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# Sugarcane vinasse as substrate for fermentative hydrogen production: The effects of temperature and substrate concentration

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ARTICLE INFO

*Article history:*
Received 25 October 2013
Received in revised form
29 January 2014
Accepted 9 February 2014
Available online 11 March 2014

*Keywords:*
Hydrogen production
Mesophilic
Thermophilic
*Clostridium*
*Thermoanaerobacterium*
Homoacetogenesis

ABSTRACT

The present study aimed to evaluate the hydrogen production of a microbial consortium using different concentrations of sugarcane vinasse (2–12 g COD $L^{-1}$) at 37 °C and 55 °C. In mesophilic tests, the increase in vinasse concentration did not significantly impact the hydrogen yield (HY) (from 1.72 to 2.23 mmol $H_2$ $g^{-1}$ $COD_{influent}$) but had a positive effect on the hydrogen production potential (P) and hydrogen production rate ($R_m$). On the other hand, the increase in the substrate concentration caused a drop in HY from 2.31 to 0.44 mmol $H_2$ $g^{-1}$ $COD_{influent}$ in the tests performed at 55 °C with vinasse concentrations from 2 to 12 g COD $L^{-1}$. The mesophilic community was composed of different species within the *Clostridium* genus, and the thermophilic community was dominated by organisms affiliated with the *Thermoanaerobacter* genus. Not all isolates affiliated with the *Clostridium* genus contributed to a high HY, as the homoacetogenic pathway can occur.


## Introduction

In Brazil, especially in the state of São Paulo, the production of ethanol is one of the most important agroindustries. A total of 23.5 billion litres of ethanol were produced in 2012 [1]. For each litre of ethanol produced, approximately 13 L of vinasse is generated. Considering these numbers, approximately 305.5 billion litres of vinasse were generated in 2012. Currently, this residue is used as a fertiliser and potassium source; however, environmental agencies are limiting the quantity of vinasse that can be applied to soil due to its high pollutant potential. Thus, biodigestion of

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http://dx.doi.org/10.1016/j.ijhydene.2014.02.058

vinasse for biogas production is an alternative means of waste disposal [2].

Due to the high chemical oxygen demand (COD) content (22–45 g $L^{-1}$), low pH (3.5–4.6), and high macronutrient content [3], the vinasse produced by biorefineries is likely to be a good substrate for hydrogen production by dark fermentation. Because this wastewater is produced at a high temperature (107 °C) [2], the use of thermophilic bacteria to produce hydrogen is attractive, as the cost of cooling the water is eliminated. Different types of waste have been successfully used for hydrogen production under thermophilic conditions, including sugar factory wastewater and molasses [4].

Although vinasse has great potential, few published reports have examined the production of hydrogen using this substrate. To our knowledge, hydrogen production using sugarcane vinasse or similar wastewaters has been evaluated by only a few authors [5–8]. The highest hydrogen yield (25 mmol $H_2$ $g^{-1}$ COD) was reported by Fernandes et al. [5] using as an inoculum a biomass from a packed-bed reactor used to produce hydrogen. Lower hydrogen yields were obtained in the other studies (ranging from 0.7 to 2.8 mmol $H_2$ $g^{-1}$ $COD_{influent}$) [8,9]. The difference in the results could be related to the source of the inoculum or the concentration of the substrate applied.

Hydrogen production using vinasse has several toxicity problems related to the content of potassium, sulphate [7], phenolic compounds [10], and melanoidins [11]. These problems could be addressed through the use of a high organic load. According to the work presented by Buitrón and Carvajal [12], the amount of hydrogen produced was affected by the initial concentration of the substrate. Thus, it is very important to test the effect of this concentration on hydrogen production.

To determine the best conditions for hydrogen production using vinasse, we evaluated hydrogen production at mesophilic and thermophilic temperatures using different vinasse concentrations. The effect of temperature and substrate concentration on the microbial community was also studied using molecular (16S rRNA genes, DGGE, and cloning libraries) and culture methods (isolation and characterisation of the predominant hydrogen-producing bacteria).

## Materials and methods

### *Acclimatisation of seed inoculum to mesophilic and thermophilic conditions*

The inoculum was retrieved from a full-scale UASB methanogenic reactor operated at 30 °C, which is part of the wastewater treatment facility of a poultry slaughterhouse (Avícola Dakar, Tietê, SP/Brazil). The granular sludge was heat-treated (90 °C for 10 min) to select endospore-producing microorganisms, which may have better hydrogen-producing capabilities, and to inhibit methanogenic hydrogen-consuming microorganisms [13]. Next, this biomass was adapted to the acidic (pH 5.5), mesophilic (37 °C), and thermophilic (55 °C) conditions of incubation using a sterile mineral medium (see Section 2.5, Culture medium) with sucrose (Sigma–Aldrich, 2 g $L^{-1}$) as a substrate. At this step, the mesophilic or thermophilic spore-forming microorganisms could be enriched and adapted to the substrate used in further experiments.

### *Enrichment of mesophilic and thermophilic consortia using vinasse as a substrate*

The biomasses adapted to mesophilic and thermophilic conditions were used to inoculate a mineral medium with sugarcane vinasse (2 g COD $L^{-1}$) as the substrate instead of sucrose. The enrichments were carried out in two glass bottles (2 L) (one each for mesophilic and thermophilic conditions) containing 0.8 L of synthetic medium. The media were inoculated with 0.2 L of adapted biomass, which was obtained by separating the cells by centrifugation (10,000 rpm at 4 °C for 5 min), washing them with sterile water, and suspending them in 0.2 L of synthetic medium. The cultures were incubated at 37 °C (for the mesophilic biomass) and 55 °C (for the thermophilic biomass) without agitation for approximately two months. During this period, the culture medium was replaced twice a week to replenish the substrate for biomass growth and remove the end products. The same volume of medium (0.8 L) and pre-grown washed biomass (as inoculum) (0.2 L) were always used.

### *Substrate: sugarcane vinasse*

The sugarcane vinasse used as a substrate was provided by the Nova Era Distillery in Ibaté (Ibaté, SP, Brazil) and kept frozen (−20 °C) until use. The characteristics of the vinasse used are presented in the Supplementary material (Table S1). Before use, the vinasse was centrifuged at 10,000 rpm for 5 min at 4 °C to remove coarse solids. The supernatant was used to supplement the media without sterilisation by autoclave to avoid alteration of the components due to heat.

### *Batch tests at different substrate concentrations*

To study the effect of substrate concentration, hydrogen production tests were performed under mesophilic and thermophilic conditions. The experiments were conducted in triplicate in 2 L glass bottles with a working volume of 1.2 L and 0.8 L of headspace filled with $N_2$ (100%) (White Martins). Before each test, the anaerobic consortia were reactivated for 24 h in fresh culture medium using sugarcane vinasse (2 g COD $L^{-1}$) as the substrate. The reactivated biomass (0.24 L) was centrifuged at 10,000 rpm for 5 min at 4 °C, and the pellet was used as an inoculum for the different tests. Different volumes of sugarcane vinasse were used to obtain four different substrate concentrations (approximately 2, 5, 7, and 12 g COD $L^{-1}$). The four different concentrations were tested at mesophilic (incubated at 37 °C) and thermophilic (incubated at 55 °C) conditions. During the incubation, the $H_2$ production was determined in the head space, and liquid samples were taken to determine the fermentation products and sugar consumption.