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A *Rhodopseudomonas palustris nifA** mutant produces H_2 from NH_4^+-containing vegetable wastes

Alessandra Adessi [a], *James B. McKinlay* [b,1], *Caroline S. Harwood* [b], *Roberto De Philippis* [a,c,*]

[a] *Department of Agricultural Biotechnology, University of Florence, Italy*
[b] *Department of Microbiology, University of Washington, Seattle, WA 98195, USA*
[c] *Institute of Chemistry of Organometallic Compounds (ICCOM), CNR, Florence, Italy*

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ABSTRACT

Research on photobiological H_2 production processes is pointing towards the use of low cost substrates as sources of reduced carbon for H_2 generation.

Those substrates (either wastewaters or effluents derived from other fermentation processes) are often rich not only in carbon, but also in fixed nitrogen. NH_4^+ is an inhibitor of nitrogenase-mediated H_2 production in purple non sulfur bacteria.

A *Rhodopseudomonas palustris* mutant strain (NifA*), which constitutively expresses nitrogenase genes, was utilized in order to test the use of NH_4^+ containing fermentation products for photobiological production of H_2. The strain was grown on both synthetic and waste-derived NH_4^+ containing media.

The *nifA** mutant produced H_2 in the presence of high concentrations of NH_4^+, both in a synthetic medium and in a real vegetable waste-derived medium resulting in higher H_2 levels than the wild-type strain. Thus, this study demonstrates that the NifA* strain is well suited to overcome the effects of inhibitory naturally occurring NH_4^+ as it converts agricultural waste into biofuel.

1. Introduction

The role of H_2 gas in the future energy market is expected to be crucial, as it is a clean energy vector [1]. When H_2 reacts with oxygen, energy and water are generated, with minimal emissions or even none when using fuel cells. It also has the highest gravimetric energy content of any potential fuel [2]. However, in order to consider H_2 as a sustainable energy vector it has to be produced via sustainable processes. None of the currently available renewable H_2 production technologies, such as photovoltaic-electrolysis or gasification of biomass, is economically affordable [3].

This is the reason for the widespread interest in investigating biological routes to H_2 production that involve microbial activity. Among the various opportunities, photofermentation is frequently cited as a promising process, as it has the potential to use waste material as a substrate source, thus combining the remediation of waste effluents with the simultaneous production of clean energy [4]. The use of wastes as substrates is one option to achieve an economically viable process.

Most research looking at the production of H_2 from food and vegetable waste has focused on dark fermentation [5]. However, fermentations produce large quantities of

* *Corresponding author*. Department of Agricultural Biotechnology, University of Florence, Piazzale delle Cascine 24, Firenze, Italy. Tel.: +39 0 553288284; fax: +39 0 553288272.
E-mail address: roberto.dephilippis@unifi.it (R. De Philippis).
[1] Department of Biology, Indiana University, Bloomington, IN 47405, USA.
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electron-rich products that cannot be fermented further into H_2. Purple non-sulfur bacteria (PNSB) can naturally convert fermented waste into H_2 using the enzyme nitrogenase. Thus, photofermentation often stands as a downstream process, following other fermentation processes where carbohydrates are converted to organic acids. Indeed PNSB can overcome the thermodynamic barrier of converting organic acids into H_2 by using light energy [6].

These materials, either wastewaters or effluents derived from other fermentation processes, are frequently rich not only in desired carbon sources but also in amino acids and other compounds that are broken down to NH_4^+. NH_4^+ acts at several levels of regulation to inhibit H_2 production by PNSB via nitrogenase.

The regulation has been modeled as a three-level control mechanism, as described by Masephol et al. [7] for *Rhodobacter capsulatus*. This regulatory cascade is often used as a general model for the regulation of nitrogenase in PNSB though there are many variations on this regulatory scheme. The general scheme is as follows: at the first level fixed nitrogen signals (e.g., NH_4^+) are sensed through the NtrBC two-component system to prevent the transcription of *nif*A, a gene encoding for an RNA polymerase sigma 54-dependent transcriptional activator. At the second level, the presence of NH_4^+ affects NifA, inducing structural changes that prevent the transcriptional activator from binding to its binding site and activating nitrogenase (*nif*) gene transcription. At the third level, the presence of NH_4^+ affects nitrogenase itself causing a "switch-off" of the enzyme through ADP-ribosylation mediated by DraT [7–10].

Recently Redwood et al. [6] and Kars and Gunduz [11] reviewed the use of mutants insensitive to NH_4^+, indicating that genetic manipulation is a potential route to overcome nitrogenase inhibition by fixed-nitrogen-containing compounds including NH_4^+. A number of strains bringing mutations in *nif*A have been obtained [8,12], all bringing single point mutations. The *nif*A* phenotype of the strain used in this study is attributed to a 48 nucleotide deletion in *nif*A, thus it is expected to more stable in a practical setting than mutants with single nucleotide changes.

The aim of this study was to use a *Rhodopseudomonas palustris nif*A mutant strain that is insensitive to NH_4^+, for phototrophic conversion of substrates in vegetable waste to H_2.

2. Materials and methods

2.1. Bacterial strains, growth media and culture parameters

All experiments were conducted with *Rp. palustris* strains CGA009 and its derivative NifA* mutant strain CGA676 constructed as described [9]. The strain CGA009 is defective in uptake hydrogenase activity [13]. CGA676 has constitutive nitrogenase activity allowing it to produce H_2 in the presence of NH_4^+ [9]. *Rp. palustris* was grown anaerobically in front of a 60-W light bulb at 30 °C in 16 mL volumes in sealed 27-mL anaerobic culture tubes (Bellco) with an argon (Ar) headspace. Cultures were grown in PM mineral medium [14] with $(NH_4^+)_2SO_4$ at the concentration indicated in the text, under an Ar headspace, or with vegetable waste derived (VWD) medium (described below) at dilutions and with additions as indicated in the text. Sodium acetate was supplied at a final concentration of 20 mM, when using PM mineral medium.

The VWD medium was obtained by the spontaneous fermentation of vegetable residues carried out by the microflora residing on vegetables, as previously described [15].The main products contained in the fermentation broth, were: acetic acid (2.19 g L^{-1}; 36.4 mM), lactic acid (7.71 g L^{-1}; 85.6 mM) and NH_4^+ (110 mg L^{-1}; 6.1 mM).

Before being used for the experiments with PNSB, the VWD medium was (where indicated) diluted with distilled water; it was supplemented with Fe(III) as ferric-citrate (5 mg L^{-1}) and the pH was adjusted from 3.2 to 6.8 with NaOH as previously described in [16]. After H_2 production stopped SO_4^{2-} was added to VWD medium in the form of Na_2SO_4 (0.124 mM, as in NF medium [17]), and Fe (III) was added in the form of ferric-citrate; this addition of citrate resulted in only in a small addition of carbon (0.020 mM of citrate) in comparison with the concentrations of acetate and lactate present in the VWD medium.

2.2. Analytical methods

H_2 was quantified by gas chromatography as indicated in [18]. Organic acids and culture supernatants were analyzed by HPLC as in [18].

The NH_4^+ content of VWD medium was determined with Nessler method [19]. The concentration in the various dilutions was estimated to be half and a third respectively for the two and the three fold dilutions.

Cell growth was quantified by optical density at 660 nm; as a blank, the medium without cells was used for the tests with VWD medium (Abs_{660} undiluted 0.77; two-fold diluted 0.39; three fold diluted 0.27). A calibration curve for optical density (OD) versus dry weight (d.w.) was built ($R^2 = 0.97$) and the equation resulted to be d.w. = 462.4*OD.

The intensity of the light reaching the front of the culture tubes was 35 μmol (photons) m^2 s^{-1}, measured using a quantum meter (Spectrum Technologies, Plainfield, IL, USA).

2.3. Substrate conversion and photosynthetic efficiency

The conversion yield of the carbon substrate to H_2 was calculated as the percentage of the maximum amount of H_2 theoretically obtainable from the complete conversion of the substrate consumed to H_2 that was actually produced as H_2. As the main components of the VWD medium were lactate and acetate, the theoretical reactions used were (equations (1)–(2)):

$$C_3H_6O_3 + 3H_2O \rightarrow 6H_2 + 3CO_2 \quad (1)$$

$$C_2H_4O_2 + 2H_2O \rightarrow 4H_2 + 2CO_2 \quad (2)$$

The photosynthetic efficiency (PE), i.e. the efficiency of conversion of light energy to H_2 energy, was calculated assuming that all the incident light was absorbed by the culture; the following equation (3), based on [20] was applied:

$$PE\% = \frac{\text{Combustion enthalpy of } H_2 \times H_2 \text{ production}}{\text{Absorbed light energy}} \times 100 \quad (3)$$