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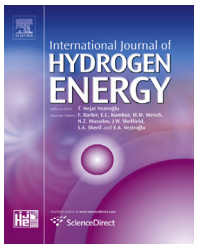

Role of homo-and heterofermentative lactic acid bacteria on hydrogen-producing reactors operated with cheese whey wastewater

Bruna Carrer Gomes [a], *Paula Rúbia Ferreira Rosa* [b], *Claudia Etchebehere* [c], *Edson Luiz Silva* [b], *Maria Bernadete AmâncioVaresche* [a,*]

[a] *Department of Hydraulics and Sanitation, Laboratory of Biological Processes, Engineering School of São Carlos – University of São Paulo (EESC – USP), Campus II, Av. João Dagnone, 1100, CEP 13563-120 São Carlos, SP, Brazil*

[b] *Department of Chemical Engineering, Federal University of São Carlos, Rod. Washington Luis, km 235, CEP 13565-905 São Carlos, SP, Brazil*

[c] *Laboratorio de Ecología Microbiana, Instituto Clemente Estable, Microbiología, Facultad de Química y Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay*

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ABSTRACT

The prevalence of lactic acid bacteria (LAB) and the effects of their antimicrobial peptides on H_2 production in anaerobic fluidized bed reactors (AFBRs) operated with cheese whey (AFBR1 and AFBR2) were verified in this study. The AFBR1 received 5 g COD L^{-1} of cheese whey with decreasing hydraulic retention times (HRT) of 14 to 8 h. The AFBR2 was operated with 3–10 g COD L^{-1} of cheese whey with an HRT of 6 h. Next, 152 colonies were selected from de Man, Rogosa and Sharpe MRS agar plates, and 45 strains were classified as LAB. The counts oscillated between 6.6 and 8.1 log CFU mL^{-1}, indicating that the LAB survived and persisted in the AFBRs. Pure cultures were identified using 16S rRNA gene sequencing, and *Lactococcus lactis* was the prevalent LAB (70%) in both reactors. The highest H_2 yields (1.9 and 2.3 mol H_2 mol lactose^{-1}) were obtained during the first operational phase in both reactors when a low organic loading rate (OLR) was applied, and when the growth of *Lactococcus* spp. was associated with *Leuconostoc pseudomesenteroides*. The bacteriocin-producing LAB (mostly *Lactobacillus* spp.) found on the specific phases of reactors AFBR1 and AFBR2 exerted a remarkable influence on H_2 yield.

Introduction

Hydrogen (H_2) is considered an energy carrier of the future as an alternative to conventional fossil fuels [1]. The use of mixed cultures for H_2 production by dark fermentative processes without substrate sterilization is advantageous because these cultures are widely available, resulting in greater H_2 yields and the co-production of metabolites of commercial interest [2–4].

It is accepted that *Clostridium* spp. and *Enterobacter* spp. are the main hydrogen producers in fermentative consortia [5]. However, the presence of other non-spore-forming bacteria, such as propionate bacteria and lactic acid bacteria (LAB), has been suggested to retard hydrogen production due to

* *Corresponding author*. Tel.: +55 16 33738356.
E-mail addresses: bruna.carrer@gmail.com (B.C. Gomes), varesche@sc.usp.br (M.B. AmâncioVaresche).
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competition for carbohydrates and the production of antimicrobial peptides [6–9]. Bacteriocins are antimicrobial peptides that interact with target cells through specific receptors, leading to the permeabilization of the cytoplasmic membrane, the dissipation of the proton motive force, cell lysis, inhibition and even the degradation of biological molecules [10–12]. Bacteriocins produced by LAB are widely used in the food industry due to their known efficacy against the spore-forming bacteria *Clostridium and Bacillus* [13,14].

LAB are gram-positive, catalase-negative cocci or rods with ability to produce lactic acid as the main products of carbohydrate fermentation, and naturally dominate the microbiota in many foods during storage [1,15].

Brazil is among the major milk producers worldwide with an annual production of approximately 33 billion liters [16]. Cheese whey is considered one of the most important and valuable by-products generated in the dairy industry, with a high organic load and chemical oxygen demand (COD) of 25–80 g L^{-1} due to its elevated concentrations of lactose and fat [16,17]. The use of cheese whey for fermentative hydrogen production brings economic advantages to the process, since it is considered an inexpensive effluent rich in carbohydrates, besides reducing the deleterious effect of such residual water to the environment [18,19]. However, it is widely known that LAB are part of the autochthonous microbiota of this wastewater [1,7,20].

Moreover, to the best of our knowledge, no study has enumerated viable LAB cells in fermentative reactors throughout the operational fermentative process. Considering the need to improve the systems for obtaining biohydrogen from organic residues in anaerobic reactors, the present study isolated and identified LAB throughout the operational process of two anaerobic fluidized bed reactors (AFBRs) fed with cheese whey for H_2 production. The effects and prevalence of this community was correlated with the H_2 yield in the reactors. The LAB was evaluated regarding their potential for producing antimicrobial peptides (bacteriocins).

Materials and methods

Sampling

The AFBR1 and AFBR2 were operated over 160 days and ten effluent samples (end of operational phases) and one initial inoculum sample were included in the study. The cheese whey powder was supplied by Cargill Agrícola S/A (Brazil). Additionally, on the last operational day, samples of the adhered cells of biomass on polystyrene particles were analyzed.

Operational conditions

The operational conditions of AFBRs are described in Table 1. The cheese whey was supplemented with micronutrients [21]. Both reactors were inoculated with a heat-treated sludge (10% v/v) from a UASB reactor used to treat swine wastewater and were operated at 30 °C [22]. The pH of the effluent was periodically monitored and kept between 4.0 and 4.5 using sodium bicarbonate. Nitrogen gas was used for sparging the fermentation medium to create an anaerobic environment. Steady state conditions were attained when the gas production rate did not change with time (Table 1).

Table 1 – Operational conditions of AFBRs fed with cheese whey.

Reactor	Phase	HRT (hours)	Substrate (g COD L^{-1})	OLR (kg m^{-3} d^{-1})
AFBR1	I	14	5.0	8.5
	II	12	5.0	10
	III	10	5.0	12
	IV	8	5.0	15
AFBR2	I	6	3.0	12
	II	6	5.0	20
	III	6	7.0	28
	IV	6	10.0	40

Analytical methods

The H_2 in biogas was measured by gas chromatography (Shimadzu GC-2010) as previously described [22] and the volumetric hydrogen production was measured using a Ritter MilliGas-Counter (Type: MGC-1) [22]. The acid and alcohol contents were determined using a liquid chromatograph (HPLC Shimadzu) [23]. The total sugar concentration was determined using the phenol-acid spectrometric method [24].

Isolation and enumeration of lactic acid bacteria

The polystyrene particles sampled in the last operational day were rinsed with 20 mL of sterile phosphate buffered saline (PBS) to remove the non-adherent cells. Adhered cells were removed by successive manual agitation (5 times) using 10 mL of PBS [25]. Next, 10 mL of the supernatant was homogenized with sterile peptone water 0.1% (w/v) for 1 min. The effluent samples (10 mL) were homogenized with 90 mL of sterile peptone water 0.1% (w/v) for 1 min. Aliquots of 100 μL of each 10-fold serial dilutions were surface plated in duplicate onto MRS agar plates (MRS - Oxoid, UK), and incubated at 30 °C for 72 h under anaerobic conditions (*AnaeroGen, Oxoid*). The square root of the total number of CFUs per plate was submitted to phenotypic tests. The counts were reported as the log CFU per milliliter of sample (log CFU mL^{-1}).

Presumptive identification of LAB

Pure cultures were submitted to Gram staining and a catalase test [26]. Typical gram-positive and catalase-negative cocci were transferred to MRS broth (Oxoid, UK) and stored at −80 °C (supplemented with glycerol 20% vol/vol) for posterior molecular identification.

Isolation of anaerobic bacteria

Samples of effluents and biomass from the biofilms from both AFBRs were streaked onto Reinforced Clostridia Medium plates (Oxoid, UK) and incubated at 37 °C for 48 h for presumptive enumeration of *Clostridium* spp. Anaerobic