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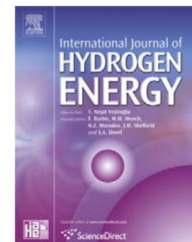

Evaluation of hydrogen production by *Rhodobacter sphaeroides* O.U.001 and its *hupSL* deficient mutant using acetate and malate as carbon sources

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ABSTRACT

Rhodobacter sphaeroides O.U.001 is one of the candidates for photobiological hydrogen production among purple non-sulfur bacteria. Hydrogen is produced by Mo-nitrogenase from organic acids such as malate or lactate. A *hupSL* in frame deletion mutant strain was constructed without using any antibiotic resistance gene. The hydrogen production potential of the *R. sphaeroides* O.U.001 and its newly constructed *hupSL* deleted mutant strain in acetate media was evaluated and compared with malate containing media. The *hupSL*⁻ *R. sphaeroides* produced 2.42 l H_2/l culture and 0.25 l H_2/l culture in 15 mM malate and 30 mM acetate containing media, respectively, as compared to the wild type cells which evolved 1.97 l H_2/l culture and 0.21 l H_2/l culture in malate and acetate containing media, correspondingly. According to the results, *hupSL*⁻ *R. sphaeroides* is a better hydrogen producer but acetate alone does not seem to be an efficient carbon source for photoheterotrophic H_2 production by *R. sphaeroides*.

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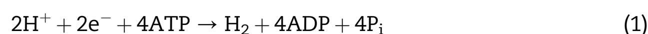
1. Introduction

Hydrogen, the candidate for the worldwide future alternative energy carrier, can be produced through photofermentation by photosynthetic bacteria, such as *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas palustris* [1,2]. Under anaerobic conditions, these bacteria are able to use simple organic acids such as malate, acetate and lactate as energy and electron sources. In these strains H_2 is evolved as a by-product during N_2 fixation. Under nitrogen fixing conditions, in the presence of alternative nitrogen source, nitrogenase acts as ATP dependent hydrogenase and the electrons taken from electron carrier ferredoxin/flavodoxin (Fd, Fn) are utilized for H_2 production [3,4]. For each electron, two ATPs are used and therefore four ATPs are consumed by nitrogenase for one H_2 produced:



$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \quad (1)$$

Total hydrogen production is constrained due to several reasons in photosynthetic bacteria, such as consumption of hydrogen by uptake hydrogenase [5], limited electron flow to

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the nitrogenase due to the production of poly-3-hydroxybutyrate (PHB) [6] or the efficiency of substrate utilization by photosynthetic bacteria. Hydrogenases are metalloenzymes catalyzing the reversible oxidation of hydrogen. Within this enzyme family, the uptake hydrogenase is encoded by the *hupSL* genes and catalyzes the conversion of molecular hydrogen to electrons and protons and thus decreasing the hydrogen production efficiency of the nitrogenase [5]. The function of the Hup hydrogenase is generally linked to the nitrogen fixation or the presence of H_2 in the environment [5]. Therefore, the uptake hydrogenase was targeted to be eliminated in some bacteria by antibiotic resistance gene insertion or by chemical mutagenesis mostly to prevent the hydrogen uptake consequently to enhance the hydrogen production [6–10].

Hydrogen is also produced by many anaerobic microorganisms from different feed stocks by a process called dark fermentation [1]. The complete oxidation of glucose to CO_2 energetically is not possible by dark fermentation. Therefore, the combination of dark fermentation with photofermentation is energetically and economically favorable. In the first dark fermentative step a cheap substrate or raw material is converted to hydrogen and organic acids and in the second stage the latter product is utilized in a photoheterotrophic process producing hydrogen [1,11,12]. Although, the composition of the effluent from dark fermentation depends on the type of substrate and microorganism used, it is mainly acetate and butyrate [13]. Therefore, utilization of acetate by photofermentative bacteria is of crucial importance in terms of feasibility of such a two stage biohydrogen production.

In order to enhance the hydrogen productivity in this study the uptake hydrogenase was eliminated from *R. sphaeroides* O.U.001, a potential photobiohydrogen producer phototrophic bacterium. For biosafety reasons, *hup* gene was deleted without inserting any antibiotic resistant gene. Then, the ability of wild type and *hupSL* deficient *R. sphaeroides* to produce biohydrogen using acetate and malate was evaluated and compared with each other and the representatives of photosynthetic bacteria published in the literature.

2. Materials and methods

2.1. Bacterial strains, plasmids and the culture conditions for H_2 production

The bacterial strains and plasmids used in this study were listed in Table 1. *R. sphaeroides* O.U.001 was cultivated in modified Biebl and Pfenning minimal medium [14], in which four-carbon organic acid malate (15 mM) and two-carbon organic acid acetate (30 mM) were used as carbon sources. Since ammonia represses the activity of Mo-nitrogenase, it was omitted from the medium and 2 mM glutamate was used as nitrogen source. *E. coli* was cultivated in Luria-Bertani medium and antibiotics were used in the following concentrations: kanamycin (25 μg/ml), ampicillin (100 μg/ml), streptomycin (25 μg/ml) and tetracycline (10 μg/ml). The plates were prepared by solidifying the liquid media of both *R. sphaeroides* and *E. coli* with 1.5% agar.

Table 1 – The plasmids and bacterial strains used in this work.

Strains	Characteristics/genotype	Reference
E. coli		
XL1 Blue	Δ(*mcrA*) 183, Δ(*mcrCB-hsdSMR-mrr*) 173, *endA1*, *supE44*, *thi-1*, *recA1*, *gyrA96*, *relA1 lac* [F′ *proAB lacIqZ*ΔM15 Tn10 (Tet^r)]	Stratagene
S17-1(λpir)	294 (*recA pro res mod*) Tp^r, Sm^r (pRP4-2-Tc::Mu-Km::Tn7), λpir	[16]
R. sphaeroides		
O.U.001	Wild type	DSM 5864
ZK1	*hupSL*$^-$, without antibiotic resistance gene	This work
Plasmids		
pK18*mobsacB*	Km^r, *sacB*, *RP4 oriT*, *ColE1 ori*	[17]
pBluescript SK(+)	Amp^r	Stratagene
pGhup3	916 bp partial *hupS* gene cloned into pBluescript SK(+)	This work
pGhup4	844 bp partial *hupL* gene cloned into pGhup3	This work
pGhup5	1778 bp deleted *hupSL* gene cloned into pK18*mobsacB* vector	This work

2.2. Hydrogen production and measurements, hydrogenase activity measurements

The *in vivo* hydrogen production was carried out with cells grown in small bioreactors (60 ml bottles) at 30 °C under the illumination of 940 μE/m^2/s by 100 W tungsten lamp from a distance of 30–40 cm [15]. The evolved gas was collected into water-filled tubes and the gas volume was recorded. The composition of the gas was analyzed by gas chromatography [13]. The differences in H_2 gas productions between wild and mutant strains in different growth media were compared statistically using Student *t*-test ($n = 3$). The hydrogenase activity measurements were performed using an artificial electron acceptor, benzyl viologen as described in Refs. [9,18].

2.3. Construction of hupSL deleted mutant strain of R. sphaeroides O.U.001

Construction of *hupSL* deleted mutant strain of *R. sphaeroides* O.U.001 was performed in three successive steps: first, constructing the suicide vector containing the proper 5′ and 3′ regions linked in frame, second, delivering it to the wild type *R. sphaeroides* and third, selecting the *hupSL* mutant strain. The suicide vector was constructed as follows; the 916 bp long 5′ end of *hupS* gene was amplified by PCR using the hupSL3 [5′-TAACGGATTTCACCCCTTCC-3′] and hupSL5 [5′-CTTCTTGG CGTAGTCGTCGT-3′] primers. The PCR program was as follows: 2 min at 94 °C for pre-denaturation, 30 cycles of amplification step (30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C) followed by a final extension at 72 °C for 5 min. The amplified truncated *hupS* gene was polished and cloned into EcoRV cut pBluescript SK(+), yielding pGhup3 vector. Then, the 844 bp long 3′ end of *hupL* gene was PCR amplified using Rshusq4 [5′-GCAATCTCGAG-GAAGTGCAT-3′] and hupSL4 [5′-GAATGGCGAGCAGTTTCTTC-3′] primers using the same PCR program. The amplified *hupL* fragment was cloned into the SmaI digested pGhup3, resulting

in pGhup4. In this construct, the remaining parts of the *hupS* and *hupL* genes were joined in frame. Finally, the 1778 bp long, defective *hupSL* genes were amplified using pGhup4 as a template and hupSL3 and hupSL4 primers. The PCR product was blunted and cloned into SmaI cut pK18*mobsacB* vector yielding the suicide vector: pGhup5. The PCR program used was the same as above, except that the extension time was 2 min.

First, the pGhup5 vector was transformed into *E. coli* S17.1λpir, then into *R. sphaeroides* by conjugation [19]. After conjugation, the *R. sphaeroides* cells were cultivated in non-selective medium to let the suicide vector to be recombined into the native *hupSL* genes allowing the replacement of the native *hupSL* genes with defective ones. Then, the double recombinant *R. sphaeroides* cells were selected on the basis of the conditionally lethal *sacB* gene. The mutant with deficient *hupSL* genes was chosen according to the lack of its *in vitro* hydrogenase activity, Southern-blotting and hybridization experiments and DNA sequencing (see below).

2.4. *Confirmation of* hup *deletion by Southern hybridization*

Southern hybridization was performed using DIG DNA Labeling and Detection Kit (Roche). The DIG-labeled probe was prepared by PCR according to the manufacturer's instructions using RshupSL6 [5′-TGGTGATGAGCTTGTCGAAG-3′] and RshupSL7 [5′-TGATGGACACGATCGAGAAA-3′] as primers and pGhup5 as a template (Roche DIG DNA Labeling and Detection Kit, Instruction Manual, Version October 2004) (Fig. 1A). The PCR program was the same as above, except that the extension time was 90 s. After the probe preparation, the NcoI–SmaI double digested gDNA fragments of the putative *hup* mutants and wild type cells separated in 0.7% agarose gel and then blotted to positively charged nylon membrane (Roche). After blotting, the hybridization of labeled probes was carried out at 75 °C overnight. The detection was performed by using Anti-Dig-Alkaline Phosphatase conjugates (Fig. 1B).

2.5. *Confirmation of* hup *deletion by sequence analysis*

The sequence analysis is a simple and straightforward genotyping method. The defective *hupSL* genes of the putative mutant bacterium were PCR amplified using the hupSL8 [5′-ATTGGGGCCGAGTTCGTAG-3′] and hupSL9 [5′-CCCGGC GATGTTCTACAG-3′] primers using gDNA as template (Fig. 1C). The PCR program was the same as above except that the extension time was 30 s. Then, the PCR product was directly sequenced (İontek, Turkey). The gDNA of the wild type cells and water were also used as templates in the PCR but the expected product was only obtained using gDNA of the selected mutant cells.

3. Results and discussion

3.1. *The analysis of* hupSL *deleted mutant of* R. sphaeroides *by genetic and biochemical tests*

Parts of *hupSL* genes were removed by homologous recombination mediated site directed mutagenesis. During the

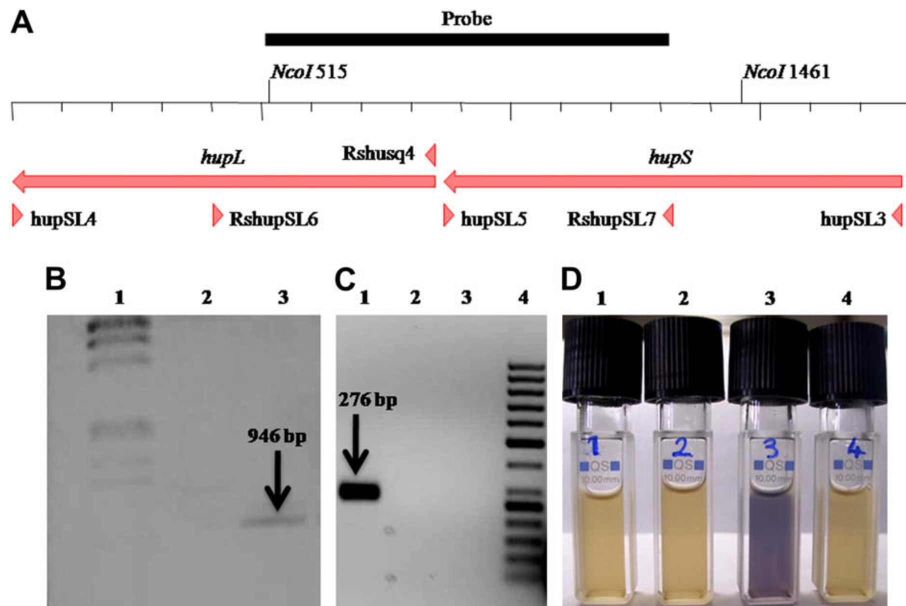

Fig. 1 – The probe whose location was shown in (A) on the deleted *hupSL* genes hybridized only to the NcoI and SmaI double digested gDNAs of *hupSL* mutant *R. sphaeroides* (B3) but not to that of wild type *R. sphaeroides* (B2). The molecular weight marker III was also loaded to the gel (B1). The deleted *hup* gene region in the *hupSL*$^-$ *R. sphaeroides* was PCR amplified using the gDNA of mutant cells as a template (C1). Then this PCR product was sequenced. gDNA of wild type cells (C2) and water (C3) were also used as templates in the PCR. The DNA ladder (C4) was run together with PCR products. The uptake hydrogenase assay was performed using *hupSL*$^-$ mutant *R. sphaeroides* ($+H_2$) (D1), *hup*$^-$ mutant *R. sphaeroides* ($-H_2$) (D2), the wild type cells ($+H_2$) (D3) and the wild type cells ($-H_2$) (D4). Hydrogen dependent appearance of the blue-purple color indicates the presence of an active hydrogenase in the assay. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

procedure, around 100 amino acids from the C-terminus of HupS and 326 amino acids from the N-terminal part of HupL were removed (Fig. 1A). The genotype of the putative *hupSL* mutant *R. sphaeroides* was analyzed by Southern hybridization and sequence analysis and its phenotype was determined in uptake hydrogenase activity assay (See Materials and methods). As the Southern hybridization in Fig. 1B shows, the probe hybridized only to a fragment of expected size in the *hupSL* deficient *R. sphaeroides* strain. The deleted *hup* gene region in the *hupSL*$^-$ *R. sphaeroides* was PCR amplified and sequenced. The sequence analysis confirmed that the sequenced DNA fragment was exactly the same as the gene fragments present in the pGhup5 suicide vector. This undoubtly proved that the truncated *hupSL* genes replaced the native one in the genome (Fig. 1C). Finally, uptake hydrogenase assay was performed to illustrate, that the uptake hydrogenase was completely inactive in the *hupSL* deleted *R. sphaeroides*. Fig. 1D demonstrates, that the mutant did not display any uptake hydrogenase activity, while wild type cells showed hydrogen dependent reduction of redox benzyl viologen displayed by a purple color. It was concluded that the uptake hydrogenase was inactive in the *hupSL* deleted mutant cells.

3.2. *The growth of wild type and* hup *deleted mutant strain of* R. sphaeroides *O.U.001 using malate and acetate*

Malate and acetate were used as carbon sources in 15 mM and 30 mM concentrations, respectively. Since, acetate is a two-carbon compound while malate is a four-carbon compound, these concentrations ensured approximately the same mol of carbon in the medium. In addition, 2 mM glutamate was also supplied as nitrogen source in the Biebl and Pfenning minimal medium. The bacteria were adapted to carbon sources (either acetate or malate) sequential passing of the culture in liquid media in 60 ml bottles. The growth and pH changes were monitored during the cultivation of the *hupSL* mutant and wild type *R. sphaeroides* in malate and acetate containing media. Both the wild type and *hupSL* mutant cells followed similar growth patterns in malate containing medium and the maximal OD_{660} values were around 1.65 (Fig. 2A). The cells grown in acetate containing media reached relatively high cell densities relative to the cells grown in malate containing media, but the growth curves were the same for both the wild type and mutant strains. In the acetate based medium, the maximal OD_{660} values of the wild type cells and the mutant strain were 3.4 and 3.0, respectively (Fig. 2B). Acetate obviously stimulated the bacteria to produce cell biomass. The by-products such as PHB formed during acetate utilization might have caused these growth profiles.

The pH values of the growth media were set to 7.0 before inoculation in all batch operations. The pH values of cultures did not change significantly and followed almost the same pattern in the case of both wild type and *hupSL*$^-$ mutant *R. sphaeroides* grown in malate/glutamate medium (Fig. 2C). However, significant increase in the pH values was observed in the case when acetate/glutamate medium was used. The pH of the medium reached to pH 9.8 and pH 9.1 for the wild type and *hupSL* knockout mutant cells, respectively (Fig. 2D). The acetate consumption during acetate utilization might have caused this pronounced increase in pH.

3.3. *Hydrogen production from malate and acetate by wild type and* hupSL$^-$ R. sphaeroides *O.U.001 strains in photobioreactor*

The hydrogen production of the wild type and *hupSL*$^-$ *R. sphaeroides* O.U.001 strains in malate and acetate containing minimal media was compared. It was observed that *hupSL* mutant cells produced significantly more hydrogen than the

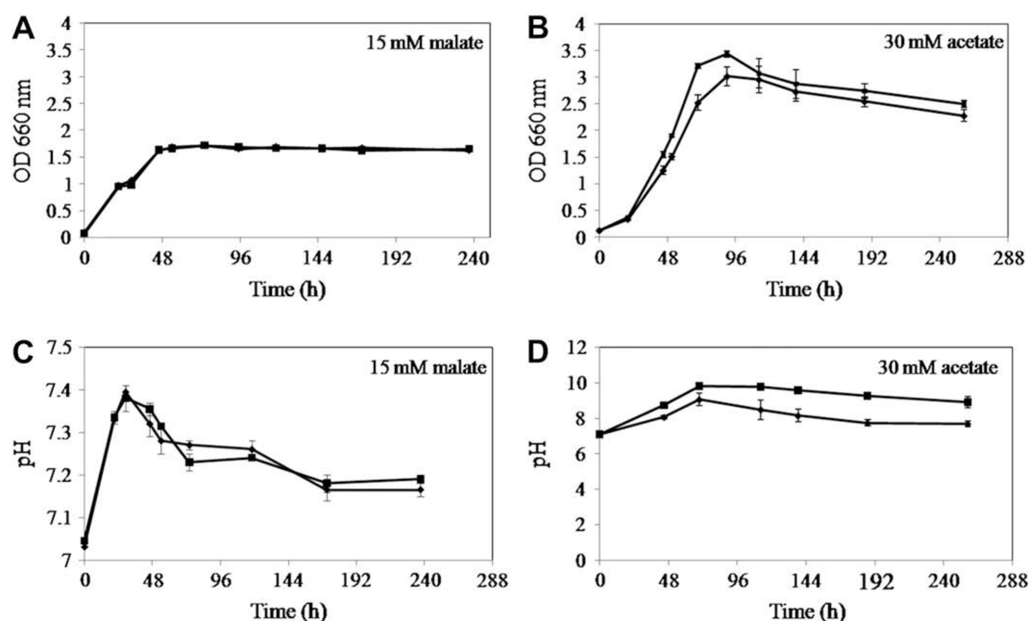

Fig. 2 – The growth of *R. sphaeroides* O.U.001 in media containing 15 mM of malate (A) and 30 mM of acetate (B). The pH of the cultures was also monitored during the cultivation of bacteria in malate (C) and acetate (D) containing media. Wild type, (■); *hup*$^-$ mutant, (◆). Each value is the mean of three times replication with ±standard deviation.

wild type cells did in both malate/glutamate and acetate/glutamate media ($p = 0.0465$ for 143 h and $p = 0.0222$ for 207 h data sets, respectively) (Fig. 3A, B). The mutant cells also had better yield (g H_2/g substrate) and substrate conversion efficiency than wild type cells (Table 2). While in malate/glutamate media, the mutant and the wild type cells produced 2.42 l H_2/l culture and 1.97 l H_2/l culture, respectively, they evolved 0.25 l H_2/l culture (wt) and 0.21 l H_2/l culture (mutant) in acetate/glutamate media. When the hydrogen production efficacies of the cells grown on malate/glutamate and acetate/glutamate media were compared, both the wild type and mutant cells produced significantly less hydrogen in acetate containing media. Within the examined period acetate is completely consumed (data not shown) and the same was observed for acetate and malate by our group [13] since it still gives an underestimated value for substrate conversion efficacy. In Table 2, three representative strains of phototrophic bacteria namely *R. sphaeroides* O.U.001 and its *hupSL*$^-$ strain, *R. capsulatus* B100 and its *hupSL*$^-$ strain and *R. palustris* R1 were compared in terms hydrogen production in malate and acetate containing media. It was observed that all three strains produced significantly less hydrogen using acetate than using malate [2,10]. In addition, all strains gave better hydrogen production yield and substrate conversion efficacy using malate than using acetate except for the *R. capsulatus* ST410 which gave better substrate conversion efficacy using acetate. It is to note that, if the malate utilization is not complete the H_2 production/mol consumed malate is even higher.

The difference in hydrogen production by wild type and *hupSL*$^-$ *R. sphaeroides* O.U.001 using malate and acetate is very pronounced. One possible explanation might be derived from the distinct pH profiles of the acetate and malate based cultures. The pH of the medium was much higher in the case of cells grown on acetate as compared to strains cultivated on malate. The higher pH might have an inhibitory effect on H_2 production. However, a study on another *R. sphaeroides* strain revealed that the increased pH did not have negative effect on the *in vivo* H_2 production [8], hence this hypothesis seems to be unlikely. Furthermore, if the increased pH would inhibit the H_2 evolution, alternative routes must be switched on to maintain the redox balance of the cells. Another, more plausible elucidation for the inability of *R. sphaeroides* to produce the expected amount of hydrogen may come from the acetate assimilation pathway present in the bacterium that is different from the known glyoxylate cycle. The genes coding for the two key enzymes (isocitrate lyase and malate synthase) in glyoxylate cycle are absent in the genomes of *R. sphaeroides* and an alternative ethylmalonyl-CoA pathway has been proposed for the acetate assimilation in this bacterium [20,21]. In relation to biohydrogen production, the ethylmalonyl-CoA pathway has few disadvantages. On the one hand, the pathway is strongly connected to CO_2 fixation, which, by requiring reducing power, diminishes the electron output of the overall process. On the other hand, the acetate assimilation pathway shares common elements with the polyhydroxybutyrate biosynthetic route. According to the proposed pathway, acetoacetyl-CoA and 3′-hydroxybutyrate are common intermediates for both polyhydroxybutyrate 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. In a previous study, a PHB synthase deficient strain of *R. sphaeroides* KD131 was shown to use only 19% of the 30 mM initial acetate, while the parental strain consumed about 60% of initial acetate and hydrogen production was unchanged due to high pH [8]. Therefore, simultaneous PHB synthesis is very likely during acetate assimilation. PHB white globules within the cells could be seen by electron microscopic observations [22] and PHB accumulation was measured by Yiğit et al. [23] as well. This proposal coincides with the fact that, PHB production is more pronounced when using acetate as carbon source than malate in several microorganisms including *R. sphaeroides* [24,25]. In previous investigations, in order to increase the hydrogen production, the *phaC* gene encoding PHB synthase was disrupted [6,25]. It was observed that the difference in hydrogen production between PHB synthase deficient mutant and wild type was only pronounced if acetate was the carbon source and there was no significant difference when malate or lactate was used. Interestingly, it was reported that no hydrogen was produced by *R. sphaeroides* ATCC 17023 using 30 mM of acetate. However, 0.45 l H_2/l culture was produced by PHB synthase deficient strain of this bacterium [25]. Therefore, it seems that PHB synthase deficiency may also impair acetate utilization in some cases.

In *R. capsulatus*, the acetate assimilation goes through a special pathway, named as citramalate cycle [26]. The pathway is similar, but more complex than the glyoxylate cycle. The process is independent of CO_2 fixation and PHB biosynthesis, which might explain the better performance of *R. capsulatus* in conversion of acetate to hydrogen.

Therefore, it can be concluded that the PHB synthesis and hydrogen production by nitrogenase compete with each other

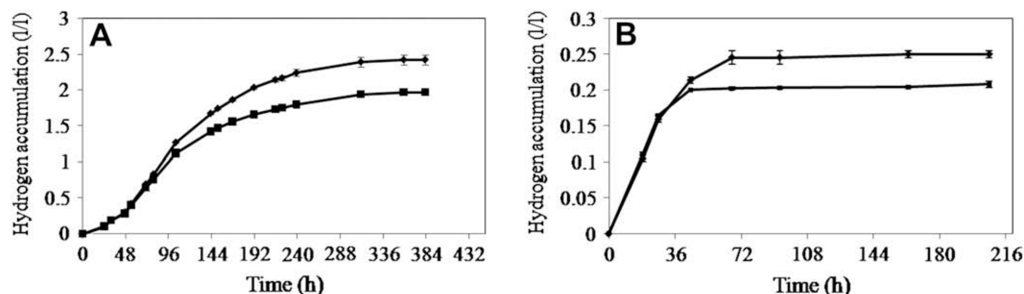

Fig. 3 – The hydrogen production by wild type (■) and *hup*$^-$ mutant (◆) *R. sphaeroides* O.U.001 in media containing 15 mM of malate (A) and 30 mM of acetate (B). Each value is the mean of three times replication with ±standard deviation.

Table 2 – The hydrogen production by three representatives of photofermentative bacteria *R. sphaeroides* O.U.001 and its *hup*⁻ strain *R. sphaeroides* ZK1, *R. capsulatus* B100 and its *hup*⁻ strain *R. capsulatus* ST410 and *R. palustris* R1 using malate and acetate as carbon sources.

Microorganism	Initial carbon source	H_2 production[a] (l H_2/l culture)	Yield[b] (g H_2/g substrate)	Substrate conversion efficiency[c] (%)	Reference
R. sphaeroides O.U.001	Malate, 15 mM	1.97	0.052	58	This work
R. sphaeroides ZK1	Malate, 15 mM	2.42	0.064	71.5	This work
R. capsulatus B100	Malate, 30 mM	2.1	n.a.[b]	73	[10]
R. capsulatus ST410	Malate, 30 mM	3.3	n.a.	46	[10]
R. palustris R1	Malate, 15 mM	1.05	n.a.	36	[2]
R. sphaeroides O.U.001	Acetate, 30 mM	0.21	0.009	4.6	This work
R. sphaeroides ZK1	Acetate, 30 mM	0.25	0.011	5.5	This work
R. capsulatus B100	Acetate, 30 mM	1.6	n.a.	53	[10]
R. capsulatus ST410	Acetate, 30 mM	2.5	n.a.	84	[10]
R. palustris R1	Acetate, 22 mM	0.37	n.a.	14.8	[2]

n.a., Not available.
a Volumetric hydrogen production. The volume of hydrogen produced/volume of culture.
b Produced mass of hydrogen/g substrate added to the medium.
c Percentage of stoichiometric maximum resulting from complete conversion of substrate to H_2 and CO_2.

for reducing equivalents. This competition is more pronounced in those bacteria which have the ethylmalonyl-CoA acetate assimilation pathway. Moreover, in the acetate assimilation pathway proposed for *R. sphaeroides* species, both the acetoacetyl-CoA reductase and crotonyl-CoA carboxylase/reductase are NADPH dependent enzymes, consequently they significantly reduce the hydrogen evolving capacity of the cells grown on acetate.

4. Conclusions

A *hupSL* loss of function mutant of *R. sphaeroides* was created by recombination based deletion mutagenesis technique in which neither antibiotic resistance gene nor any foreign DNA was inserted into the genome of *R. sphaeroides*. The strains were tested for hydrogen production in malate/glutamate and acetate/glutamate media. The *hupSL* deficient cells produced significantly more hydrogen than the wild type cells in both malate/glutamate and acetate/glutamate media. However, the cells had better hydrogen producing capacity using malate as carbon source than using acetate. Based on these results and previous works, it can be concluded that acetate alone does not support the hydrogen production efficiently in *R. sphaeroides*. This phenomenon is probably due to the special acetate assimilation pathway of this strain which has few unprofitable properties in view of hydrogen production, such as its strong linkage to CO_2 fixation and its overlap with PHB biosynthetic route. The elimination of PHB synthesis also does not seem to adequately improve the biohydrogen production by this strain. Therefore, additional carbon sources such as lactate or malate should be supplemented to a system where acetate is to be utilized by *R. sphaeroides* O.U.001. It was also recently reported that the maximum hydrogen production rates and substrate conversion efficiencies were improved for substrate mixtures compared to single substrate cases in *R. sphaeroides* O.U.001 [13]. Acetate might serve as carbon source for biomass production while the other substrates can be used for hydrogen production.

Acknowledgments

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