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PHOTOFERMENTATIVE PRODUCTION OF HYDROGEN BY *THIOCAPSA ROSEOPERSICINA* FROM SIMPLE ORGANIC SUBSTRATES

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H_2 is an ideal, clean and potentially sustainable energy carrier for the future due to its large energy content per weight, abundance and non-polluting nature. The selection of optimal H_2 production technology depends on the H_2-producing enzymes available. *Thiocapsa roseopersicina* contains a nitrogenase and several [NiFe] hydrogenases, which participate in H_2 metabolism. In the present study, H_2 production by the Hox1 soluble hydrogenase and the nitrogenase were investigated. The amount of H_2 evolved by the nitrogenase enzyme was much higher than the amount produced by the Hox1 hydrogenase enzyme. By comparing the H_2 production by nitrogenase from five short-chain organic acids (acetate, citrate, pyruvate, succinate, formate) the highest productivity of H_2 (~3 times) was observed in the presence of 4 g/l pyruvate. In this case, the pyruvate consumption was 100%, the biomass growth was equal to that of the control, therefore the produced H_2 derived from pyruvate.

Keywords: hydrogenase, nitrogenase, photofermentation, *Thiocapsa roseopersicina,* biohydrogen

Introduction

Biohydrogen can be produced by anaerobic micro-organisms and considered as a potential energy carrier of the future [1]. The anaerobic, non-photosynthetic bacteria decompose organic compounds (most frequently carbohydrates) into organic acids, carbon dioxide and H_2, but the subsequent anaerobic conversion of the organic acids is not feasible energetically in dark fermentation [2]. However, some photosynthetic bacteria manage to further exploit these organic acids, driven by solar energy due to the light-harvesting pigments found in the bacterial cell membrane. Being intimately linked to light energy, the process that makes possible the decomposition of organic acids into H_2 and carbon dioxide is called photofermentation [3].

Hydrogenase enzymes play pivotal role in photo-fermentative H_2 production as a biocatalyst of the reversible oxidation of molecular H_2. H_2 is also generated by nitrogenases, which catalyze the reduction of molecular nitrogen into ammonia and that is accompanied by the reduction of protons to H_2 [4, 5].

Photosynthetic bacteria have long been studied for their capacity to produce H_2 [1]. The nitrogenase based H_2 production in purple non-sulphur bacteria is the major field of research [6], while the study of nitrogenase mediated H_2 production in purple sulphur bacteria seems to be a novel approach.

Our model organism, *Thiocapsa roseopersicina* BBS is an anaerobic, purple sulphur phototrophic bacterium which contains at least four distinct and active [NiFe] hydrogenases: two membrane-bound (HynSL, HupSL) and two soluble (Hox1EFUYH, Hox2FUYH) enzymes as well as a nitrogenase enzyme [7, 8]. In this study, the H_2 productivity of the Hox1 soluble hydrogenase and the nitrogenase were compared. Moreover, the basic growth medium was supplemented with various organic carbon substrates in order to identify the ones the bacteria could use as electron source to produce H_2. The organic acids are utilized in diverse metabolic pathways. Electrons formed by biochemical reactions are transferred by cofactors as long as they get to the enzyme and become reduced to H_2.

Materials and methods

Bacterial strains and growth conditions

Mutant strains of *T. roseopersicina* BBS (wild type) used in this study were GB1121 (*ΔhynSL, ΔhupSL*) and M539 (*ΔhypF*) [9] for Hox1-mediated and nitrogenase-based H_2 production measurements, respectively. As negative control GB112131 (*ΔhynSL, ΔhupSL, Δhox1EFUYH*) and M539 (*ΔhypF*) under non-nitrogen fixing conditions were applied. HypF is an accessory

protein that is required for the biosynthesis of all active [NiFe] hydrogenases. All strains were grown anaerobically in liquid cultures with continuous illumination (35 µmol photons·m^{-2}·s^{-1} light intensity) at 25ºC in Pfennig's mineral medium (2% NaCl, 0.1% KH_2PO_4, 0.1% $MgCl_2$, 0.1% KCl, 0.1% NH_4Cl as nitrogen source, 0.2% $NaHCO_3$ as carbon source, 0.4% $Na_2S_2O_3$, 20 µg/µl vitamin B_{12}, 3.3 mg/l Fe-EDTA, 2.975 mg/l Na_2-EDTA, 0.3 mg/l H_3BO_3, 0.2 mg/l $CaCl_2$, 0.1 mg/l $ZnSO_4$, 0.03 mg/l $MnCl_2$, 0.03 mg/l Na_2MoO_4, 0.713 mg/l $NiCl_2$, 0.01 mg/l $CuCl_2$). For nitrogen-fixing conditions, the NH_4Cl was omitted.

The growth media were supplemented with various carbon sources (acetate, citrate, formate, pyruvate, succinate) and tested at different initial concentrations (2 g/l, 4 g/l, 6 g/l).

In vivo H_2 evolution activity measurements

Cultures (20 ml and 60 ml) were grown in 27 ml and 100 ml hypovials; the gas phase was flushed with N_2 after inoculation. The produced H_2 was determined daily by gas chromatograph [9] (Agilent Technologies 6890N equipped with Molesieve 30 m x 0.53 mm x 25 µm column and thermal conductivity detector; oven and detector temperature 160 °C, mobil phase: N_2).

Organic-acid analysis

In order to determine the residual organic acid concentrations in the culture medium, 1 ml of cell suspension was centrifuged at 13000 rpm for 10 min, and 50 µl of the supernatant was analyzed by HPLC (Hitachi Elite, equipped with ICSep ICE-COREGEL 64H column and refractive index detector L2490) using the following parameters: solvent 0.1 N H_2SO_4, flow rate 0.8 ml/min, column temperature 50 °C, detector temperature 41 °C.

Results and discussion

Biohydrogen production by Hox1 hydrogenase and nitrogenase enzymes

The H_2-evolving enzymes used by most biohydrogen evolving systems are nitrogenases and hydrogenases. In order to compare the H_2 productivity of the Hox1 [NiFe] hydrogenase and the nitrogenase enzymes in *T. roseopersicina*, two mutant strains were used.

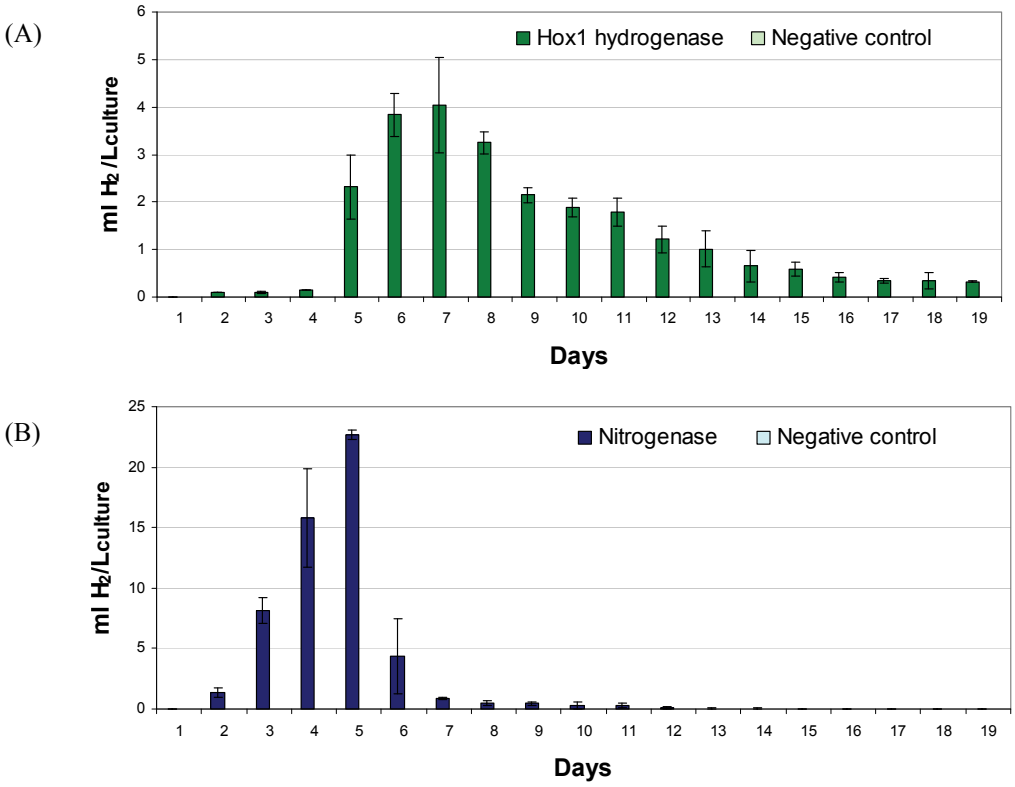

Figure 1: In vivo daily H_2 production by Hox1 hydrogenase (A) and by nitrogenase (B)

The quantity of the H_2 produced by Hox1 hydrogenase (GB1121 strain) and nitrogenase (M539 strain) enzymes was measured daily. It has to be noted, that although the GB1121 strain contains the genes encoding the Hox2 hydrogenase too, this enzyme does not evolve H_2 in the circumstances studied [8]. It was observed that the amount of H_2 produced by the nitrogenase enzyme under nitrogen-fixing conditions was much higher than the amount produced by the Hox1 hydrogenase enzyme under nitrogenase repressed conditions *(Fig. 1)*. However, almost no nitrogenase-based H_2 production was observed after the 7th day, while the Hox1 hydrogenase-based H_2 production lasted for two more weeks. Moreover, the daily removal of H_2 from the headspace of the culture

which produces H_2 via Hox1 hydrogenase resulted in a relatively high H_2 production rate (1.018 ml H_2/l culture/day). It has to be noted that, in all experiments, the strains used as negative control did not produce detectable amounts of H_2.

Substrates for biohydrogen production

Photosynthetic bacteria are able to produce H_2 from various reduced compounds; therefore, they are favourable substrates for biological H_2 production. The bacteria are capable of evolving H_2 from a wide range of organic acids, as well as from hydrogen sulphide, elemental sulphur or thiosulphate if CO_2 is supplied as carbon source. The conversion of different organic acids into H_2 – which would be advantageous for coupling clean, bioenergy production with organic waste-treatment – by different purple non-sulphur bacteria is well documented [10, 11], but little is known about the affinity of the purple sulphur bacteria to these organic acids. Therefore, by supplementing the growth medium (20 ml) with various organic acids, the conversion efficacies of these substrates by *T. roseopersicina* strains were tested. The experiments were repeated 3 times (except the asterisk labeled data in *Table 1*) using different initial concentrations of the substrates as listed in Table 1. The values given in Table 1 are the mean value of the data measured at day 9 of growth. The relative H_2 production refers to the H_2 produced by the same culture without supplementation. The H_2 produced by the cultures without supplementation in different runs are taken as 100%.

The data clearly show, that the Hox1 hydrogenase mediated H_2 production was negatively influenced by the organic acids present in the culture medium containing elevated amount of thiosulphate. However, at lower thiosulphate concentration, acetate driven hydrogen production could be observed (data not shown).

Supplementation of the nitrogen-fixing medium used for nitrogenase-based H_2 production with different organic acids resulted in higher H_2 production in case of the pyruvate and succinate. The increase of the initial concentration of pyruvate from 2 g/l to 4 g/l, increased the amount of H_2, but further increase of the substrate concentration to 6 g/l decreased the H_2 production. Additionally, almost all of the added pyruvate was consumed in the first two cases (2 g/l and 4 g/l), while in the third case (6 g/l) the substrate consumption was only 77% in the first 9 days of the experiment.

Similar results were found in case of supplementing the growth medium with succinate with the difference that the volumes of H_2 produced were less, and the substrate consumption rates were lower, too.

In the course of experiments, the bacterial growth was measured beside the H_2 production. The application of 2 and 4 g/l pyruvate did not result in higher biomass growth (8.65 g cell/l culture and 8.4 g cell/l culture, respectively) compared to the pyruvate-free control (8.8 g cell/l culture); therefore it is likely that the extra H_2 derived from the pyruvate. Nevertheless, in the case of 6 g/l pyruvate not only the biomass concentration was increased (18.5 g cell/l culture), but also the specific H_2 production was enhanced.

Table 1: Hydrogen production by hydrogenase (Hox1) and nitrogenase (N_2ase)

Organic compound	Initial conc. (g/l)	H_2 production (ml H_2/l culture)		Relative H_2 production (%)		Relative substrate consumption (%)	
		Hox1	N_2ase	Hox1	N_2ase	Hox1	N_2ase
Acetate	2	0.51	110	16	90	74	74
	4	0.59	49	9	68	54	53
	6	0.46*	-	17*	-	46*	-
Citrate	2	2.27	110	65	94	-36	-30
	4	4.60	76	60	97	13	18
	6	0.90*	-	33*	-	44*	-
Pyruvate	2	2.48	219	63	**185**	100	99
	4	0.55	251	9	**307**	85	97
	6	0.59*	227*	22*	**211***	97*	77*
Succinate	2	1.19	178	39	**151**	98	40
	4	2.31	214	30	**263**	71	32
	6	0.85*	226*	32*	**209***	70*	31*
Formate	2	0.60	62	17	53	39	34
	4	0.81	33	14	45	45	47

The effect of pyruvate addition on the nitrogenase-based biohydrogen evolution

In order to test the observation that in the case of nitrogenase, the extra H_2 was derived from the pyruvate, the same experiment was repeated with 2 g/l pyruvate in 60 ml culture volume. On day 13, when no H_2 production was observed, further pyruvate was added. During the experiments the total H_2 production, pyruvate concentration and the amount of biomass were measured daily. *Fig. 2A* illustrates the H_2 production with or without pyruvate and the variation of pyruvate concentration in time.

Visible improvement in the H_2 evolution was observed after supplementing with pyruvate, moreover the *in vivo* H_2 generation started at day 3, 2 days earlier than the control. This result is in agreement with the tendency of the bacterial growth *(Fig. 2B)*.

The supplementation with pyruvate enhanced the bacterial growth, but at day 9 both culture reached the same level.

Further addition of pyruvate to the culture in stationary phase (day 13) resulted in a lower rate increase of H_2 production, which is caused by the repression of nitrogenase due to the production of organic nitrogen compounds.

Remarkably, a 2-fold difference in the amount of H_2 produced by nitrogenase from 2 g/l pyruvate in 20 ml and 60 ml cultures (220 ml H_2/l culture and 420 ml H_2/l culture, respectively) was observed. The pyruvate-free control did not show any difference in 20 ml and 60 ml culture (98 ml H_2/l culture and 96 ml H_2/l culture, respectively). These results indicate that accumulation of H_2 in the headspace may have an inhibitory effect on biohydrogen production, which has to be considered in scale-up experimental design.

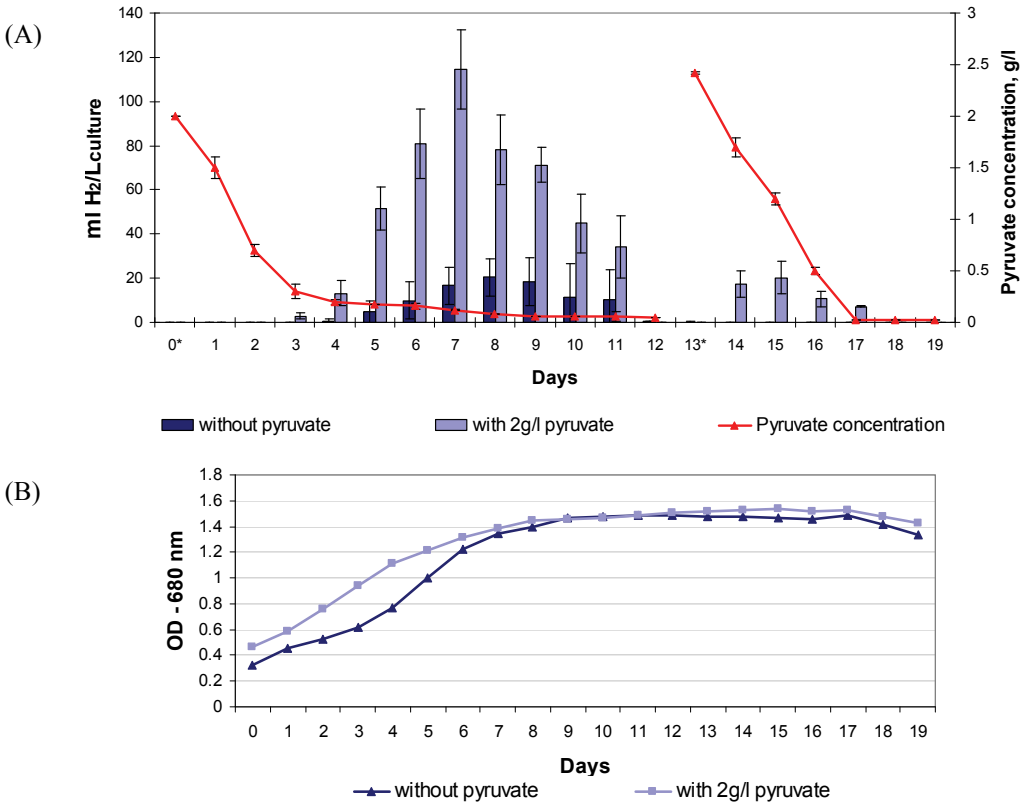

Figure 2: Effect of 2 g/l pyruvate on *in vivo* H_2 production by nitrogenase (A) and biomass growth (B)

In summary both nitrogenase and Hox1 hydrogenase can catalyze biohydrogen production from simple and inexpensive organic substrates in photoheterotrophic mode of growth. The overall H_2 yield generated by the nitrogenase system is higher, i.e., about 55 ml H_2/l culture compared to the 25 ml H_2/ l culture for Hox1. Nitrogenase evolves H_2 intensively in short term while Hox1 [NiFe] hydrogenase is capable of sustained *in vivo* biohydrogen production, which may have important ramification for large scale exploitation.

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