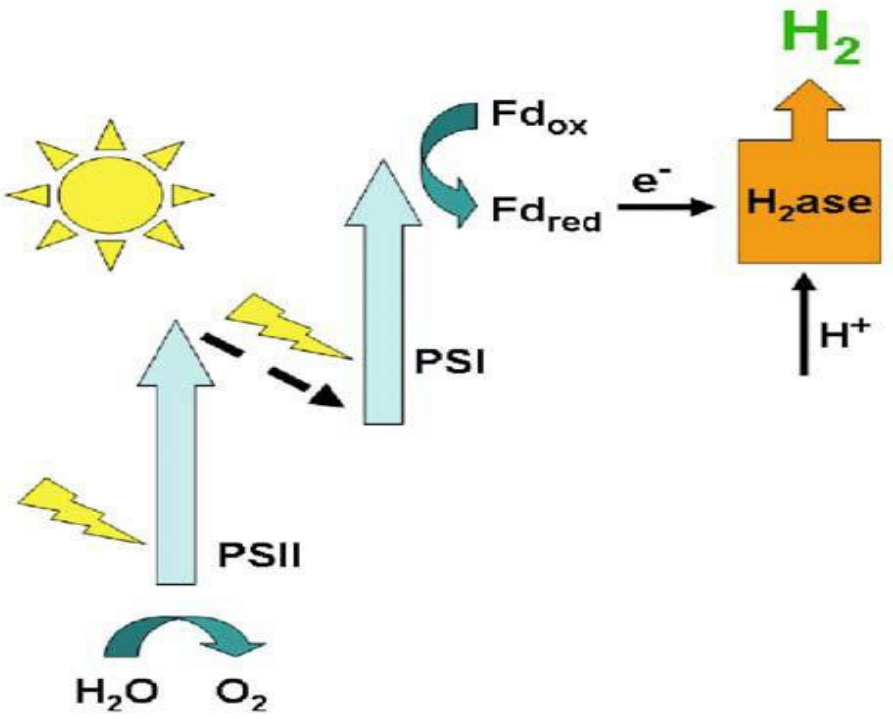


Figure 2. Biophotolysis (adapted from Hallenbeck and Ghosh, 2009, used with permission).

1.4.1.1 Direct biophotolysis:

In direct photolysis, the electrons generated through water splitting by PS2 are utilized directly under anaerobic conditions to produce hydrogen. The whole hydrogen production process is carbon-metabolism independent, and electrons flow from water through PS2 and I to ferredoxin, and finally to H_2, the last step catalyzed by hydrogenase according to the following reaction equation (8) (Seifert et al, 2012; Levin et al, 2004; Chandrasekhar et al, 2015; Eroglu and Melis, 2011):

$2H^+ + 2FD^- \leftrightarrow H_2 + 2FD$ (8)

1.4.1.2 Indirect biophotolysis:

Algae or cyanobacteria can produce H_2 directly under anaerobic phototrophic conditions.

In green algae, under deprivation of sulfur (sulfate) which is important for photosynthesis, and its absence in the medium leads to degradation of PS2 and reduction in oxygen production, the "hydrogenase pathway" is induced, producing hydrogen photosynthetically using the captured light energy and previously fixed carbon (Show and Lee, 2013).

Cyanobacteria can also synthesize and evolve H_2 through photosynthesis via the following processes (Seifert et al, 2012, Levin et al, 2004; Chandrasekhar et al, 2015):

$12H_2O + 6CO_2 + \text{light energy} \rightarrow C_6H_{12}O_6 + 6O_2$ (9)

$C_6H_{12}O_{26} + 12H_2O + \text{light energy} \rightarrow 12H_2 + 6CO_2$ (10)

1.4.2 Dark-fermentation:

Fermentative bacteria can produce H_2 when growing on carbohydrate- rich media anaerobically in the dark. Dark-fermentation processes produce a mixed biogas containing primarily H_2 and carbon dioxide (CO_2) (Levin et al, 2004) among other products such as liquid metabolites such as simple volatile fatty acids (VFA) and simple alcohols. (Seifert et al, 2012) while direct and indirect photoelectrolysis produce just pure hydrogen and oxygen. The most well-known bacterial species that are used for fermentative hydrogen production are species of *Enterobacter*, *Bacillus*, and *Clostridium*. The preferred substrates for fermentative H_2 producing bacteria are carbohydrates such as; glucose, other hexose isomers, or polymers in the form of starch or cellulose (Levin et al, 2004). In the presence of reversible hydrogenase an organic compound is transformed to pyruvate during glycolysis process (11). Next, it is oxidized to acetyl-Co-A with the reduction of ferredoxin (12). In the third step ferredoxin is oxidized with the derived electrons directed to proton reduction

with the formation of molecular hydrogen (13) as shown in (fig 3).

Glucose → pyruvate (11)

Pyruvate + CoA + 2Fd(ox) → acetyl-CoA + 2 Fd (red) + CO_2 (12)

2Fd (red) → 2Fd(ox) + H_2 (13)

Theoretically, one mole of glucose should generate four moles of hydrogen and acetic acid in a dark fermentation process.

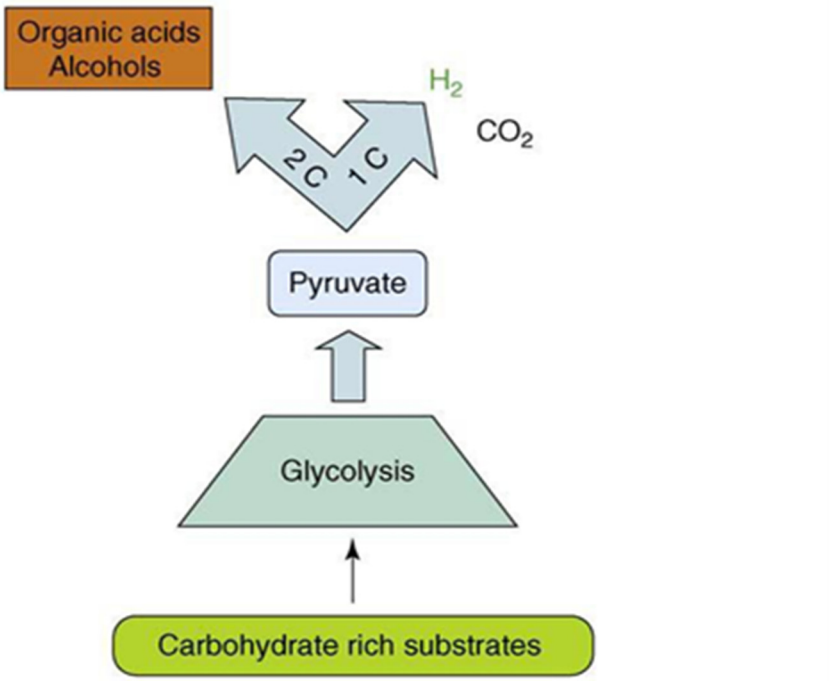


Figure 3. Dark fermentation process (adapted from Hallenbeck and Ghosh, 2009, used with permission)

1.4.3 Hybrid system:

Hybrid systems, which are attractive technologies designed to increase total hydrogen yields, can be carried out in two approaches: a one-step hybrid system or two-step hybrid system. In a one-step hybrid system, which is also called co-cultures, both types of bacteria are grown in one pot. Although, this single stage system gives high rate and yield of hydrogen compared to dark fermentation performed by one culture only, unfavorable VFAs accumulate in the medium. High

concentrations of VFA in the medium leads to substrate inhibition as well as to a lowering of the pH value which therefore reduces the hydrogen yield or completely stops hydrogen production (Saratale et al, 2013). A two-stage system combines dark and photofermention in separate steps to increase hydrogen yield. It is well documented that the decomposition of substrates cannot be completely done during dark fermentation because of thermodynamic limitations, leaving H_2, CO_2, and short chain organic acids as products. Thus, it is suggested that the two-stage system is the best solution for overcoming the drawbacks of dark fermentation. As well, the application of this system allows the use of wastes containing inhibitors of photofermentation process (e. g. ammonium ions). These inhibitors are neutral for bacteria engaged in the dark fermentation. In the second stage of this system (photofermentation), photosynthetic bacteria, which have the ability to capture light and convert it to chemical energy, would stoichiometrically convert the organic acids to hydrogen and carbon dioxide (Hallenbeck et al, 2009; Hallenbeck et al, 2012; Saratale et al, 2013). This kind of experiment can be run in continuous mode for several days. Theoretically, this system can use 1 mole of glucose to produce 12 moles of hydrogen. Therefore, combining photofermentation with dark fermentation (fig 4) can produce the maximum yield of hydrogen. Thus, the advantages of two-stage systems are the ability to use organic wastes and wastewaters, and decreasing the time and volumes required for initial substrate conversion (Hallenbeck et al, 2009; Keskin and Hallenbeck, 2011). There are several possible combinations of a hybrid system including:

(i) Dark fermentation with photofermentation.

(ii) Dark fermentation plus microbial electrolysis cell.

(iii) Dark fermentation plus cell-free enzymatic system.

(iv) Dark fermentation plus anaerobic digester (Ding et al, 2016; Das and Veziroglu, 2008).

Even though the two-stage hybrid system has been well studied, there are still a few technical limitations including; sensitivity to fixed nitrogen, low light conversion efficiencies, inability to use high light intensities, and the need for low cost, transparent, hydrogen impermeable photobioreactors (Hallenbeck et al, 2012). Finally, it may be hard to justify the use of any type of co-culture system. Thus, a two-stage system, where the two bacterial cultures are separated, could be a promising application in achieving cost- effective hydrogen production in large scale bioreactors (Hallenbeck et al, 2009).

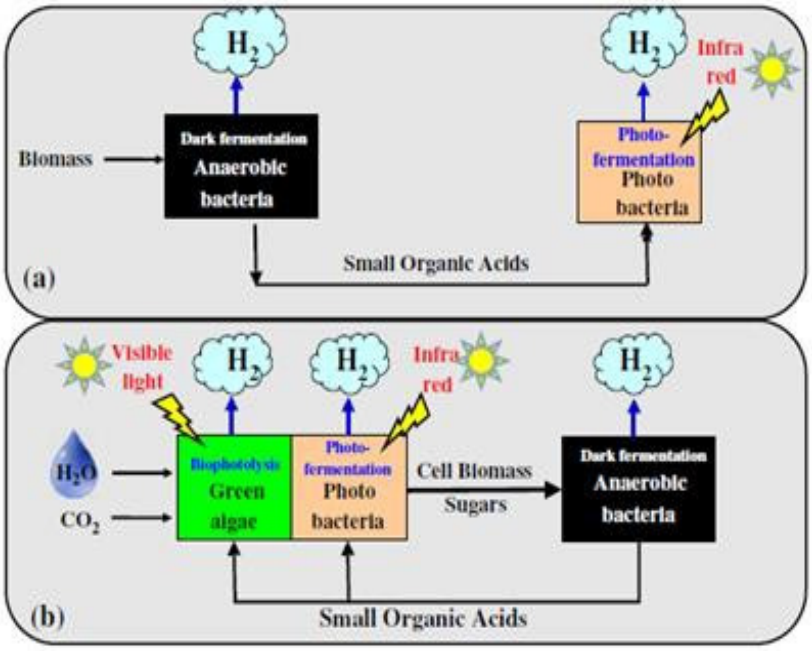


Figure 4. Simplified schematics for integrated hydrogen production processes: (a) Dark fermentation followed by photo-fermentation process. (b) Photosynthetic process (co-cultivated green algae and photo-fermentative bacteria) followed by dark fermentation process (adapted from Eroglu, and Melis, 2011, used with permission).

1.4.4 Microbial electrolysis cells (MECs):

Microbial electrolysis cells (MECs), a recent method to produce H_2 from a wide range of substrates, have been under research for decades. A microbial electrolysis cell is conceptually an extension of the concept of a microbial fuel cell (MFCs), which has been rapidly developed and

fundamentally adapted. MEC technology is also called electrofermentation or biocatalyzed electrolysis cells. However, the performance of MECs is determined by the type of microorganism, electrode supplied, type of the membrane used, applied potential range, structure, concentration of the substrate, and MEC design. A MEC produces hydrogen by supplementing the voltage generated at the anode with voltage from an external power source, driving hydrogen evolution at the cathode (fig 5), allowing hydrogen production from substrates whose redox potential would normally not allow it (Chandrasekhar et al, 2015; Hallenbeck, 2012). Two research groups independently found that bacteria could produce hydrogen in an electrolysis process based on a microbial fuel cell (MFC) (Logan et al, 2008). The device is called a microbial electrolysis cell (MEC), and the microbes are exoelectrogens because they release electrons instead of hydrogen. Methanogenesis is always associated with hydrogen production by MECs because the whole system is anaerobic (Ding et al, 2016).

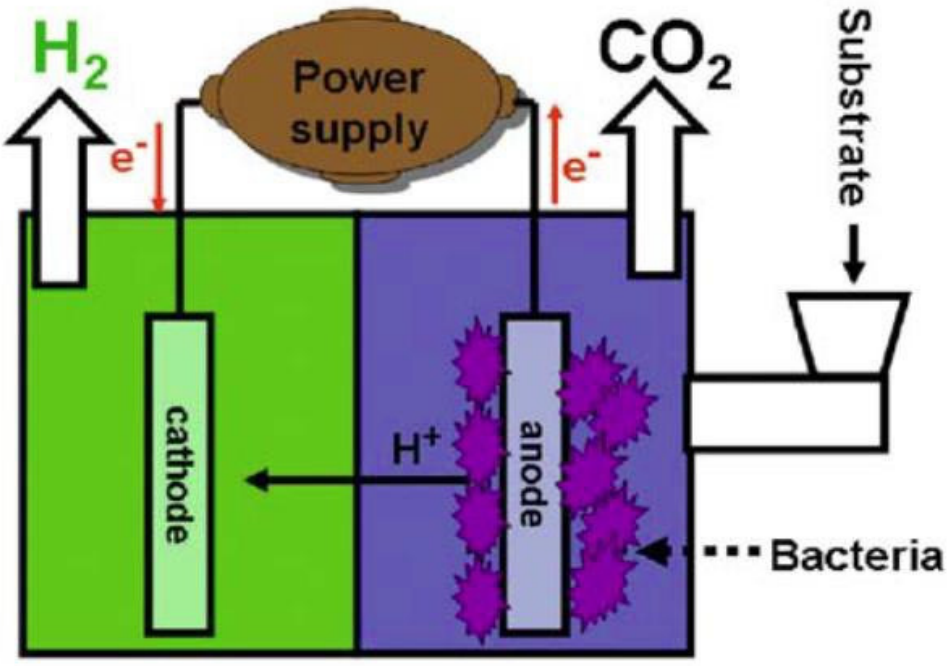


Figure 5. Hydrogen production by a microbial electrolysis cell (adapted from Hallenbeck et al, 2009, used with permission).

1.4.5 Photofermentation:

Photo fermentative hydrogen production is a microbial process carried out by purple non-sulfur photosynthetic bacteria (PNSB), which produce hydrogen under anaerobic, photoheterotrophic, and nitrogen-limited conditions using organic substrates as electron donor and sunlight as energy source (fig 6). The process is mainly mediated by the nitrogenase enzyme, which catalyzes H_2 and converts N_2 to NH_3. Hydrogen production is an inherent activity of the nitrogenase enzyme, which forms 1 mole of H_2 per mole of N_2 fixed as shown in equation 14.

$N_2 + 8H+ + 8e^- 16ATP \rightarrow NH_3 + H_2 + 16ADP + 16Pi$ (14).

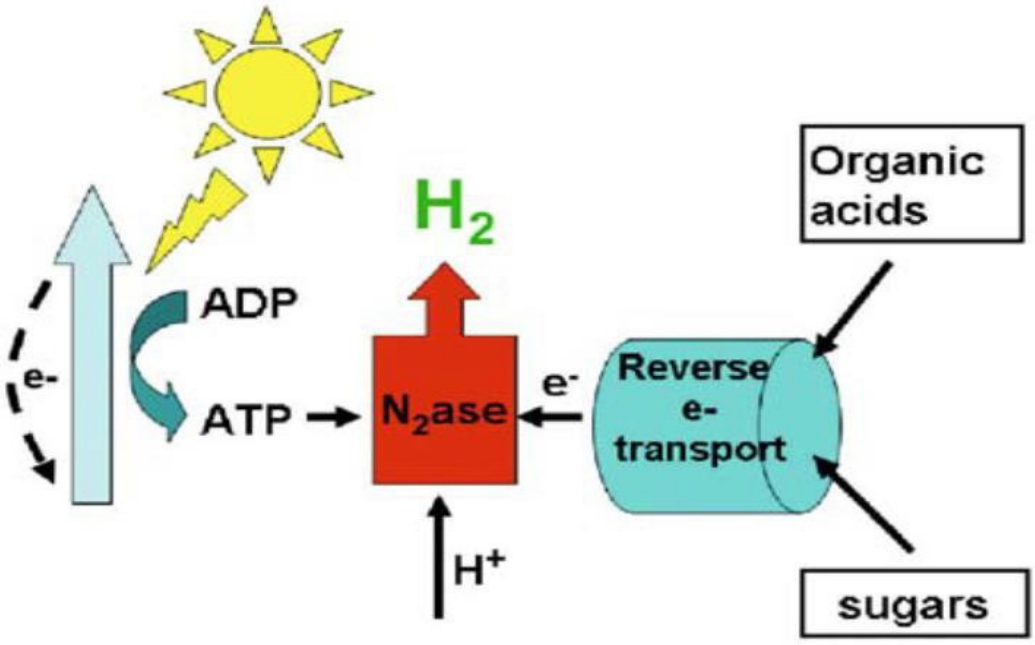


Figure 6. Scheme of hydrogen generation in photofermentation process (adapted from Hallenbeck and Ghosh, 2009, used with permission).

However, under limiting nitrogen source, the enzyme functions as a hydrogenase and catalyzes the reduction of protons to form molecular hydrogen at the expense of 4 moles of ATP as shown in equation 15.

$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4Pi$ (15)

1.4. 5.1 Purple Non-Sulfur Photosynthetic Bacteria:

PNSB are facultative anoxygenic phototrophs belonging to the class of Alphaproteobacterial and include several genera within order of *Rhodobacterales, Rhodospiralles,* and *Rhizobiales* (Androga et al, 2012). Purple non-sulfur bacteria (PNS) are capable of H_2 production due to their ability to: (i) reach high substrate conversion efficiencies, (ii) operate anaerobically, bypassing the oxygen sensitivity issue that adversely affects the FeFe hydrogenase, the NiFe hydrogenase, and nitrogenase enzymes, (iii) utilize sunlight proficiently, i.e., being able to absorb and utilize both the visible (400–700 nm) and near infrared (700–950 nm) regions of the solar spectrum, (iv) show flexibility in organic substrate utilization, including small organic acids from a wide variety of waste material (Eroglu and Melis, 2011). The most widely studied and characterized PNSB strains are *Rhodobacter sphaeroides*, *Rhodospirillum rubrum*, *Rhodobacter capsulate, R. sulfidophilus*, and, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* (Seifert et al, 2012; Androga et al, 2012). It is known that PNSB have the ability to carry out N_2 fixation which is related to H_2 production (Adessi and De Philippis, 2012). Purple non- sulfur bacteria are less capable of tolerating and using toxic sulfur compounds such as sulfide compared to the PSB, since sulfide inhibits growth at low concentrations (Dahl, 2017).

Cultures of PNSB vary from yellowish to greenish to brown in color when growing under anaerobic phototrophic conditions; however, the culture color becomes reddish when grown aerobically, due to the conversion of carotenoids to ketocarotenoids in the presence of oxygen (van Niel, 1944). PNS bacteria have relatively simple photo transduction machinery, with a single photosystem (lack of photosystem II). They are found in various areas in nature, particularly freshwater, marine habitats, and soil. Growth of PNS bacteria requires a pH of 6-9, and temperatures of 25-35ºC.

PNS bacteria have the ability to grow in different growth modes such as aerobic/anaerobic respiration, fermentation, photoautotrophy and photoheterotrophy depending on available conditions, and they can readily switch from one growth mode to another. PNS bacteria generally prefer to grow in the photoheterotrophic mode and this mode of growth is the only mode resulting in hydrogen production (Koku et al, 2002). This versatility of growth modes attracted research interest for many years, and made PNSB a model organism to study the metabolic regulation of carbon, nitrogen, and energy metabolism. PNSB are capable of growth on a variety of organic carbon sources including sugars (glucose, sucrose), short chain organic acids (acetate, malate, succinate, fumarate, format, butyrate, propionate, lactate), amino acids, alcohols, and even polyphenols. They can use also CO_2 as a source of carbon after transformation of metabolism into a photoautotrophic one. However, if the light intensity is too low to reduce CO_2 then the cell can use H_2 and even H_2S (at low concentrations) as a source of electrons (Soon et al, 2014; Adessi and De Philippis, 2012). The unique characteristic of purple bacteria is their ability to form their energy carrier (ATP) in the absence of oxygen by using sunlight as source of energy. Hydrogen can also be an electron donor for purple bacteria, oxidized by a membrane bound hydrogenase. The reaction can take place in both directions, depending on the presence or absence of the substrates (Adessi and De Philippis, 2012). In PNSB, the photosynthetic apparatus is in the intracytoplasmic membranes, the photosystem contains light harvesting complex 1 (LH1) and 2 (LH2), and a reaction center, which are protein-pigment complexes that contain different types of carotenoids and bacteriochlorophyll a. Growth and total carotenoid production in PNSB are generally influenced by pH, temperature, dissolved oxygen, nutrients, and light intensity (Soon et al, 2014). Depending on the metabolic mode PNS bacteria carry out, carbon compounds have different roles being not only a carbon source but also a source of reducing power. *R. capsulatus*

has been described as the most versatile of prokaryotes because it can grow photoautotrophically, chemoautotrophically, photoheterotrophically, and chemoheterotrophically (fig 7) with a variety of electron acceptors as well as by fermentation of sugars (McEwan,1994). Also, *R. capsulatus* can grow with many different nitrogen sources including ammonium, urea, most amino acids, and N_2 (Masepohl, 2017).

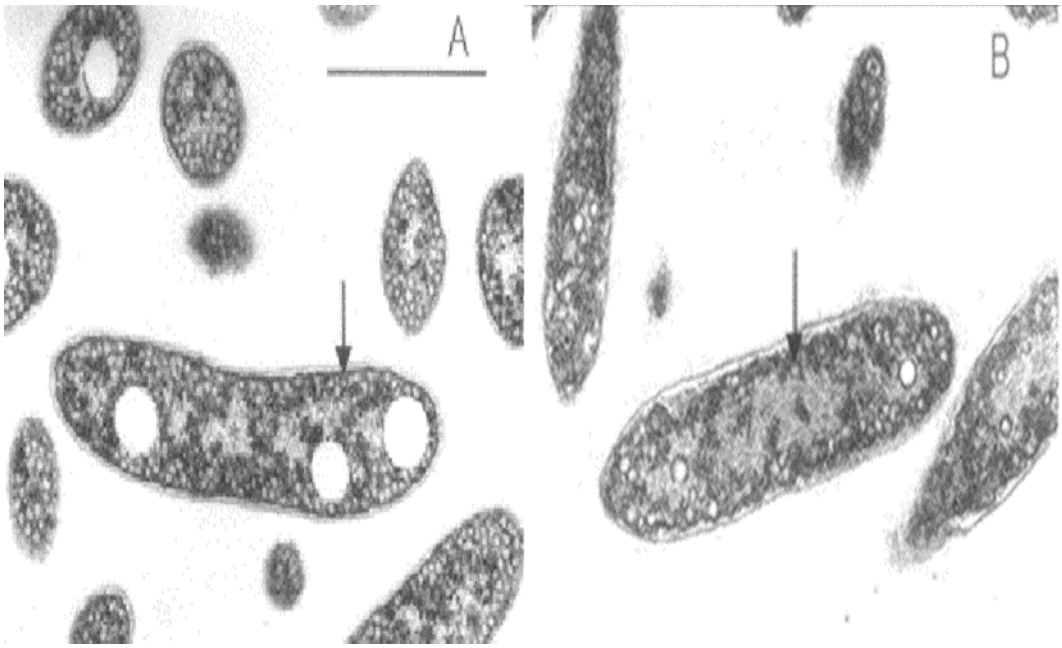


Figure 7. Electron micrograph of *R. capsulatus* BIO grown (A) chemoautotrophically in darkness with H_2 and (B) growing photosynthetically with low light intensity (adapted from Madigan and Gest, 1979, used with permission).

1.4.5.2 PNSB hydrogen production mechanism:

In the PNSB, hydrogen is the by-product of nitrogenase activity, which is induced under nitrogen- deficiency conditions and facilitated by sunlight as the energy source and small organic molecules as the carbon substrate. PNS photosynthetic bacteria, are able to reduce H^+ ions to gaseous H_2, using both the reducing power derived from the oxidation of organic compounds, and the energy derived from light. The preferred substrates for hydrogen production are the low-molecular weight organic acids that can easily enter the TCA cycle, which is very active during

anaerobic photosynthetic growth (Adessi and De Philippis, 2012). When PNSB use such simple organic molecules such as acetate, lactate, or glucose, the maximum theoretical yields of conversion of these compounds to hydrogen are described by the following equations (16-18):

(acetic acid) $C_2H_4O_2 + 2\ H_2O \rightarrow 2CO_2 + 4H_2$ (16)

(lactic acid) $C_3H_6O_3 + 3\ H_2O \rightarrow 3CO_2 + 6H_2$ (17)

(glucose) $C_6H_{12}O_6 + 6\ H_2O \rightarrow 6CO_2 + 12H_2$ (18)

The conversion of lactate occurs easily with relatively high yields, on the other hand acetate and glucose conversion is much more difficult and gives low hydrogen yields. The difference between theoretical values in hydrogen yields can be explained by the different pathways of carbon metabolism that are used by the PNS bacteria (fig 8). There are three important external factors that determine the metabolic route: the carbon source, light, and O_2 availability (Androga et al, 2012). In addition, the differences in molecular structure of organic acids can lead towards a completely different metabolic pathway. For example, in the conversion of acetate to H_2, the photosynthetic bacteria can use different metabolic pathways, such as: the glyoxylate cycle, the citramalate cycle, and the ethylmalonyl-CoA pathway (Saratale et al, 2013; Androga et al, 2012). Hydrogen production may be obtained using different approaches such as: batch, repeated-batch, fed-batch, and continuous operation (Basak et al, 2014). Although hydrogen production by photosynthetic degradation of organic compounds has been amply demonstrated, considerable efforts are still required to make a large-scale process attractive economically. Even though substrate conversion is generally high, the production rate of H_2 is slow, and hydrogen yields are often far from the theoretical maximum. There are several issues that affect photo- fermentative H_2 production including, the low rate of H_2 production, reoxidation of the produced H_2 by uptake hydrogenase, and the generation of a high cellular ATP level for efficient H_2 production.

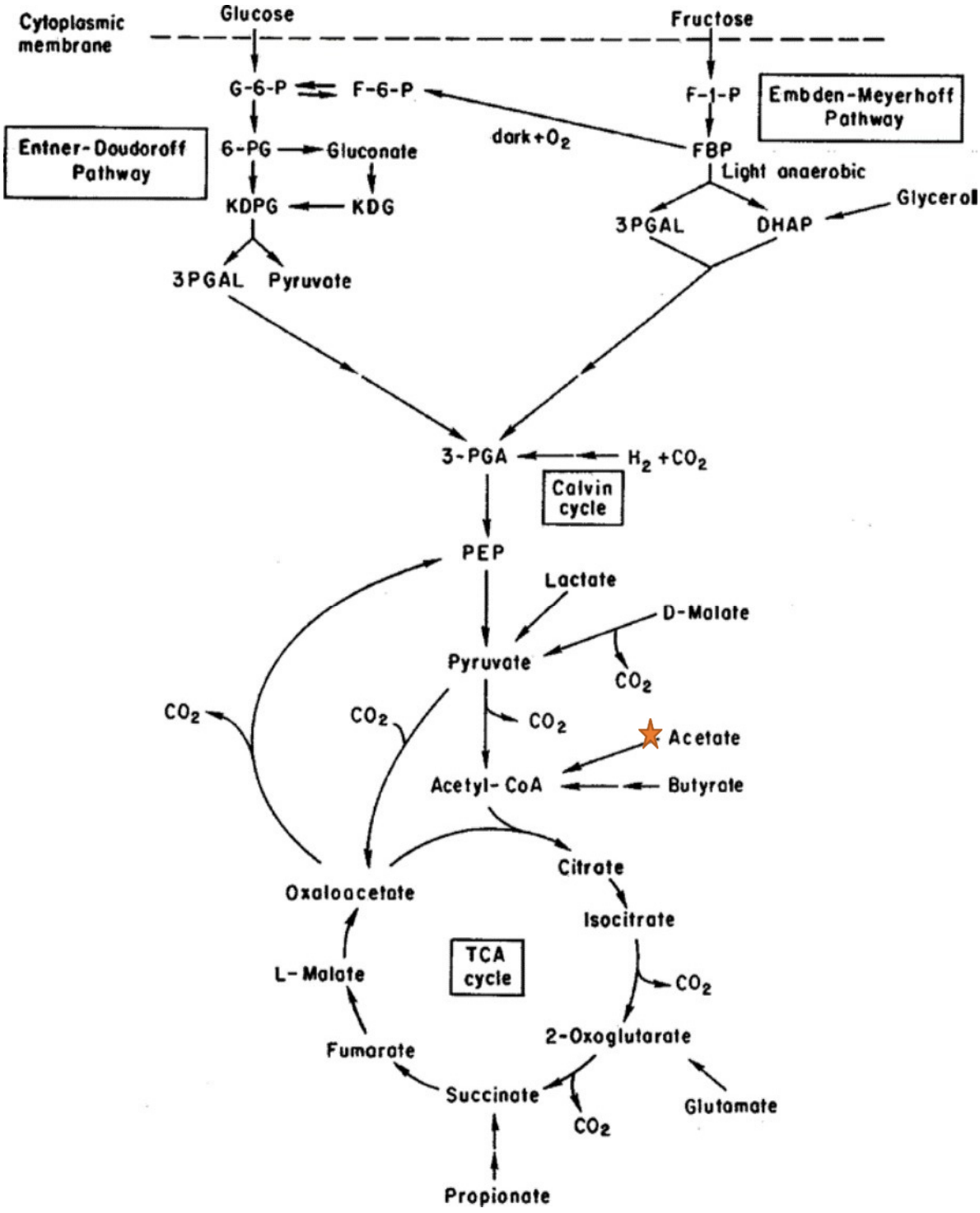


Figure 8. The metabolism of carbon in PNS bacteria. This ★star indicates where acetate will be converted to Acetyl CoA and goes through TCA cycle (adapted from Koku et al, 2002, used with permission).

1.4.5.3 Factors affecting H_2 Production:

Many parameters can play a role in the photo-fermentative H_2 production process and can affect both the rate of H_2 production and the yield, (Levin et al, 2004) including; pH, temperature, substrate to inoculum ratio, C/N ratio, inoculum age, light intensity, iron (Fe^{2+}) concentration, and the dark/light cycle. Several *Rhodobacter* species have different capacities to metabolize carbon and nitrogen sources. Thus, the C to N ratio should be adjusted depending on the strain to achieve effective H_2 production. Maximizing hydrogen yields greatly depends on the carbon and N source used. Lactic acid and malic acid seem to be the most suitable organic acids, and glutamate is the most common nitrogen source used. Light conversion efficiency is one of the core parameters determining system productivity (Keskin and Hallenbeck, 2011). Increasing light intensity can have, up to a certain point, a positive effect on hydrogen yield as well as production rate, whereas it has a counter effect on light conversion efficiencies (Keskin et al, 2011; Seifert et al, 2012). Temperature is also an important environmental factor influencing the growth rate, enzymatic and metabolic activities of hydrogen-producing bacteria. The suitable temperature values vary between 30ºC and 40ºC depending on the strain. Another major factor is pH which influences the activity of the iron-containing hydrogenase enzyme, nitrogenase activity, and the metabolic pathway (Androga et al, 2014; Cai and Wang, 2012; Uyar et al, 200; Tsygankov et al, 1997; Das and Veziroglu, 2008). The appropriate pH values vary between 6.8 and 9 (Keskin and Hallenbeck, 2011). Fe^{2+} is a fundamental component of the ferredoxin that is required for nitrogenase, the key enzyme for photo-hydrogen production (Eroglu et al, 2011). In addition, the optimum phosphate (P) concentration is also important to increase the H_2 yield since phosphorus is an essential element for the production of H_2, in the form of adenosine triphosphate (ATP), has a major part in energy generation in the bacterial cell, and is also involved in the system that

controls the buffering capacity as substitute to carbonate (Chandrasekhar et al, 2015). The dark/light cycle has a great effect on the yield and rate of photo-hydrogen production (Li et al, 2011) due to illumination time constraints. In the dark period, the substrate is used for cell maintenance rather than hydrogen production. Thus, these parameters must be optimized and the limiting factors must be overcome in order to achieve maximum hydrogen production. It has been suggested that combining two processes may make photosynthetic hydrogen production economically viable (Hallenbeck et al, 2009).

1.5 Metabolic Pathways that compete with H_2 production for electrons:

The production of H_2 is related to various metabolic pathways that deal with ATP generation including nitrogen fixation and carbon metabolism (TCA cycle and CO_2 fixation) (fig 9). Generally, all the processes involved in energy generation, such as photosynthesis and H_2 oxidation, and energy consumption, as N_2 and CO_2 fixation, are altogether controlled by the two-component system RegB–RegA. There are mainly three pathways that can compete for electrons: CO_2 fixation, N_2 fixation/H_2 production and polyhydroxybutyrate (PHB) biosynthesis. PNSB use CO_2 as an electron acceptor under photoheterotrophic conditions to get rid of excess reducing equivalents and balance redox homeostasis. They use the Calvin-Benson-Bassham (CBB) pathway to fix CO_2 at the expense of ATP and NADPH. Another electron consumer is molecular nitrogen (N_2), which is fixed to NH_3 by a nitrogenase enzyme at the expense of 16 moles of ATP and 8 moles of electrons (Androga et al, 2012).

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow H_2 + 2NH_3 + 16\ ADP + Pi \qquad (19)$$

Poly-β-hydroxybutyrate (PHB) synthesis in PNSB is another major competitor to nitrogenase for electrons (Koku et al, 2002), since it involves massive NADH consumption for the synthesis of this carbon and energy storage material from acetyl-CoA under carbon replete conditions (Ryu et al, 2014). There are several strategies that researchers have attempted in order to improve hydrogen production via metabolic engineering of existing pathways such as blocking competing pathways (Hallenbeck and Ghosh, 2009; Oh et al, 2011).

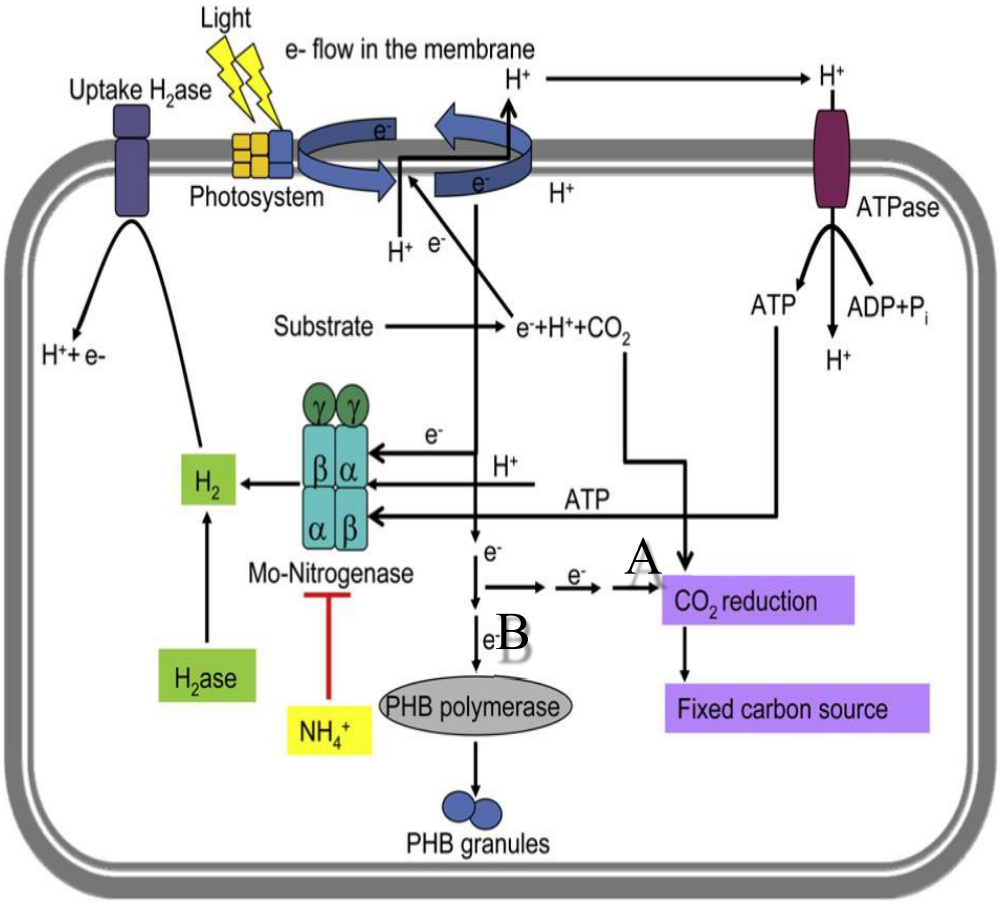


Figure 9. Metabolic pathways that compete nitrogenase for electrons and reducing equivalents. (**A**) CO_2 fixation, (**B**) PHB synthesis during photofermentation (adapted from Kars and Gunduz, 2010, used with permission).

1.6 Polyhydroxybutyrate (PHB):

Many species of gram positive and negative bacteria including cyanobacteria, PSB and PNSB are well-known for their capability of producing polyhydroxyalkanoic acid (PHAs). Polyhydroxybutyrate (PHB) is the most common PHA produced by the PNS bacteria (Sudesh et al, 2000; Wu et al, 2002). PHB is usually formed during the stationary phase of growth when the cells are starving for essential nutrients such as; sulfur, N_2, phosphorus, or iron but have an excess of carbon. It has been reported that sulfur deprivation in *R. rubrum* leads to inhibition of nitrogenase activity, as well as N_2 fixation and H_2 production, so that PHB accumulation is enhanced (Andrago et al, 2012). PHB is a widespread intracellular energy and carbon- storage compound typically found in prokaryotic organisms (fig 10) (Brandle et al, 1990; Aslim et al, 1998).

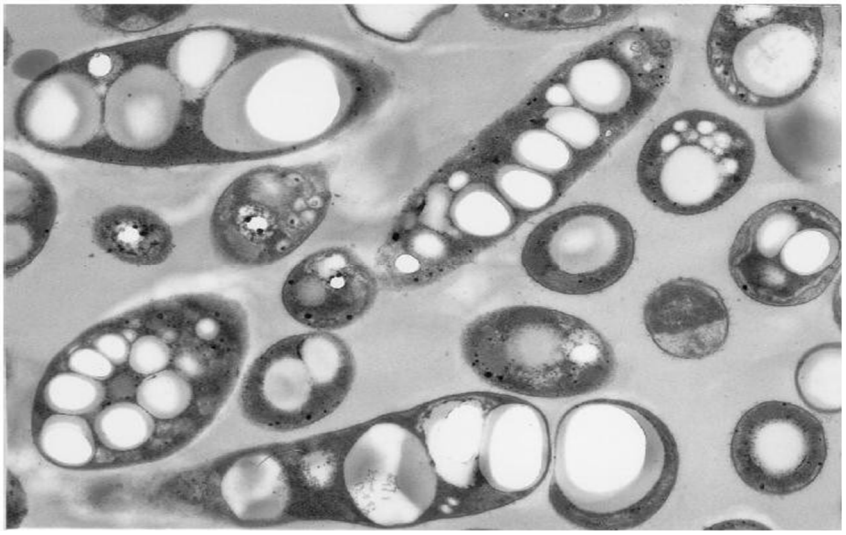


Figure 10. PHB accumulation in *R. capsulatus* SB1003 (adapted from Kranz et al, 1997, used with permission).

In PNSB, PHB synthesis consumes many electron and reducing equivalents that are required for H_2 production. PNSB can also use PHB for survival when carbon and energy sources are limited.

(Wu et al, 2012). It was stated by Cetin et al (2006) that PHB is one of the by-products of hydrogen production in *R. sphaeroides* O.U. 001 when it is cultivated in minimal media containing malate and sodium glutamate under anaerobic phototrophic conditions. PHB is also considered to be of commercial importance due to its thermoplastic properties which are resistantto water and its ability to undergo complete biodegradation. Biosynthesis of PHB is done from acetyl-coenzyme A (acetyl-CoA) in 3 steps. A PHA-specific β-ketothiolase, encoded by *phbA*, catalyzes the condensation of 2 molecules of acetyl-CoA to form acetoacetyl-CoA. Acetoacetyl-CoA is then reduced by acetoacetyl-CoA reductase, encoded by *phbB*, to produce β-hydroxybutyryl-CoA which is then polymerized by PHB synthase, encoded by *phbC* to become high molecular weight PHB (fig 11) (Yang et al, 2006).

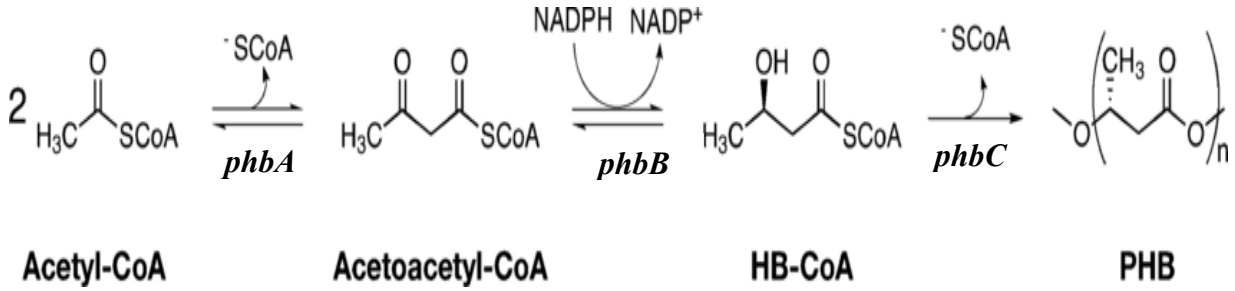


Figure 11. PHB biosynthesis pathway (adapted from Kranz et al, 1997, used with permission)

It was reported by Kranz et al (1997) *Rhodobacter capsulatus* SB1003 *phbA* and *phbAB* mutants could synthesize PHA when they were grown on acetone while *phbC* mutants were unable. These results indicate that PHB synthase (*phbC*) is the key enzyme for PHB synthesis. It was stated that the regulation of the PHB pathway seems to be complex (Luengo et al, 2003).

Moreover, the PNS bacteria, such as *R. sphaeroides* and *R. rubrum*, were able to produce more PHB polymers when the carbon source was acetate. It was reported that PHB was higher when the culture is grown on acetate as acetyl-CoA is the substrate for PHB biosynthesis (Androga et al, 2012). In addition, four strains of PNS bacteria, *R. sphaeroides, R. capsulatus, R. palustris* and *R.*

rubrum, were revealed to have diverse acetate assimilation pathways (fig 12) (Kars and Gunduz, 2010). These acetate assimilation pathways are the ethylmalonyl-CoA pathway for *R. sphaeroides*, the citramalate cycle for *R. capsulatus,* the glyoxylate cycle for *R. palustris* and an unknown pathway for *R. rubrum*. However, another study demonstrated that the PNS photosynthetic bacterium *R. rubrum*, synthesize PHB from acetate through the following pathway:

acetate → acetyl-CoA→ acetoacetyl-CoA →L-(+) --hydroxy butyryl-CoA → crotonyl-CoA→ D-(−) --hydroxy butyryl-CoA →PHB (Koku et al, 2002).

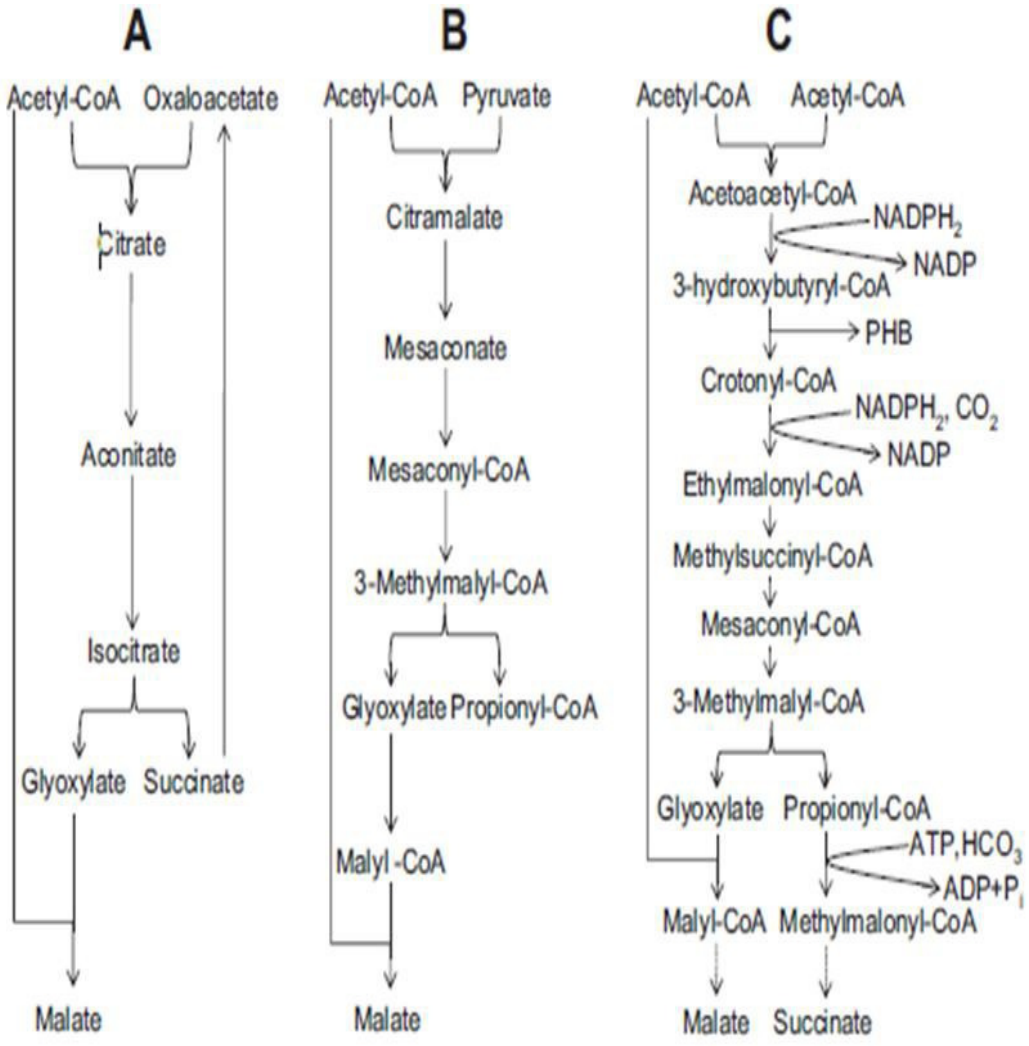


Figure 12. Acetate assimilation pathways in PNS bacteria such as glyoxylate cycle (A), citramalate cycle (B), and ethylmalonyl-CoA pathway(C). (Adapted from Kars and Gunduz, 2010, used with permission).

1.6 The relationship between H_2 and PHB production by PNS photosynthetic bacteria:

Hydrogen and PHB production have similar physiological roles, disposing of excess energy, and reducing equivalents in the cell. Both processes occur when there is a high C/N ratio in the environment. Consequently, these processes compete for reducing equivalents in the cell. Similarly, H_2 production has in common several parameters that have positive or negative effects on PHB accumulation such as; pH, temperature, C/N ratio, substrate concentration, and light/dark cycle etc.

A new study showed that *R. capsulatus* accumulated more PHB under light- dark cycles than under continuous light conditions (Corona et al, 2017). pH is another factor affecting the relative amount of PHB accumulated. Researchers have found that when the initial pH of the medium was 7.5 or more, hydrogen production decreased significantly and the amount of accumulated PHB increased three times (Khatipov et al, 1998; Kim et al, 2006). The partition of metabolism between hydrogen and PHB production seems to be related to the nature of the substrate. PHB is mainly produced from substrates, e.g. acetate and butyrate, which are simply converted to acetyl units without forming pyruvate (Koku et al, 2002). In other words, acetate provides more acetyl moieties to form more acetyl-CoA, thus PHB accumulation is enhanced over the photoautotrophic growth using CO_2 as the sole carbon sources. On the other hand, acetate does not show great stimulation of cellular growth.

A possible explanation is that the formed acetyl-CoA readily transferred into substances that benefit normal cell growth. Likewise, glucose stimulates cell growth significantly, but PHB content does not increase to the same extent, suggesting that most of the glucose is assimilated into cellular substances rather than the desired product, PHB. These results indicate that not all the carbon and energy escaping from the glycogen biosynthesis goes to the PHB biosynthesis pathway (Wu et al, 2002).

Another study showed that various substrates effect PHB and H_2 production. PHB accumulation was ambiguous when strain *R. palustris* WP3-5 was incubated with malate and was small on lactate (1.4% increases). In contrast, PHB was obviously synthesized on acetate and propionate, and the maximum PHB content that could be achieved was 10.2% and 4.2% of cell dry weight on acetate and propionate, respectively. The difference in the PHB formation by strain WP3-5 for the four organic acid substrates may be caused by the different metabolic routes of their assimilation and the precursor contents for PHB synthesis. Intracellular PHB was beneficial to H_2 production in strain WP3-5 in a pH-stressed environment, indicating that PHB likely has multiple functions rather than only competing with H_2 production for reducing equivalents (Wu et al, 2012).

In relation to biohydrogen production, the acetate assimilation pathway shares common elements with the polyhydroxy butyrate biosynthetic route with regards the proposed pathway, acetoacetyl-CoA and 3-hydroxybutyrate are common intermediates for both PHB synthesis and acetate assimilation. This means that the initial steps of both pathways are the same and they branch only at the PHB polymerization/crotonyl-CoA formation steps (Kars and Gunduz, 2010). A constructed *R. sphaeroides* PHB synthase deficient mutant did not show a great increase in H_2 production on lactate, malate, and succinate, which are efficiently used in the production of hydrogen. In contrast, a considerable increase in H_2 production from acetate was observed, and acetate is known to be a very efficient substrate for PHB synthesis (Koku et al, 2002). In *R. capsulatus*, acetate assimilation goes through a special pathway called the citramalate cycle, a pathway that is similar to the *R. sphaeroides* pathway (glyoxylate cycle.), but is more complex (Kars and Gunduz, 2010). A study examined hydrogen production of *R. sphaeroides* KD131 and confirmed that the mutant strain produced thirty percent more H_2 than the wild type. In addition to this study, there are many studies which report increases in hydrogen production by *phbC* mutated *R. sphaeroides* strains (Kim et al, 2011; Franchi et al, 2004).

1.8 Metabolic engineering to improve photo-fermentative hydrogen production:

Metabolic engineering is a powerful tool that can redirect the metabolic pathways of hydrogen-producing organisms to enhance hydrogen production, eliminate unwanted products, or the degradation of unwanted molecules. (Ding et al, 2016; Mathews and Wang, 2009). Several approaches have been applied to understand, analyze, and modify the metabolic pathways of PNSB to improve H_2 production yields including molecular biology, modern analytical and genomic techniques. In other words, metabolic engineering has been applied to resolve H_2 production issues by providing ways to increase carbon flow to the hydrogen- producing pathway, increase substrate utilization, and engineer more efficient and/or oxygen- resistant hydrogen evolving enzymes (Mathews and Wang, 2009). Therefore, it is proposed that such genomic modification should be designed based on the understanding of the content of bacterial genome of PNSB (Hallenbeck, 2013). The first genetic exchange of photosynthetic bacteria *R. capsulatus* system was done in 1974 and the complete genome sequencing of *R. capsulatus* genome was completed by Haselkorn et al (2001). Currently, many genomes of PNS bacteria have been sequenced which contributes greatly toward successful genetic modification to enhance the rate and yield of H_2 production (Kars and Gunduz, 2010). A variety of genetic manipulation strategies have been applied to improve photo-fermentative H_2 production including the following:

1-Reducing the size of light-harvesting complexes and quantity of photosynthetic pigments in photosynthetic bacteria in order to increase the efficiency of light utilization have been applied in *R. sphaeroides* RV, and a mutant with a lower LH2 content yielded 50 % more H_2 compared to the wild type in a plate-type photobioreactor (Androga et al, 2012; Allakhverdiev et al, 2010).
Ma et al (2012) showed in their study that the mutant MC1417 obtained by transposon mutagenesis had a lower light harvesting pigment content and a photo-fermentative H_2 production

was improved by 24.4% compared to the wildtype (Ma et al, 2012).

2- Enhance energy flow to nitrogenase by overexpressing the *rnf* operon which is thought to transport electrons to nitrogenase (Ding et al, 2016) or by inactivating the uptake hydrogenase which catalyzes the conversion of molecular hydrogen to electrons and protons, thereby decreasing the hydrogen yield (Kars and Gunduz, 2010).

It has been reported by Kars et al (2008) that inactivation of uptake hydrogenase has led to an increase in hydrogen production by *R. sphaeroides* O.U.001 (Kars et al, 2008).

3- Develop ammonia-insensitive photosynthetic bacteria so that it produces hydrogen in the presence of NH_4. Nitrogenase expression is strictly controlled at the transcriptional and posttranscriptional levels in response to the availability of a nitrogen source (Ding et al, 2016). In the presence of NH_4, two PII-like proteins, GlnB and GlnK, activate DraT, which inhibits nitrogenase by ADP- ribosylation. The two PII- like proteins also control the activity of NifA a transcriptional activator of nitrogenase structural genes, so nitrogenase is fully repressed in the present of NH_4. Many studies attempted to overcome nitrogenase repression by deletion of regulatory proteins (GlnB and GlnK) to develop strains that are able to produce hydrogen in the presence of NH_4. It has been reported that the synthesis of nitrogenase, and its enzymatic activity is greatly enhanced in a double GlnB and GlnK mutant of *R. capsulatus* during cultivation in the present of 200 mM NH_4 with a resulting 1.5- fold increase in hydrogen production over the wild type (Oh et al, 2011).

Additionally, Ozturk and Gokce (2012) reported that a pseudo-revertant strain starts to produce hydrogen earlier and with a hydrogen productivity that is 1.6-fold higher than that of the parent strain. Moreover, the pseudo-revertant strain produces a comparable amount of hydrogen in the presence of ammonium (Ozturk and Gokce, 2012).

4- Block metabolic pathways that compete with H_2 production for reducing equivalents, such

as poly-β-hydroxybutyrate (PHB) synthesis, CO_2 fixation, CBB pathways so the energy can be directed to produce hydrogen (Androga et al, 2012). Kim et al (2011) investigated the improvement in photo-fermentative H_2 production by using *R. sphaeroides* KD131 and its PHB synthase deleted-mutant resulting in increased H_2 production despite lower substrate degradation when acetate and butyrate are the carbon source (Kim et al, 2011).

Recently, several studies have made multiple mutations in one single strain to make super hydrogen producing photosynthetic bacteria. Ryu et al (2014) constructed a superior hydrogen-producing strain HPCA (fig 13) of the photosynthetic bacteria *R. sphaeroides* by combining multiple mutations in a single strain, including modifications in uptake hydrogenase, poly-β-hydroxybutyrate synthesis, light-harvesting complex, *rnf* operon, and the *nifA* gene. It was found that mutations in the *nifA* gene have the most significant effects on total hydrogen yield and production rate. In another study, a double mutant of *R. sphaeroides* KD131 that had both uptake hydrogenase (*hup)* and poly-β-hydroxybutyrate (PHB) synthase (*phbC)* inactivated showed a much higher H_2 production of 3.34 ml H_2/mg cell over that of the wildtype parent strain (1.32 ml H_2/ mg cell) (Ryu et al, 2014).

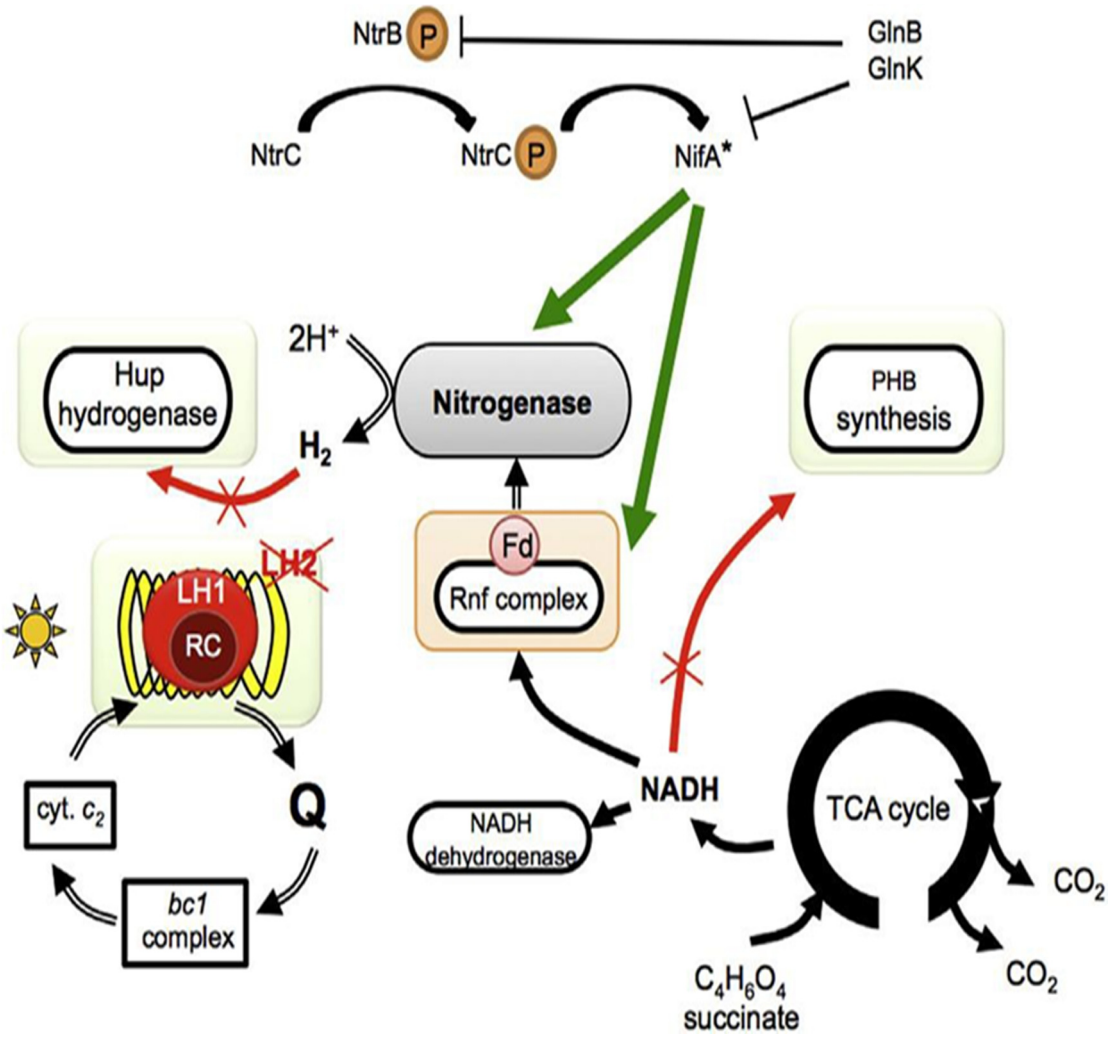


Figure 13. *R. sphaeroides* HPCA mutant (adapted from Ryu et al, 2014, used with permission).

1.9 The Aim of Present Study:

Despite the success of some aspects of photo-fermentative hydrogen production, it still suffers from low hydrogen rates and yields. Recently, metabolic engineering has been applied to improve the rate and yield of hydrogen production.

The main purpose of the current study was to apply a metabolic engineering approach to improve H_2 production with the photosynthetic bacterium *Rhodobacter capsulatus* JP91 by inactivation of the PHB synthase (*phbC)*, since under photoheterotrophic conditions PHB synthase competes with nitrogenase for electrons. In theory, by blocking PHB production, more metabolic flux could be directed to hydrogen production, thus a *phbC* mutant should show greater H_2 production that the wildtype and the size of this effect will be different depending upon the substrate since different metabolic pathways are involved.

Objective 1: Construct a *phbC* mutant by disruption of the *R. capsulatus* JP91 *phbC* gene.

Objective 2: Determine the effect of the *phb*C mutation on hydrogen production with the different carbon sources, acetate, lactate, and glucose.

CHAPTER 2: - Material and Methods

2.1 Bacterial strains, storage, and inoculation:

All bacterial strains used are indicated in Table 2. *R. capsulatus* was stored as a 30 % glycerol stock at -80ºC. For cultivations, *R. capsulatus* was taken from the bacterial stock by sterile loop and streaked on a YPS plate and incubated at 30ºC for 48 h in an incubator (Canlab). After bacterial growth, a single colony was picked and transferred to a 16-ml screw capped tube filled with liquid YPS medium and incubated under anaerobic phototrophic conditions at 30ºC for 48 h (fig 14). For *E. coli* cultivation, which contained the desired plasmid, a bacterial culture was taken from a glycerol stock and streaked on LB plates with the necessary antibiotic. The plates were incubated at 37ºC overnight, and after growth, a single colony was picked and inoculated in 5 ml of liquid Luria-Bertani (LB) with the appropriate antibiotic and incubated with shaking overnight at 37ºC.

Bacterial strains	Characteristics	Reference
Rhodobacter capsulatus		
JP91	*hup*⁻ derivative of B10 wild type	Dr J. Willison
RS15	*hup*⁻, *phbC*⁻, derivative of JP91. Gm^R	This study
E. coli		
DH5 α	Δ*lac*U169 (Φ*lacZ*ΔM15), *rec*A1, *end* A1, *λpir* phage lysogen.	Invitrogen
S17.1	*λpir*, *rec*A *2-(Tc:Mu) (Km::Tn7)* integrated into the chromosome	Cebolla, 2001
Plasmids		
pBR322	Amp^R, Tet^R	Watson, 1988
pYPRUB 91	Gm^R, Km^R, insert detection *LacZ*	Dr. Bernd Masepohl
pK18mobsacB	Km^R, *sac B*, insert detection: *lacZ*'	pK18mobsacB (ATCC® 87097™)
pK15mobsacb	Km^R, Gm^R, PHB fragment (618bp) inserted in *Eco*R1 site *sac B*, insert detection: *lacZ*'	This study

Table 2. Bacterial Strains and Plasmids.

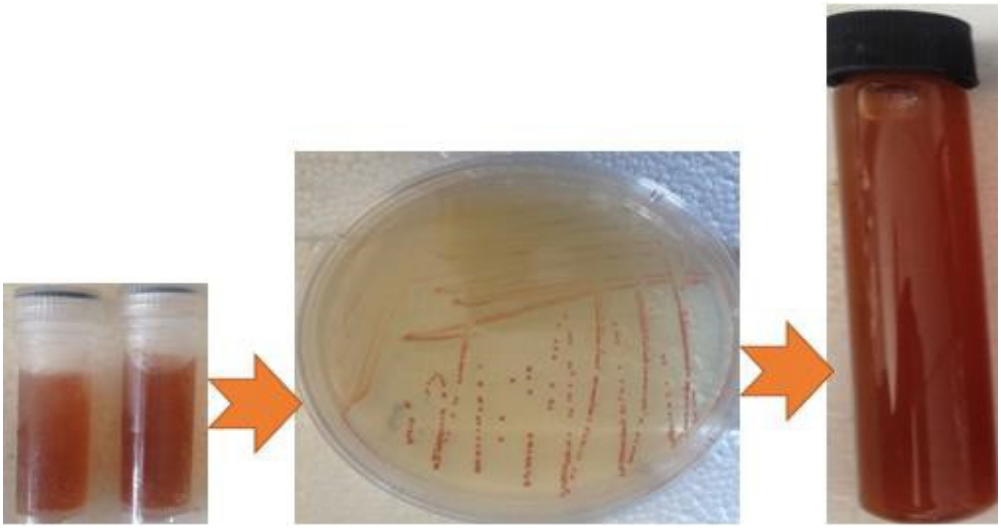


Figure 14. The above figure shows the storage, cultivation, the growth process of *R. capsulatus* strains in YPS liquid medium and on agar plates.

2.2 Growth:

R. capsulatus (JP91 and RS15) were grown in 16 ml screw capped tubes filled with RCV media containing 30 mM lactate as carbon source and 3.5 mM $(NH_4)_2SO_4$ as nitrogen source and 10 ug/ml of Gm for RS15, and incubated at 30ºC in an Biotronette Mark III (Labline Instruments) environmental chamber equipped with three 150 W incandescent bulbs. The composition of the media and solutions are given in appendix A.

2.3 Competent cells preparation:

In order to make competent cells, TB buffer should be prepared first (see appendix B). The *E. coli* strain (DH5 or S17.1) was taken from -80ºC storage and inoculated on LB agar plates without antibiotics and incubated at 37ºC overnight. After bacterial growth, 10-12 colonies were picked and inoculated into 250 ml liquid LB with 20 mM $MgSO_4$. The cultures were incubated with shaking at 18ºC or room temperature until the OD600 nm reached 0.4 – 0.6 placed on ice for 10 min. centrifuged at 2500×g (Sorval SLS 1500 Rotor) for 10 min at 4ºC. The cell pellet was resuspended in 80 ml of cold TB buffer and recentrifuged at 2500×g for 10 min at 4ºC.

The supernatant was discarded and the pellet resuspended in 20 ml of cold TB buffer. DMSO was slowly added while swirling and the cell suspension was placed on ice for 10 min. For storage, the suspension was then dispensed into sterile microcentrifuge tubes (1.5 ml), directly frozen in liquid nitrogen and stored at -80ºC for further experiments.

2.4 DNA manipulation:

Genomic DNA of *R. capsulatus* was extracted and purified using a Gel/PCR DNA fragment extraction kit (Geneaid). A Nanodrop spectrophotometer (ND1000) was used to measure the concentration and purity of DNA. High-Speed Plasmid Mini Kit (Geneaid) was used to isolate and purify the desired plasmid from DH5α.

2.4.1 Plasmid construction:

PHB synthase gene (*phbC)*, which is 618 bp, was amplified from genomic DNA of *R. capsulatus* as template and using primers: PHB-F 5'GCTGGAATTCAGCAAATGCGGGACAATC and PHB-R 5'GAAGGAATTCTTGTCCGAAAGCGTGAT which created an *Eco*R1 site at each end of the amplified gene. The PHB fragment was introduced into pBR322 using *Eco*R1 to create pBR322-PHB. In order to inactivate the PHB gene, a Gm cassette was inserted in the middle of PHB gene using *Sac*I. The Gm cassette was PCR- amplified from pYPRUP91using primers: Gm *Sac*I R 5' GCCGAGCTCGCATGCC and Gm *Sac*I F 5' GCTATGACCATGATTAC. Insertion of the Gm cassette created pBR322-PBH-GM (fig 15). The PHB-GM fragment was removed from pBR322 and inserted into the suicide plasmid pK18mobsacB using *Eco*R1 to create pK18mobsacbPHBGM (fig 16). Finally, purified pK18mobsacBPHBGM was transformed to *E. coli* S17.1, which is suitable for conjugation with *R. capsulatus*

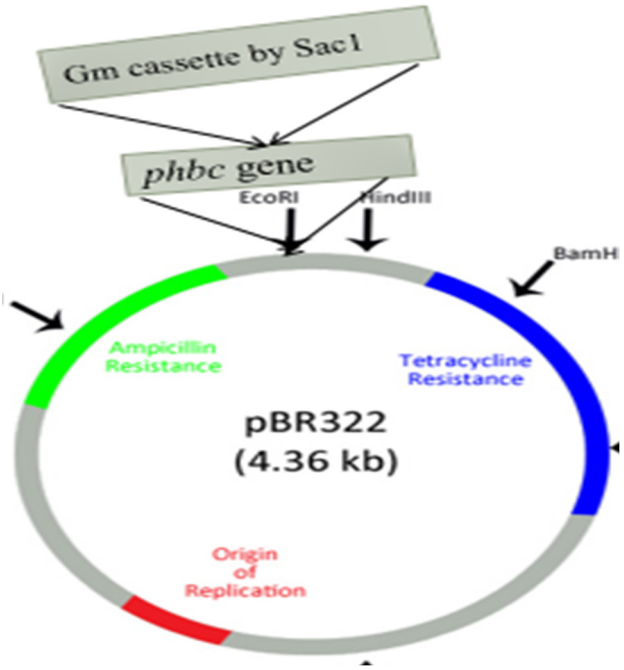


Figure 15. Plasmid construction of pBR322-*phbC-gm*.

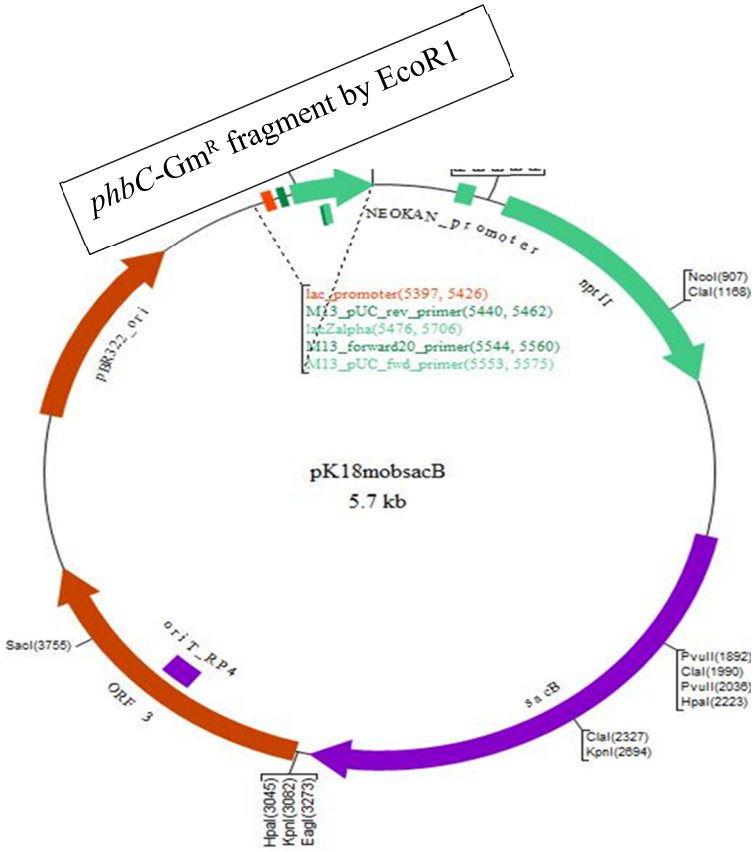


Figure 16. The insertion of *phbC-G*m^R fragment into the suicide plasmid pk18mobsacB by *Eco*R1.

2.4.2 Conjugation

E. coli S17.1 containing pK18mobsacB was grown in 5ml of LB liquid medium with 10 ug/ml Gm and 25 ug/ml Km aerobically overnight at 37ºC with shaking. *R. capsulatus* was grown in 16 ml screw capped tubes filled with YPS without antibiotics under phototrophic anaerobic conditions for 48 h. 50 ul was taken from each culture, mixed, and spread in the middle of a YPS plate without antibiotic. The plates were incubated in the dark overnight at 30ºC. The next day, the bacterial suspension was recovered with 1 ml of RCV without antibiotic and serial dilutions were made. 100 ul of each dilution was plated on RCV plates with 10 ug/ml gm and incubated in an anaerobic jar under the light at 30ºC for 3-5 days until the appearance of red colonies. Single red colonies were picked and inoculated into liquid YPS without antibiotic and incubated anaerobically under the light for 48 h. Then 100 ul was taken from each tube and inoculated again into liquid YPS with Km and Gm for 2 days under phototrophic anaerobic conditions at 30ºC. Using a sterile loop, the culture was streaked on YPS + sucrose plates and incubated in the dark for 2 days at 30ºC. the colonies that grew on the plate were picked and streaked on and YPS + Km plates and YPS + Gm plates which were incubated for 2 days at 30ºC in the dark. Colonies that grew on YPS plates with Gm and that did not grow on YPS plates with Km, were picked and inoculated into 16 ml screw capped tubes filled completely with YPS with gm and incubated at 30ºC for 48 h in the light. Colonies were also tested for sucrose sensitivity and colonies that were able to grow on sucrose were picked and grown on YPS with Gm. Prospective strains were preserved as a 30 % glycerol stock and stored at -80ºC for later experiments. Finally, positive trans conjugants were screened using the primers that had previously been used to amplify the *phbC* gene.

2.5 Growth measurements:

Growth of *R. capsulatus* wild-type and mutant was carried out on RCV with acetate, lactate, or glucose as carbon source and $(NH_4)_2SO_4$ as nitrogen source under phototrophic anaerobic conditions at 30ºC. 800 ul was withdrawn daily with a sterile syringe and optical density was read at 600 nm using a double-beam spectrophotometer (Shimadzu UV 2101 PC).

2.6 Hydrogen production

RCV media, prepared for hydrogen production, consisted of a carbon source (lactate, acetate, or glucose), sodium glutamate as a nitrogen source, 3 ml of phosphate buffer, 5 ml of RCV super salts and, as needed, 10 ug/ml of Gm. All experiments were performed in 160 mL glass bottles (100 mL working volume). The headspace of the bottles was flushed with oxygen free argon for 15 min after 5% v/v inoculation of both strains. The bottles were kept in a temperature controlled incubator at 30ºC in an Biotronette Mark III (Labline Instruments) environmental chamber equipped with three 150 W incandescent bulbs. Total PHB production was measured at the end of each run using a spectrophotometric assay.

2.7 Analytic methods

Hydrogen was measured by taking gas from head space of the experiment bottles and injected into a gas chromatograph (Shimadzu GC-8A) equipped with 1 m column packed with 5A° molecular sieve using argon as carrier; the temperature of the injector and column were 110ºC and 60ºC, respectively, and the current was 70 mA. The total volume was measured by releasing the pressure in the bottles using a gas syringe. The total hydrogen production was calculated using the percentage of H_2 produced and the total volume of biogas released. A Delta OHM photo/radiometer (HD 2102.1) was used to adjust light intensities. A pH meter was used to

measure and adjust the pH of media and solutions. The growth of bacteria was determined by measuring the optical density at 600 nm using an Evolution 200 Series UV- Visible spectrophotometer (Thermo Scientific). All data are presented in terms of mean, standard deviation and standard error using the data analysis tools of Microsoft Excel.

2.8 Determination of PHB (Poly-β-hydroxybutyrate):

PHB was extracted as described by (Slepecky and Law, 1960). Briefly, after fermentation, cells were harvested by centrifugation at 18°C and 6000×g (Sorval SLS 1500 Rotor) for 10 min and then the intracellular PHB was extracted using the chloroform–hypochlorite dispersion extraction method by suspending cell pellets in 30 % sodium hypochlorite and incubated with shaking at 30°C for 1 h. The mixture obtained was then centrifuged at 6000×g (Sorval SLS 1500 Rotor) for 10 min, the pellet was washed with water, acetone, and then ethanol. Warm chloroform was added to extract PHB from cells and then the chloroform was evaporated in a chemical hood.

The dried PHB was dissolved in 10 ml of concentrated H_2SO_4 and heated in a water bath at 100ºC for 10 min, cooled down and measured at 235 nm using a double-beam spectrophotometer (Shimadzu UV 2101 PC) against sulfuric acid as blank. For the development of a calibration curve, standard PHB (Sigma) solutions ((0.5-3 ug) /ml) were prepared as follows: 5.8 mg of pure PHB (Sigma) was added to 5.8 ml of concentrated H_2SO_4 in 15 ml clean tubes, and heated at 100°C for 10 min. The tubes were cooled down and serial of PHB concentrations were made (0.5, 1, 1.5, 2, 2.5, 3 ug/ml) and measured at 235 nm against concentrated H_2SO_4 as blank.

2.9 Analysis of organic acids and sugars:

2.9.1 Lactate measurement:

Lactate concentrations were determined as described by Figenschou and Marais (1991). Briefly, 0.1 to 1 ml of the sample was used. For the blank, deionized water was used, and then 1.5 ml of Oxidizing reagent was added to the samples in clean 15 ml tubes. After an oxidation period of 15 minutes at room temperature, 0.2 ml of Nitrite Solution was added to the tubes. After that 5ml of Alkalinizing Reagent solution was added, and finally 1ml of Oxalyldihydrazide solution was added. The samples were mixed well with all the added solutions and centrifuged for 1 min at 1000×g. For color development, the samples were incubated for 30 min at room temperature and the absorbance of the supernatant was measured against the blank at 610 nm. All solutions and calibration curves are shown in appendix B.

2.9.2 Glucose measurements:

Glucose concentrations in the culture media were determined using the DNS assay as described by Miller (1959). 3 ml of DNS reagent was added to 3 ml of the glucose sample in a lightly capped test tube. The mixture was heated at 90ºC for 5-15 minutes to develop the red-brown color. Then 1 ml of a 40% potassium sodium tartrate (Rochelle salt) solution was added to stabilize the color. After cooling to room temperature in a cold-water bath, the absorbance at 575nm was measured with a spectrophotometer. All reagent components are shown in appendix D.

2.9.3 Acetate measurement:

Acetate concentrations were determined using an acetate assay kit (Biovision).

RESULTS AND DISCUSION:

3.1 Construction and confirmation of the *R. capsulatus* RS15 mutant strain:

To simplify screening for a *phbC*¯ mutant, a mutation in *R. capsulatus* JP91 was created by the insertion of a Gm cassette into the *phbC* gene as shown in (figs 17, 18) and (Table 1). The suicide vector, pK18mobsacb, which carries Km resistance for plasmid maintenance and transfer, was introduced into *R. capsulatus* JP91 by conjugation and incorporated into the chromosome by homologous recombination. After obtaining colonies that had integrated into plasmid, double recombinants were screened on YPS plates containing Km or Gm (fig 19). The colonies that grew on Km, were not taken for further investigation since they represent single crossover events where the plasmid, including Km resistance, has integrated into the genome. After that the colonies were spread on YPS plates with 10% sucrose the assumed double crossover recombinants were picked for further investigation. The *sacB* gene found in the pK18mobsacB vector encodes the levansucrase enzyme which converts sucrose into toxic compounds (Schafer et al, 1994). Consequently, colonies that grew on YPS- sucrose plates are either double recombinants or wild type. The colonies that were Gm^R and unable to grow on sucrose were tested by PCR as follows: the *phbC* gene was PCR amplified using two primers sets that were previously used to amplify the *phbC* gene from *R. capsulatus* JP91 genomic DNA. The PHB primers:

PHB-F5'GCTGGAATTCAGCAAATGCGGGACAATC and

PHB-R 5'GAAGGAATTCTTGTCCGAAAGCGTGAT, were designed to amplify the entire *phbC* gene which is 618 bp. If the target fragment amplification was successful, the amplified fragment length would be 1600 bp due to insertion of the Gm cassette. After initial PCR screening, the PCR products were subjected to restriction enzyme digestion to confirm the

success of Gm cassette insertion (fig 20). After verifying that introduction of a mutation in *phbC* was successful, *R. capsulatus* RS15 was cultivated under anaerobic/light conditions in RCV la NH_4 at 30ºC until it reached optical densities similar to those for the parental strain (JP91) and used for further experiments.

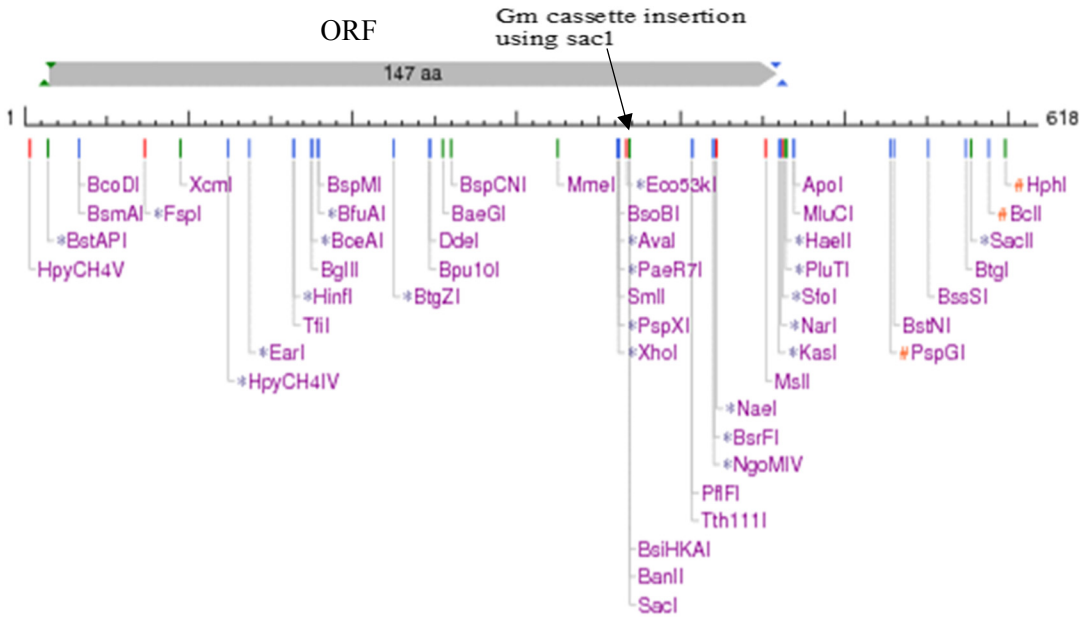


Figure 17. Linear sequence map of PHB synthase gene (Vincze et al, 2003)

#	Cut position (blunt - 5' ext. - 3' ext.)	5'... Site with flanks ...3'
1	370/366	356 TGATCGCCTC G▲AGCT▼C GATCGAGGAA

Table 3. The position of *Sac*1 cleavage sites (Vincze et al, 2003)

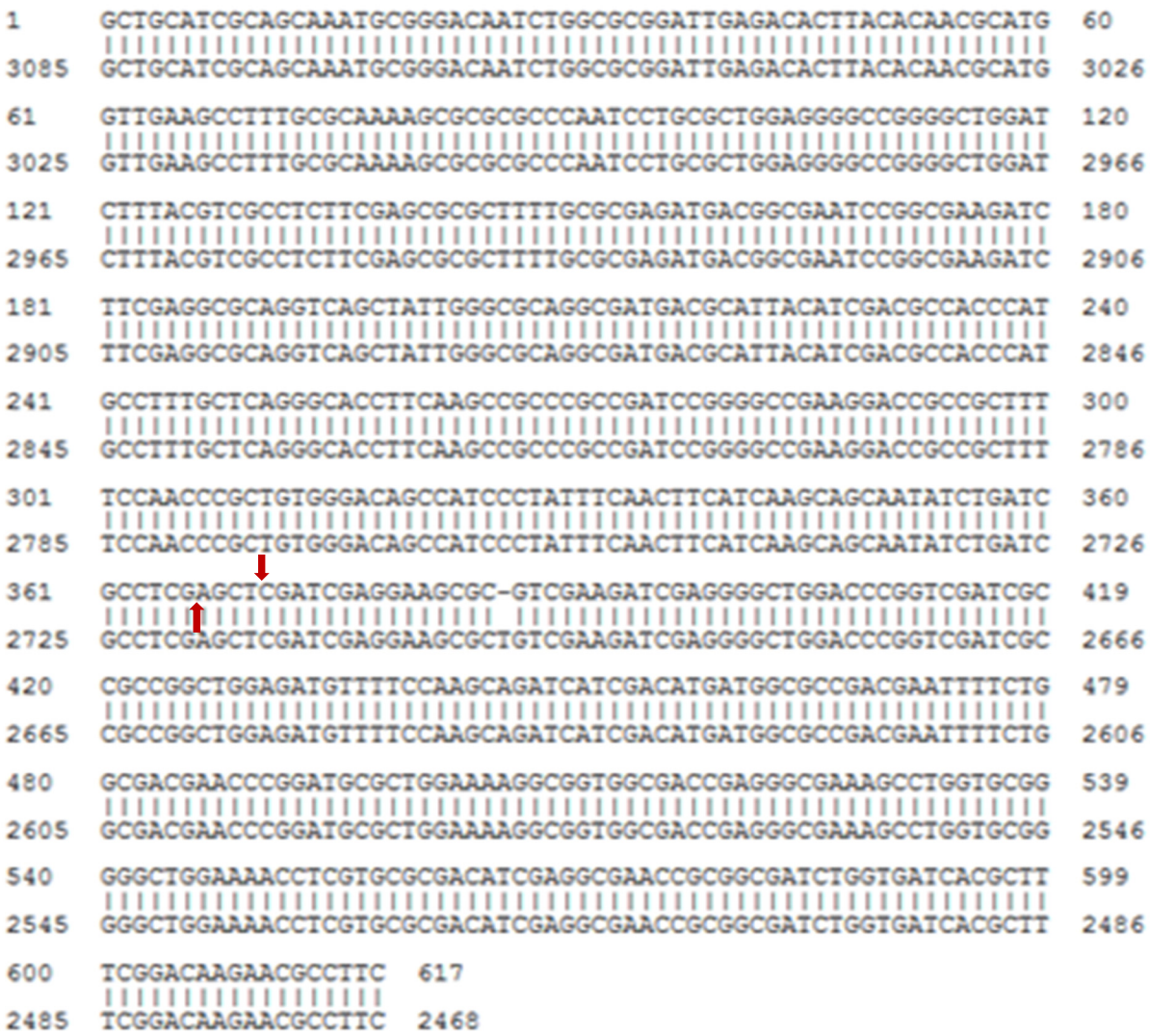


Figure 18. The sequence of PHB synthase gene of *Rhodobacter capsulatus*. The red arrows indicate the position of Gm cassette insertion (adapted from Zhang et al, 2000).

Figure 19. Physical screening for double crossover recombinants on YPS-Gm plates and YPS- km plates.

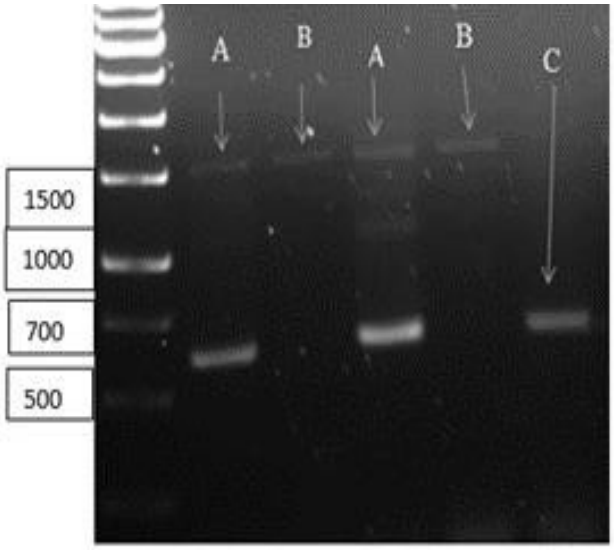


Figure 20. Analysis of recombinants by PCR, A single recombinant, B double recombinant contains p*hbC* + Gm^R cassette (1600 bp), and C *phbC* fragment (618 bp*).*

3.2 The effects of a *phbC* mutation on growth with different carbon sources:

R. capsulatus JP91 and its mutant derivative RS15 were cultured in RCV medium with ammonia as nitrogen source and lactate, acetate, or glucose as carbon source under photoheterotrophic anaerobic conditions. Growth was measured at 600 nm until there was no further change in OD reading, indicating that growth had reached stationary phase. Based on changes in OD at 600 nm it can be seen that in general, *R. capsulatus* RS15 grows slower than *R. capsulatus* JP91 (fig 21A). The best growth for both strains was observed with lactate, and the slowest growth occurred with acetate for both JP91 and RS15. In addition, *R. capsulatus* RS15 exhibited an extended lag phase, taking longer to commence growth than JP91. Growth of RS15 on acetate plus glutamate showed a similar pattern to growth on acetate and ammonia (fig 21B).

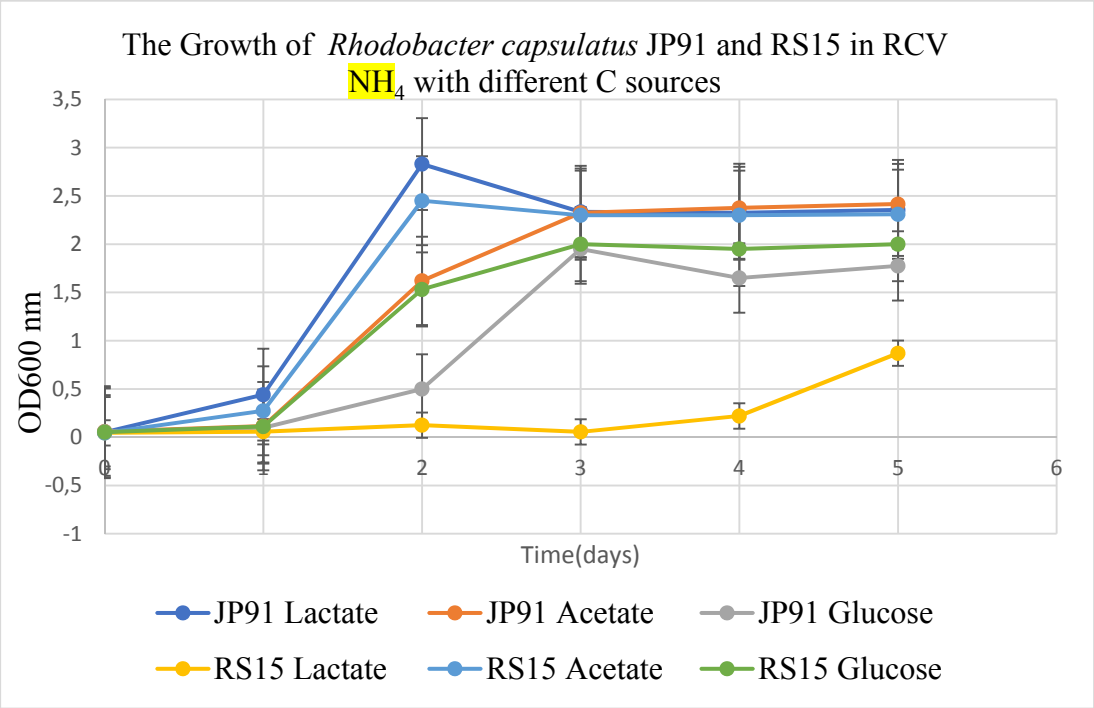


Figure 21A. Growth of *Rhodobacter capsulatus* JP91 and mutant RS15 using different carbon sources with ammonia as nitrogen source.

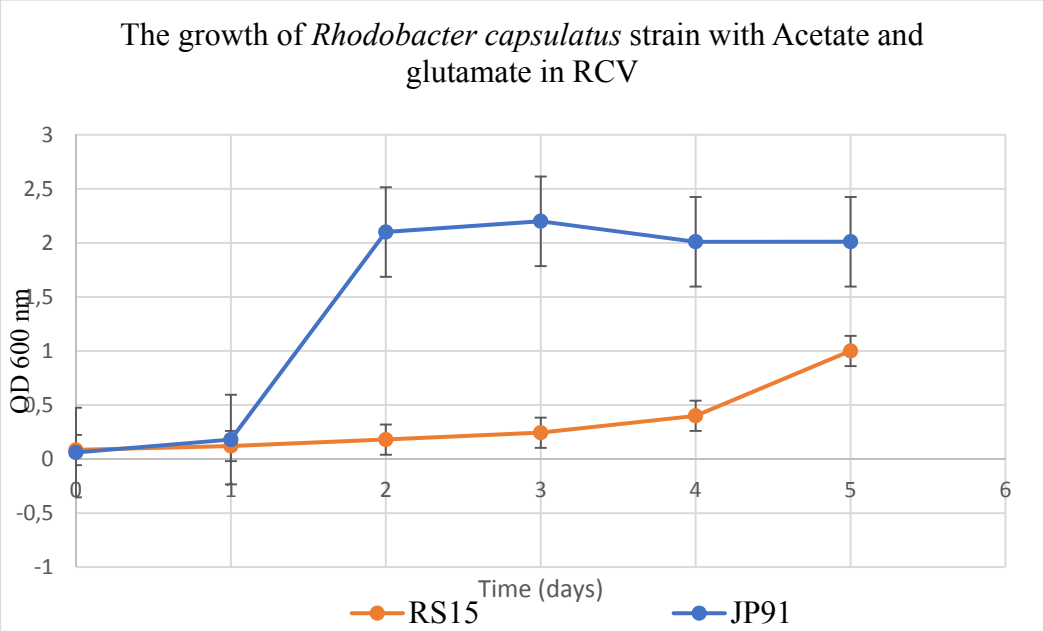


Figure 21B Growth of *Rhodobacter capsulatus* strain JP91 and mutant RS15 with acetate and glutamate as nitrogen source.

Once again, the mutant strain, RS15, showed an extended lag phase when compared with JP91 and, even after 5 days, had a much lower final OD. Therefore, the altered pattern of growth is most likely related to specific imbalances in the use of acetate brought about by the mutation in RS15 rather than an effect of the nitrogen source.

Various genera of photosynthetic bacteria show the capacity to grow on acetate as carbon source, with different assimilation pathways being involved in different organisms, with at least three different pathways being proposed; the glyoxylate shunt (*R. palustris*), the citramalate cycle (*R. rubrum*), and the ethylmalonyl-CoA (EMCoA) pathway (*R. rubrum* and *R. sphaeroides* [Hädicke et al., 2011]. At present it is unclear which pathway of acetate assimilation is used by *R. capsulatus.*

Blasco et al (1989) observed a noticeable lag phase when *R. capsulatus* ElF1 was grown on acetate as carbon source, whereas growth was unaffected when it was grown on malate, which suggests that the glyoxylate cycle is active in this bacterium and used to assimilate acetate. The enzyme isocitrate lyase (ISL), which cleaves isocitrate to glyoxylate and succinate, plays a key

role in the glyoxylate cycle when the glyoxylate cycle is used for acetate assimilation (Dunn et al, 2009). Indeed, addition of itaconate, a well-known inhibitor of bacterial isocitrate lyase (ISL or ICL), extended the lag phase in *R. capsulatus* for 2 or 3 days. Growth inhibition by itaconate has also been noted for *R. sphaeroides* and *R. rubrum* when cultivated in media containing acetate, even though in these bacterial strains only the growth rate was affected, but not the lag phase (Blasco et al 1989). Meister et al (2005) suggested that ISL activity does not exist in *R. capsulatus* and that therefore this organism does not use the glyoxylate cycle for acetate assimilation. On the other hand, Petushkova and Tsygankov (2011) reported the presence of isocitrate lyase (ISL) in *R. capsulatus* B10 and different levels of enzyme activity were found depending on inoculum age and on length of the growth on acetate medium. Since the presence of ISL activity in acetate grown cells coincided with an increase in the growth rate, it was concluded that the glyoxylate cycle might play an important role in the metabolism of acetate in *R. capsulatus* (Petushkova, and Tsygankov, 2011). As well, these researchers noted the presence in the *R. capsulatus* genome of a complete set of genes for the EMCoA pathway, suggesting that this could be operational for acetate assimilation at least under some conditions. Another study gave similar results, with maximum cell growth of a PHB synthase deleted-mutant (P1) being 1.76 and 1.25 times slower than that of parental strain *R. sphaeroides* KD131 when grown on acetate, an effect attributed to the negligible production of PHB (Kim et al, 2011). Obviously, the results of previous studies are inconclusive as to the pathways of acetate assimilation in *R. capsulatus*, and therefore it is difficult to explain with any details the inability of *R. capsultsus* RS15 to grow on acetate.

The most likely explanation is that acetate assimilation in *R. capsulatus* is indeed via the EMCoA pathway. The first two steps of this pathway require *phb*A and *phb*B (fig 22) (Tang et al, 2011).

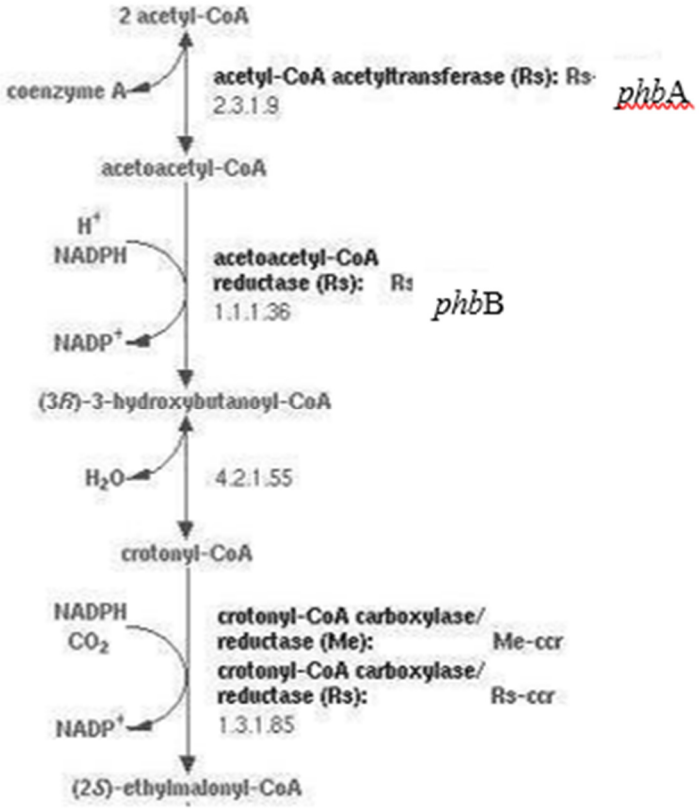


Figure 22. Acetate assimilation in *R. capsulatus* via EMCoA (pathway adapted from MetaCyc (accessed August 16th, 2017).

PHB synthase (*phbC*) is known to have multiple protein-protein interactions, in particular since it is an integral component of the carbonosome, a bacterial organelle dedicated to PHB synthesis and breakdown (Uchino et al., 2007). Thus, *phbC* could very well interact with *phb*B and help direct its function towards acetate assimilation. In this model, acetate assimilation would be compromised in the absence of *pub*. This hypothesis is supported by the prediction of *phbC*-*phb*B interaction by STRING (https://string-db.org/ accessed August 16, 2017).

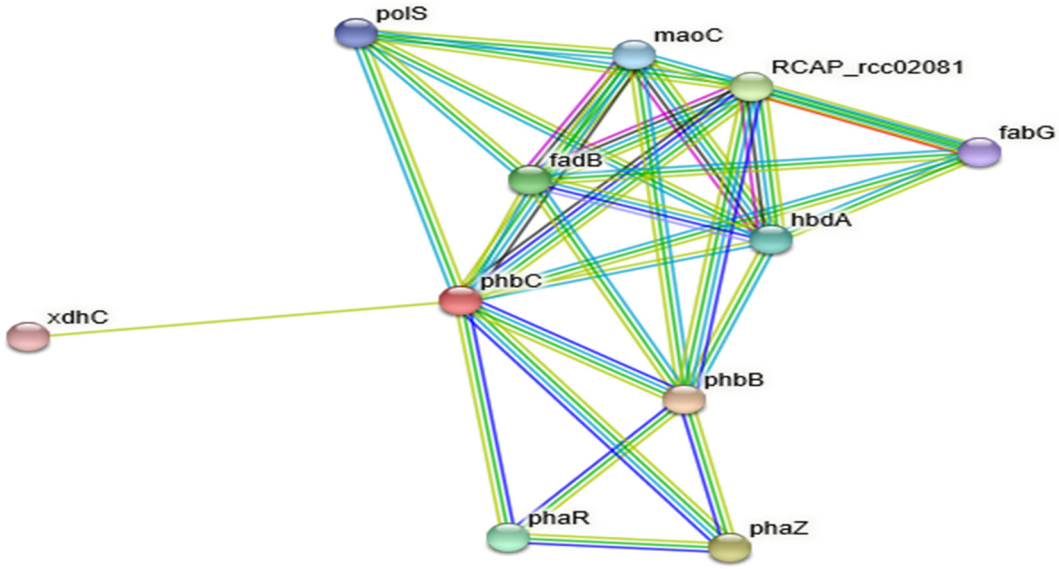


Figure 23. The figure above shows STRING view of the network of functional protein interactions with *R. capsulatus phbC.*

3.3 The effect of inactivation of *phbC* on H_2 and PHB production:

Hydrogen production experiments were carried out in triplicate, as described in the Materials and Methods section, with *R. capsulatus* JP91(*hup*¯) and RS15 (*hup*¯, *phbC*¯) cultured in RCV with 3.5 mM glutamate as nitrogen source and 30 mM acetate, lactate, or glucose as sole carbon source. *R. capsulatus JP91*, where the hydrogenase structural gene (*hup*) is interrupted by the adventitious insertion of IS21 DNA (Colbeau et al, 1990), was used as the basis for further strain construction since numerous studies from this laboratory as well as others have shown that the *hup*¯ phenotype confers greater hydrogen production.

Cumulative hydrogen production was measured daily until hydrogen production stopped after 10 days (fig 24A, B, and C). From these results, greater hydrogen production was observed for RS1 on acetate, and lactate than with the parental JP91 strain. *R. capsulatus RS15* produced 20% greater hydrogen production on acetate compared to JP91 despite its long lag phase.

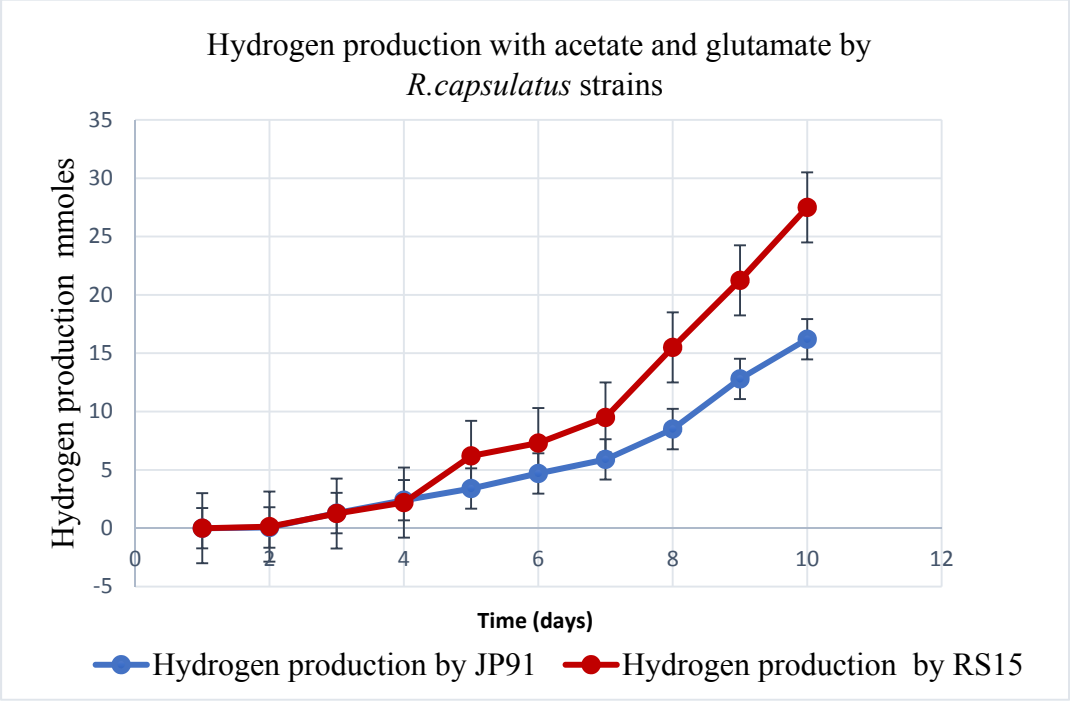


Figure 24A. Hydrogen production by *Rhodobacter capsulatus* strains JP91 and RS15 with acetate as carbon source.

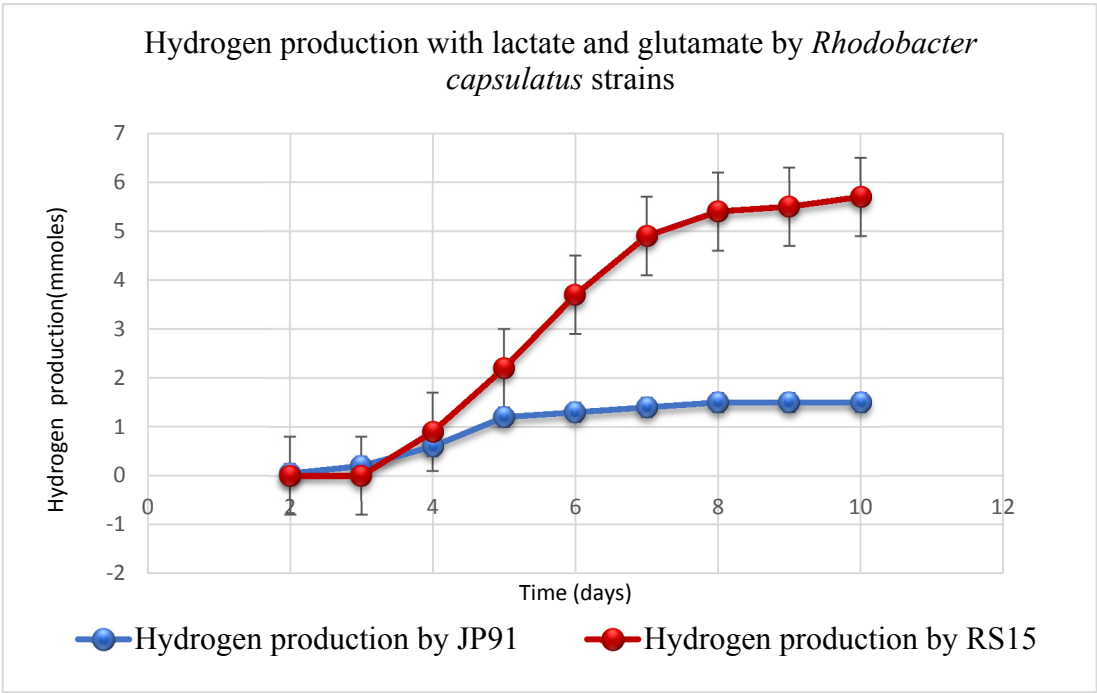


Figure 24B Hydrogen production by *Rhodobacter capsulatus* strains JP91 and RS15 with lactate as carbon source.

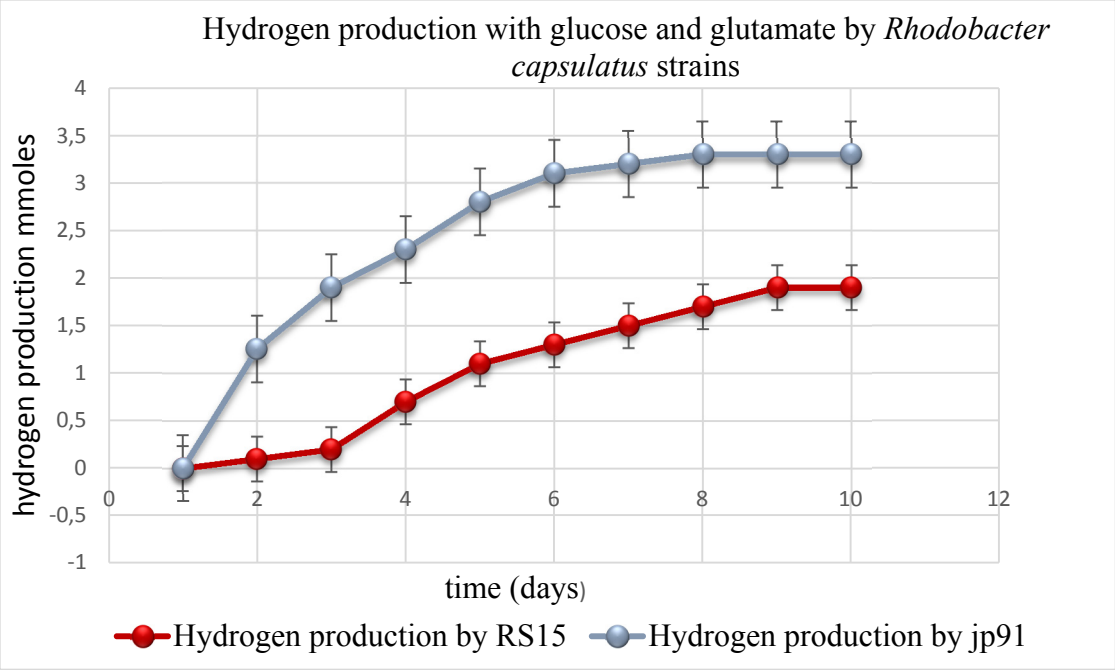


Figure 24C. Hydrogen production by *Rhodobacter capsulatus* strains JP91 and RS15 with glucose as carbon source.

Many studies have reported that the PNSB expend a significant amount of energy on PHB production, particularly when cultivated on acetate as carbon source. Also, we observed that hydrogen production by RS15 was less than JP91 when glucose was used as carbon source (fig 24C). Glucose significantly stimulates cell growth, but not PHB production, indicating that most of the glucose is assimilated into cellular substances rather than PHB. These results show that not all carbon sources are easily diverted from cellular biosynthesis into the PHB biosynthetic pathway (Wu et al, 2002).

From these results, it can be seen that both *R. capsulatus* strains JP91and RS15 produced H_2 until growth ceased. RS15 produced more hydrogen when acetate was the sole carbon source in spite of slower growth. In general, photo-fermentative hydrogen production by photosynthetic bacteria is associated with growth since the same conditions that are required for hydrogen production, the availability of a suitable source of fixed carbon, the conversion of light energy to

chemical energy in the forms of ATP and proton motive force for reverse electron flow, are also favorable for growth (McKinlay et al, 2011; Hädicke et al, 2011). A number of different studies have shown that photosynthetic bacteria produce H_2 as a way of disposing of excess electrons and maintaining redox balance depending upon the redox state of the carbon substrate, the pathways taken to produce the necessary metabolic precursors, and the other pathways that are available for maintaining redox balance (flux through the CO_2-fixing Calvin-Benson-Bassham cycle, H_2 production by nitrogenase, PHB synthesis (McKinlay et al, 2011: Hädicke et al, 2011). Modeling of the various metabolic pathways reveals that, even though in principle a large number of catabolic pathways are potentially available, biomass and CO_2 yields associated with each substrate are fixed constraints that do not depend upon the assumption of optimal growth (Hädicke et al, 2011). Thus, under some circumstances, here growth and H_2 production on acetate by strain *R. capsulatus* RS15, it is possible to have appreciable H_2 production with slowly growing cultures.

It is known that acetate is a very efficient substrate for PHB synthesis but a very unfavorable substrate for hydrogen production. In general substrates, such as acetate and butyrate, which are easily converted to acetyl CoA units without requiring pyruvate input, produce primarily PHB (Koku et al, 2002). In other words, acetate can be converted more easily to acetyl units than lactate which is naturally converted to pyruvate. Growth rate and acetate degrading rate of mutant were slightly slower than wild-type (Chen et al, 2012). So, when *phbC* is inactivated most of converted acetate will be directed to H_2 production.

The results show that there was no significant difference in hydrogen production between JP91 and RS15 when using lactate as carbon source, even though JP91 grew well on lactate containing media. Thus, these results are similar to a previous study where it was reported that PHB synthase deficient mutants showed an increase in H_2 production on acetate, but no significant difference

when lactate was used (Kim et al 2011).

In the present study, spectrophotometric analysis showed that the cellular content of PHB in JP91 reached the highest levels on acetate (fig 25), whereas on the other substrates, lactate and glucose, it was lower than acetate; thus, acetate seems to be the best carbon source for PHB production. In addition, the results show that the apparent cellular PHB content of *R. capsulatus* RS15 is, under all circumstances, much lower than that of the JP91. Although RS15 might be expected to show no PHB upon analysis due to the inactivation of *phbC* encoding PHB synthase, critical gene involved in PHB biosynthesis, a small amount of color development occurred when PHB from RS15 was assayed. This is very likely due to known interference by carbohydrates (Slepecky and Law, 1960b: Cai et al, 2000) released by the rather indiscriminate hydrolysis by hypochlorite (Law and Slepecky, 1961). At any rate, it is apparent that the mutation strategy was successful in creating a mutant affected in PHB accumulation, thus validating the main thrust of this research.

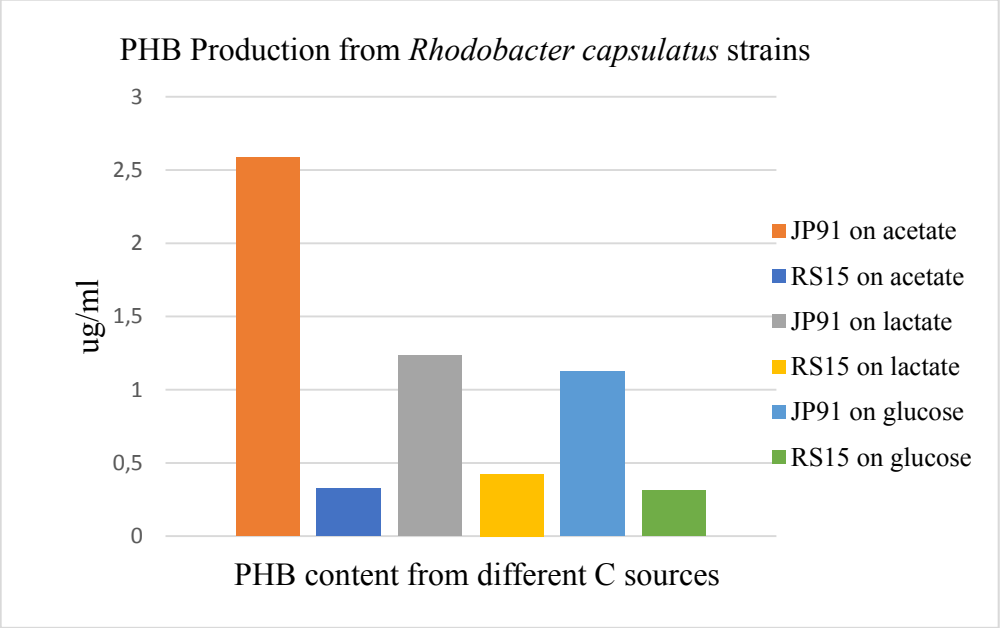


Figure 25. PHB production by *Rhodobacter capsulatus* strains JP91 and RS15 cultivated on different carbon sources. Results are reported as micrograms of PHB per ml of culture. Cultures were harvested at an OD235 of 2.1.

4- Conclusion and perspectives:

Biohydrogen is a promising energy carrier since it is environmentally friendly, renewable, and potentially costeffective. Photo-fermentative biological hydrogen by PNS photosynthetic bacteria has been demonstrated; however, it still suffers from some issues like lower H_2 yields and rates due in part to some metabolic pathways competing with N_2ase for electrons (reducing equivalents). PHB biosynthesis, creating an intracellular carbon storage, is one of the competitors of H_2 production. *R. capsulatus* is one of PNSB strains that has been widely studied for its H_2 and PHB production capabilities. Redox balance of the cells is very important for biohydrogen production, since the requirements of nitrogenase to reduce protons to hydrogen are high energy electrons and ATP. Optimizing the flow of reducing equivalents to N_2ase was carried out using genetic modifications to knockout the PHB synthesis pathway which competes with N_2ase for reducing equivalents. Hypothetically, creating *phbC*$^-$ mutants should improve H_2 yields; nevertheless, in practice this has been hard to illustrate clearly. An early study found that there was no difference between the amount of H_2 produced by a *phbC*$^-$ mutant of *R. sphaeroides* and the parental strain when lactate was used as substrate (Franchi et al, 2004). Kim et al, 2006 reported that a *phbC*$^-$ mutant of *R. sphaeroides* strain gave a 34% increase in specific hydrogen production when it grew on malate, but it gave a 55% increase in hydrogen production when grown on acetate. In addition, for reasons that are unclear, the *phbC* mutant strain grew poorly.

Recently, a compilation of the effects of a variety of carbon sources on H_2 production by *phbC*$^-$ and WT strains found that there were differences in H_2 production when acetate and propionate are used as carbon source (Hallenbeck et al, 2012). Numerous studies have reported an increase in photo-fermentative hydrogen production when a PHB mutation was coupled with

a mutation in the uptake hydrogenase (Hallenbeck et al, 2012; Adessi and De Philippis, 2012).

The purpose of the present work was to enhance the production of hydrogen by *R. capsulatus* by introducing a *phbC* mutation into a strain (JP91) that is *hup*$^{-}$, creating strain RS15, and to investigate the effects of a variety of carbon sources on a *phbC* mutant of *R. capsulatus* JP91. The results reported here show that metabolically engineered *R. capsulatus* RS15 was able to grow on different carbon sources, despite a long lag phase period when grown on acetate. Both *R. capsulatus* strains (JP91, RS15) produced H_2 until the cells reached stationary phase. Strain RS15 produced more hydrogen when acetate was the sole of carbon source in spite of slow growth. Acetate is easily converted to Acetyl CoA and the inactivation of *phbC* should therefore direct acetate directly to H_2 production. Conversion of organic acids would be advantageous as it would couple energy production with organic waste treatment (Barbosa et al, 2001).

Further investigations are required to maximize the yields and rates of H_2 production. The experimental results reported here show that acetate is a suitable carbon source for PHB production, and that production by *R. capsulatus* RS15 was higher when acetate was the sole source of carbon. Several future directions can be suggested. One possibility would be to investigate higher acetate concentrations, since only one acetate concentration (30 mM) used in the present work, and it has been previously reported that high acetate concentrations increase intracellular PHB content (Sangkharak and Prasertsan, 2007).

Another possibility would be to enhance the growth of *R. capsulatus* RS15 on acetate by overexpression of isocitrate lyse since a previous study mentioned that the presence of ISL activity in cells grown on acetate gives high growth rates (Petushkova, and Tsygankov, 2011). A third possibility, optimization of different parameters such as light intensity, temperature, and pH would be advantageous for strain cultivation. Finally, it would also be important to analyze the expression levels of key enzymes for H_2 (nitrogenase) and PHB production (PHB synthase,

phbC). Overall, *R. capsulatus* RS15 (*hup*$^{-}$, *phbC*$^{-}$) seems to be a promising candidate for use in two - stage hybrid systems since the main dark fermentation effluents are acetate and butyrate. Such a process could potentially make biohydrogen production technology feasible on an industrial scale.

Appendix A

YPS medium:

2 mM $CaCl_2$	(4 ml of 7.5% $CaCl_2 \cdot 2H_2O$ per Liter)
2 mM $MgSO_4$	(2.4 ml of 10% $MgSO_4$ per Liter)
Yeast extract	3 g/L
Peptone	3 g/L
Agar	15 g/L

RCV / liter

10% lactate	40 ml
Super salts	50 ml
Phosphate buffer	30 ml
NH_2SO_4 (for growth)	11.9 ml
Sodium glutamate (for H_2 production)	12 ml
Agar for solid plates if needed	15 g

LB media/ liter

Bacto tryptone	10 g
Yeast extract	5 g
$NaCl_2$	10 g
ddH_2O to	1 L

Super Salts solutions

1% EDTA + 0.24 g $FeSO_4$ $7H_2O$	40 ml
20% $MgSO_4$	20 ml
7.5% $CaCl_2$ $2H_2O$	20 ml

Trace elements	20 ml
Thiamine HCl (1mg/l)	20 ml
ddH_2O to	1000 ml

Trace Element Solution

$MnSO_4$ H_2O	0.3975 g
H_3BO_3	0.7000 g
$Cu(NO_3)_2$ $3H_2O$	0.0100 g
$ZnSO_4$ $7H_2O$	0.0600 g
$NaMoO_4$ $2H_2O$	0.1875 g
ddH_2O to	250 ml

0.64 M Phosphate buffer

KH_2PO_4	20 g
K2HPO4	30 g
ddH_2O to	500 ml

pH adjusted to (6.8)

Antibiotics:

Kanamycin	25 ug/ml
Gentamicin	10 ug/ml
Tetracycline	1 to 1.5 ug/ml
Ampicillin	100 mg/ml

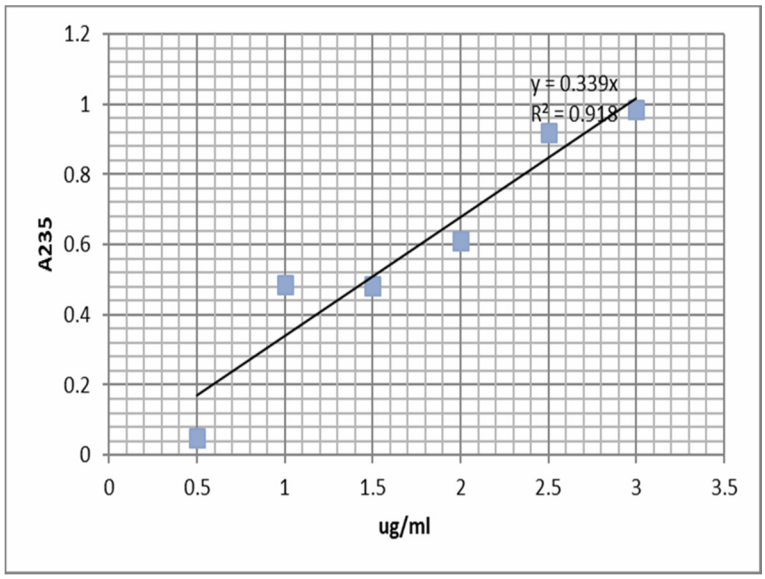
Appendix B

TB buffer /L

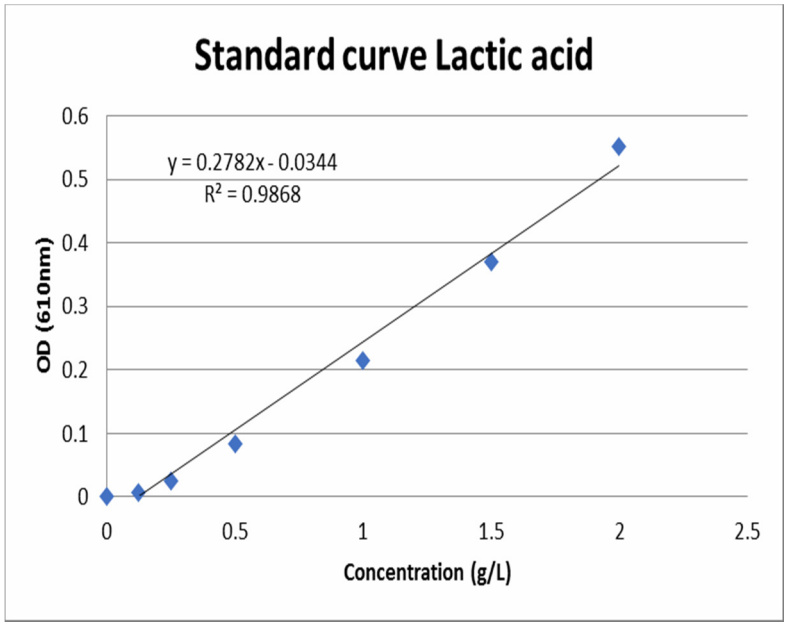
10 mM PIPES Na salt	3.35 g
15 mM $CaCl_2$	2.2 g
250 mM KCl	18.64 g
55 mM $MnCl_2$	10.9 g
ddH_2O to	1 L

Appendix C

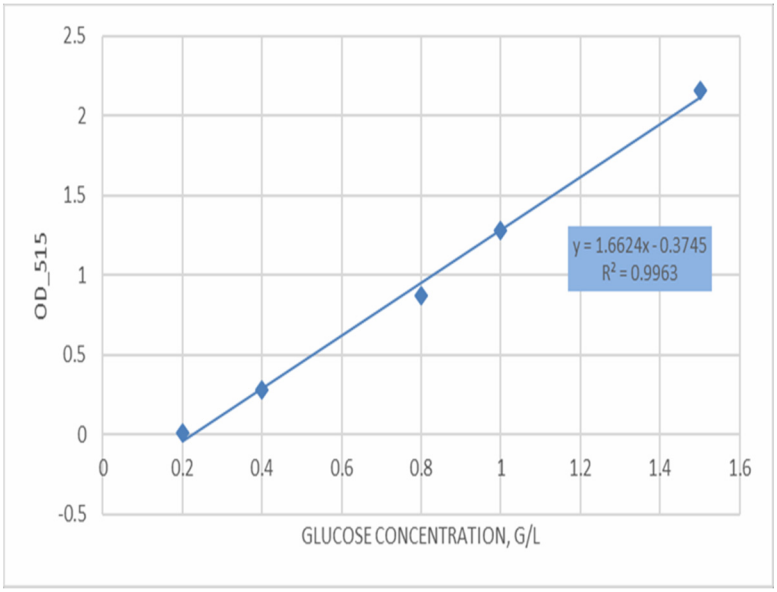
Calibration curves



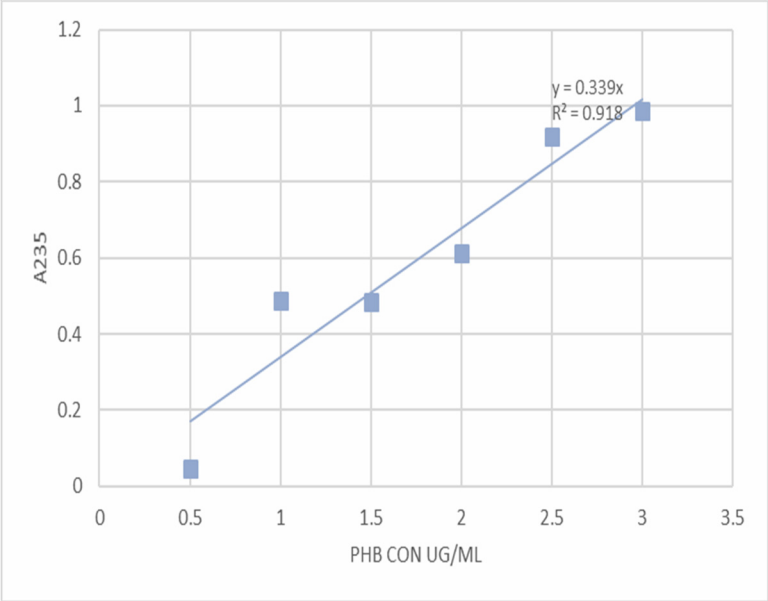
Acetate calibration curve



Lactic acid calibration curve



Glucose calibration curve



PHB calibration curve

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