SCREENING OF *CLOSTRIDIUM* STRAINS PRODUCING BIOMOLECULES FROM THE PERSPECTIVE OF BIOREFINERIES

Pamela Baptista Ferreira[1], Vanessa Kristine de Oliveira Schmidt[2], Maikon Kelbert[3] & Débora de Oliveira[3*]

[1] *Chemical Engineering/Technological Center/Department of Chemical Engineering and Food Engineering/Federal University of Santa Catarina, Florianópolis, Brazil.*
**pamelabapferreira@gmail.com*
vanessa.schmidt@posgrad.ufsc.br
maikonkelbert@gmail.com
debora.oliveira@ufsc.br

ABSTRACT

The production of biomolecules such as hydrogen and volatile fatty acids (VFAs) from lignocellulosic materials of agro-industrial waste is crucial both for converting waste into high-value products and for mitigating waste disposal issues. Hydrogen stands out as an efficient and clean energy alternative, while VFAs are crucial for the production of chemicals, fuels, and polymers. *Clostridium* strains are recognized for their capacity to produce these compounds via ABE fermentation. However, the industrial implementation of these bioprocesses still faces challenges in scalability and optimization, highlighting the importance of prospecting and evaluating new strains capable of producing these compounds for future applications. This study identified *Clostridium* strains with high potential for biohydrogen production, reaching a maximum production of 6.15 ± 1.15% hydrogen through fermentation. Additionally, the production of butyric acid and butanol was observed through volatile compound analysis.

Keywords: *Clostridium*. Biohydrogen. Fermentation. Prospection. VFA.

1 INTRODUCTION

Short-chain volatile fatty acids (VFAs), such as acetic acid and butyric acid, constitute relevant raw materials for various industrial sectors, including food, pharmaceutical, chemical, and biofuel industries (SUN; ZHANG; LOH, 2021). Hydrogen (H_2), in turn, is widely recognized as a promising alternative energy source due to its environmentally friendly nature and high energy yield (122 kJ/g) (Rambabu et al., 2020). Although various technologies have been developed for the production of these compounds, the biotechnological method has garnered interest, mainly due to the use of lignocellulosic material (LCM) derived from agro-industrial residues. This method is particularly notable for its approach of integrated bioprocesses that employ innovative microorganisms (Rambabu et al., 2020).

The bacterial genus *Clostridium* is recognized for its ability to generate various bioproducts through ABE fermentation (Acetone-Butanol-Ethanol). Among these bioproducts, volatile fatty acids (VFAs) (e.g., butyric and acetic acid) and biohydrogen (bioH_2) stand out, which can be produced via the acidogenic route. However, achieving industrial-scale production of these compounds with high yields and productivity through biotechnological pathways remains a significant challenge.

Recently, researchers have turned their attention to the prospecting of new strains with the potential for producing these biocompounds for possible large-scale implementation. According to Fonseca *et al.* (2016)., it was possible to isolate a new H_2-producing bacterial culture from anaerobic sludge. The isolated culture produced the highest amount of H2 at 35 °C and an initial pH of 7, utilizing various carbon sources, including glucose, galactose, and mannose from algal biomass.

Although some studies have identified *Clostridium* strains that produce VFAs and bioH_2 (JIANG et al., 2018), comparative research between these strains is lacking, especially in the context of a biorefinery utilizing lignocellulosic material (LCM). Therefore, the objective of the study is to prospect new strains of Clostridium capable of efficiently synthesizing bioproducts of high industrial value, such as VFAs and bioH_2.

2 MATERIAL & METHODS

The anaerobic granular sludge was taken from an anaerobic reactor from a food industry in the Santa Catarina state. The sludge (volatile solids: 42.73 ± 0.44 g/L) was pre-treated by decreasing the pH to 3.0 before plating. The acidification was performed by adding the sludge sample (5 mL) to 0.1 M citrate phosphate buffer at pH 3.0 (35 mL) and incubating for 12 h at 37 °C with an agitation of 150 rpm.

After pre-treatment, 100 µL of sludge were diluted in 900 µL NaCl solution (0.9% w/v). Subsequently, 100 µL of the diluted sample were plated on sterile Petri dishes covered with 25 mL Differential Reinforced Clostridial Medium (DRCM) to culture *Clostridium* species at pH 7.0. Petri dishes were incubated in an anaerobic jar at 37 °C until colonies appeared. To ensure colony isolation, the process was repeated three times. The colonies were resuspended in saline solution, and 1.0 mL was inoculated into serum

bottles containing 50 mL of RCM medium. Nitrogen gas was bubbled into the bottles before sealing with a rubber stopper and aluminum seal and incubated at 37°C for 72 h. After this period, the headspace gas composition was analyzed by gas chromatography. With the liquid portion, we conducted the analysis of VFAs (with gas chromatography-mass spectroscopy). The organisms producing VFA-H2 were then separated for identification. The methodology used was adapted as described by Fonseca *et al.* (2016).

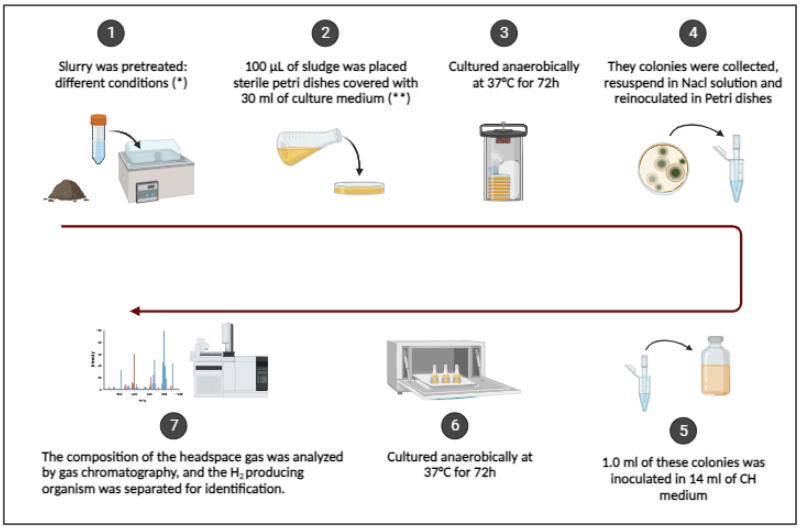

Figure 1: Description of the methodology used.

3 RESULTS & DISCUSSION

The acid pretreatment of the sludge favored the isolation of three strains producing H_2 and VFAs, named ISO02, ISO07, and ISO10. The DRCM revealed the presence of *Clostridium* by darkening of the medium due to sulfite reduction, common in *Clostridium spp*.

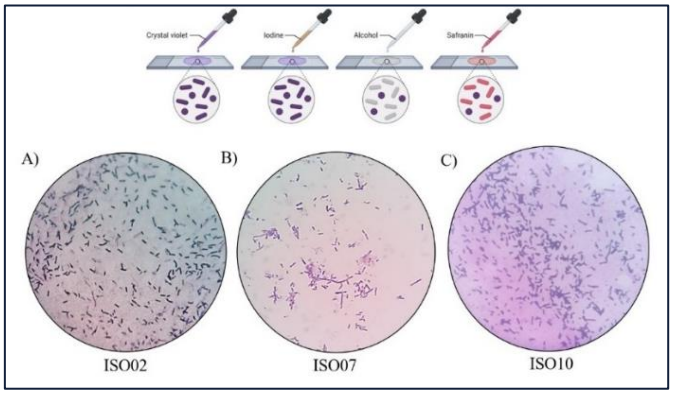

Figure 2: Gram analisys of isolated strain.

Microscopy images at 100x magnification show a positive result for the Gram test and the rod-shaped morphology of the isolated bacteria. The samples are: (A) ISO02, (B) ISO07, and (C) ISO10.

Preliminary results of gas chromatography confirmed H_2 (5.57±0.82, 6.15 ± 1.15, and 5.62 ± 0.5%) and CO_2 (30.40 ± 0.91, 20.23 ± 1.06, and 23.23 ± 0.75%) production by the strains (Table 1).

Table 1: Quantification of hydrogen and carbon dioxide produced by the isolated stains by gas chromatografy.

Samples	Hydrogen (% mol/ mol)	Carbon Dioxide (% mol/ mol)
ISO02	5.57 ± 0.82	30.40 ± 0.91
ISO07	6.15 ± 1.15	20.23 ± 1.06
ISO10	5.62 ± 0.5	23.23 ± 0.75

VFA analysis identified products such as butyric acid. Additionally, the presence of alcohols such as butanol was noted in Table 2.

Table 2: The main volatile organic compounds produced by ISO02, ISO07, ISO10.

				ISO 02	ISO 07	ISO10
Compound name	**Formula**	**MW (g/mol)**	**RT**	**Average Peak area (%)**	**Average Peak area (%)**	**Average Peak area (%)**
Alcohols						
Ethanol	C_2H_6O	46,07	1,98	1,98		0,35
1-Butanol	$C_4H_{10}O$	74,12	3,45	2,85	0,38	0,25
3-Thietanol	C_3H_6OS	90,14	3,96	10,17		
1-Hexanol	$C_6H_{14}O$	102,17	7,37	5,16		
1-Butanol, 3-methyl-	$C_5H_{12}O$	88,15	4,60	1,11	2,88	1,62
1-Nonanol	$C_9H_{20}O$	144,25	8,81	1,34		
1-Octanol	$C_8H_{18}O$	130,23	11,61	1,68		
Alkane						
Cyclotetrasiloxane, octamethyl-	$C_8H_{24}O_4Si_4$	296,62	14,59	0,49	2,19	2,45
Benzene, (1-hexylheptyl)-	$C_{19}H_{32}$	260,46	23,79	0,10		
Chlorinated compound						
Phenol, 2-chloro-4-(1,1-dimethylpropyl)-	$C_{11}H_{15}ClO$	198,69	18,03	0,15		13,00
Ester						
Phytol, acetate	$C_{22}H_{42}O_2$	338,57	23,88	0,23		
Di-sec-butyl phthalate	$C_{16}H_{22}O_4$	278,34	24,35	0,13		
Acetic acid, hexyl ester	$C_8H_{16}O_2$	144,21	10,43	0,14		
Propanoic acid, 2-methyl-, 3-methylbutyl ester	$C_9H_{18}O_2$	158,24	11,34			15,45
Butanoic acid, 3-methyl-, 3-methylbutyl ester	$C_{10}H_{20}O_2$	172,26	12,29		0,24	3,81
Butanoic acid, 3-methylbutyl ester	$C_9H_{18}O_2$	158,24	11,31	8,55	2,43	0,32
Organic Acid						
Butanoic acid	$C_4H_8O_2$	88,11	6,70	3,78	2,34	
Butanoic acid, 3-methyl-	$C_5H_{10}O_2$	102,32	7,62		0,83	

Soluble metabolites analysis revealed the presence of various compounds, as shown in Table 2. Changes in pH significantly affect the byproducts of fermentative processes. A pH below 6.0 favors the production of butyric and acetic acids, while a higher pH results in acetic acid and ethanol. In *Clostridium* species, the transition from pH 7 to 4.5 shifts the phase from acidogenic to solventogenic. The accumulation of organic acids and bioH_2 reduces pH, leading to the solventogenic phase and decreasing the biosynthesis of these compounds.

4 CONCLUSION

The results confirm the isolation of *Clostridium* strains with the potential for biomolecules production, as well as demonstrating the production of bioH_2 and VFAs by these isolated strains.

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