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# Characterization and antimicrobial activity of lactic acid bacteria from fermentative bioreactors during hydrogen production using cassava processing wastewater

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## HIGHLIGHTS

- Hydrogen production anaerobic fluidized bed reactors from cassava wastewater.
- 2.0 mmol $H_2$ $g^{-1}$ COD and 2.1 L $H_2$ $d^{-1}$ $L^{-1}$ were produced.
- Elevated counts of LAB were found in the effluent samples.
- The LAB community were similar to *Lactobacillus* sp. and *Lactococcus lactis*.

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## ABSTRACT

Fermentative hydrogen production was evaluated using two anaerobic fluidized bed reactors (AFBRs). The reactors were fed cassava processing wastewater and operated with varying organic loading rates (OLR: 4–30 kg COD $m^{-3}$ $d^{-1}$) for up to 160 d under mesophilic conditions. The effects and roles of lactic acid bacteria (LAB) and their antimicrobial peptides in hydrogen-producing bioreactors and on hydrogen producers were evaluated. A maximum hydrogen yield of 2.0 mmol $g^{-1}$ COD and a maximum hydrogen production rate of 2.1 L $H_2$ $d^{-1}$ $L^{-1}$ were achieved with OLRs of 10 kg COD $m^{-3}$ $d^{-1}$ and 14 kg COD $m^{-3}$ $d^{-1}$, respectively, which coincided with the absence of bacteriocin-producing lactic acid bacteria. The major metabolites were lactic acid, ethanol, methanol, acetic acid and butyric acid. After growth on MRS agar, gram-staining and catalase tests, 47 strains were classified as presumptive lactic acid bacteria, with counts ranging from <10 to 1.5 × $10^9$ CFU $mL^{-1}$ for both reactors, providing evidence that lactic acid bacteria are able to survive and persist in the reactors. Thirty-nine pure cultures of the LAB community were successfully identified by 16S rRNA sequencing. For AFBR1 (4–12 kg COD $m^{-3}$ $d^{-1}$), there was a prevalence of *Lactobacillus* sp. (45.4%), followed by *Lactococcus lactis* (31.8%). *Lactobacillus* sp. (78.6%) was the prevalent genus for AFBR2 (14–30 kg COD $m^{-3}$ $d^{-1}$).



## 1. Introduction

Increased energy demands associated with increased economic activity has resulted in a higher consumption of fossil fuels and environmental contamination. As an energy carrier, hydrogen is an alternative to conventional fossil fuels; furthermore, hydrogen is a valuable raw material for various branches of the chemical industry [1]. A significant amount of research on hydrogen production via dark fermentation has been performed due to high $H_2$ production rates (HPR) and the ability to use pretreatment liquors derived from underutilized agricultural residue [2].

According to the Food and Agriculture Organization (FAO) of the United Nations, the cassava plant, a tropical root crop originally from the Brazilian Amazon, is the staple food of an estimated 800 million people worldwide. The global production of cassava in 2012 was estimated to be 262,585,741 tons, with the top producers being Nigeria, Indonesia, Brazil, and Thailand [3]. After 1 kg of fresh cassava root is peeled, washed, grated and mixed with water during starch production processes, approximately 0.2 kg of

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starch, 0.4–0.9 kg of residue, and 5–7 L of carbohydrate-rich wastewater are generated [3,4]. The generated wastewater is rich in biodegradable carbohydrates and is potentially useful as a substrate for fermentative hydrogen production.

Lactic acid bacteria (LAB) are members of the autochthonous microbiota of cassava and are responsible for the fermentation of this root; furthermore, LAB reduces cassava toxicity and prevents post-harvest deterioration [5,6]. However, in hydrogen-producing reactors, a few LAB strains may have an inhibitory effect due to their bacteriocins, which are antimicrobial peptides that have a deleterious effect on $H_2$-producing bacteria [7]. Ren et al. [8] evaluated the dynamics of these communities in three continuous stirred-tank reactors (CSTRs) that were used for $H_2$ production from molasses wastewater at three pH ranges (4–4.5, 5.5–6, and 6–6.5) and found that the worst $H_2$ yields were obtained for reactors operated at 5.5–6.0. This pH range may favor the growth of *Lactococcus* sp., an LAB that produces ethanol under some conditions but cannot generate $H_2$.

Batch bioreactors have been frequently used in the production of hydrogen from cassava wastewater (CW) [9,10] and for determining the biohydrogen potential. Continuous reactors, such as CSTR [11] and anaerobic sequencing batch reactors (ASBR) [4], have also been used with CW and have shown the greatest potential for hydrogen production. In our recent studies, we operated an anaerobic fluidized bed reactor (AFBR) to evaluate the effect of the hydraulic retention time (HRT) on fermentative hydrogen gas production from a CW and glucose mixture [12]. The effects of HRTs (10–12 h) and different sources of inoculum were reported. From the same sludge in this study, the maximum hydrogen yield (HY) was 0.7 mmol $g^{-1}$ COD (HRT of 10 h). However, the methane production was high (5.13 L $CH_4$ $d^{-1}$ $L^{-1}$), which affected the performance of the reactors. Furthermore, a phylogenetic analysis of 16S rRNA showed that 98.2% of the analyzed sequences corresponded to the family Lactobacillaceae, and 76.8% of the clones were affiliated with the genus *Lactobacillus* sp. Based on the results of this study, it would be necessary to evaluate the effect of lactic bacteria on hydrogen production.

A few factors that affect hydrogen production in AFBRs have been studied, such as (a) differing ratios of carbon sources [13], (b) the organic loading rate (OLR) [14], (c) the support material [15], and (d) the upflow velocity [16]. These studies were performed using simple substrates, such as glucose, and real wastewater, such as cheese whey and sugarcane stillage. However, there have been no studies in the literature that have evaluated the influence of LAB on bioreactors with a continuous CW feed, which naturally contains a significant population of LAB. Thus, in the production of hydrogen from CW, this study evaluated the direct effect of LAB and their antimicrobial peptides in AFBRs. The reactors were operated over a diverse range of OLRs under optimum conditions, as determined in a previous study [12].

## 2. Materials and methods

### 2.1. Hydrogen production from cassava processing wastewater

This study was performed by using two AFBRs (for a total volume of 770 $cm^3$, a reactor height of 80 cm and an internal diameter of 3.5 cm) for biohydrogen production. Polystyrene was used as a support material for biomass immobilization. The AFBRs were made of acrylic and jacketed with a water bath [12]. The reactor temperature was maintained at 30 °C.

Anaerobic sludge from a UASB reactor was used for the treatment of swine wastewater. The sludge was subjected to thermal pre-treatment by using a methodology adapted from Kim et al. [17].

The CW feeding substrate was subjected to acid hydrolysis with sulfuric acid and heated at 120 °C for 30 min before being used in the reactor feeds [12]. In the reactors, the biomass was activated using a mixture of glucose and CW in the influent [14].

The ratios of the carbon sources were varied by changes in the OLRs as shown in Table 1. The operation was continuous for 160 d. The pH was adjusted to 5.0 with NaOH. Nitrogen was injected to remove trace oxygen from the liquid and headspace. The start-up conditions of the AFBRs were described in a previous study [12].

### 2.2. Isolation of lactic acid bacteria and the evaluation of antimicrobial peptide production (bacteriocins)

At least 50 mL of the effluents was collected in sterile tubes at each operational phase change. Additionally, samples of the biomass that adhered to polystyrene were collected at the end of operational phase 5. All samples were immediately stored on ice and processed.

The isolation of LAB from the effluent and biomass samples was performed using De Man Rogosa-Sharpe agar plates (MRS; Oxoid, UK). At least 25 g of the biomass that adhered to the support was transferred to 50-mL sterile flasks and washed (five times) with 20 mL phosphate-buffered saline (PBS, pH 7.2) to remove non-adherent cells. Adherent cells were resuspended by vortexing the samples five times in 10 mL PBS [18]. The suspended cells then underwent serial dilution with peptone water (0.1% w/v). One hundred microliters of the serial dilutions were spread-plated on the surface of the MRS agar and incubated at 30 °C for 72 h under anaerobic conditions (AnaeroGen, Oxoid) [19]. Plates containing between 25 and 250 colonies were selected for the enumeration of the LAB, and the colonies underwent phenotypic tests (Gram staining and catalase testing corresponded to the square root of the total CFUs per plate). The counts were reported as CFU per milliliter of sample (CFU $mL^{-1}$), as previously reported in Crispim et al. [21]. Typical gram-positive and catalase-negative cocci [20] were transferred to MRS broth (Oxoid, UK) supplemented with 20% v/v glycerol and stored at 80 °C for later molecular identification.

After isolation and purification, 20 μL aliquots of each LAB strain were evaluated for the ability to produce bacteriocin in trypticase soy agar with 0.6% w/v yeast extract (TSAYE) (Oxoid, UK) under anaerobic conditions by using the indicator microorganisms *Lactobacillus sakei* ATCC 15521 and *Listeria monocytogenes* ATCC 19115 [22]. After anaerobic incubation, the presence of a clear inhibition zone around the colonies was considered positive [22]. Tests were performed in independent duplicates.

### 2.3. Molecular identification of lactic acid bacteria

The protocol of Griffiths et al. [23] was used to obtain the genomic DNA of the presumptive LAB. The DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Polymerase chain reaction (PCR) assays were used to amplify the genes encoding the 16S rRNA sequence. The universal primers 27F (5′-AGAGTTTGATCCTGCTCAG) and 1492 R (5′-CTACGGCTACCTTGT TACGA) were used to amplify the genes encoding the 16S rRNA sequence, according to Liu et al. [24]. In brief, each reaction mixture (50 μL) contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 25 pmol of each primer, 0.625 U Taq polymerase, and 60 ng DNA template. The amplification conditions were 94 °C for 5 min, followed by 30 denaturation cycles at 94 °C for 1 min, annealing at 50 °C for 45 s, and extension at 72 °C for 1 min 45 s. A final extension step was conducted at 72 °C for 10 min.

The PCR products were confirmed by electrophoresis on 1.2% agarose gels stained with Blue Green Loading Dye I (LGC Biotecnologia) in a 1× TAE buffer. The gels were visualized on a UV tran-