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VALORIZATION OF VINASSE AS BROTH FOR BIOLOGICAL HYDROGEN AND VOLATILE FATTY ACIDS PRODUCTION BY MEANS OF ANAEROBIC BACTERIA

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Abstract

Vinasse is the liquid waste removed from the base of sugarcane ethanol distillation columns at a ratio of 12-15 liters per liter of alcohol, resulting in an estimated production of approx. 370 billion liters in 2012/2013 in Brazil. Vinasse has a low pH and high chemical oxygen demand, which can cause land desertification when indiscriminately used as fertilizer. Also, underground water contamination is being observed in some regions. We evaluated the potential of vinasse as nutrient source for biohydrogen and volatile fatty acids production by means of anaerobic consortia. Two different vinasse-based media were proposed, using sugarcane juice or molasses as carbon source, and were compared to fermentation in a sucrose-supplemented medium. Pure cultures (4) and consortia (7) were cultured in the propose media and evaluated for volatile fatty acids (VFAs) and biohydrogen production. The consortium LPBAH1 was selected for fermentation of vinasse supplemented with sugarcane juice, resulting in a higher H_2 yield of 7.14$mol_{H2}/mol_{sucrose}$ and hydrogen content in biogas of approx. 31% after process optimization. Similarly, the optimized process using the consortium LPBAH2 resulted in 3.66 $mol_{H2}/mol_{sucrose}$ and 32.7% hydrogen content in biogas. The proposed process is of great importance for giving a more rational destination to vinasse and expanding Brazilian energy matrix, reducing the dependence of fossil fuels.

CHAPTER 1

An Introduction to the metabolism of Biohydrogen and Volatile Fatty acids of anaerobic bacteria

1. Introduction

Almost 100% of our (increasing) energetic demand is supplied by carbon-containing fossil sources such as oil, coal and natural gas. The environmental concerns involving the use of such sources of energy are related to the increase in atmospheric carbon concentration, which is the main cause of global warming and climate change.

A reduction of CO_2 emissions by more than 50% is recommended by the Intergovernmental Panel on Climate Change (IPCC) in order to stabilize the CO_2 level in the atmosphere at 550 parts per million volume (ppmv) to curb negative climate effects. In this context the scientific community is doing great efforts to develop renewable cost-effective sources of energy.

The Framework Convention on Climate Change, sign in Rio de Janeiro in 1992, made global warming a major focus and development of technologies for reducing/absorbing greenhouse gases (GhG) gained importance. Rubin et al (1992) divided the greenhouse gases emissions reductions alternatives into three groups: conservation, direct mitigation and indirect mitigation. Conservation measures reduced electricity consumption and thus GhG emissions, direct mitigation techniques capture and remove CO_2 emitted by specific emissions sources, and indirect mitigation involve offsetting actions in which GhG producers support reductions in GhG emission.

The gradual introduction of fuels with an increasingly lower carbon content per unit of energy (wood → coal → oil → natural gas) results in a continuous decarbonisation of the global fuel mix, the main objective of the international agreement cited before. This chain of lower carbon content fuel ends in Hydrogen. Hydrogen has a higher gravimetric energy density than any other known fuel and is compatible with electrochemical and combustion processes for energy conversion without producing the carbon-based emissions that contribute to environmental pollution and climate change (36).

2 Hydrogen and Volatile Fatty Acids (VFA) Production

Anaerobic acidogenesis is known as the first step in the anaerobic digestion of soluble organic materials to methane and CO_2, during which hydrogen is produced. Because many kinds of bacteria are involved in this process several kinds of organic acids and alcohols can be produced (70), representing around 55% of the carbon destination (56).

2.1 Hydrogen Production Processes

Hydrogen does not exist alone in nature. Natural gas contains hydrogen (about 95% of natural gas is methane, CH_4), as does biomass (cellulose), water and hydrocarbons. The carbon-hydrogen and oxygen-hydrogen bonds present in these substances, however, have low energy. On the other hand hydrogen-hydrogen bonds contain much more energy. Methods for producing high-energy content hydrogen-hydrogen bonds includes a diverse array of primary energy sources such as wind, solar, geothermal, nuclear and hydropower, can be used to extract hydrogen from water or other feedstock. This diversity of options enables hydrogen production almost anywhere in the world.

At present, hydrogen is mainly produced from fossil fuels, either by thermal and chemical methods (Table 1). About 40% is produced from natural gas, 30% from heavy oils and naphtha, 18% from coal, and 4% from electrolysis and about 1% is produced from biomass (51). Nearly 50 million tons of hydrogen is traded annually worldwide with a growth rate of nearly 10% per year (58).

Table 1 – Most common hydrogen production processes.

Method	Process	Feedstock
Thermal	Steam reformation	Natural gas
	Thermochemical water splitting	Water
	Gasification	Coal, biomass
	Pyrolisis	Biomass
Electrochemical	Electrolysis	Water
	Photoelectrochemical	Water
Biological	Photobiological	Water and algae
	Anerobic digestion	Biomass
	Fermentative microrganisms	Biomass

2.2 Biotechnological Biohydrogen Production

Hydrogen obtained from physicochemical methods usually cannot be regarded as an alternative pollution free energy source. Regarding a sustainable energy production the biological production of hydrogen represents a particularly pollution free and energy-saving process, since it is possible to use industrial wastes. As a consequence it has received special attention by the scientific community during the last years. Between the years 2000 and 2006 only 391 articles were published regarding the biological production of hydrogen, while between 2006 and 2012 these numbers were approx. 6 times greater. Only at the first two months of 2013, more than 150 articles in this field were published.

Several processes are currently under development, ranging from biomass fermentations to photobiological processes. Table 2 gives a more

detailed overview of biological hydrogen production processes that are being explored in fundamental and applied research.

Table 2 - Overview of biological hydrogen production processes (2).

	Process		General reaction	Microorganisms used
1	Direct Biophotolysis		$2\ H_2O + light \rightarrow 2\ H_2 + O_2$	Microalgae
2	Photo-fermentations		$CH_3COOH + 2\ H_2O + light \rightarrow 4\ H_2 + 2\ CO_2$	Purple bacteria, Microalgae
3	Indirect biophotolysis	a	$6\ H_2O + 6\ CO_2 + light \rightarrow C_6H_{12}O_6 + 6\ O_2$	Microalgae, Cyanobacteria
		b	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$	
		c	$2\ CH_3COOH + 4\ H_2O + light \rightarrow 8\ H_2 + 4\ CO_2$	
			Overall reaction: $12\ H_2O + light \rightarrow 12\ H_2 + 6\ O_2$	
4	Water Gas Shift Reaction		$CO + H_2O \rightarrow CO_2 + H_2$	Fermentative bacteria, Photosynthetic bacteria
5	Two-Phase $H_2 + CH_4$ Fermentations	a	$C_6H_{12}O_6 + 2\ H_2O \rightarrow 4\ H_2 + 2\ CH_3COOH + 2\ CO_2$	Fermentative bacteria + Methanogenic bacteria
		b	$2\ CH_3COOH \rightarrow 2\ CH_4 + 2\ CO_2$	
6	High-yield Dark Fermentations		$C_6H_{12}O_6 + 6\ H_2O \rightarrow 12\ H_2 + 6\ CO_2$	Fermentative bacteria

The advantages of the fermentative hydrogen production are the broad spectrum of applicable substrates as well as high hydrogen production yields (3). The possibility of coupling the energetic hydrogen production from biomass with the simultaneous treatment of waste materials is an addition crucial advantage. Both biohydrogen production and methane from anaerobic digestion are CO_2-neutral since the carbon released by their combustion is derived, directly or indirectly, from recently fixed atmospheric CO_2 (2). Moreover, the emitted carbon associated with hydrogen produced by microbial fermentation is released during the fermentation rather than during its utilization, thus potentially allowing easy capture of CO_2. Large scale production will allow recovery of the CO_2 for use in microalgae cultures, greenhouses, storage in chemical form (e.g. as carbonates) or in underground reservoirs. In this scenario, biological hydrogen production could even be a carbon negative technology (61). In fuel cells, hydrogen can be converted to electricity very efficiently, producing only water as a waste product, thus drastically reducing CO_2, NO_x, particulate and other emissions that accompany the use of fossil fuels.

As shown in table 2, biohydrogen may be produced biotechnologically by photo-fermentations, two phase fermentations and dark fermentations. In these cases a specific environment needs to be created in which hydrogen producing bacteria flourish and others perish (38). Each approach has distinct advantages and disadvantages with challenging technical barriers to practical application.

The processes for the production of biohydrogen differ primarily concerning the involved microorganisms, the substrates and the light dependence.

2.2.1 Photo-fermentations

Towards the end of the 1930s it was discovered that under certain conditions unicellular green algae are able to produce hydrogen (4, 5) due to the presence of a specific enzyme called hydrogenase. Since then hydrogenases and indeed H_2 production have been found to be ubiquitous throughout the prokaryotic and eukaryotic kingdoms.

Physiological studies of cyanobacteria have identified many producing strains, such as *Spirulina platensis* (122), *Anabaena cylindrica* (123)*, Cycas revoluta* (124) and others. One of the greatest drawbacks of this technology, besides dependence of light (which influences in bioreactors development, difficulties in large scale production, among others) is that hydrogen production by cyanobacteria occurs in such a limiting environment that that cell death is a natural consequence.

Despite that, it can be used as a coupled process to dark anaerobic process.

2.2.2 Dark Anaerobic Biohydrogen Production

Dark hydrogen production is a ubiquitous phenomenon under anoxic or anaerobic conditions. Anaerobic fermentative bacteria produce hydrogen without photo energy, and so the cost of hydrogen production is 340 times lower than the photosynthetic process (6).

A wide variety of bacteria use the reduction of protons to hydrogen to dispose of reducing equivalents which result from primary metabolism. This

oxidation generates electrons which need to be disposed of for maintaining electrical neutrality. In aerobic environments, oxygen is reduced and water is the product. In anaerobic or anoxic environments, other compounds need to act as electron acceptor, e.g. protons, which are reduced to molecular hydrogen (H_2). The capacity to reduce other electron acceptors than oxygen requires the presence of a specific enzyme system in the micro-organisms: hydrogenases.

2.2.3 Two phase

The idea of two- and multi-stage systems is that the overall conversion process of the waste stream to biogas is mediated by a sequence of biochemical reactions which do not necessarily share the same optimal environmental conditions (52). The principle involves separation of digestion, hydrolysis and acidogenesis from the acetogenesis and methanogenesis phases.

There are three major advantages to a two-phase design. In a two-phase system, acid formation is promoted during the acid phase. Therefore the methane phase is constantly receiving acids to encourage maintenance of high populations of these methanogen microorganisms. The second advantage is that biomass production, acidogens and methanogens, can be maintained each at their optimal growth conditions. The third advantage is higher methane content in the methanogenic phase reactor (52).

3 Dark Fermentation Metabolism of Biohydrogen producers

Dark hydrogen fermentation is a ubiquitous phenomenon under anoxic or anaerobic conditions (i.e., no oxygen present as an electron acceptor). The advantages of dark fermentation over other processes are: (i) better process economy for lower energy requirements, (ii) process simplicity, (iii) higher rates of hydrogen production, and (iv) utilization of low-value waste as raw materials (49). Figure 1 illustrates the biochemical pathway for conversion of renewable biomass in to hydrogen via fermentation.

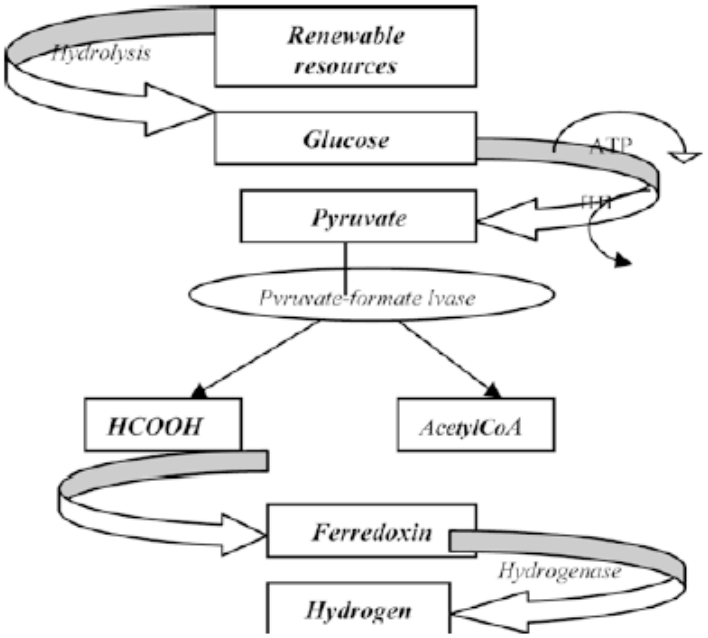

Figure 1 - A schematic pathway for conversion of renewable to hydrogen via fermentation (53).

Dark Fermentation is an incomplete oxidation. The profile of the fermentation products is closely related to biohydrogen yields. In respect to fermentation products, family Clostridiaceae members include pH-neutral solvent producers, mixed acid, homoacidogenic and alcohol producers (butyric, acetic and/or lactic acids, ethanol, propanol or butanol. Among the wide range of by-products of diverse microbial metabolism, the two pathways producing hydrogen from carbohydrates are associated with acetate and butyrate. The theoretical yield of H_2 per mole of glucose associated to the production of acetate and butyrate is described in the following reactions:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COO^- + 2CO_2 + 4H_2 \quad \Delta G'0 = -206 \text{ kJ.mol}^{-1}$$

$$C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3CH_2CH_2COO^- + 2CO_2 + 2H_2 \quad \Delta G'0 = -264 \text{ kJ.mol}^{-1}$$

A maximum of 4 moles of H_2 per mole of glucose can be produced concurrently with the production of energy (206 kJ per mole of glucose) and acetate, which is sufficient to support microbial growth. The thermodynamical explanation for this limitation is based on the substrate level, since phosphorylation must produce whole numbers of ATP and the yield of ATP from glucose must be at least 1 mol/mol for the cell to survive (11). However, microbial fermentation typically

generates more than 1 mol ATP and less than 4 mol H_2/mol hexose, quantities that vary according to the metabolic system and conditions. Figure 2 is a general representation of the metabolic pathways associated to dark fermentation.

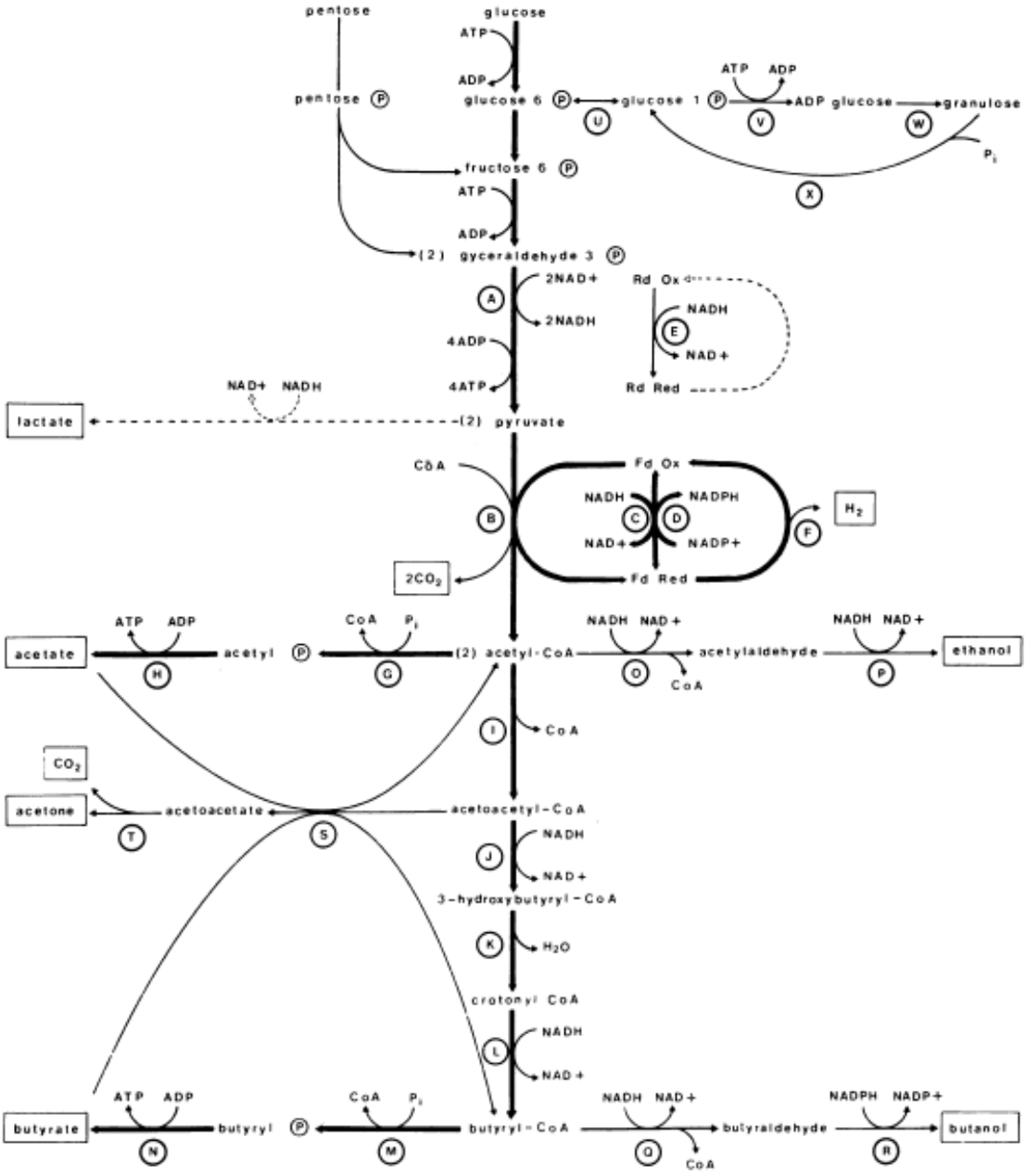

Figure 2 - Metabolic pathway of the production of acids and solvents from glucose in Clostridium acetobutylicum. From Jones and Woods (50).

The production of hydrogen occurs due to the cleavage of hexoses to pyruvate through Embden-Meyerhof pathway, with the formation 2 mol of

reduced nicotinamide adenine dinucleotide (NADH). Part of the electrons generated during the oxidation of glucose is involved in the production of butyrate and ethanol, while the rest is recycled to produce NAD and hydrogen, maintaining the electrical neutrality.

Three enzymes compete for pyruvate: pyruvate:ferredoxin oxidoreductase (PFOR), pyruvate:formate lyase (PFL) and the fermentative lactate dehydrogenase (LDH). The nature of the fermentation depends to a large extent on these enzyme activities (55). Pyruvate is predominantly cleaved by PFOR to form acetil-CoA, CO_2 and reduced ferredoxin (FdH_2). Both PFOR and Fd are iron-sulfur proteins which contains 4Fe-4S clusters. The released H_2 yield is dependent upon the fate of pyruvate, which differs among species due to varying activities of PFL, PFOR and LDH (12).

This reduced ferredoxin is able to transfer electrons to an iron-containing hydrogenase which permits the use of protons as a final electron acceptor, resulting in the production of molecular hydrogen (50). This assures the production of two moles of hydrogen per mole of glucose consumed. The overall reaction of the processes can be described as follows:

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \rightarrow \text{Acetyl-CoA} + 2\text{Fd(red)} + \text{CO2}$$

$$2\text{H+} + \text{Fd(red)} \rightarrow H_2 + \text{Fd(ox)}$$

There are two main types of hydrogenases which are phylogenetically distinct and contain different active sites where the relevant chemistry occurs; Ni–Fe hydrogenases and [FeFe] hydrogenases. In general, NiFe hydrogenases are poised to catalyze hydrogen oxidation and [FeFe] hydrogenases are extremely active in proton reduction. In Clostridia, hydrogen evolution is catalyzed by a soluble [FeFe] hydrogenase.

The remainder of the hydrogen in the hexose is conserved in the byproduct acetate and butyrate, and under non-ideal circumstances, more reduced products like ethanol, lactate or alanine. These reduced products are produced to satisfy metabolic needs. Acetate allows ATP synthesis, and the reduced products permit the reoxidation of NADH (which is necessary for continuing glycolysis) (65).

Under abnormal conditions (inhibition of hydrogenase, depletion of iron, for example), lactate can be produced from pyruvate. This pathway only appears to operate as a less efficient alternative to allow energy generation and the oxidation of NADH to continue when the mechanisms for the disposal of protons and electrons by the generation of molecular hydrogen is blocked.

Acetyl-CoA produced by the phosphoroclastic cleavage is the central intermediate, leading to both acid and solvent production (figure 1). The generation of hydrogen by fermentative bacteria also accompanies the formation of organic acids as metabolic products. Highest release of hydrogen is observed when more oxidized products are produced (acetate and butyrate), which occurs during the initial growth phase (acidogenic phase). Acid accumulation causes a sharp drop of culture pH leading to a subsequent inhibition of bacterial hydrogen production; it is thus required a way to reduce acid production or to neutralize protons outside of the cells, (53). Inhibition of biohydrogen production can also be caused, and in practice is the main barrier to achieve high yields, by high H_2 partial pressure. According to the model developed by Ruzicka (1996) (72), as the concentration of dissolved H_2 increases in the liquid phase, the transfer of electrons from glucose to H_2 becomes increasingly unfavorable.

During acid-producing metabolism there is a rapid flow of electrons derived both from the phosphoroclastic cleavage of pyruvate and from NADH to ferredoxin (50). Since NADH has a higher potential than H_2, the dehydrogenation of triose phosphate to produce 2 mols of H_2 can occur only when the partial pressure of H_2 is lower than $6x10^{-4}$ atm, while the production of H_2 via the oxidation of pyruvate and ferredoxin can generate another 2 mols of H_2 at higher H_2 pressure up to 0.3 atm (71). Thus, in order to obtain H_2 yields greater than 2 $mol_{H2}/mol_{glucose}$ the production of H_2 via triose phosphate dehydrogenation and NADH must be achieved. Since two moles of NADH are produced during glycolysis, up to a maximum of two additional molecules of H_2 could potentially be generated by NADH pathway.

The formation of relatively reduced organic molecules (e.g. acetate, butyrate) can inhibit H_2 production if these metabolites are allowed to accumulate (12). These reduced end-products contain additional H atoms that

are not liberated as gas (48). This is the reason why practical production of hydrogen is lower than the theoretic maximum. For example, the H_2 yield from *C. butyricum* could in theory reach 4 mol H_2/mol hexose although a detailed metabolic analysis of *C. butyricum* gives a calculation of a maximum of 3.26 mol H_2/mol hexose and practical yields obtained using clostridia rarely exceed 2 mol H_2/mol hexose (12).

4 Organic Acids Microbial Production

Low-molecular-mass carboxylic acids are important intermediates and metabolites in biological processes. Known as volatile fatty acids (VFAs) these homologues and corresponding structural isomers include acetic, propionic, iso- and *n*-butyric and iso- and *n*-valeric acid. The presence of VFAs in a sample matrix is often indicative of bacterial activity.

Organic acids are some of the end products of anaerobic metabolism to produce biohydrogen, especially C2 and C4 acids. Generally they are not recovered, but used in sequential processes as substrate for microbial methane or solvent production.

If recovered from the broth, organic acids can be produced and sold as commodity chemicals or further processed into higher value chemicals, biofuels, or bio-products. Among the acids produced during biohydrogen production are acetic, butyric, succinic, lactic, formic and propionic acids. Usually, in biohydrogen processes it is observed a preferential production of acetic and butyric acids. Considering the economic issues associated to biohydrogen production systems, the recovery or reuse of such VFAs are of great interest, since H_2 production is high.

Butyric acid has many uses in different industries, and currently there is a great interest in using it as a precursor to biofuels, more specifically biobutanol. Butyric acid has also applications in the production of low-molecular-weight esters which have pleasant aromas (perfume industry) or tastes (food flavoring), in animal feed and in the production of Cellulose Acetate Butyrate (a biopolymer used in high impact plastics).

Acetic acid is an important feedstock for many chemicals such as vinyl acetate monomer (for polymers), cellulose acetate, acetic acid esters and acetic anhydride. Lactic acid is largely used as preservative in food industry (soft drinks, essence, extracts, fruit juices), as well as propionic acid. Succinic acid is used as building blocks for chemicals, such as polymers, while formic acid is largely used in leather industry (prevention of mold), in agriculture (silage preservation) and in animal feed.

CHAPTER 2

Preliminary studies on biohydrogen production in vinasse-based media by anaerobic bacteria

1 Introduction

1.1 Substrates for Biohydrogen Production

Currently, the cost of H_2 generated from biological processes is very high. Intensive research on biohydrogen is underway, and in the last few years several novel approaches have been proposed and studied in order to surpass economical drawbacks that prevent its industrial production (61). Environmental concerns and evolving legislations on international scale, and considerations about increasing energy prices, request more participation of net energy producing waste treatment processes for sustainable pollution control (37). Since the carbon dioxide produced during the fermentation is derived, directly or indirectly, from recently fixed atmospheric CO_2, the net CO_2 charge in dark fermentation processes using agroindustrial wastes is zero.

In respect to the range of potential substrates which can be utilized by the broad range of hydrogen producing bacteria it can be stated that, at present, it is vast and open for further exploration. The major problem in developing large scale technologies using such wastes is their availability and coverage. In this terms, domestic and industrial waste waters are good examples, since they will be produced wherever there is industrial and human activity. The energy accumulated in wastes can be harvested and converted to hydrogen through dark fermentation. The energy, now accumulated in hydrogen molecules, can be then converted to electricity or heat or be stored for further use.

Recently, complex carbon sources, such as molasses (114), food wastes (45), dairy wastewater (115), mushroom waste (116), rice slurry (116), cheese wey (117), lignocellulosic materials, glycerol waste (118), vegetable waste (119) and many others were proved to be susceptible for dark fermentation (Table 3). The more carbohydrate the wastewater/biomass contains, more suitable it is for biohydrogen production. Most of times pre-treatment of the complex-carbohydrate source (usually thermal treatment) is necessary to generate high production rates, otherwise biohydrogen production is limited by the microorganism(s) hydrolytic activity.

Table 3 – Some examples of yields of biohydrogen production achieved using agroindustrial residues as carbon sources.

Microorganism	$Y_{(H2/S)}$ (mol.mol^{-1})	Carbon source	Reference
Caldicellulosiruptor saccharolyticus	2.3	bagasse	108
Clostridium butyricum	0.76	Rice straw hydrolisate	109
Clostridium butyricum	0.75	Sugarcane bagasse hydrolisate	110
Clostridium thermocellum	1.47	Delignified wood fibers	111
Ruminococcus albus	2.59	Sorghum residues	112
Thermoanaerobacterium thermosaccharolyticum	2.4	Corn stover hydrolisate	113

Because of the complex nature of the substrates frequently used and the often no identification of mixed microbial cultures it is difficult to compare one study with another (61). The highest H_2 yields have been achieved using *Clostridia*, enteric bacteria and hyperthermophiles. The strict anaerobic *Clostridia* are said to produce hydrogen in higher yields than facultative anaerobes. Extreme thermophiles achieved yields of approximately 83-100% of the maximal theoretical value of 4 mol/mol (38), but usually grow to low biomass concentrations (resulting in low production rates). The proper choice of microorganism(s) and substrate is crucial in the development of a feasible biohydrogen and VFAs production technology.

The use of mixed cultures in the production of hydrogen is an alternative that is being actively studied by the scientific community. High yields of 2,6 $mol_{H2}.mol^{-1}_{glucose}$ (125) and productivities of up to 150 $mmoles_{H2}.L^{-1}.h^{-1}$ were described (126). The main advantages related to mixed culture fermentations are the considerable low susceptibility to contamination and less toxicity to

oxygen, which favor process handling. Moreover, when complex substrates are used the presence of different microorganisms generally improves substrate degradation and consequently hydrogen production. On the other side, issues associated to process stability are noted (modifications on the process or variation on the composition of the substrate may lead to changes in the microbial community.

1.1 Sugarcane Vinasse

In Brazil, ethanol is produced through a classic fermentation process, in which yeasts transform sugarcane juice, molasses, or a molasses-juice mixture into ethanol. This is a biological process that can be represented by the stoichiometric equation of Gay Lussac:

$$C_{12}H_{22}O_{11} + H_2O \rightarrow C_6H_{12}O_6 + C_6H_{12}O_6 \text{ (a)}$$
$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 + 23{,}5 \text{ kcal (b)}$$

At the end of the fermentation, practically 100% of the sugar (sucrose) present in the culture media is consumed by the yeast (usually a *Saccharomyces*), resulting in a liquid called wine. The wine has a concentration of ethanol (% in volume) between 6 and 10°GL, which is recovered by distillation in the top part of distillation columns, where the present volatile substances are separated based on their different boiling points.

Vinasse is removed from the base of the distillation columns. It is nothing more than the fermented broth free of ethanol. It contains some organic solids in suspension as well as minerals, residual sugar and some volatile compounds. Considering the ethanol concentration in the wine, vinasse is generated in an average proportion of 12 to 15 liters for each liter of alcohol produced. According to Monteiro (33), the physicochemical characteristics of vinasse are: pH 3.8-5.0; Total solids (g/l) 21.0-85.0; Soluble solids (g/l) 4.0-31.0; Non-soluble solids (g/l) 3.0-13.0; C.O.D. (mg/l) 15,000-27,000; Water (%) 89-96; Organic matter in total solids (%) 70; Nitrogen (g/l) 1.0-3.5; Phosphorus (g/l) 0.4-4.0; Potassium (g/l) 9.0-13.0; Magnesium (g/l) 0.8-1.5; but this varies

considerably and should be analyzed case by case. Because of its production rate and its chemical characteristics vinasse constitutes the largest pollution source of the Brazilian ethanol industry.

Currently, the destination given to vinasse is its aspersion over sugarcane plantations. Vinasse is usually stored in depuration lagoons (Figure 3) prior use. Channels are built through sugarcane plantations where vinasse drains and a motor pump truck is responsible to sprinkle the liquid (Figure 4). Its application as fertilizer has some advantages, especially in terms of productivity, but the amount used might be well determined. There is a maximum rate of vinasse application in the field, based on soil composition (but in practice soil characterization is not carried and inspection by environmental organizations is very difficult to be handled).

Figure 3 – Depuration LPB AH2on where vinasse is stored at Usina Catanduva (Catanduva, São Paulo, Brazil). At the day this picture was taken, the flow of vinasse was $350 m^3.h^{-1}$.

Figure 4 - Channels for the distribution of vinasse along the field at Usina Catanduva (Catanduva, São Paulo, Brazil. It can also be seen a pump used for vinasse aspersion.

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When used in excess, productivity reduction, late maturation and low sucrose content are commonly observed (120). When vinasse is produced in excess and cannot be used as fertilizer, which is very common, industries throw it in areas called “sacrifice zones”. In this area the soil becomes very salty and acid causing desertification and rendering it unusable for any other purpose. In long-term these characteristics are also noted in productive land, causing productivity decrease, late maturing and decrease in sucrose content (120). In 1986 40% of the vinasse produced in Brazil was not used as fertilizer and was thrown in sacrifice zones (121). Unfortunately no updated data collection is available (informal conversations with the environmental manager of an industry in São Paulo indicates that this number is approx. 25%).

Seiju Hassuda (34) identified infiltration problems due to vinasse aspersion in Bauru Aquifer (SP-Brazil), the most important aquifer in Brazil. This problem is not only related to the sacrifice zones, since it can be seen in the Figure 4 that no protection is given to avoid vinasse infiltration in the soil. New government regulations are now forcing the industries to coat the channels (Figure 5), but inspection is very limited. Mellissa et al (35) stated vinasse can promote changes of soil physical properties in two different ways: (i) improving

aggregation, consequently raising the capacity of infiltration of water in the soil, thus causing ions leaching and contamination of the groundwater; and (ii) promoting the dispersion of soil particles, reducing the rate of infiltration and increasing the runoff, resulting in possible contamination of surface water.

Figure 5 - Coated channel at Usina Catanduva (Catanduva, São Paulo, Brazil) folowing the new legislation.

In this context, it is of great importance to give a more rational destination to vinasse or at least reduce its toxicity.

During the last decades, ethanol production has increased very rapidly. Brazil is, nowadays, the second higher ethanol producer in the world. Recent international incentive and demand for biofuels production influenced Brazilian ethanol industries, increasing production. Thus, the problem of vinasse disposal will worsen. Indeed, its continuous discharge onto land can endanger the chemical and physical structure of the soil, reduce yields and lead to serious groundwater pollution problems.

Usina Sao Martino (Sao Paulo – Brazil) installed a pilot plant for the biodigestion of vinasse, obtaining biogas, which is used to burn as fuel in the boilers of the plant. The technology has reached a reasonable degree of maturity due to the successive experiments, but some uncertainties decelerated its scale up (42). In the year 2012 a 612 MWh biogas plant was installed at Companhia Alcoolquímica Nacional (Vitória do Santo Antão, Pernambuco, Brazil) for the processing of 20% of the vinasse produced daily.

Regarding the composition depicted in Table 4, vinasse is an interesting substrate for microorganism growth because it presents a great amount of micronutrients. Iron, magnesium, phosphorus and nitrogen content are interesting for the development of biohydrogen production. The fact that some successful cases of methane production are described also reinforced the possibility of hydrogen production.

Table 4 - Physico-Chemical characterizations of Vinasse (media of 64 samples from 28 ethanol industries from São Paulo – Brazil) (54).

Parameter	Unity	Medium Value
pH		4,15
Brix	ºB	18,65
DBO_5	mg/L O_2	16494,76
DQO	mg/L O_2	28450,00
Calcium	mg/L CaO	515,25
Chloride	mg/L Cl	1218,91
Cooper	mg/L CuO	1,20
Iron	mg/L Fe_2O_3	25,17
Phosphorus	mg/L P_2O_4	60,41
Magnesium	mg/L MgO	225,64
Manganese	mg/L MnO	4,82
Nitrogen	mg/L N	356,63
Ammonia Nitrogen	mg/L N	10,94
Potassium	mg/L K_2O	2034,89
Sodium	mg/L Na	51,55
Sulfate	mg/L SO_4	1537,66
Sulfite	mg/L SO_4	35,90
Zinc	mg/L ZnO	1,70
Ethanol- CG	mL/L	0,88
Glycerol	mL/L	5,89

Because low amounts of fermentable carbon are present in its composition, vinasse might be enriched with a carbohydrate source to allow the production of great quantities of hydrogen. Some cheap fermentable carbon sources are available in Brazil, especially in the ethanol industries, where vinasse is generated: sugarcane molasses and sugarcane juice. Molasses arises from sugar production, after the sugarcane juice concentration and centrifugation. Usually it is used in yeast fermentation for ethanol production, together with sugarcane juice.

Considering the usage of molasses or sugarcane juice as carbon sources they do not burdens on the cost of the medium for biohydrogen production. At this point, promotion and maintenance of anaerobic environment are the processes that will probably impact most significantly the price of the final product. If purified, biohydrogen can be used in chemical industry or in fuel cells for the production of electricity. Otherwise, the hydrogen-rich biogas can be used for heat generation through direct combustion or in boilers.

Preliminary studies on the evaluation of using vinasse as culture medium for biohydrogen and VFAs production by anaerobic bacteria were carried at the Laboratoire de Gènie Chimique et Biochimique (LGCB) at the Université Blaise Pascal - Clermont-Ferrand, France, and are described in this chapter.

2 Material and Methods

2.1 Anaerobic Medium Preparation

The procedures for promoting an anaerobic culture were based on the technique developed by Ralph S. Wolfe during the mid-1970s, which is generically referred to as “the Balch technique”.

The removal of oxygen and lowering the redox potential of culture media by the addition of a reducing agent are the two crucial parts of the technique. The removal of oxygen was achieved by boiling the medium under an anoxic ambient (CO_2 atmosphere) (Figure 6). The CO_2 was scrubbed free of oxygen in a heavy-walled copper tube packed with copper turnings and heated to 150–200ºC in a tube furnace.

Figure 6 - Technique of producing an anaerobic medium. Boiling under anoxic environment is one important step.

Bicarbonate was added at the temperature of 85ºC and Cysteine-HCl at 65ºC as reducing agents to lower the redox potential of medium. To assure oxygen removal Resazurin was used as indicator. After naturally cooling to room temperature the medium was distributed into 15ml Hungate tubes under pure CO_2 atmosphere and autoclaved.

The experiments were carried out in 15 ml Hungate tubes, with working volume of 6 ml, sealed with autoclavable Bakelite lids with rubber stopper and incubated in a shaker at 37ºC and 30 rpm.

Fermentation medium was constituted by pure vinasse supplemented with 10g/L of one of the following carbon sources: glycerol, sucrose and glucose. The cultures were maintained at these conditions for 1 week and then inoculated in a new medium. Each new culture will be called “generation”.

2.2 Microorganisms

Two known *Clostridium* strains, *C. saccharoperbutylacetonicum* and *C. beijerinckii* purchased from ATCC (ATCC #27021 and #8260, respectively), two isolated *Clostridium* strains (C2 and C6) and one natural vinasse consortium (VINA) were used.

The two ATCC strains are potential hydrogen and VFAs producers able to use sucrose as carbon source. The isolated Clostridium strains, C2 and C6, were chosen based in hydrogen and VFAs production among other isolated strains from the Laboratory Génie Chimique et Biochimique (LGCB). C6 is capable of using sucrose as carbon source, while C2 can only growth in glucose medium. The vinasse natural consortium, VINA, was obtained directly by incubating anaerobic pure vinasse supplemented with sucrose.

2.3 Biogas Production and Composition Analysis

Biogas production in Hungate tubes cultures was periodically measured using 60 mL plastic syringes (Figure 7). Gas production was measured and analyzed twice in a week or daily, according to the experiment. Those cultures degassed daily were considered free of H_2 partial pressure. Hydrogen total production and production rate was calculated based on the volume of medium, gas composition and intervals of analysis.

Figure 7 - Biogas quantification using a 60ml syringe.

The biogas sampled from the headspace was analyzed using a MicroGC Agilent 300A with 2 channels for gas analysis. Hydrogen (H_2), oxygen (O_2), nitrogen (N_2) and methane (CH_4) was measured through a MoleSieve 5A (10mx0.32mm) column operated at 100ºC, at injector temperature of 95 °C,

using argon as the carrier gas at 30ψ. Carbon dioxide (CO_2), hydrogen sulfite (H_2S), air and water vapor ($H_2O_{(v)}$) were measured in a PLOT U (8mx0.32mm) column operated at 70ºC, at injector temperature of 70 °C, using hydrogen as carrier gas at 15ψ. Each column was connected to a separated TCD for detection.

2.4 Ion Chromatography (IC)

Ion chromatography (761 Compact IC 817 Bioscan chromatograph) was used for the determination of vinasse mineral composition. For cations analysis a Metrohm METROSEP C3 250/4.0 (250 mL x 4.0 mmID) column was used. Analytical conditions were: 3.5 mM HNO3, 1.0 mL/min, 40ºC, 20 µL sample volume, 11.2 MPa. A standard chromatogram was prepared with the following cations: Ca, Mg, K, Na, Zn, NH_4 and Fe. Anions analyses were made in a Metrosept A Supp 5 250/4.0 column (250 mL x 4.0mmID). Analytical conditions were: 3,2mM Na_2CO_3 + 1mM $NaHCO_3$, 1.0 mL/min, 40ºC, 20 µL sample volume, 10.2 MPa. A standard chromatogram was prepared with the following anions: F, Br, NO_3, PO_4, SO_4 and Cl. All reagents used were analytical grade (Sigma–Aldrich).

2.5 High Performance Liquid Chromatography (HPLC)

Organic components were determined through High Performance Liquid Chromatography (HPLC). Before injection the samples (2 ml) was treated with 0.25 ml of BaOH (0.3M) and 0.25 ml of $ZnSO_4$ (5%), centrifuged for 10 min at 10^4xg and filtered (Milipore 0,2µm), to avoid column obstruction by suspended solids.

The HPLC equipment used was an Agilent 1100, equipped with 2 ion exclusion columns (Phenomenex Rezex ROA 300 x 7.8 nm) placed in series in a 50ºC oven. A 2mM sulfuric acid in ultrapure water solution (Millipore, MilliQ plus) was used for elution at 0.7 ml flux (pomp HP 1100 series, Agilent Technologies). The chromatograph is equipped with an automatic injector (Agilent Rhéodyne). Detection was done through a refractive index detector (HP

1100 series) and the signals integrated (HP 1100 series). The acquisition is done by the HPChem program (Agilent Technologies). The compounds quantified by this method are cellobiose, glucose, fructose, succinate, lactate, formate, acetate, propionate, isobutyrate, butyrate, isovalerate and valerate.

2.6 Nuclear Magnetic Resonance

Measurements of NMR spectra were performed at 27 °C on a 300 or 500 MHz Avance Bruker spectrometer equipped with 5mm TXI ^{1}H, ^{13}C, ^{15}N probe with inverse detection.

Samples were centrifuged (10000 rpm, 10min) and to 540 µl of supernatant, 60 µl of a solution $TSPD_4$ (2,08 ml TSPD4 10mM + 7,92ml D_2O - used as internal reference for chemical shift and quantification).

2.7 Proteins and Aminoacids Quantification

Proteins were quantified by the method of Bradford. The Dye stock was prepared by dissolving 100 mg of Coomassie Blue G in 50 ml of methanol, followed by the addition of 100 ml of 85% H_3PO_4 and dilution to 200 ml with distilled water. Due to the natural color of vinasse, the methodology was adapted. The procedure was made by adding 1 ml of dye stock to 4 ml of sample. The absorbance was read at 595 nm. A standard curve was made using vinasse instead of water by adding known quantities of BSA to each sample, in order to minimize the effect of vinasse's color on the results. The amount of protein in vinasse was determined based on the equation obtained by the linearized curve.

3 Results and Discussion

3.1 Vinasse Analysis

The mineral composition of vinasse was analyzed by ion chromatography and is presented in Table 5. The ions that could not be determined were

considered based on the analysis made by Neto and Nakahodo in 1995 (54). The organic composition of vinasse was determined by HPLC and RMN (Table 6).

Table 5 – Mineral composition determined by Ion Chromatography. Those considered following the studies of Neto and Nakahodo, 1995 (54), are signaled with a (*).

Mineral	**mg/L**
Ca	515,25
Cl	1218,91
P	120,82
Mg	244,71
N	356,63
K	1750,9
Na	51,55
SO_4	1537,66
N_{NH3}*	10,94
Cu*	1,2
Fe*	25,17
Mn*	4,82
SO_3*	35,9
Zn*	1,7

Table 6 – VFAs composition of vinasse by HPLC and RMN analysis.

VFA	**mg/L**
Ethanol	0
Butyrate	1300
Propionate	1100
Acetate	700
Lactate	200

Proteins were quantified by the method of Bradford and resulted in approximately 670 mg/L. Aminoacids quantified by the ninhydrin method resulted in 470 mg/L. Since no carbohydrate was detected, it was expected the

necessity to supplement vinasse medium with an organic source of carbon for feasible biohydrogen production.

As expected, vinasse analysis indicated that it is a rich residue, containing a great variety of mineral compounds. This is interesting for bacterial growth and also in promoting hydrogen production (especially iron). The presence of some VFAs is not ideal but they are present in low amounts and might not be a problem for biohydrogen production.

3.2 Effect of carbon source in biohydrogen and VFAs production

Since vinasse analysis indicated absence of sugars, different carbon sources were added to vinasse. The choice of the carbon source to be added is of great economic importance to the process. The use of pure carbon sources in these preliminary experiments was carried in order to evaluate the metabolism and the potential of each strain prior to the use of complex substrates.

The following substrates were evaluated in these preliminary experiments:

(i) Sucrose: sucrose is present in high concentrations in sugarcane molasses, a residue from industrial sugar production, and also in sugarcane juice, which is extracted for both alcohol and sugar production. Because of its availability sucrose (or alternative sources of sucrose) is probably the most interesting carbon source to be used.

(ii) Glycerol: glycerol is another interesting carbon source because it is produced in great amounts in biodiesel industries, which are largely increasing in the last years. The fate of the glycerol generated in biodiesel industries is object of great concern due to the enormous amounts produced, making it an interesting substrate for the process proposed in this work.

(iii) Glucose was also tested to serve as model as it is the most easily assimilated source of carbon by the majority of microorganisms. It can be obtained from complex substrates through hydrolysis.

3.2.1.1 Hydrogen production in vinasse medium supplemented with pure carbon sources

During 20 generations the gas produced during fermentation was measured and analyzed twice a week (4th and 7th days of fermentation). Results of average hydrogen production rate (in $mL.L^{-1}.day^{-1}$) and average total production (in $mL_{H2}.L^{-1}$) of each strain are showed in Table 7.

Table 7 – Hydrogen production by 5 strains grown in vinasse medium supplemented with different carbon sources. Results represent an average of 20 generation measurements.

Strain	Carbon Source	H_2 (ml/L/day)	Total H_2 (ml/L)	Hydrogen in Gas Phase (%)
C2	Glucose	104.0±46.5	728	10
C2	Glycerol	7.9±1.9	55.3	3
C2	Sucrose	0	0	0
C6	Glucose	237.6	1663.2	9
C6	Glycerol	7.9±2.7	55.3	3
C6	Sucrose	197.3±11.5	1381.1	13
VINA	Glucose	643.4	4503.8	25
VINA	Glycerol	20.0±7.0	140.0	2.5
VINA	Sucrose	262.6±66	1838.2	12
ATCC 27021	Glucose	730.5	5113.5	35
ATCC 27021	Glycerol	0	0	0
ATCC 27021	Sucrose	587.8±160	4114.6	36
ATCC 8260	Glucose	780.3	5462.1	40
ATCC 8260	Glycerol	0	0	0
ATCC 8260	Sucrose	635.3±89	4447.1	34

It is can be observed that the pure strain C2 was not capable of growing in sucrose vinasse medium, while ATCC 8260 and ATCC 27021 were not capable of growing in medium supplemented with glycerol.

Hydrogen production was higher in vinasse medium supplemented with glucose for all the strains tested. The lower yields were achieved when glycerol was used as carbon source. For all strains, a fluctuation in H_2 production was

observed, which might be a consequence of the high complexity of natural vinasse.

The consortium VINA presented a great difference in terms of hydrogen production when grown in glucose and sucrose medium. This indicates that this consortium is composed by some microorganisms which cannot use sucrose or fructose as carbon source to produce hydrogen.

The pure strains ATCC 27021 and ATCC 8260 presented the best results for biohydrogen production. The higher volume of hydrogen produced were accompanied by higher hydrogen concentration on the gas phase, which is also important for future gas purification processes.

In those media supplemented with sucrose, H_2 production was considerable high and not much lower than when glucose was used, except for VINA consortium. An interesting point that might be considered is the availability of cheap sucrose sources in Brazilian Ethanol Industries (molasses and sugarcane juice). For these reasons sucrose was chosen as the carbon source for the following experiments.

3.2.1.2 Liquid phase analysis of cultures carried in vinasse medium with sucrose as carbon source

In the 7th day of the cultures carried in sucrose supplemented vinasse medium samples were withdrawed and analyzed. Results of HPLC and RMN analysis of the fermented broth are presented in

Table **8**.

Table 8 – VFAs produced (in g/L) by different strains and the consortium VINA in vinasse based medium.

Strain	Acetate	Formate	Butyrate	Ethanol	Propionate	Lactate
ATCC 27021	1.79±0.25	0	3.53±0.14	0	1.42±0.0	0.79±0.09
ATCC 8260	1.64±0,11	0	4.28±0.21	0	1.39±0.05	0.59±0.0
VINA	1.7±0.2	0.6±0.2	2.3±0.31	1.8	0.9±0.0	0.25±0.05
C2	1.3±0.0	0.15±0.05	1.6±0.09	2.4	1.4±0.3	0.1
C6	1.4±0.2	0.5±0.3	1.3±0.11	2.6±0.11	1.0±0.1	0.2±0.15

Acetate and butyrate were the main VFA products by VINA, C2 and C6. These strains also produced ethanol and propionate. ATCC 27021 and ATCC 8260 presented acetate, butyrate and propionate as main products. Lactate was also produced in significant amount, suggesting that the metabolism of Acetyl Co-A (and consequently H_2 and VFAs) was blocked. Valerate and isobutyrate were found in trace concentrations and are not showed. The presence of more reduced products, such as ethanol, is an evidence of a metabolic shift caused by hydrogen partial pressure caused by non-continuous gas measurements.

3.2.1.3 Metabolism effect when minimizing H_2 partial pressure

Knowing that hydrogen partial pressure is central in hydrogen production an experiment was carried withdrawing (and analyzing) daily the gas produced during fermentation. Results are showed in

Table **9**.

Table 9 – Hydrogen production by 5 strains grown in Natural Vinasse Media (NVM) supplemented with different carbon sources avoiding hydrogen accumulation. Results represent an average of 20 generation measurements.

Strain	Carbon Source	H_2 (ml/L/day)	Total H_2 (ml/L)	Hydrogen in Gas Phase (%)
C2	Glucose	100.0	700.0	7
C6	Sucrose	202.8	1419.6	10
VINA	Sucrose	403.9	2827.3	13.4
ATCC 27021	Sucrose	2526.3	17684.1	33
ATCC 8260	Sucrose	1895.8	13270.6	24

A great increase of hydrogen production was observed for both ATCC strains and for the consortium VINA in comparison with the experiments described in the previous section (Table 7).

Since gas production was greatly increased by minimizing H_2 partial pressure, analysis of the liquid phase was also carried. The VFAs analysis at the 4th day of cultivation is showed in Table 10. At this point no sugars were detected in cultivations of C6, C2 and VINA, while in cultivations of ATCC 27021 and ATCC 8260 3.5g/L and 0.37g/L, respectively, were detected. The negative concentrations found for propionate and lactate indicate the consumption of this metabolites in comparison to the non-fermented medium.

Table 10 – VFAs concentrations (g/L) at the 4th day of fermentation in vinasse based medium.

Strain	Acetate	Formate	Butyrate	Ethanol	Propionate	Lactate	Succinate
ATCC 27021	0.90	0	2.49	0	-0.38	-0.37	0.16
ATCC 8260	0.98	0	3.13	0.08	-1.10	-0.37	0.16
VINA	1.05	0.4	0.41	2.60	-0.70	-0.37	0
C2	0.82	0.32	0.49	2.00	-0.38	-0.37	0
C6	0.98	0.4	0.17	2.64	-0.70	0	0.08

Another VFAs analysis was carried in the last day of culture (7th day), when the substrate was completely consumed in all cultures. Results are showed in Table 11.

Table 11 – VFAs concentration (g/L) at the 7th day of fermentation in vinasse based medium.

Strain	Acetate	Formate	Butyrate	Ethanol	Propionate	Lactate	Succinate
ATCC 27021	1.14	0	3.61	0	-1.10	-0.37	0.16
ATCC 8260	1.94	0.08	2.55	0.16	-1.10	-0.37	0.24
VINA	0.98	0.40	0.41	2.48	-0.70	-0.37	0
C2	0.98	0.4	0.17	2.60	-0.70	0	0.08
C6	1.14	0.48	0.25	2.88	-0.62	-0.13	0

Strains ATCC 27021 and ATCC 8260 produced acetate and butyrate as main VFAs, while C2, C6 and the consortium VINA produced mostly acetate and ethanol. All strains presented the capacity of consuming lactate and propionate under the conditions tested.

Comparing the VFAs production in 4th and 7th day it can be observed a considerable increase in butyrate concentration in ATCC 27021 and in C2 (although butyrate concentration in C2 is low) and in acetate for ATCC 8260. The other VFAs didn't show considerable variation, which is compatible with the presence of sugar in 4th day and with the consumption of propionate between the 4th and 7th day.

It is also interesting to note the effect of the H_2 partial pressure in VFAs production by comparing tables 8 and 11. When H_2 partial pressure was minimized it was observed an enhanced production of more oxidized products.

Based on VFAs and gas analysis it was noted that high butyrate/acetate ratio is related to higher hydrogen content in the gas phase (Table 12). At the same time there is a relation between the amount of gas produced and the hydrogen concentration in the gaseous phase.

Table 12 – Butyrate/acetate ratio, gas produced and hydrogen content in the gas phase in the experiments carried with each strain. Butyrate/Acetate ratio was considered based on VFAs analysis of the 7th day of fermentation.

	C2	C6	VINA	ATCC 8260	ATCC 27021
Butyrate/Acetate	0,17	0,22	0,42	1,31	3,17
H_2 (%)	7	10	13,4	24	33
Gas (L_{gas}/L_{medium})	10	14,19	21,10	55,29	53,59

4. Conclusion

Vinasse has proved to be an interesting base medium for biohydrogen and VFAs production by anaerobic bacteria. Higher yields were achieved when glucose was used as substrate, closely followed by the yields achieved in sucrose vinasse medium. Due to process economics (higher availability and no need of processing) sucrose was chosen as the substrate for VFAs biohydrogen production in vinasse based medium.

CHAPTER 3

Metabolic analysis of potential strains and consortia for the production of biohydrogen and VFAs in vinasse medium

1 Introduction

The anaerobic degradation of organic matter by heterotrophic microorganisms can liberate H_2 at high rates, depending on the particular organisms and conditions. Hydrogen producing microorganisms can be divided in four groups: strict anaerobes (I), facultative aerobes (II), aerobes (III), co- and mixed cultures (IV) (2). The first group is the most studied, and the most important microorganisms are *Clostridia*, Rumen bacteria, thermophiles and methanogens. Known facultative anaerobes include *Enterobacter*, *E. coli* and *Citrobacter* and known aerobes are *Alcaligenes* and *Bacillus*.

Most studies described in the literature use glucose and sucrose as carbon sources for biohydrogen production via dark fermentation (The search for endogenous microorganisms in the development of bioprocess technologies is of great importance due to their increased adaptation to specific conditions. It is believed that for future applications of biohydrogen processes the use of mixed cultures from industrial wastes might have more advantages because they are less susceptible to contamination by H_2-consuming bacteria and are more sensitive to process variations.

Other advantages of making use of a diverse microbial community when (agro)industrial wastes are used as substrate are: (i) development of a food web where specific groups of organisms maintain low concentration of critical intermediate products and promote flux of carbon and electrons from the feedstock material to the desired end product by reducing direct inhibition of microbial activity by metabolic intermediates (121); (ii) higher adaptation to substrate variation, which is an intrinsic characteristic of (agro)industrial wastewaters, due to the presence of alternative metabolic pathways.

Microflora from various sources has been used as inoculum for hydrogen production (see some examples in

Table **14**). Digester sludge from the treatment of urban wastewater, livestock waste, anaerobic sludge effluent and soil are common sources of inoculum. To avoid methane producers, heating (75-121^{o}C for 15-120min) or chemical (2-bromoethanesulfonate) treatments are frequently used.

Table 13).

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Table 13 – Yields of biohydrogen production of microorganisms grown in pure carbon sources.

Microorganism	$Y_{(H2/S)}$ (mol.mol^{-1})	Carbon source	Reference
Clostridium acetobutylicum	1.39	glucose	93
Clostidium beijericnkii	1.86	glucose	94
Clostidium beijericnkii	4.20	sucrose	95
Clostidium butyricum	1.35	sucrose	96
Clostidium *saccharoperbutylacetonicum*	1.72	glucose	97
Clostridium sp.	3.24	lactose	98
Clostridium beijericnkii	3.9	cellobiose	95
Clostridium butyricum	0.72	xylose	99
Enterobacter aerogenes	1.89	sucrose	100
Enterobacter aerogenes	0.83	lactose	100
Enterobacter aerogenes	0.39	fructose	101
Escherichia coli	1.95	glucose	102
Klebsiella oxytoca	1.5	sucrose	103
Ruminococcus albus	2.11	glucose	104
Ruminococcus albus	1.44	arabinose	105
Thermoanaerobacterium thermosaccharolyticum	7.44	lactose	106
Thermoanaerobium thermosaccharolyticum	2.42	glucose	107

Table 14 - Some yields achieved by using consortia for in fermentation of different substrates.

Culture	$Y_{(H2/S)}$ (mol.mol^{-1})	Carbon source	Reference
Anaerobic Digester	2.18	Glucose	87
Rice Rhizosphere microflora	2.3	Apple pomace wastes	88
Activated and Digested sludge	1.16	Glucose	89
Digested wastewater sludge	6.12	Sucrose	90
Methanogenic granules	1.2	Glucose	91
Anaerobic mixed culture	5.15	Sugar-beet pulp	92

Since the choice of the microorganism is of great importance, this chapter reports the search and evaluation of potential strains and consortia from the Brazilian environment for the production of biohydrogen and VFAs. Samples were taken from environments capable of supporting anaerobic forms of life. The metabolic behavior of each strain/consortium was evaluated in vinasse medium supplemented with cheap sources of sucrose (sugarcane juice and sugarcane molasses) and under the presence/absence of hydrogen partial pressure.

2 Material and Methods

2.1 Microorganisms

Besides the strains ATCC 8260, ATCC 27021, C2, C6 and the consortium VINA used in the experiments described in the previous chapter, 9 samples of Brazilian environments with proper conditions for the development of methane producers (and consequently, hydrogen producers) were collected. The name of the strains and origin are described in Table 15.

Table 15 – Origin of the samples collected with potential for methane/biohydrogen production.

Name	Origin
LPB AH1	Faeces from fruit bat (unknow species)
LPB AH2	Lake of a dairy farm
LPB AH3	Soil used for Sugarcane cultivation
LPB AH4	Domestic sewage
LPB AH5	Swine faeces
LPB AH6	Mangrove from Matinhos-Paraná
LPB AH7	Cow feaces
LPB AH7	Puddle in a cave at São Paulo

2.2 Medium Composition and Culture Conditions

The experiments were carried out in 15 ml Hungate tubes, with working volume of 6 ml, sealed with autoclavable Bakelite lids with rubber stopper and incubated in a shaker at 37ºC and 30 rpm. The cultures were maintained at these conditions for 1 week and then inoculated in a new medium. 1 ml of culture was, then, inoculated in 5 ml of medium. Each new culture will be called "generation".

Anaerobic environment and medium was carried according to the Balch technique. Bicarbonate was added at 85ºC and Cysteine-HCl at 65ºC as reducing agents to lower the redox potential of medium. Otherwise stated, medium pH was adjusted to 6.8 with 1N KOH.

Anaerobic media containing vinasse and different sucrose sources were used: i) Sucrose + vinasse, ii) Sugarcane molasses + vinasse and iii) Sugarcane juice + vinasse. Carbon source concentration in the media was fixed in 10g/L. Sugarcane molasses addition to reach 10g/L was based on ^{o}brix while sugarcane juice sugar content was quantified by the phenol sulphuric method.

All strains and consortia were cultivated in this media during 15 generations before analysis.

2.3 Culture media and Medium Analysis

Because the vinasse used at the preliminary studies (presented in chapter 2) was concentrated and then reconstituted prior to use, a new fresh vinasse was used in media preparation. This decision was based on the unknown effects of concentration in vinasse composition and the necessity to carry the experiments (described in chapters 3, 4 and 5) with the same vinasse.

The new vinasse was a courtesy of Usina Moreno (located in Planalto-SP) and was collected from the first storage tank situated after the distillation unit. The industrial process carried at Usina Moreno involves the use of the excess molasses from sugar production together with sugarcane juice to produce ethanol.

Vinasse composition was determined by BioAgri Laboratories (registration number 278887/2011-0) and is presented in It can be noticed the presence of important ions for the production of biohydrogen, such as iron, manganese, magnesium and phosphorus. Moreover, the low content of nitrogen indicates that microbial growth will be greatly limited unless it is added to the medium.

Regarding this, total nitrogen content in the medium supplemented with molasses and sugarcane juice was determined by the Kjeldahl method. In molasses supplemented medium, nitrogen content was approx. 73 mg/L while in sugarcane it was approx. 27mg/L.

Table 16.

Experiments were also carried in a synthetic medium, known as Clostridium acetobutylicum medium (CAB), largely used in cultivation of Clostridia. CAB medium contains, per liter: 4.0 g yeast extract, 1.0 g tryptone, 1.5 g K_2HPO_4, 0.5 g asparagine, 1 ml of 0.2%(v/v) resazurin, 0.1 g $MgSO_4.7H_2O$, 0.1 g $MnSO_4.H_2O$, 15 mh $FeSO_4.7H_2O$, 0.1 g NaCl, 10 g sucrose. pH was adjusted to 7,0 with KOH.

2.4 High Performance Liquid Chromatography (HPLC) and Ethanol quantification.

Organic components were determined through High Performance Liquid Chromatography (HPLC). Before injection the samples (2 ml) were centrifuged and filtered (Milipore 0,2µm).

The HPLC equipment was an Shimadzu Liquid Chromatograph equipped with a Aminex® HPX-87H 300 x 7,8mm (Bio-Rad) column and a refractive index detector (RID-10A). The column was kept at 60^oC and a 5mM H_2SO_4 at 0,6 ml/min was used as mobile phase. The compounds quantified by this method are glucose, fructose, succinate, lactate, formate, acetate, propionate and butyrate. All chemicals used were of analytical grade.

Because the retention time of butyrate and ethanol are very similar, it was impossible to differentiate them by HPLC. The method used for determining ethanol content was based on the titration of excess $K_2Cr_2O_7$ from the oxidation of ethanol (with potassium dichromate in an acidic medium) with Ferrous Ethyleneammonium Sulfate (FEDS). A solution containing 10.00-mL 0.003M $K_2Cr_2O_7$, 5 mL concentrated sulfuric acid and 0,5mL samples. was titrated with FEDS to a salmon color using ferroin as indicator.

2.5 Gas Analysis

Gas was collected by inserting a graduated syringe through the flange-type butyl rubber septum (Figure 8). Cultures degassed daily were considered free of H_2 partial pressure, different from those degassed twice a week (4th and 7th days).

Figure 8 – The use of a graduated syringe in the quantification of the gas produced during fermentation

The gas from some generations (those that were analyzed for VFAs) had the produced gas purified for hydrogen content estimation. Purification was carried by an adaptation of a widely used technique that involves the pulverization of the biogas a column containing a 10% NaOH solution. This system was used because carbon dioxide and H_2S reacts instantly reacts with NaOH, but hydrogen do not. The tower used was made of glass and was filled 50% of its volume with different sized glass beads in order to increase gas contact time with the basic solution. Gas was injected at approximately 2 ml/s through a porous stone. Hydrogen content was estimated by dividing the volume obtained after and before purification.

3 Results and Discussion

3.1 Vinasse composition

The vinasse used in these experiments was analyzed for its composition and is presented in It can be noticed the presence of important ions

for the production of biohydrogen, such as iron, manganese, magnesium and phosphorus. Moreover, the low content of nitrogen indicates that microbial growth will be greatly limited unless it is added to the medium.

Regarding this, total nitrogen content in the medium supplemented with molasses and sugarcane juice was determined by the Kjeldahl method. In molasses supplemented medium, nitrogen content was approx. 73 mg/L while in sugarcane it was approx. 27mg/L.

Table 16.

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Table 16 – Complete composition of the vinasse used during the experiments was carried by BioAgri Laboratory.

Parameter	mg/L
Iron	41,8
Manganese	3,7
Lead	<0,1
Cadmium	<0,1
Mercury	<0,00035
Arsenium	<0,1
pH	4,52
Nitrate	<10
Total Nitrogen (Kjeldahl)	2,15
Sodium	20,1
Calcium	791
Potassium	2386
Magnesium	203
Sulphate	1700
Total Phosphorus	104,9
DBO	8358
DQO	29600

3.2 Strains metabolism analysis

Metabolic behavior in terms of VFAs and hydrogen production of each strain/consortia is depicted in this section. Due to the complexity of the metabolism of hydrogen producers and the use consortia a metabolic analysis is very intricate. Moreover, the synergetic effects of hydrogen partial pressure and carbon source on hydrogen producer metabolisms further increase this complexity.

3.2.1 ATCC 8260

According to the described methods, VFAs and gas production analysis were carried and the results are shown in Table 17. It can be noticed a great difference in the profile of VFAs when a complex substrate was used in comparison with pure sucrose. Moreover, different sources of carbon resulted in different profile of VFAs.

Table 17 – Metabolic products of the cultivation of ATCC 8260 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
ATCC 8260	**CAB**	0	8,070	0	0.089	0	0.230	0	<0,40
	Juice	0	0	0	1,196	0,074	4,485	0	1,35±0,33
	Molasses	0	0	0	0,352	0,241	3,982	0	0,73±0,17
	Sucrose	0,285	0	2,695	0,14	-0,707	3,211	0	1,84±0,34
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
ATCC 8260	**Juice**	0	0,299	0	3,446	0,624	7,594	0	1,70±0,3
	Molasses	0	0	0	-1,032	0,988	4,242	0	1,04±0,12
	Sucrose	0	0	0	1,263	-0,41	5,798	0	2,77±0,21

It can be noticed that ATCC8260 is mainly an acetate-butyrate producer, except when cultured in the synthetic CAB medium. In medium containing sucrose and avoiding H_2 pressure, only these two VFAs were produced, while in CAB medium high amounts of lactic acid was produced. Sucrose was the carbon source that gave best hydrogen and VFAs yields.

In terms of hydrogen production, cultivation of ATCC 8260 in artificial medium resulted in very low amounts when compared to vinasse-based medium. Considerable improvement in H_2 production was noticed on fermentations avoiding H_2 pressure, which was expected.

3.2.2 ATCC 27021

The strain ATCC 27021 presented the best results for biohydrogen production in the preliminary experiments (Chapter 2). A great effort was made in order to keep that productivity but the strain showed to be very sensible and hard to work with (at frequent time intervals the culture showed no gas production). This was also noted by partners that started working with this strain at Blaise Pascal University. Because this would result in difficulties at manipulation in industrial scale, this strain was no longer used.

3.2.3 C6

Volatile fatty acids and hydrogen production by this strain is showed in Table 18. This strain is a potential ethanol producer, which was also observed in the experiments described in Chapter 2.

Table 18 - Metabolic products of the cultivation of the strain C6 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
C6	**CAB**	0	7,932	0	0	0	0	0	0,48±0,08
	Juice	0	0	0	2,677	0,172	3,612	2,71	0,99±0,32
	Molasses	0	0	0,172	1,231	0,065	2,895	1,77	0,95±0,23
	Sucrose	0,716	0	0,906	-0,292	-0,146	4,895	1,07	1,36±0,22
Without H2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
C6	**Juice**	0	0	0,335	2,375	0,513	6,557	2,21	1,83±0,55
	Molasses	0	0	0,816	-1,052	-0,3875	3,099	0,32	1,99±0,45
	Sucrose	0,129	0	0,466	-0,274	-0,392	5,448	1,11	1,68±0,23

High hydrogen production was achieved using molasses as carbon source and avoiding hydrogen partial pressure, situation which resulted in lower ethanol production. When sugarcane juice was used as carbon source a great

amount of butyric acid was produced, but the presence of other VFAs would result in laborious purification process.

As observed for ATCC 8260, large amounts of lactic acid were produced when CAB medium was used. Accompanied by this, very low amounts of hydrogen were produced.

3.2.4 VINA

The metabolic analysis (

Table **19**) of the consortium VINA showed a great proportional effect of hydrogen partial pressure in ethanol production. Because this consortium was originated from the vinasse itself, the ethanol production observed was expected.

The use of molasses and sugarcane juice also caused changes in metabolism, probably due to variations in the consortium composition caused by the different composition of such complex substrates. Pure sucrose was the best carbon source for hydrogen production, followed by molasses.

It is interesting to note that when the synthetic medium was used, again a completely different profile of VFAs was noted. At the same time, and as observed in the previous strains and consortia, lower amount of hydrogen and great amounts of lactic acid was produced when compared to vinasse-based medium.

Table 19 - Metabolic products of the cultivation of the consortium VINA in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
VINA	**CAB**	0,352	7,724	1,371	0	0	0	0	<0,40
	Juice	0	0	0,058	2,803	0,123	2,999	3,19	0,78±0,32
	Molasses	0	0	1,793	1,932	0,508	2,908	1,99	1,13±0,31
	Sucrose	1,428	0	0,507	-0,801	-0,288	3,821	2,04	1,66±0,35
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
VINA	**Juice**	0	1,837	0	-1,144	3,135	1,729	2,99	0,51±0,18
	Molasses	0	0	0	0,95	-0,1125	1,324	0,52	1,84±0,26
	Sucrose	0,499	0	0,671	3,219	-0,208	1,75	2,33	2,58±0,41

3.2.5 LPB AH3

When cultured in vinasse medium the consortium LPB AH3 presented a very high production of butyric acid for all substrates tested. Sucrose was the best carbon source for hydrogen production in vinasse medium, with yields slight higher than those of sugarcane juice (

Table **20**).

The use of molasses resulted in very low hydrogen yield but high amount of butyric acid. The highest amount of butyric acid (10 $g.L^{-1}$) among all strains evaluated was produced by this consortium (in sugarcane juice supplemented medium).

Table 20 - Metabolic products of the cultivation of the consortium LPB AH3 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH3	**CAB**	0,105	0	0,627	2,150	0	1,5105	0.3450	0,58±0,06
	Juice	0	0	0	2,167	0,627	6,073	1,78	0,83±0,26
	Molasses	0	0	0	-1,046	0,267	4,127	1,06	<0,40
	Sucrose	0	0	0	3,113	0,576	5,238	0,99	1,37±0,18
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH3	**Juice**	0	0	0	-1,236	1,243	10,088	2,21	1,04±0,14
	Molasses	0	0	0	0,917	1,3435	7,13	1,09	0,60±0,19
	Sucrose	0	0	0	3,069	0,798	7,896	1,02	1,63±0,10

Because the amount of metabolites produced is greater than the available substrate for fermentation, we can conclude that this consortium is capable of using other components from vinasse, sugarcane juice and molasses as carbon source.

3.2.6 LPB AH1

The consortium LPB AH1 presented a high capacity to produce biohydrogen in vinasse medium, especially when sucrose or sugarcane juice was used as carbon source (Table 21). When CAB medium was used, about half of the biohydrogen production achieved in vinasse medium supplemented with sugarcane juice and sucrose was achieved.

From Table 21 it can be noticed that in vinasse medium supplemented with sugarcane juice the effect of H_2 partial pressure in H_2 production was minimum. This is very interesting considering industrial application because facilitates process handling. On the other hand, the profile of VFAs under and avoiding H_2 pressure was very different, resulting in propionic acid accumulation in the first condition. The effect of the synthetic medium in the consortia

development can also be noticed by the production of formic acid instead of propionate.

Table 21 - Metabolic products of the cultivation of the consortium LPB AH1 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH1	**CAB**	0.14	0	0.285	1.324	0	1.216	0	0,83±0,1
	Juice	0	0	0	0,599	1,321	6,793	0	2,03±0,31
	Molasses	0	0	0,067	1,157	0,118	4,322	0,2	1,15±0,24
	Sucrose	0	0	0,163	-0,581	-0,2	6,995	0	2,08±0,19
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH1	**Juice**	0	0	0,185	3,525	0,408	7,642	0	2,25±0,29
	Molasses	0,05	0	0	-2,085	-0,4025	4,421	0	1,97±0,26
	Sucrose	0	0	1,393	1,049	-0,451	4,824	0	2,94±0,31

3.2.7 LPB AH2

Metabolic analysis of the consortium LPB AH2 showed a great potential for biohydrogen production in molasses and sugarcane juice supplemented media (Table 22). The use of molasses as carbon source together with the maintenance of a low H_2 partial pressure environment resulted in the exclusive production of butyrate as VFA.

Higher H2 production was observed in fermentations carried under reduced H_2 pressure, except in sucrose supplemented medium where no statistical difference was noted, which is interesting because facilitates the management of the process in an industrial scale.

Table 22 - Metabolic products of the cultivation of the consortium LPB AH2 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH2	**CAB**	0	0	0,06	2,12	0	1,028	0	0,99±0,08
	Juice	0	4,197	0	2,956	0,311	6,313	0	1,74±0,42
	Molasses	0	0	0	3,783	0,383	6,396	0	1,45±0,29
	Sucrose	1,17	0	0,876	1,333	-0,398	7,044	0	2,29±0,42
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH2	**Juice**	0	0	0	-2,62	0,478	8,000	0	2,16±0,35
	Molasses	0	0	0	-1,793	-0,1355	6,067	0	2,17±0,25
	Sucrose	2,749	0	0,518	4,709	-0,72	6,809	0	2,37±0,20

3.2.8 LPB AH4

The VFAs profile generated by the fermentation of vinasse based medium with the consortium LPB AH4 is presented in

Table **23**. It can be noticed that a mix of acetic, propionic, butyric and ethanol (and formic acid in sucrose supplemented medium) was produced. It is interesting to observe that in synthetic CAB medium lactate was produced, which was not noted in vinasse-based medium.

In terms of hydrogen production, we can note that vinasse medium resulted in higher yields, which is consistent with the theory depicted in Chapter 1 (more reduced products results in less hydrogen yield).

Table 23 - Metabolic products of the cultivation of the consortium LPB AH4 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH4	**CAB**	0,4395	8,76	0	0	0	1,0612	0,487	0,55±0,1
	Juice	0	0	0	1,574	0,045	2,649	1,612	0,81±0,20
	Molasses	0	0	0	0,671	1,173	2,248	0,83	0,82±0,26
	Sucrose	0	0	0,639	-0,439	-0,53	2,59	0,99	1,25±0,26
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH4	**Juice**	0	0	0	1,06	1,269	4,466	1,1	0,93±0,28
	Molasses	0	0	1,505	1,394	-0,2125	2,138	0,88	1,07±0,23
	Sucrose	0,649	0	0,564	-0,052	0,042	6,317	0,41	1,34±0,30

3.2.9 LPB AH5

The consortium LPB AH5 didn't presented capacity to use molasses and sugarcane juice as carbon sources for growth. Because sucrose was consumed both in synthetic (CAB) and vinasse media it is possible that some constituent(s) of molasses and juice is(are) toxic to this consortium. Even in sucrose based media the amount of VFAs and hydrogen produced was too low.

Table 24 - Metabolic products of the cultivation of the consortium LPB AH5 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH5	**CAB**	0	0,083	0,573	0,451	0	0,677	0	<0,40
	Juice	0	0	0	0	0	0	0	0
	Molasses	0	0	0	0	0	0	0	0
	Sucrose	0	0	0,088	2,058	2,125	0,658	1,43	0,52±0,05
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH5	**Juice**	0	0	0	0	0	0	0	0
	Molasses	0,363	0	0	-0,659	0,287	0,02	0	<0,40
	Sucrose	0	0	0	3,192	2,579	1,469	0,54	0,54±0,10

*not possible to determine (above limit of detection of the method used)

3.2.10 LPB AH6

When cultivated in the media supplemented with sucrose, the consortium LPB AH6 presented a high hydrogen production. In vinasse based medium this condition was achieved since a low H_2 pressure was kept.

The consortium LPB AH6 was the only consortium to presented adaptation to molasses but no adaptation to sugarcane juice, which was not expected because molasses is usually more toxic to some microorganisms.

Table 25 - Metabolic products of the cultivation of the consortium LPB AH6 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH6	**CAB**	0	9,591	0,99	0	0	0	0	0,61±0,08
	Juice	0	0	0	0	0	0	0	0
	Molasses	0	0	0,26	1,363	1,041	2,57	1,05	1,15±0,32
	Sucrose	1,006	0	0,606	0,662	-0,53	2,717	0,76	1,47±0,35
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH6	**Juice**	0	0	0	0	0	0	0	0
	Molasses	0	0	0	-2,103	2,2065	2,004	0,88	1,58±0,27
	Sucrose	1,321	0	0,604	0,301	0,797	3,291	0,82	2,31±0,38

3.2.11 LPB AH7

The consortium LPB AH7 showed a behavior similar to the observed for the consortium LPB AH5: hydrogen and VFAs production was observed only in those media where pure sucrose was used as carbon source.

Table 26 - Metabolic products of the cultivation of the consortium LPB AH7 in vinasse medium containing different carbon sources. Results include cultivation allowing and avoiding H_2 partial pressure. VFAs concentration is shown in $g.L^{-1}$. Results are the average of 5 analyses.

With H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH7	**CAB**	0	9,030	0	0	0	0	0	0,62±0,11
	Juice	0	0	0	0	0	0	0	0
	Molasses	0	0	0	0	0	0	0	0
	Sucrose	0	0	0	3,169	3,756	2,054	0	0,37±0,10
Without H_2 partial pressure									
Strain	**Carbon source**	**Succinic**	**Lactic**	**Formic**	**Acetic**	**Propionic**	**Butyric**	**Ethanol**	**Hydrogen (L/L)**
LPB AH7	**Juice**	0	0	0	0	0	0	0	0
	Molasses	0	0	0	0	0	0	0	0
	Sucrose	1,321	0	0,823	0	2,954	0,185	0	0,51±0,08

3.3 Conclusions

Because our objective was to develop an economic feasible process of biohydrogen production with the possibility to take advantage of the VFAs produced, the selection of strains was carried considering the capacity to produce biohydrogen in vinasse medium supplemented with complex carbon sources (molasses and sugarcane juice) and the profile of VFAs produced.

The consortium LPB AH2 presented the highest H_2 production capacity in vinasse medium with molasses (2.17 L_{H2}/L_{medium}). At this condition, only butyrate was produced, at considerable amount (6.1 $g.L^{-1}$), which is interesting and facilitates its recovery.

In terms of butyric acid production, the consortium LPB AH3 achieved the highest value (10 $g.L^{-1}$), but because of considerable amounts of propionate and ethanol production, the H_2 productivity was low in comparison to others.

In sugarcane juice supplemented medium the consortium LPB AH1 presented the best results. Hydrogen production reached 2.25 L_{H2}/L_{medium}, accompanied by considerable amounts of acetate and butyrate production (3.5 and 7.6 $g.L^{-1}$, respectively), which is relevant in coupling to methane or solvent production.

Two consortia presented considerable ethanol production: C6 and VINA, both in sugarcane supplemented medium and in environment with high H_2 partial pressure. The first one achieved a production of 2.71 $g.L^{-1}$ while the other reached 3.19 $g.L^{-1}$. This is approx. 40% of the ethanol that is produced by yeast fermentation through traditional fermentation. Ethanol associated to hydrogen production in vinasse medium may be interesting due to the possibility of this ethanol recuperation be facilitated since it is quite possible that the bioH_2 facilities are installed coupled to the ethanol plant (more specifically the distillation unit). On the other hand this technology competes with traditional ethanol production due to use of sugarcane juice as substrate and can probably be considered if someday greater restrictions for vinasse disposal are imposed.

It might be also considered the possibility to produce large amounts of lactic acid using the synthetic CAB medium (further studies should be carried on this theme). At the same time, those consortia that produced lactic acid in the synthetic medium but didn't on vinasse medium indicates that changes in process conditions (in vinasse composition, for example) can result in the generation of undesirable products instead of hydrogen.

Based on these observations, the consortium LPB AH2 was chosen for biohydrogen and VFAs production in vinasse medium supplemented with molasses and LPB AH1 in vinasse medium with sugarcane juice.

CHAPTER 4

Optimization of culture conditions of the consortia LPB AH2 and LPB AH1 cultivated in vinasse-based medium for biohydrogen and VFAs production under anaerobic conditions.

1 Introduction

Based on the metabolic analysis of each strain/consortia described in chapter 3, 2 strains were selected as potential biohydrogen and VFAs producers: LPB AH2 and LPB AH1. Before process scaling up an optimization step was carried in order to achieve highest biohydrogen production.

Many factors that fall under the topic of bioprocess parameters have been studied including type of organism/organisms, pH, substrate loading (OLR – organic loading rate), type of reactor/growth conditions (batch, sequencing batch, continuous; CSTR, UASB, etc.), type of substrate (pure carbohydrate, various waste streams), media composition, ions availability, etc. Several approaches that can be considered to increase hydrogen yields in the dark fermentation will be discussed in this chapter.

The yield of hydrogen during dark fermentation is severely affected by the partial pressure of the product. At high H_2 partial pressures a metabolic shift to production of more reduced products, like lactate or ethanol occurs, decreasing the yield of H_2. The formation of relatively reduced organic molecules is an integral part of all dark fermentations and some of these molecules (e.g. acetate) can inhibit H_2 production if allowed to accumulate (12). Metabolic engineering of hydrogen producing microorganisms to minimize production of other more reduced products by blocking their biosynthetic pathways is an alternative to provide higher H_2 yields (13, 14, 15). Gas sparging has also been found to be a useful technique to reduce hydrogen partial pressure in the liquid phase for enhancement of its yield (32) but results in difficulties in hydrogen purification.

In terms of carbon source, only acids are produced when carbon source is limited in the medium (75). Unlike carbon-limited cultures, solvents are produced by cultures grown in phosphate- or sulfate-limited media. pH is also an important factor, as high fermentation rates lead to strong acidification due to the production of organic acids. This can affect both product distribution and biomass production. Higher hydrogen yields will most probably be achieved by limiting cell growth through nutrient limitations, thereby enhancing catabolic

processes but high cell densities are needed to maximize hydrogen production rates.

The balance of the medium to reach this optimal point is crucial in process development. The determination of the composition of complex media for industrial applications plays, thus, an important role in development and maintenance of an industrial H_2 process. Yu et al (57b) reported, for example, that the production of acetate was inhibited by Zn and Cu; but production of propionate and hydrogen was favored at low concentrations of Zn (up to 80 mg l^{-1}) and Cu (up to 40 mg l^{-1}). Other studies indicate that nitrogen, phosphorous and iron are the most important essential nutrients for hydrogen gas production (59). Magnesium ion is also an important cofactor that activates almost 10 enzymes including hexokinase, phosphofructokinase and phosphoglycerate kinase during glycolysis process (66).

Hawkes et al (67) reviewed the media composition for hydrogen production. They found that apart from N and P source, only K, Mg and Fe are common in all recipes in analyzed. A 20-fold variation in the amount of Fe added with respect to hexose concentration was also observed. One or more workers did not add one or more of the elements Ni, Ca, B, Mo, Zn, Co, Cu, Mn or I. Hydrogen production described in the literature showed large variation and most of the time no relation is established with inorganic nutrients consumption. More information on minimum amounts of these nutrients for continuous operation is needed.

The use of hyper-thermophiles and extreme temperatures in hydrogen production represents some gains in terms of hydrogen yields, since at increased temperatures hydrogen production becomes more exergonic (17). Therefore, extreme- and hyper-thermophiles show a better resistance to high hydrogen partial pressures (18). Another advantage of fermentations at extreme temperatures is that the process is less sensitive to contaminations by hydrogen consumers. The major problems are (i) to achieve an economical relation between the energy used in order to heat and maintain the reactor at high temperatures and the H_2 production, and (ii) that extreme thermophiles anaerobic bacteria usually grow to low densities resulting in low production rates.

In this chapter, the optimization of culture parameters was conducted considering the fact that biohydrogen technology faces economical drawbacks. It was described that some micronutrients play an important role in biohydrogen production and could had been considered. But since the main goal is to develop an economic and simple-to-handle process, the smaller the changes made in medium composition, the better for process economics. In this context only pH and the carbon/nitrogen ratio were optimized.

pH and carbon are probably the most important factors to be regulated in anaerobic digestion processes. They play a critical role in governing the metabolic pathways of microbial H_2 production [77] and the composition of the microbial community.

Process optimization was carried by using the Response Surface Methodology (RSM), a widely used technique to model processes in which the response of interest (in this case, biohydrogen production) is influenced by several variables (pH and C/N). Because fist-order models won't be enough, a central composite design (CCD) was chosen in order to estimate with more accuracy the mathematical behavior of biohydrogen production.

2 Material and Methods

2.1 Medium Composition and Culture Conditions

The experiments were carried out in 15 ml Hungate tubes, with working volume of 6 ml, sealed with autoclavable Bakelite lids with rubber stoppers and incubated in a shaker at 37ºC and 30 rpm. The cultures were maintained at these conditions for 1 week and then inoculated in a new medium. 1 ml of culture was, then, inoculated in 5 ml of medium. Each new culture will be called "generation".

Medium pH was adjusted with 1N KOH. Anaerobic environment and medium was carried according to the Balch technique. Bicarbonate was added at 85ºC and Cysteine-HCl at 65ºC as reducing agents to lower the redox potential of medium.

Biohydrogen and VFAs production by the consortium LPB AH2 was carried using vinasse medium supplemented with sugarcane molasses, while the consortium LPB AH1 was cultivated in vinasse medium supplemented with sugarcane juice. Vinasse, molasses and sugarcane juice used in these experiments were the same used in the previous chapter.

2.2 High Performance Liquid Chromatography (HPLC)

Organic components were determined through High Performance Liquid Chromatography (HPLC). Before injection the samples (2 ml) were centrifuged for 10 min at 10^4 g and filtered (Milipore 0.2µm).

The HPLC equipment was an Shimadzu Liquid Chromatograph equipped with a Aminex® HPX-87H 300 x 7,8mm (Bio-Rad) column and a refractive index detector(RID-10A). The column was kept at 60^{o}C and a 5mM H_2SO_4 at 0.6 ml/min was used as mobile phase. The compounds quantified by this method are glucose, fructose, succinate, lactate, formate, acetate, propionate and butyrate. All chemicals used were of analytical grade. Ethanol quantification was carried as described in chapter 3.

2.3 Gas Measurement and Analysis

Before analysis, 7 successive cultivations were made in order to achieve a balanced microbial community (resulting in a theoretical stability of the process). Hydrogen partial pressure was minimized by daily degassing. Total gas production (L_{gas}/L_{medium}) was considered as the sum of the gas produced and quantified daily divided by the volume of medium.

Gas analysis was carried twice a week, more precisely in the 4th and 7th day of culture. Gas was collected by inserting a graduated syringe through the flange-type butyl rubber septum. The gas collected in the 4th day was purified for hydrogen content estimation, as follows.

Since it was noted in chapter 2 that there is a direct relation between gas production and hydrogen content in the gas phase, the optimal conditions was considered as the one that resulted in higher (bio)gas production.

2.4 Strains

The strains used in this experiments were those selected based on the results of chapter 3. The consortium LPB AH2 was chosen for biohydrogen and VFAs production in vinasse medium supplemented with molasses, while the consortium LPB AH1 was chosen for biohydrogen and VFAs production in vinasse medium supplemented with sugarcane juice.

2.5 Optimization and data analysis

Optimization was carried using a statistical tool called “Essential Experimental Design”, version 2.213. An inscribed central composite design with 2 factors at 3 levels and 3 center points was used for each strain. The response used for optimization was total gas produced (in L_{gas}/L_{medium}) since it was noted in chapter 2 that there is a direct relation between gas production and hydrogen content in the gas phase. The statistical plan is showed in Table 27.

Table **28** shows the values assigned to each level.

Table 27 – Statistical plan used for the optimization of conditions for biohydrogen and VFAs production by the chosen consortia.

Exp #	***Carbon Source (g/L)***	***pH***
1	-1	-1
2	0	0
3	1	-1
4	-1	1
5	1	1
6	0	0
7	0	-1,414
8	0	1,414
9	-1,414	0
10	0	0
11	1,414	0

Table 28 – Values of pH and carbon source assigned to each level of the optimization plan.

Carbon Source (g/L)					
Level	-1,414	-1	0	1	1,414
Value	7,93	10	15	20	22,07

pH					
Level	-1,414	-1	0	1	1,414
Value	4,88	5,5	7	8,5	9,12

3 Results and Discussion

3.1 Consortium LPB AH1 cultivated in vinasse medium supplemented with sugarcane juice.

The experimental results for gas production by the consortium LPB AH1 are presented in Table 29. The effect of pH and carbon source concentration on hydrogen production are represented in the 3-D and Contour plots presented in Figure 9. A maximum production of biogas of respectively 8,29L_{gas}/L_{medium} occured at pH 7,0 and 12g/L carbon source.

Table 29 – Gas production achieved by cultivating the consortium LPB AH1 under conditions according to the statistical model used for optimization.

Exp #	*Carbon Source (g/L)*	*pH*	*Gas (L/L_{medium})*
1	-1	-1	7,38
2	0	0	8,25
3	1	-1	2,04
4	-1	1	7,46
5	1	1	3,00
6	0	0	8,29
7	0	-1,414	2,88
8	0	1,414	2,89
9	-1,414	0	6,33
10	0	0	8,27
11	1,414	0	2,25

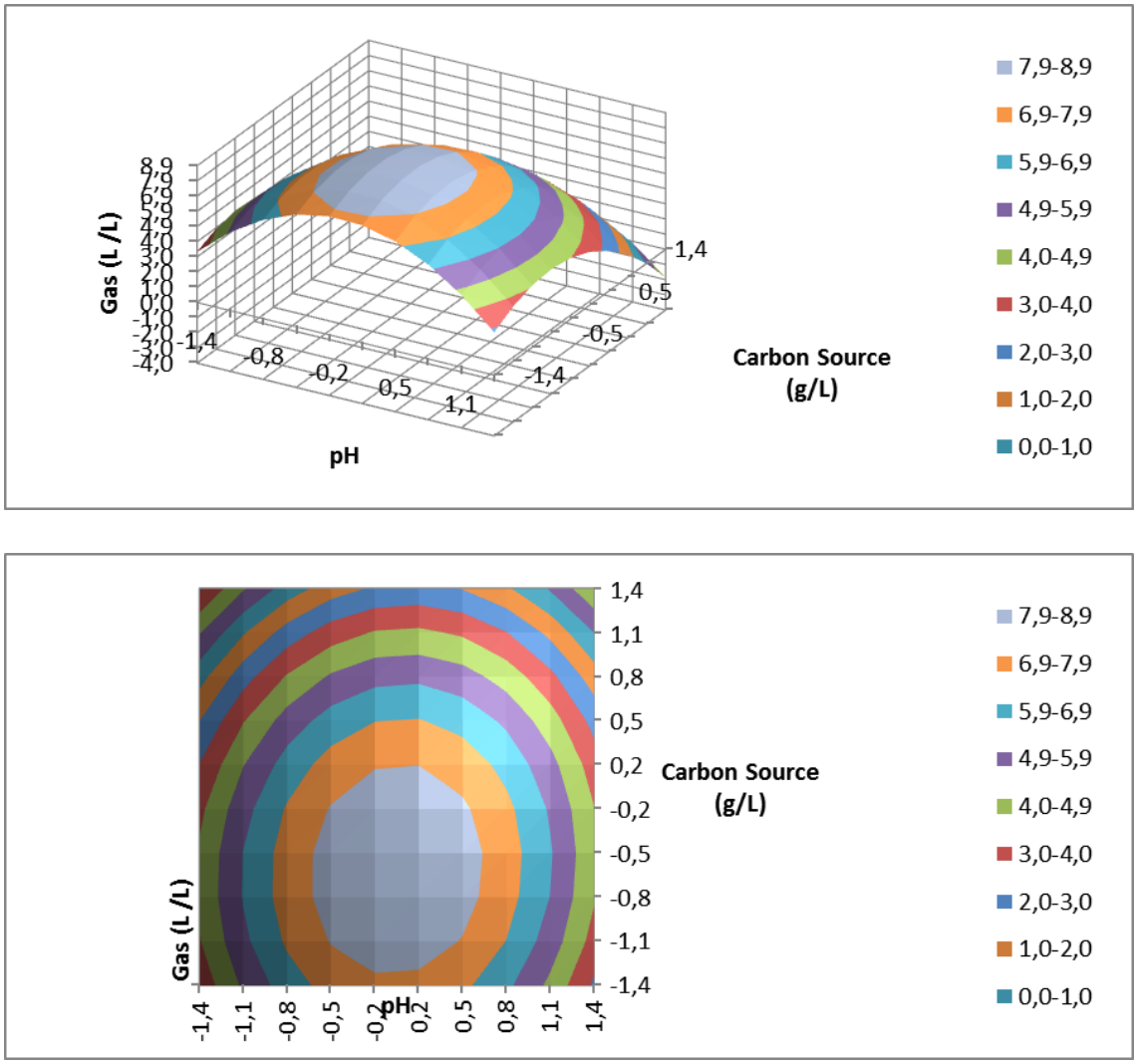


Figure 9 – Graphical 3-D and contour displays of the achieved results for optimization of gas production by LPB AH1 consortium cultivated in vinasse medium supplemented with sugarcane juice.

The best mathematical model that fit satisfactory to the results is a full quadratic model (Table 30), presenting a R^2 higher than 0,91. This means that it is possible to predict hydrogen production by the consortium LPB AH1 grown in terms of pH and substrate concentration.

Low coefficient of variation approx. 20% and standard error (1,095) were observed, which was impressive since higher variation was expected because of the complex composition of the medium (sugarcane juice and vinasse). VIF value under 5 indicates the inexistence of multicollinearity among the regressors (Table 30).

Table 30 - The equation of the full quadratic model that fit best to the results achieved in this optimization is presented. Coefficient values, standard errors, 95% interval of confidence and T student are also shown.

Gás_(ml) = b0 + b1*Fonte de Carbono (g/L) + b2*pH*pH + b3*Fonte de Carbono (g/L)*Fonte de Carbono (g/L) + b4*Fonte de Carbono (g/L)*pH + b5*pH							
		P value	Std Error	-95%	95%	t Stat	VIF
b0	8,270	4,65041E-05	0,632	6,645	9,894	13,09	
b1	-1,946	0,00400	0,387	-2,941	-0,952	-5,029	1,000
b2	-2,347	0,00379	0,461	-3,531	-1,163	-5,095	1,095
b3	-1,644	0,01606	0,461	-2,829	-0,460	-3,569	1,095
b4	0,220	0,704	0,547	-1,187	1,627	0,402	1,000
b5	0,132	0,747	0,387	-0,863	1,127	0,341	1,000

The Durbin-Watson statistic test was carried but was inconclusive for the detection of autocorrelation in the residuals (dL<d<dU; 0.758<1.094<1.604; interval of confidence = 95%). A first order autocorrelation (Pearson's r) value of 0.358 was observed and indicates a weak positive autocorrelation between residuals. This is important because high positive autocorrelation means biased estimated coefficients in the mathematical model and suggests that other variables should be included.

The ANOVA analysis presented in Table 31 showed a low percentage of residuals, indicating that the predicted responses are close to the obtained ones. The F test confirms that the model is valid in a confidence interval of 99% (Fsignif<confidence interval).

Table 31 – The ANOVA analysis showed low content of residuals and indicates that the full quadratic equation proposed is valid.

ANOVA						
Source	*SS*	*SS%*	*MS*	*F*	*F Signif*	*df*
Regression	67,36	92	13,47	11,24	0,00946	5
Residual	5,991	8	1,198			5
LOF Error	5,990	8 (100)	1,997	4991,4544	0,000200	3
Pure Error	0,000800	0 (0)	0,000400			2
Total	73,35	100				10

Table 32 presents the VFAs produced in each condition of optimization. It can be observed that conditions where low quantities of biogas was produced was related with high amounts of lactic acid production, excepted the one of pH 7 and 7,93 $g.L^{-1}$ substrate, in which low amount of gas is probably related to low content of fermentable carbon. It was also noticed that conditions of high pH and/or high carbon source concentration favored the development of lactic acid bacteria; in these conditions formic acid was also produced.

Table 32 – Volatile fatty acids production of the consortium LPB AH1 during optimization. Substrate, succinic, lactic, formic, acetic, propionic and butyric acids are showed in $g.L^{-1}$.

pH	Substrate	Succinic	Lactic	Formic	Acetic	Propionic	Butyric	*Gas (L/L_{medium})*
4,88	**15,00**	0,000	7,994	3,175	0,969	-0,576	1,331	2,88
5,5	**10,00**	0,000	0,000	0,000	2,430	0,046	6,123	7,38
5,5	**20,00**	0,000	8,323	3,218	0,772	-0,371	2,321	2,04
7	**7,93**	0,000	0,000	0,000	1,995	-0,187	5,150	2,88
7	**15,00**	0,000	0,000	0,000	2,280	0,146	7,638	8,27
7	**22,07**	0,000	9,257	3,564	0,952	-0,009	2,589	2,25
8,5	**20,00**	0,000	12,998	4,946	1,050	-0,456	1,916	3,00
8,5	**10,00**	0,000	0,615	0,000	0,000	-0,218	6,728	7,46
9,12	**15,00**	0,000	10,737	4,146	1,343	-0,150	2,874	2,89

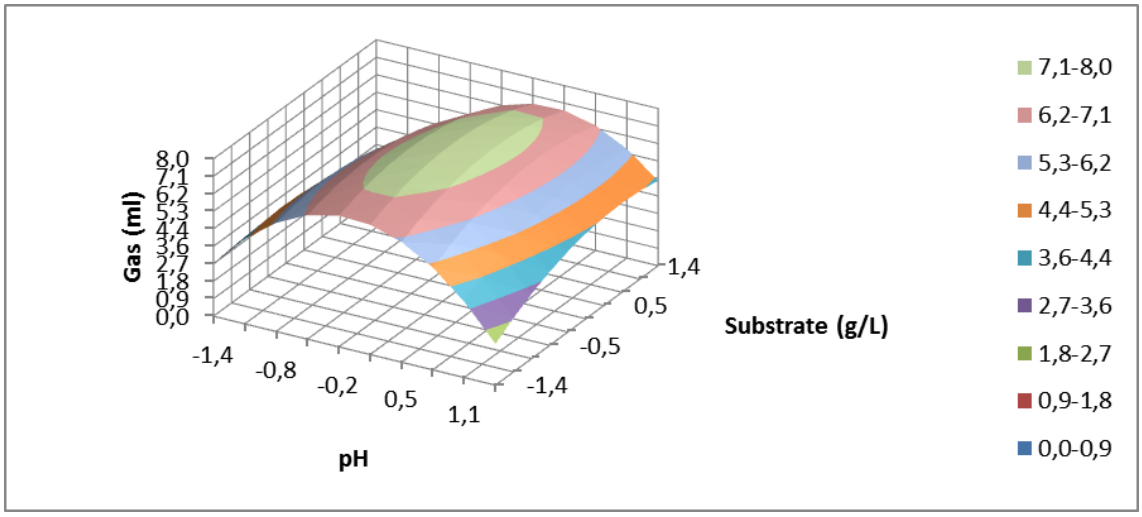
3.2 Consortium LPB AH2 cultivated in vinasse medium supplemented with sugarcane molasses.

The experimental results for gas production by the consortium LPB AH2 are presented in Table 33. The lowest production observed was 1,83 L_{gas}/L_{medium} when the consortia was cultivated at the lowest pH, while the

highest (7,67 L_{gas}/L_{medium}) was achieved at the central point (pH 7,0 and 15g.L^{-1} substrate). Figure 10 presents the effects of the conditions on biohydrogen production.

Table 33 – Gas production achieved by cultivating the consortium LPB AH1 under conditions according to the statistical model used for optimization.

Exp #	*Carbon Source (g/L)*	*pH*	*Gas (L_{gas}/L_{medium})*
1	-1	-1	5,88
2	0	0	7,61
3	1	-1	3,88
4	-1	1	5,92
5	1	1	6,33
6	0	0	7,56
7	0	-1,414	1,83
8	0	1,414	3,72
9	-1,414	0	5,67
10	0	0	7,67
11	1,414	0	6,44



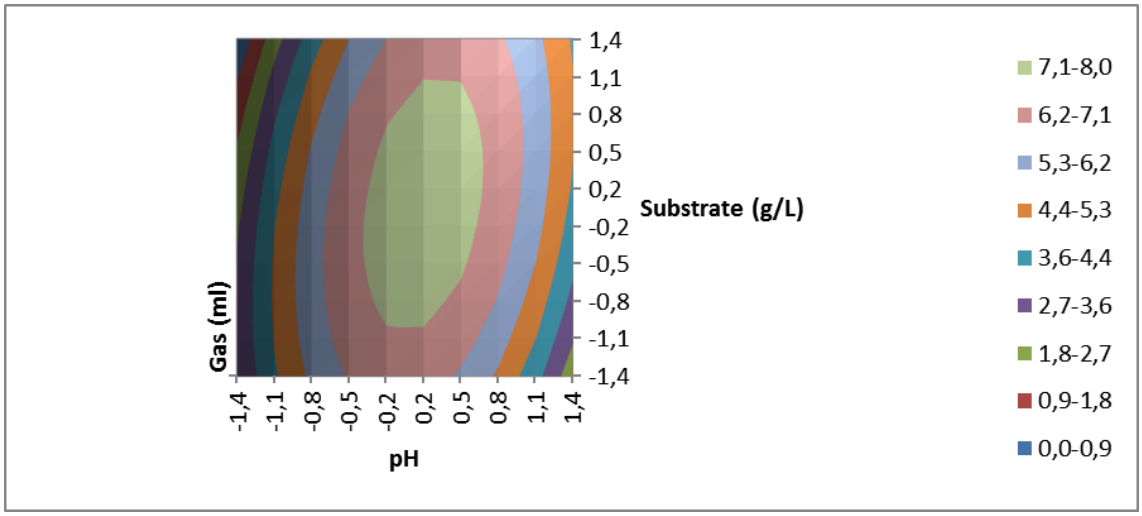


Figure 10 - Graphical 3-D and contour displays of the achieved results for optimization of gas production by LPB AH2 consortium cultivated in vinasse medium supplemented with sugarcane molasses.

The best mathematical model that fit satisfactory to the profile of the results achieved is a full quadratic model (Table 34) with R^2 of approx. 0,90. It means that it is possible to predict hydrogen production by the consortium LPB AH2 grown in vinasse medium supplemented with sugarcane molasses.

Table 34 - – The equation of the full quadratic model that fit best to the results achieved in this optimization is presented. Coefficient values, standard errors, 95% interval of confidence and T student are also shown.

Gas_(ml) = b0 + b1*pH*pH + b2*pH + b3*substrate (g/L)*substrate (g/L) + b4*substrate (g/L)*pH + b5*substrate (g/L)							
		P value	Std Error	-95%	95%	t Stat	VIF
b0	7,615	1,58788E-05	0,467	6,413	8,816	16,29	
b1	-2,148	0,00148	0,341	-3,024	-1,273	-6,306	1,095
b2	0,645	0,07381	0,286	-0,09032	1,381	2,255	1,000
b3	-0,508	0,196	0,341	-1,384	0,368	-1,491	1,095
b4	0,603	0,197	0,405	-0,438	1,643	1,489	1,000
b5	-0,06266	0,835	0,286	-0,798	0,673	-0,219	1,000

As observed for the LPB AH1 consortium, a low coefficient of variation and a low standard error were achieved (14% and 0,809, respectively). The Durbin-

Watson statistic test was again inconclusive for the detection of autocorrelation in the residuals (dL<d<dU; 0,758<1,057<1,604; interval of confidence = 95%). A first order autocorrelation (Pearson's r) value of 0,347 was observed and indicates a weak positive autocorrelation between residuals.

The ANOVA analysis presented in Table 35 showed a low percentage of residuals. The F test showed that the model is valid in a confidence higher than 98%.

Table 35 – The ANOVA analysis showed low content of residuals and indicates that the full quadratic equation proposed is valid.

ANOVA						
Source	*SS*	*SS%*	*MS*	*F*	*F Signif*	*df*
Regression	30,96	90	6,193	9,452	0,01384	5
Residual	3,276	10	0,655			5
LOF Error	3,270	10 (100)	1,090	360,3381	0,00277	3
Pure Error	0,00605	0 (0)	0,00303			2
Total	34,24	100				10

In terms of VFAs production (Table 36), it can be observed that in extreme conditions of pH and carbon source lactate production was observed, especially in high pH (above 8,5). It was possible to note that in fermentations that lactic acid was produced in great quantity low amount of gas was released. In most fermentations only butyric acid was observed, which is in accordance to results achieved in chapter 3.

Table 36 – Volatile fatty acids production of the consortium LPB AH2 during optimization. The concentration of the carbon source, succinic, lactic, formic, acetic, propionic and butyric acids are showed in $g.L^{-1}$.

pH	Carbon Source	Succinic	Lactic	Formic	Acetic	Propionic	Butyric	*Gas (L/L_{medium})*
4,88	15	0,000	2,017	0,000	-0,493	1,390	6,794	2,88
5,5	10,00	0,000	0,000	0,000	-0,450	-0,877	1,850	7,38
5,5	20	0,000	0,000	0,000	-0,643	-0,755	3,675	2,04
7	7,93	0,000	0,364	0,000	0,092	-0,756	1,538	6,33
7	15,00	0,000	0,000	0,000	-0,846	-0,293	6,065	7,61
7	22,07	0,000	5,817	0,000	-0,655	-0,628	4,296	2,25
8,5	20	0,000	8,095	0,000	-0,948	-0,696	3,403	3,00
8,5	10,00	0,000	0,368	0,000	0,659	0,514	6,289	7,46
9,12	15,00	0,000	7,936	0,000	-0,012	-0,558	0,836	2,89

4 Conclusions

The optimization of the conditions of culture resulted in higher biohydrogen production close to the central points for both consortia. In terms of pH it was expected since the experiments described in chapters 3 and 4 were conducted at pH 7,0. Anyway it was not observed flourishing of hydrogen-producers resistant to extreme pHs. The methodology chosen and the design proposed can predict through a mathematical model how biohydrogen is produced in relation to pH and carbon source concentration.

It was not expected that such a uniform behavior could be achieved. Because the consortia are composed by more than one microorganism its adaptation to different conditions is facilitated, which is confirmed considering that hydrogen production was observed even at very low or high pHs. It would be of great value if a considerable biohydrogen production was achieved in low pH because vinasse's natural pH is usually close to 5.

CHAPTER 5

Scaling up: bioreactor cultivation of consortia under optimized conditions for biohydrogen and VFAs production in vinasse-based medium

1 Introduction

Dark fermentative biohydrogen processes is found to be most often performed in closed vessels. Closed batch mode is generally used as the first step to examine physical factors (type of substrate, carbon content, temperature, gas pressure) affecting the process (78) as a first step in process development. Generally H_2 production and growth kinetics are successfully investigated through this technique.

Regarding industrial application (large scale operations), biohydrogen processes are expected to work in continuous mode in most cases. CSTR (continuous stirred tank reactor) is the most commonly studied, where the hydraulic retention time (HTR) is the parameters of greatest influence. They are also preferred in terms of ease of operation. The concentrations of volatile fatty acids in the digester are proportional to the organic loading rate (OLR) and to HRT.

A variety of organic load rates (OLR) have been tested and although the results are highly variable given the different substrates used. It is obvious that high substrate concentrations are to be preferred from an operational standpoint since they potentially lead to high volumetric production rates. The effect of OLR, at least in mixed cultures, on hydrogen yields is somewhat contradictory with no easy explanation for the disparity in the results. In pure culture fermentations hydrogen yields are favored at low carbon concentrations whereas hydrogen productivity is favored at high carbon concentrations. Recent studies with mixed cultures also generally support this idea, although the relationship seems more complex (61). Kim et al. (68) reported that short HRT would favor hydrogen production as methanogens require more than approx. 3 days HRT before they were washed out from a CSTR reactor.

Low HTR generally results in low operation costs and is used to eliminate methane producers. On the other hand the efficiency of the process is reduced (biomass growth and hydrogen production is limited, especially in CSTR) and it is observed loss of fermentable sugar in the wastewater. The optimal HTR for each process must be evaluated because it changes according to substrate and

inoculum. Generally CSTR generates higher H_2 productivity but with lower yields when compared to batch mode.

To overcome the low biomass production (and consequently low hydrogen productivity) in continuous operating reactors, the use of immobilized cells or methods to allow formation of granules or flocs is being considered. Examples are the use of fixed-bed (79) and membrane reactors (80).

In batch reactors the highest yield described was achieved using the thermophile *Caldicellulosiruptor owenensis* (4.0 $mol_{H2}/mol_{glucose}$) (82) while non-thermophile strains can reach up to 3,10 $mol_{H2}/mol_{glucose}$ (83). The highest evolution rate of 35 $mmol\,L^{-1}\,h^{-1}$ was described in a culture of *Enterobacter cloacae* II BT-08 grown in sucrose-rich synthetic medium in batch mode ($Y_{H2/S}$ = 6,0) (84), less than half the amount achieved with the same strain cultured in continuous mode (77 $mmol\,L^{-1}\,h^{-1}$) ($Y_{H2/S}$ not described) (85).

This chapter describes the scaling up of the proposed biohydrogen process using vinasse as medium and the optimized conditions as described in previous chapter to a bench reactor operated in batch mode. The objective is to evaluate the metabolism of both consortia selected to obtain valuable information for a future development of continuous operation. An economical discussion is also carried based on the results achieved.

2 Material and methods

2.1 Culture Conditions and Strains

The experiments were carried out in a 2L bioreactor, with working volume of 1,5L, adapted for anaerobic cultivation (Figure 11). Batch fermentations were maintained at 37ºC and without agitation during 5 days.

Figure 11 – 2L Bioreactor used in scaled up production of biohydrogen and VFAs by the consortium LPB AH2 (cultivated in vinasse medium supplemented with sugarcane molasses) and LPB AH1 (cultivated in vinasse medium supplemented with sugarcane juice).

Medium pH was adjusted with 1N KOH. Anaerobic environment and medium was carried according to the Balch technique. The reactor was autoclaved and a anaerobic environment was created by CO_2 injection in the headspace. Bicarbonate was added when the medium temperature reached 85ºC and Cysteine-HCl at 65ºC as reducing agents to lower the redox potential of medium. The bioreactor was then kept overnight under CO_2 environment prior inoculation.

Carbon source concentration and pH were set according to the results achieved in the previous chapter. Biohydrogen and VFAs production by the consortium LPB AH2 was carried in vinasse medium supplemented with 15g/L sugarcane molasses (based on ^{o}brix), while cultivation of the consortium LPB AH1 was carried in vinasse medium supplemented with 12 g/L sugarcane juice (based on ^{o}brix). The initial pH for each strain was 7.0.

Inoculum production was carried through serial inoculations. A 6 mL daily degassed culture was inoculated in 50 ml new medium. After 5 days of culture and daily degassing, this culture was used as inoculum for a 300ml culture, which was then inoculated in the bioreactor.

2.2 Vinasse

The vinasse used in bioreactor scale was the same used in the previous chapter. Its composition is presented in Table 16, chapter 3.

2.3 High Performance Liquid Chromatography (HPLC)

Organic components were determined through High Performance Liquid Chromatography (HPLC). Samples were withdrawed daily, centrifuged for 10 min at 10^4 g and filtered (Milipore 0,2µm) before injection.

The HPLC equipment was a Shimadzu Liquid Chromatograph equipped with a Aminex® HPX-87H 300 x 7,8mm (Bio-Rad) column and a refractive index detector (RID-10A). The column was kept at 60^oC and a 5mM H_2SO_4 at 0.6 ml/min was used as mobile phase. The compounds quantified by this method are glucose, fructose, succinate, lactate, formate, acetate, propionate and butyrate. All chemical used were of analytical grade.

2.4 Gas Measurement and analysis

Gas measurement was carried using an inverted beaker (Figure 12) connected by a rubber hose to the bioreactor gas exit. Gas production was considered equal to the volume of displaced water.

Figure 12 – The system of gas measurement (foreground) adapted to the bioreactor (background).

At the end of the fermentation, 40ml of the accumulated gas was sampled and analyzed. Gas analysis was carried at the Institute for Technology Development (Instituto de Tecnologia para o Desenvolvimento – LACTEC) in a Thermo Gas Chromatographer equipped with the following analytical columns: Petrocol DH150 (50mx0,25mm), DC 200 (1,8m) and Porapak-N (2,0m x 1/8''), which were placed in by-pass series flow path of gas chromatograph system. The columns were connected to a TCD detector (block temperature: 120°C, transducer temperature: 120°C, filament temperature: 190°C). This system allowed the measurement of oxygen (O_2), nitrogen (N_2), carbon dioxide (CO_2) and methane (CH_4). Hydrogen (H_2) content was then considered as the amount to reach 100%.

2.5 Other Analysis

Biomass was quantified daily by centrifuging 10ml samples at 16500g and drying at 60^{o}C until constant weight. Total carbohydrate was quantified

daily by the Phenol-Sulfuric method. pH was monitored daily in a digital pHmeter.

3 Results

3.1 Biohydrogen and VFAs production in bioreactor scale by the consortium LPB AH1

Biohydrogen and VFAs fermentation process using the consortium LPB AH1 was carried using vinasse and sugarcane juice as medium at the conditions described in Material and Methods. Initial sugar content in the fermentation medium was equal to 11.48g.L^{-1}, being completely exhausted by the end of fermentation. Biomass production was equal to 0.25g.L^{-1}.

The VFAs and biomass production profile during the 5 days of fermentation are showed in Figure 13, while gas production, carbohydrate consumption and pH variation is showed in Figures 13 and 14.

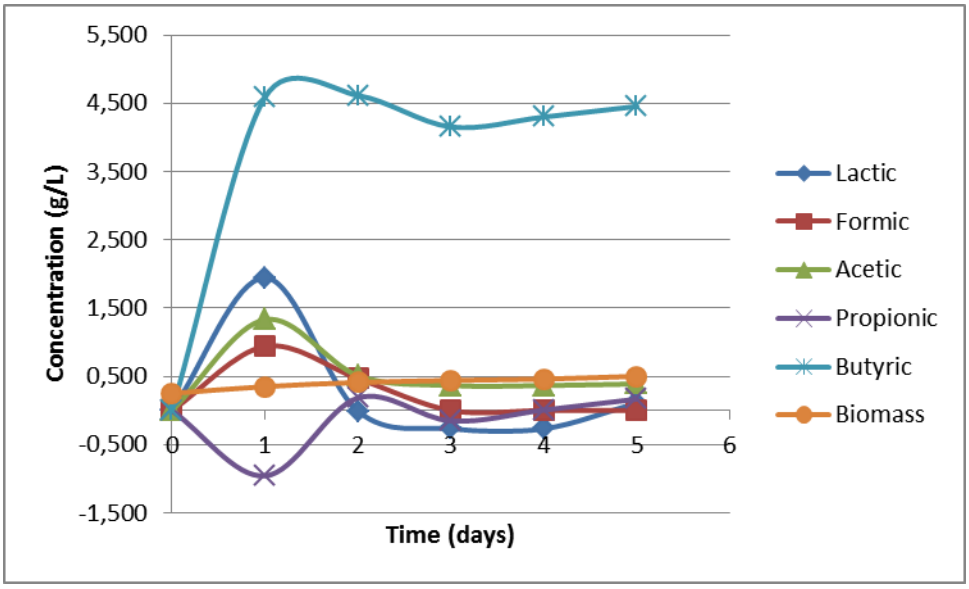


Figure 13 - Curves of biomass and VFAs production during the cultivation of the consortium LPB AH1 in vinasse medium supplemented with sugarcane juice.

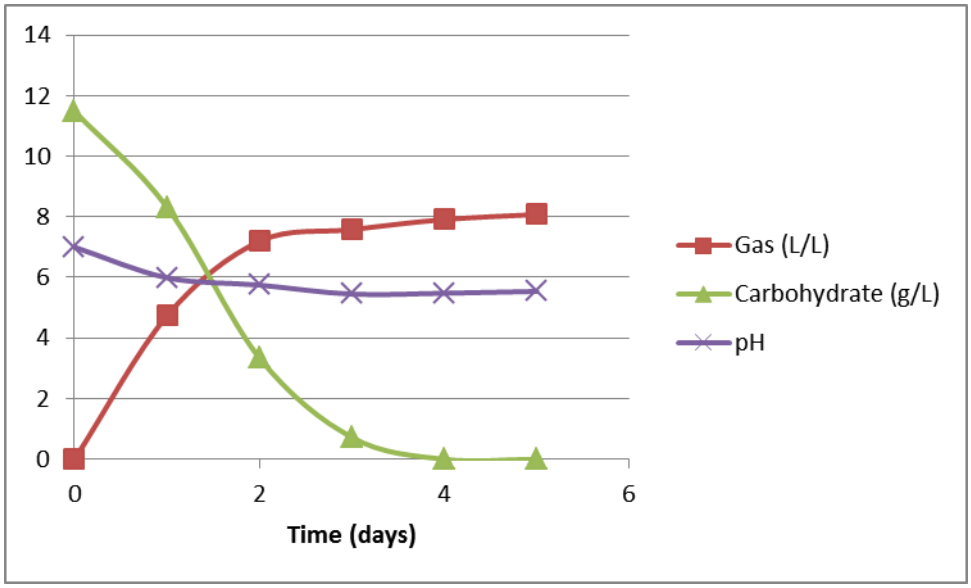


Figure 14 - Biogas production, substrate consumption and pH variation during fermentation of vinasse supplemented with sugarcane juice by the consortium LPB AH1.

It was noticed a high production of VFAs, mainly butyrate and lactate, on the first 24 hours, which was accompanied by a high rate of sugar and propionic acid consumption, pH decrease and biogas production. More than 60% of the gas that has been produced until the end of fermentation was produced in this first 24hours time interval.

On the second day of fermentation, it was noticed the consumption of the carbon source and some of the VFAs produced in the first day (lactic, acetic and formic acids) resulting in propionic acid, gas and biomass production. This might be a consequence of flourishing of different microorganism(s) (apparently propionic bacteria) from those present in the first 24 hours of fermentation.

Because no considerable differences in VFAs profile was noticed after the second day of fermentation, we can state that the consumption of such VFAs was directed towards hydrogen and biomass production (which is confirmed by comparing Figures 10 and 11).

These results indicate that a continuous fermentation process for biohydrogen and butyric acid by the consortia LPB AH1 can be carried with a hydraulic retention time of 48 hours (since butyric acid is a desired product). In this process very low concentration of lactic, acetic and propionic acid would be found and more than 90% of the carbohydrate would be consumed. This proposed reduction of fermentation time for a continuous process was carried

by Zhang et al. (2006) (81) and resulted in a reduction of the diversity of microbial community associated with an elimination of propionate production without affecting the existence of dominant pure cultures.

On the other hand, conducting fermentation during only 24 hours will result in higher H_2 yield and productivity but a mixture of VFAs that will demand a more laborious downstream prior purification, if this pure specific acid(s) is (are) desired.

3.1.1 Metabolic analysis

According to the results presented in figures 13 and 14, a μmax of 0,15 d^{-1} was achieved. The substrate consumption rate in the first 48 hours was equal to 4,07 $g.L^{-1}.d^{-1}$. Approximately 2,2% of the substrate was used in biomass production ($Y_{X/S}$), while almost 39% was used for butyrate production (44,6% considering every acid produced – $Y_{VFAs/S}$). This means that 53,2% of the consumed substrate was probably used in CO_2 production ($Y_{CO2/S}$) and cellular maintenance ($Y_{m/S}$).

At the end of the fermentation, 8,08L_{gas}/L_{medium} was produced, an amount very close to the one predicted during optimization in chapter 4 (8,85 L_{gas}/L_{medium}). The biogas composition is presented in Table 37. The presence of very low quantities of oxygen indicates insignificant contamination of the gas before analysis and shows that an anaerobic condition for cultivation was successfully achieved.

Table 37 – Composition of the biogas produced during the fermentation by the consortium LPB AH1.

Biogas components	Content (%)
Nitrogen	2,13
Carbon dioxide	66,2
Oxygen	0,62
Methane	0,00
Hydrogen*	31,05

*Hydrogen content was estimated by the amount to reach 100%

Considering the carbon dioxide percentage on the biogas and considering that it is behaving as an ideal gas, we can estimate the amount of substrate used in CO_2 as follows:

If P.V=n.R.T where P=1atm, V=(66,25%*8,08L_{gas}/L_{medium}), R= 0,082057 atm.L.$mol^{-1}.K^{-1}$ and T (K)=37°C+273,15°C, the value for n_{CO2} is 0,21 mol/L (9,25 g_{CO2}/L). If all the 53,2% of the cited consumed substrate was destined for CO_2 production, the amount of the carbon dioxide produced should be:

CO_2 = 0,532*11,48$g_{substrate}$/L*1,54g$CO_2/g_{sucrose}$ = 9,41g_{CO2}/L

which means that approx. 98% of the 53,2% consumed substrate was destined for CO_2 production. The conclusion is, finally, that $Y_{CO2/S}$ = 52% while $Y_{m/S}$ is approx. 1,0%. A general representation of the destination of the substrate consumed by the consortium LPB AH1 is showed in Figure 15.

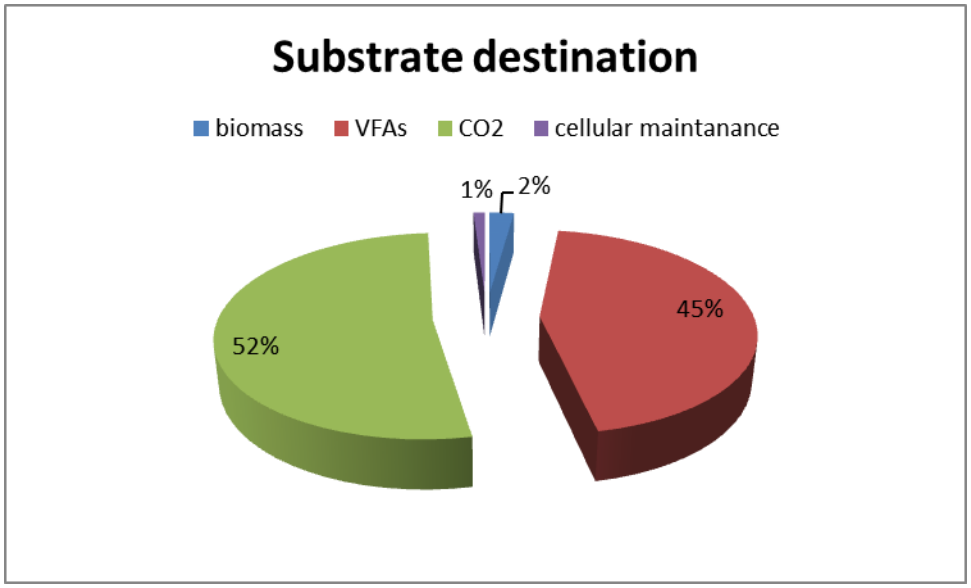


Figure 15 - Representation of the destination of the substrate in terms of VFAs, biomass and CO_2 production and cellular maintanance.

3.1.2 Hydrogen production potential analysis

The maximum productivity of hydrogen was achieved considering the first 24 hours of fermentation, reaching 61,5 $ml_{H2}.L^{-1}.h^{-1}$ (which means 2,75 $mmol_{H2}.L^{-1}.h^{-1}$). This productivity can be considered low, since it is quietly normal to find productivities of 5-20 $mmol_{H2}.L^{-1}.h^{-1}$ in the literature.

On the other hand, a yield of 7,14 $mol_{H2}.mol_{sucrose}^{-1}$ was achieved, which is as high as 89,25% of the theoretical maximum yield. This is very high and is achieved more frequently using thermophiles.

This opposite behavior of achieving high $mol_{H2}.mol_{Csource}^{-1}$ and low productivities is well described in the literature. In order to achieve high conversion rates, generally long times are needed, which consequently results in low productivity. As example, a very high productivity of $50mmol.L^{-1}.h^{-1}$ was achieved but with yields as low as $0,09mol_{H2}.mol_{glucose}^{-1}$ by culturing *Bacteroides fragilis* in glucose rich medium (86). Probably higher productivities (with lower conversion rate) could be achieved if hourly analysis were carried during the exponential H_2 production phase.

Considering the hydrogen content in the biogas, the inferior and superior calorific powers (ICP and SCP) were calculated and estimated as 9050 $kcal.kg^{-1}$ and 10730 $kcal.kg^{-1}$, respectively. In comparison to a methane rich biogas (65% CH_4 – ICP = 7735 $kcal.kg^{-1}$ and 8612 $kcal.kg^{-1}$), the calorific power presented by the hydrogen rich biogas is superior (17% higher).

At this point, a reflexion might be carried in terms of the feasibility of usage of hydrogen-rich biogas as heat source instead of methane-rich biogas. The difference in terms of calorific power is considerable and indicates that hydrogen-rich biogas is better, but the complexity of the technology to produce it is greatly superior to the one to produce methane. This suggests that to became feasible, considering that hydrogen content in the biogas could not be greatly increased, the proposed technology might consider the purification of hydrogen (adding value to the final product).

3.2 Biohydrogen and VFAs production in bioreactor scale by the consortium LPB AH2

Biohydrogen and VFAs fermentation process using the consortium LPB AH2 was carried using vinasse and sugarcane juice as medium, according to the results achieved in previous chapters. Carbohydrate concentration at the beginning of the fermentation was quantified as 13.42 g/L and the initial pH was 7,0.

Almost 50% of the carbon source was consumed in the first 24 hours and was exhausted in the last day of fermentation (Figure 16). Biomass production was equal to 0.65g/L and final pH was 5.15.

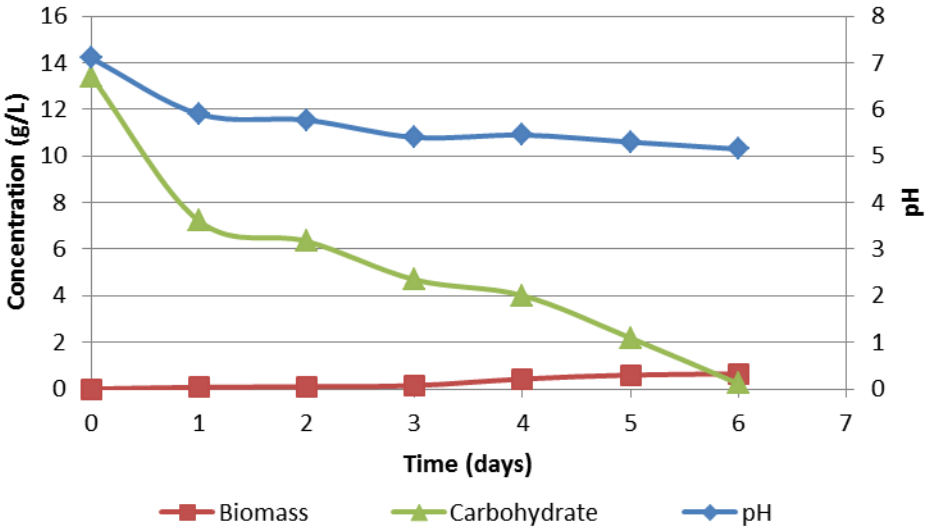


Figure 16 - Biogas production, substrate consumption and pH variation during fermentation of vinasse supplemented with sugarcane molasses by the consortium LPB AH2.

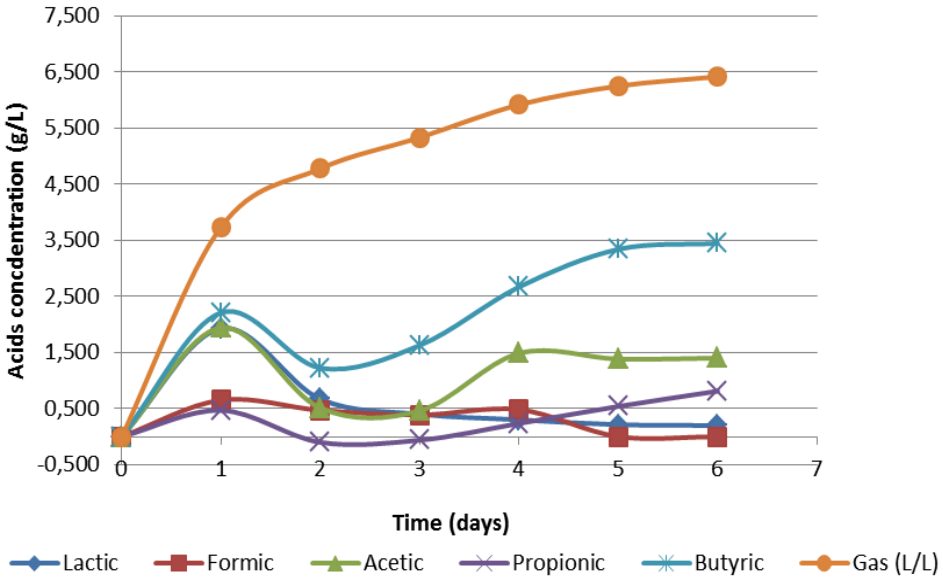


Figure 17 - Curves of biomass and VFAs production during the cultivation of the consortium LPB AH1 in vinasse medium supplemented with sugarcane molasses.

The HPLC analysis of the samples withdrawed daily showed a complex behavior of VFAs production (Figure 17). In the first 24 hours butyric, acetic and lactic acids were produced in large amounts, while low quantities of formic and propionic acid were identified. This was accompanied by great carbohydrate uptake (6.21g.L^{-1}), a great biogas production (58% of the gas that would be produced by the end of the fermentation) and a pH drop to 5.9. In the second day of fermentation, the VFAs were consumed and gas production lowered,

which is possibly an effect of consortia composition variation. During the third day of fermentation little changes was noticed, but after that butyric, acetic and low amounts of propionic acids were observed together with carbohydrate consumption. Biomass production was greatly increased in this time interval (4^{th}-6^{th} day of fermentation) and gas production rate was kept relatively constant.

3.2.1 Metabolic analysis

According to the results presented in figures 16 and 17, a μmax of 0.375 d^{-1} was achieved. The maximum substrate consumption rate (first 24 hours) was equal to 6.21 $g.L^{-1}.d^{-1}$ ($Y_{X/S}$). About 4.9% of the substrate was used in biomass production ($Y_{X/S}$), while almost 41.5% was used for VFAs production ($Y_{VFAs/S}$). This means that 53.6% of the consumed substrate was probably used in CO_2 production ($Y_{CO2/S}$) and cellular maintenance ($Y_{m/S}$).

In comparison to the optimization prediction of gas production in chapter 4 (7.76 L_{gas}/L_{medium}), 6.41L_{gas}/L_{medium} was produced at the end of the fermentation in the bioreactor. This difference illustrate the expected variation of biogas (and consequently biohydrogen) production caused by the use of complex medium and a consortium of microorganisms.

The biogas composition is presented in Table 38. The presence of very low quantities of oxygen indicates an insignificant contamination of the gas before analysis and shows that an anaerobic condition for cultivation was successfully achieved.

Table 38 – Composition of the biogas produced during the fermentation by the consortium LPB AH1.

Biogas components	Content (%)
Nitrogen	3.93
Carbon dioxide	62.4
Oxygen	0.97
Methane	0.00
Hydrogen*	32.7

*Hydrogen content was estimated by the amount to reach 100%

The mass balance to determine $Y_{CO2/S}$ and $Y_{m/s}$ was carried as described in section 3.2.1. If P.V=n.R.T where P=1atm, V=(62.4%*6.41L_{gas}/L_{medium}), R= 0.082057 $atm.L.mol^{-1}.K^{-1}$ and T (K)=37°C+273.15°C, the value for n_{CO2} is 0.157

mol/L (6.91 g_{CO2}/L). If all the 53.6% of the cited consumed substrate was destined for CO_2 production, the amount of the carbon dioxide produced should be:

$$CO_2 = 0.532*13.42g_{substrate}/L*1.54gCO_2/g_{sucrose} = 10.99g_{CO2}/L$$

which means that approx. 82% of the 53.6% consumed substrate was destined for CO_2 production. The conclusion is, finally, that $Y_{CO2/S}$ = 43.9% while $Y_{m/S}$ is approx. 9.7%. A general representation of the destination of the substrate consumed by the consortium LPB AH1 is showed in Figure 18.

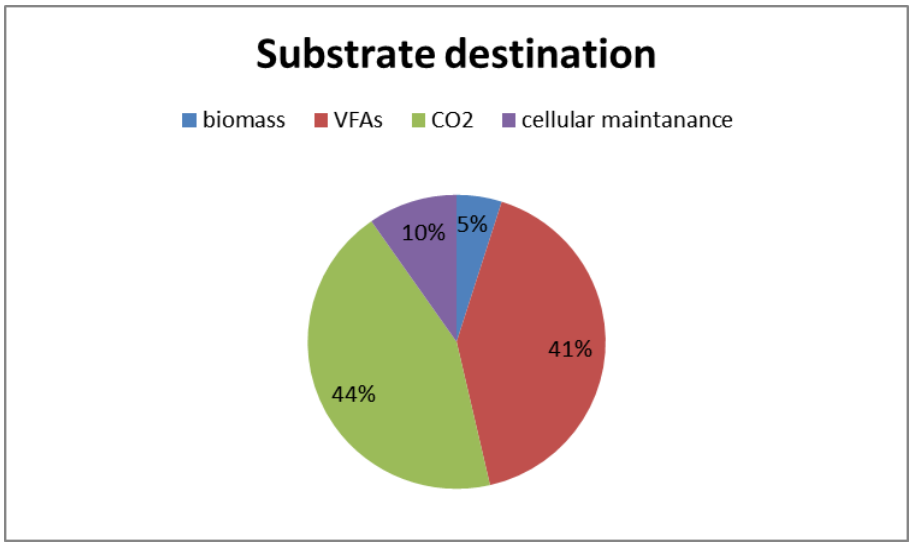


Figure 18 - Representation of the destination of the substrate in terms of VFAs, biomass and CO_2 production and cellular maintanance.

3.2.2 Hydrogen production potential analysis

The maximum productivity of hydrogen was achieved considering the first 24 hours of fermentation, reaching 55.07 $ml_{H2}.L^{-1}.h^{-1}$ (which means 2.46 $mmol_{H2}.L^{-1}.h^{-1}$) with a yield of 3.25 $mol_{H2}.mol_{sucrose}^{-1}$ (41% of the theoretical maximum yield). The highest yield was achieved considering 2 days of fermentation (3.66 $mol_{H2}.mol_{sucrose}^{-1}$), but the productivity was as low as 35.2 $ml_{H2}.L^{-1}.h^{-1}$.

Considering the hydrogen content in the biogas, the inferior and superior calorific powers (ICP and SCP) were calculated and estimated as 9483 $kcal.kg^{-1}$ and 11248 $kcal.kg^{-1}$, respectively. In comparison to a methane rich biogas (65%

CH_4 – ICP = 7735 kcal.kg^{-1} and 8612 kcal.kg^{-1}), the calorific power presented by the hydrogen rich biogas is superior (22.6% higher).

4. Conclusion

According to the results achieved in the experimentations described in this chapter showed the feasibility to produce hydrogen in the conditions described in a rector scale. The metabolic analysis by daily analysis of biogas production and VFAs provided important information to the development of a continuous process, which is more feasible to the proposed technology.

Through material and energy balance it was possible to estimate how the energy of the substrate is distributed during the fermentation. Moreover, growth indicators were calculated are of great value in further development of this process.

Considering the possibility of associating this technology to a biogas or solvent production process (for biogas production, VFAs works as substrates), it is important to note that there is a production of considerable amounts of VFAs. The process developed with the consortium LPB AH1 has potential for butanol production since butyrate concentration in the broth is much higher than other VFAs.

In terms of how the hydrogen produced can be used, a deep analysis might be carried. The first point is that the proposed technology depends on sugarcane molasses or juice, which are used in ethanol production. In order to be considered promising (and to be transferred to the industry) the proposed technology should provide substantial economic gains. Thus, 3 scenarios of usage of hydrogen by the ethanol industry are proposed:

i) Use in direct heat generation: Ethanol industries use sugarcane bagasse in boilers to produce heat. Sugarcane bagasse (with 20% water) PCS is 3641 kcal/kg, almost 3 times lower than the biogas generated by using vinasse supplemented with sugarcane juice.

A ethanol plant that produced 1000 m³ of ethanol/day uses approx. 12000 tons of sugarcane, resulting in approx. 1800tons of sugarcane bagasse, which are capable to generate 6,55*10^9 kcal. In this scenario, the increase in the energy generated by using biogas as a

complementary source of energy will be insignificant (hydrogen-rich biogas can produce up to $8,5*10^6$ kcal/day considering the production rate and biogas composition achieved using LPB AH1 consortium) .

This suggests that at the current level of development the use of hydrogen for direct heat production is unfeasible.

ii) Use in fuel cells: Ethanol industries generally produce more energy than they use, selling the surplus electricity to local electric companies. Anyway, hydrogen could be used to enhance this energy production.

Considering a 1,2 kW proton exchange membrane fuel cell that uses hydrogen with purity of 99.99%, at a consumption rate of 18.5 L/min (31), 20 m^3 of vinasse based medium was needed to produce enough hydrogen to operate when considering fermentation of vinasse and molasses with the consortium LPB AH2 at the conditions described in this chapter. When considering the use of the consortium LPB AH1 (vinasse supplemented with sugarcane juice) the volume needed is of approx. 19 m^3.

Considering the realistic daily production of 1000m^3 ethanol by an ethanol plant, which means the daily generation of 12 thousand m^3 of vinasse, approx. 720kW of energy could be produced. The average price of the KWh in 2012 in Brazil was R$0,333 (U$0,17), which means that monthly approx. U$3600 in energy could be produced using hydrogen in fuel cell, which is very low.

iii) Purification of hydrogen: the price of pure analytic hydrogen is approx. U$ 56,50/m^3 (White Martins, Brazil). By the proposed technology, fermenting 1 m^3 of vinasse generates daily approx. 1,5 m^3 of pure hydrogen (rate achieved using LPB AH1 consortium). Considering an ethanol plant generating 12000 m^3 of vinasse, approx. U$ 1 million dollar/day can be obtained by selling pure H_2.

General Conclusion

The scientific advances for the reuse of industrial reuse for the production of compounds promote the recovery of the energy and nutrients that were lost in wastewater treatments. Since there are limitations of compounds produced from these residues and wastewaters, because in most cases they are not feasible to be used in food/feed or as pharmaceuticals, the development of technologies for biofuels production is an important alternative.

Among the biofuels feasible to produce through biological methods (oils, biodiesel, CH_4, H_2) hydrogen is the one with higher energy density. Hydrogen production through fermentation of agroindustrial wastes (liquids and solids) issues zero extra carbon to the atmosphere, and its combustion results in water and oxygen. It is thus a eco-friendly source of energy and is told to be the fuel of the future.

In present, fermentation technologies to produce hydrogen are in a basic level of development and faces economical drawbacks. At this context it is unanimous that the use of agroindustrial residues (solid and liquid) is the main alternative to this economic dilemma. In recent years this topic is gaining importance and significant advances will come.

In Brazil, vinasse is the industrial liquid residue produced in most quantity. Despite presenting benefits when used as fertilizer, the issues that are being observed as consequences of its disposal will certainly result in increasing government (environment) restrictions. Furthermore most ethanol industries generate vinasse in excess and gives not rational destination to it. The production of biohydrogen from vinasse is interesting because of the possibility to use it as source of energy within the industry in an integrated process. The suggested process needs improvements, but the main objective of giving important step in this theme and to open alternatives to be studied were accomplished.

Future works

It would be of great interest to carry studies in the following topics:

- Development of a continuous process for biohydrogen production in bioreactor using the consortia LPB AH1
- Development of a continuous process for biohydrogen production in bioreactor using the consortia LPB AH2;
- Molecular characterization of both consortia (LPB AH1 and LPB AH2);
- Use of the liquid waste from anaerobic fermentation of vinasse as fertilizer for sugarcane;
- Biohydrogen production from vinasse coupled to hydrogen cell;
- Sequential production of biohydrogen and biomethane from vinasse;

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