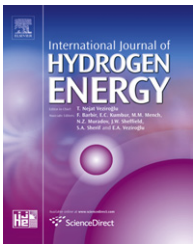
Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/he



# Hydrogen-producing purple non-sulfur bacteria isolated from the trophic lake Averno (Naples, Italy)

*Lucia Bianchi, Francesca Mannelli, Carlo Viti, Alessandra Adessi, Roberto De Philippis**

*Department of Agricultural Biotechnology, University of Florence, Piazzale delle Cascine 24, I 50144 Florence, Italy*

ARTICLE INFO

*Article history:*
Received 29 June 2010
Received in revised form
5 August 2010
Accepted 7 August 2010
Available online 15 September 2010

*Keywords:*
Hydrogen production
Photofermentation
Purple non-sulfur bacteria
Trophic lake
Poly-β-hydroxybutyrate
Vegetable residues

ABSTRACT

Seventeen purple non-sulfur bacterial strains, isolated from the trophic lake Averno, Naples, Italy, were phylogenetically classified and their $H_2$-producing performances were tested utilizing various synthetic substrates and the fermentation broth derived from the spontaneous fermentation of vegetable residues. All the strains showed the capability to produce hydrogen on at least one of the four carbon substrates tested (malic, lactic, acetic and succinic acid). On lactate, *Rhodopseudomonas palustris* strain AV33 showed the best maximum production rate (50.7 ± 2.6 mL ($H_2$) $L^{-1}$ $h^{-1}$), with a mean rate, calculated on the whole period of production, of 17.9 mL ± 0.7 ($H_2$) $L^{-1}$ $h^{-1}$. In the presence of acetate, AV33 produced only few mL of $H_2$, but intracellularly accumulated poly-β-hydroxybutyrate up to a concentration of 21.4 ± 3.4% (w/w) of cell dry weight. *Rp. palustris* AV33 also produced $H_2$ on the fermentation broth supplemented with Fe, with a maximum production rate of 16.4 ± 2.3 mL ($H_2$) $L^{-1}$ $h^{-1}$ and a conversion yield of 44.2%.



## 1. Introduction

Biological hydrogen production is generally considered a promising process owing to both its low impact on the environment and the possibility to use organic wastes as substrate for the production of the gas [1,2].

Amongst the microorganisms that can be utilized for biological hydrogen production, purple non-sulfur bacteria (PNSB) are generally considered as good candidates, being characterized by a very versatile metabolism [3,4] and by a high substrate to $H_2$ conversion efficiency [1]. This feature can be profitably exploited for producing $H_2$ from organic wastes or from agricultural residues [5–8], thus making this process economically more favourable. Furthermore, PNSB are also capable to produce poly-β-hydroxybutyrate (PHB) [9,10], a valuable by-product which is a biodegradable thermoplastic having industrial and medical interest [11], even if it was demonstrated a competition between the metabolic pathways involved in the production of $H_2$ and PHB [9,12].

In order to make the process of biological hydrogen production economically feasible, PNSB strains capable of efficiently utilizing the reducing equivalents coming from the oxidation of the organic substrates and the energy coming from the solar light for synthesizing hydrogen are needed. Thus, an intensive effort for searching new PNSB strains in environments suitable for harbouring this kind of microorganisms is highly desirable.

PNSB are widely distributed in nature in all the aquatic environments characterized by low oxygen concentration and by the availability of light and soluble organic matter [13]. These very common environmental conditions and the extreme metabolic versatility of PNSB bacteria have as consequence that these bacteria are widely distributed not only in freshwater and marine habitats but also in sediments

* *Corresponding author*. Tel.: +39 0553288284; fax: +39 0553288272.
E-mail address: roberto.dephilippis@unifi.it (R. De Philippis).
0360-3199/$ – see front matter © 2010 Professor T. Nejat Veziroglu. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.ijhydene.2010.08.038

and moist soils. However, the largest number of PNSB species has been found in eutrophic lakes, both in the water column and in the sediments [13].

Averno Lake is a trophic lake located in a volcanic basin in the area named *Campi Flegrei*, close to Naples (Italy). It is occasionally polluted by urban waste waters coming from the overflows of the pipelines connecting the city sewer system to the local depuration plant [14]. The lake presents stratification during the warmer season, whereas the water column is occasionally overturned in winter by the cooling of epilimnion due to weather conditions [15]. When the stratification occurs, it is possible to discriminate three zones through the water column, an upper aerobic zone in the first 6 m, a second zone (from −6 to −15 m) characterized by a deep decrease of oxygen concentration and a third anaerobic zone (from −15 to the bottom of the lake) with high concentration of $H_2S$, $CO_2$ and $CH_4$ and strongly affected by anoxic microbial activity both in the water column and in the sediments [15]. In particular, this third zone, characterized by high concentrations of organic substances, anoxic conditions and a residual amount of light sufficient for phototrophs, was considered to be an environment suitable for harbouring PNSB capable of efficiently utilizing the low MW fatty acids, produced by the anaerobic chemotrophic microbial community, for producing $H_2$.

This research was aimed at finding, in the water column and in the sediments of the trophic Averno Lake, new PNSB strains possessing good hydrogen producing properties. The newly isolated strains were phylogenetically classified and their $H_2$-producing performances were tested utilizing various synthetic substrates and the fermentation broth derived from the spontaneous fermentation of vegetable residues.

## 2. Materials and methods

### 2.1. Sampling and isolation procedures

The samples were withdrawn from the Averno Lake (geographical coordinates: 40°50′18″N 14°04′30″E) at nine different depths of the water column (−1, −3, −5, −6, −9, −15, −21, −27, −32 m) and from the sediment, at −33 m, using a Niskin bottle. The samples were shacked in tubes containing the enrichment medium (RPN, see below). In order to create anaerobic conditions, the tubes were incubated at 30 °C in the darkness, thus favoring the consumption of the oxygen by PNSB. After 1 h, the tubes were exposed to a continuous light with an intensity of 20 μmol (photons) $m^{-2}sec^{-1}$. After 24 h, the light intensity was increased to 40 μmol (photons) $m^{-2}sec^{-1}$. The enriched cultures that showed, after four days of incubation, a red or pink pigmentation were serially diluted and the PNSB were isolated by the streak plate method. The plates were aerobically incubated at 30 °C for 1 h in the dark and then anaerobically under a light intensity of 20 μmol (photons) $m^{-2}sec^{-1}$, for 24 h, and of 40 μmol (photons) $m^{-2}\,s^{-1}$ for the following days. After six to ten days incubation, the red colonies formed were individually transferred into screw cap tubes filled with RPN nutrient medium and incubated as above reported.

### 2.2. Cultivation media

The cultures were carried out in RPN medium containing (g $L^{-1}$): DL-malic acid, 2.0; $NH_4Cl$, 0.5; $K_2HPO_4$, 0.5; $KH_2PO_4$, 0.3; $MgSO_4.7H_2O$, 0.4; NaCl, 0.4; $CaCl_2.2H_2O$, 0.075; Ferric citrate, 0.005; yeast extract, 0.4. Trace elements were provided by adding 10 mL per liter of a solution containing (mg $L^{-1}$): $ZnSO_4.7H_2O$, 10; $MnCl_2.4H_2O$, 3; $H_3BO_3$, 30; $CoCl_2.6H_2O$, 20; $CuCl_2.2H_2O$, 1; $NiCl_2.6H_2O$, 2; $Na_2MoO_4.2H_2O$, 30. The pH of the medium was adjusted at 6.8 with NaOH before autoclaving.

The hydrogen production medium (RPP) contained (g $L^{-1}$): organic substrate (malic acid, 4.0, lactic acid, 3.6, acetic acid, 3.6, or succinic acid, 3.6, alternatively used); Na glutamate, 0.4; $K_2HPO_4$, 0.5; $KH_2PO_4$, 0.3; $MgSO_4.7H_2O$, 0.4; NaCl, 0.4; $CaCl_2.2H_2O$, 0.075; Ferric citrate, 0.005. The trace elements were added as above reported. The vitamins necessary for bacterial growth were supplied as a solution (1 mL per liter of medium) containing (mg per 100 mL): biotin, 10; niacin, 35; thiamine dichloride, 30; p-aminobenzoic acid, 20; pyridoxolium hydrochloride, 10; Ca-pantothenate, 10; Vitamin B12, 5.

The fermentation broth was obtained by the spontaneous fermentation of vegetable residues carried out by the microflora residing on the vegetables, as previously described [6]. The main products contained in the fermentation broth, depending on the stock, were: acetic acid (in the range 2.0–3.0 g $L^{-1}$), lactic acid (in the range 8.0–9.0 g $L^{-1}$) and ammonia (80–100 mg $L^{-1}$). Before being utilized for the experiments with PNSB, the fermentation broth was diluted 1:3 with distilled water and the pH was adjusted from 3.2 to 6.8 with NaOH.

### 2.3. Enzymatic amplification of 16S rDNA

Bacterial isolates were identified by 16S rDNA sequencing. Total DNA was extracted from cultures anaerobically grown on solid media under continuous light for six–seven days.

DNA was extracted from samples by using the Instagene matrix according to the manufacturer's protocol (Instagene DNA Matrix, Bio-Rad Laboratories, USA). PCR amplification of 16S rDNA was performed under the same conditions for all strains using universal primers: primer forward 27 and primer reverse 1392. PCR amplicons were directly sequenced, forward and reverse, with their respective oligonucleotide primers. Sequencing was performed by the BMR Genomics (Padova, Italy). 16S rDNA sequences were compared with sequences of 9 validly described PNSB species from the NCBI database GenBank. These species and their accession numbers are: *Rhodobacter capsulatus* ATCC 11166 (D16428); *Rhodobacter blasticus* ATCC 33485 (DQ342322); *Rhodopseudomonas palustris* ATCC17001 (D25312); *Rhodobacter sphaeroides* ATCC 17023 (DQ342321); *Rhodospirillum rubrum* ATCC 11170 (D30778); *Rhodobacter azotoformans* KA 25 (D70846); *Rhodovulum sulfidophilum* ATCC 35886 (DQ342323); *Rhodospirillum molischianum* ATCC 14031 (M59067); *Rhodospirillum salinarum* ATCC 35394 (M59069).

Sequences were aligned using Clustal X (Version 1.81) and dissimilarities were converted to evolutionary distances according to Jukes and Cantor [16]. The construction of neighbouring joining trees and bootstrap analysis of 1000 resamplings was performed using the software package TREECON for Windows Version 1.3b [17], including *Escherichia coli* (K02555) 16S rDNA as the single-sequence (forced) outgroup.