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Vegetable waste as substrate and source of suitable microflora for bio-hydrogen production

Antonella Marone [a,*], Giulio Izzo [a], Luciano Mentuccia [a], Giulia Massini [a], Patrizia Paganin [b], Silvia Rosa [a], Cristiano Varrone [a], Antonella Signorini [a]

[a] ENEA-Italian Agency for New Technologies, Energy and Sustainable Development, Dep. of Renewable Energy Sources, Laboratory of Biomass and Bioenergy, Rome, Italy
[b] ENEA-Italian Agency for New Technologies, Energy and Sustainable Development, Technical Unit for Sustainable Development and Innovation of Agroindustrial System, Rome, Italy

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ABSTRACT

Self-fermentation of cellulosic substrates to produce biohydrogen without inoculum addition nor pretreatments was investigated. Dark fermentation of two different substrates made of leaf-shaped vegetable refuses (V) and leaf-shaped vegetable refuses plus potato peels (VP), was taken in consideration. Batch experiments were carried out, under two mesophilic anaerobic conditions (28 and 37 °C), in order to isolate and to identify potential H_2-producing bacterial strains contained in the vegetable extracts. The effect of initial glucose concentration (at 1, 5 and 10 g/L) on fermentative H_2 production by the isolates was also evaluated.

H_2 production from self-fermentation of both biomasses was found to be feasible, without methane evolution, showing the highest yield for V biomass at 28 °C (24 L/kg VS). The pH control of the culture medium proved to be a critical parameter. The isolates had sequence similarities ≥98% with already known strains, belonging to the family *Enterobacteriaceae* (γ-*proteobacteria*) and *Streptococcaceae* (*Firmicutes*). Four genera found in the samples, namely *Pectobacterium*, *Raoultella*, *Rahnella* and *Lactococcus* have not been previously described for H_2 production from glucose. The isolates showed higher yield (1.6 −2.2 mol H_2/mol $glucose_{added}$) at low glucose concentration (1 g/L), while the maximum H_2 production ranged from 410 to 1016 mL/L and was obtained at a substrate concentration of 10 g/L. The results suggested that vegetable waste can be effectively used as both, substrate and source of suitable microflora for bio-hydrogen production.

1. Introduction

Being a sustainable energy carrier, hydrogen is considered to be the fuel of the future, mainly due to its high energy yield (122 kJ/g), recyclability and non polluting nature. Among the methods for its production, dark fermentation of organic wastes seems to be the most sustainable and environmentally friendly one, as it could solve both the problem of clean energy production and waste disposal [1]. Organic waste self-decomposes, if left at ambient temperature for prolonged time, because of the presence of abundant indigenous microflora, including hydrogen-producing bacteria [2]. Despite, at present only few studies reported bio-hydrogen production from wastes self-fermentation. According to literature overview [2–21], biological hydrogen production by self-fermentation is defined as a heterotrophic anaerobic fermentation of waste without inoculum addition, regardless of whether the substrate/inoculum is subjected to chemical and physical treatments. In most of the published researches, heat pretreatments (including sterilization) of substrate/inocula were performed to improve hydrolysis process, to select H_2-producing community and to kill non spore-forming microorganism (mainly methanogens). However, these pretreatments also eliminate useful microorganisms, like facultative anaerobic H_2-producing bacteria, that can promote hydrogen production by maintaining a strict anaerobic environment and by breaking down complex substrates [22]. Ohnishi and colleagues [3], for instance, characterized a fermentative bacterial community of leaf litter cattle compost able to produce hydrogen from food waste. Neither inoculum nor substrate underwent pretreatment or active control of anaerobic condition.

* Corresponding author. ENEA-Italian Agency for New Technologies, Energy and Sustainable Development, Dep. of Renewable Energy Sources, Laboratory of Biomass and Bioenergy, C.R. Casaccia, Via Anguillarese 301, 00123 Rome, Italy. Tel.: +39 0630486343; fax: +39 0630484554.
E-mail addresses: antonella.marone@enea.it, antonella.marone1980@gmail.com (A. Marone).

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The results showed that *Megasphaera elsdenii* was the dominant H_2-producing bacterium and lactic acid-producing bacteria (LaB) were prevalent. In particular *Lactobacillus* spp proved to consume oxygen and produce lactate, while *M. elsdenii*, an anaerobic non spore-forming gram-negative bacterium, produced hydrogen by using lactate. Obviously, both strains would have been lost, in case of heat shock-pretreatment. Bearing in mind that an ideal reactor design should consider removing, as much as possible, oxygen from feedstock, before it enters the bioreactor (and considering the size of industrially feasible operations), the co-metabolism within the microbial community for oxygen depletion was not only advantageous, but played a pivotal role in bio-hydrogen production [22].

It is also worth noting that natural selection of desirable native species can be carried out by controlling the reactor operating conditions such as pH, temperature, hydraulic retention time and organic loading rate, both in batch and in continuous mode [4–7,23]. However, so far only few studies on H_2 production by self-fermentation, without pretreatments, have been reported. To our knowledge, Yokoyama et al. [5] were the first authors that implemented this practice, cultivating cow waste slurry at various temperatures, without inoculum addition nor pretreatments. The highest H_2 production yield by self-fermentation (137.2 mL H_2/g VS) was obtained from food waste in thermophilic condition (50 °C) [21]. Nevertheless, Perera and colleagues [24] showed that negative net energy balance was achieved when the fermentation temperature exceeded 30 °C, thus underlining the importance of enhancing the efficiency of mesophilic processes.

Therefore, this study had two main objectives. First, to investigate the feasibility of biological H_2 production by dark self-fermentation of common domestic vegetable waste, without lengthy and costly pretreatments. To this purpose two mesophilic temperature regimes were tested: below and above 30 °C (28 and 37 °C, respectively). The addition of chemicals was limited to buffer compounds, to reduce the large pH fluctuations during the initial growth period [25] and to contrast the pH decrease due to the accumulation of acids during fermentation. In this view, the efficiency of KH_2PO_4/Na_2HPO_4, 0.1 M buffer was tested and the H_2 production performances were compared with those obtained without buffer.

The second objective was to explore the diversity of cultivable H_2-producing bacteria in waste extracts under mesophilic anaerobic conditions (28 °C). The effect of initial glucose concentration (1–5–10 g/L) on fermentative H_2 production by the isolates was also evaluated.

2. Materials and methods

2.1. Feedstock preparation and culturing media

Vegetable waste was collected from a cafeteria at the ENEA Casaccia Research Centre, Rome. Two different types of feedstock were utilized: leaf-shaped vegetables refuses (V) and a mixture (wet mass) composed by 80% of leaf-shaped vegetables and 20% of potato peels (VP). The mixture composition reflected the average weekly production of these wastes in the cafeteria. The total solid (TS), volatile solid (VS), Chemical Oxygen Demand (COD expressed as g O_2/TS and g O_2/g Wet Weight) and the heating value (kJ/g Wet Weight) of the substrates were estimated, according to standard methods [26].

Basal Fermentation Medium (BFM) was used for isolation and batch H_2 production experiments from glucose. It consisted of (g/L): peptone 3; yeast extract 1; $FeSO_4 \cdot 7H_2O$ 0.1; $MgCl_2$ 0.1; 10 mL mineral salt solution (g/L: $MnSO_4 \cdot 7H_2O$ 0.01; $ZnSO_4 \cdot 7H_2O$ 0.05; H_3BO_3 0.01; $CaCl_2 \cdot 2H_2O$ 0.01; Na_2MoO_4 0.01; $CoCl_2 \cdot 6H_2O$ 0.2; $AlK(SO_4)_2 \cdot 12H_2O$ 0.01; $NiCl \cdot 6H_2O$ 0.001) and 5 mL vitamin solution (g/L: cobalamin 0.01, vitamin C 0.025; riboflavin 0.025; citric acid 0.02; pyridoxal 0.05; folic acid 0.01; creatine 0.025) dissolved in 1 L of KH_2PO_4–Na_2HPO_4 buffer (final concentration 0.1 M pH 6.70) [27]. After sterilization of the medium (121 °C for 15 min), mineral salts, vitamins and glucose, previously sterilized by filtering with 0.22 μm filter, were added. Agar 15 g/L, was added for isolation plates.

Preculture basal medium was used to revive the bacterial strains as reported in Pan et al. [27].

2.2. H_2 production by self-fermentation

Batch experimental trials were carried out to examine H_2 production from non pretreated vegetable waste, using the microflora naturally present within the waste. The experiments were carried out in 500 mL reactors, with a working volume of 250 mL, incubated anaerobically at 28 and 37 °C, with magnetic stirring (120 rpm). V and VP wastes were crumbled to less than 2 cm particle size and were diluted a) using 0.1 M phosphate buffer (KH_2PO_4–Na_2HPO_4) pH 6.70, and b) without buffer, using tap water, pH 7.00, for a final 0.4 w/v ratio. The experiments were performed in duplicate.

During fermentation, the total amount of biogas produced (mL) and biogas composition (%) were determined. At the end of fermentation the composition of soluble products was analyzed.

2.3. Isolation and selection of H_2-producing bacteria

The isolation was performed on two extracts obtained by homogenizing 1 g of the samples V and VP in sterile saline solution (0.9% v/v). Serial dilutions of the two extracts were plated on agar BFM (glucose 10 g/L). Plates were incubated at 28 °C for 48 h in an anaerobic jar. Single colonies, showing different morphologies, were picked up from plates of 10^3, 10^4, 10^5-fold dilutions, in an anaerobic chamber. Each colony was re-streaked on fresh plates more than three times to ensure the purity of the isolates. Bacterial cells were picked up from the surface of BFM plates and aseptically transferred in 25 mL serum bottles containing 10 mL of BFM liquid medium (glucose 10 g/L). Cultures were incubated at 28 °C.

Bacterial isolates exhibiting H_2 production activity ($\geq$0.2 mol H_2/glucose$_{added}$) were selected for further studies and cryopreserved in glycerol (30% v/v) at −80 °C until use.

2.4. 16S rRNA gene sequencing and analysis

DNA of each selected bacterial isolate was prepared by lysis of 2–3 colonies grown overnight on BFM plates, according to the procedure described by Vandamme et al. [28].

PCR amplification of the 16S rRNA gene was carried out using eubacterial universal primers 27f (5′-GAGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-CTACGGCTACCTTGTTACGA-3′) with 2 μL of each cell lysate suspension in 20 μL of Quiagen Taq buffer, with 0.5 U of Taq DNA polymerase, as reported by [29]. PCR products were purified using the Sephadex™ G-100 resin, according to the supplier's instructions, and quantitatively analyzed using the spectrometer Thermo Scientific NanoDrop™.

Sequencing reactions were prepared using the sequencing kit Applied Biosystem Big Dye Terminator® version 3.1, according to the manufacturer's instructions, and analyzed with an ABI PRISM 310 Genetic Analyzer Perkin-Elmer, at the ENEA Genome Research Facility DNA Sequencing Laboratory (GENECHRON, ENEA C.R. Casaccia, Italy). Thermal cycling was performed with a gene Amp PCR System 9700 instrument (Applied Biosystems).

Sequence similarity searches were performed using the BLAST network service of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and taxonomic analysis of 16S rRNA gene sequences was performed using the RDP Classification Algorithm (http://rdp.cme.msu.edu/classifier/classifier.jsp). Sequences obtained in this