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INFLUENCE OF NICKEL ON BUTANOL PRODUCTION BY *Clostridium beijerinckii* USING HYDROLYZATE FROM GREEN COCONUT SHELL

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ABSTRACT

The improvement of biotechnological processes capable of transforming agro-industrial waste into products with high added value has stood out in the area of renewable energies, promoting positive impacts to the environment. Thus, the present work evaluated the influence of nickel on the conversion of fermentable sugars, present in the green coconut shell hydrolyzate (GCSH), into butanol and other products. Fermentation assays were performed at 37 °C, starting with 19.4 $g.L^{-1}$ of sugars and 1.0 $g.L^{-1}$ of inoculum (*C. beijerinckii*). The GCSH was supplemented with tryptone, yeast extract, ammonium acetate, minerals and phosphate buffer. Two conditions were tested: with and without addition of nickel. Concentrations of sugars (glucose and xylose), intermediate products (organic acids), acetone, butanol, and ethanol were determined by high performance liquid chromatography (HPLC). The results show that the butanol production was higher from GCSH without addition of nickel, reaching a concentration of 2.14 $g.L^{-1}$ of butanol. Therefore, the presence of nickel in the hydrolyzate was not favorable in the production of butanol under the studied process conditions.

Palavras-chave:
Resíduos agroindustriais
Hidrólise
Ni catalisador
Fermentação ABE

INFLUÊNCIA DO NÍQUEL NA PRODUÇÃO DE BUTANOL POR *Clostridium beijerinckii* UTILIZANDO O HIDROLISADO DA CASCA DO COCO VERDE

RESUMO

O aprimoramento de processos biotecnológicos capazes de transformar resíduos agroindustriais em produtos de alto valor agregado tem se destacado na área de energias renováveis, promovendo impactos positivos ao meio ambiente. Este estudo avaliou a influência do níquel na conversão de açúcares fermentescíveis, presentes no hidrolisado da casca do coco verde (HCCV), em butanol e outros produtos. Foram realizados ensaios de fermentação a 37 °C, iniciando com 19,4 $g.L^{-1}$ de açúcares e 1,0 $g.L^{-1}$ de inóculo (*C. beijerinckii*). O HCCV foi suplementado com triptona, extrato de levedura, acetato de amônio, minerais e tampão fosfato. Duas condições foram testadas: com e sem adição de níquel. As concentrações de açúcares (glicose e xilose), produtos intermediários (ácidos orgânicos), etanol, acetona e butanol foram determinados por cromatografia líquida de alta eficiência (CLAE). A produção de butanol foi maior no HCCV sem adição de níquel, alcançando uma concentração de 2,14 $g.L^{-1}$ de butanol. Portanto, a presença do níquel no hidrolisado não foi favorável à produção de butanol nas condições de processo estudadas.

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INTRODUCTION

Brazil is an agricultural country where many residues from the agribusiness are improperly disposed or underutilized, even though there are important energy reserves in the form of cellulose and hemicellulose (PADILHA *et al.*, 2019). Coconut production, for example, occupies the 5th position in the world ranking, 2.33 million tons/year (FAOSTAT, 2019), being part of the production destined to the processing of coconut water and/or direct consumption, generating urban waste. About 70 to 80% of the fruit corresponds to green coconut shell and takes around 8 years to decompose (NOGUEIRA *et al.*, 2018). However, it is necessary to hydrolyze this material to make the fermentable sugars available (NOGUEIRA *et al.*, 2018) to microorganisms that convert them into products, such as butanol.

In this context, pretreatments along with enzymatic hydrolysis are steps prior to the transformation of lignocellulosic material, source of renewable energy, that can make them products of industrial interest. However, it is important to highlight that the choice of pretreatment (physical and/or chemical) of the lignocellulosic material can lead to the generation of undesirable by-products, e.g., furfural (FF) and hydroxymethylfurfural (HMF), in greater or lesser proportions, affecting the growth of microorganisms (AMIRI; KARIMI, 2018). Butanol, for example, is an alcohol that has attracted the attention of researchers for presenting properties, such as higher miscibility with gasoline, higher energy density, lower volatility, and better biodegradability (ZETTY-ARENAS *et al.*, 2019), being a promising biofuel, beyond other applications in plastics, solvents, paints and adhesives industry (BURHANI *et al*., 2019; ZHEN *et al*., 2020).

Anaerobic bacteria of the genus *Clostridium* can convert some sugars, such as pentoses and hexoses, into acetone, butanol and ethanol (ABE) through condensation reactions present in the biochemical route of the fermentative process (MAGALHÃES, 2015). Many microorganisms could extract electrons from hydrogen molecules to fuel their metabolism or remove excess low-potential electrons from the environment, reducing protons and releasing hydrogen. In these microorganisms, the inter-conversion between the consumption and release of hydrogen is efficiently mediated by specific enzymes, known as hydrogenases (TRAN; BARBER, 2012), which are responsible for catalysis of the reversible reaction of hydrogen oxidation. These enzymes can be grouped into two distinct classes, based on the type of metal found in their respective catalytic centers: contain only iron, being called [FeFe]-hydrogenases and those that contain nickel and iron denominated [NiFe]-hydrogenases. Some enzymes can also have selenium, in addition to nickel and iron in the catalytic center, being called [NiFeSe]-hydrogenases (SÁ *et al.*, 2014).

Transition metals such as nickel are used as dehydrogenating agents for the production of alcohols, including butanol (KETABCHI *et al.*, 2020a). According to Trchounian *et al.* (2017), single (Fe^{2+}, Fe^{3+}, Ni^{2+}) or mixed supply of metals (Fe^{2+} and Ni^{2+}) can increase the specific hydrogenase activity by 50 %. The aim of this study was to evaluate the influence of nickel powder on the fermentation by *Clostridium beijerinckii*, using the green coconut shell hydrolyzate as substrate. The intention was to obtain a chemical product, i.e., cellulosic butanol of comparable quality to that obtained by conventional chemical process, however, in a sustainable way and with benefits to the environment. It is noteworthy that there is no study in the literature on the influence of nickel on the production of butanol using *Clostridium beijerinckii* from green coconut shell hydrolyzate.

MATERIAL AND METHODS

Microorganism, maintenance and inoculum preparation

This study used the *Clostridium beijerinckii* ATCC 10132 strain, acquired from the Tropical Cultures Collection (Campinas, SP). The strain, obtained in a lyophilized form, was reactivated in fluid thioglycolate medium (BD, USA) and cultivated in RCM (Reinforced Clostridial Medium, BD, USA) at 37 °C for 18-24 h. Both media were purged with nitrogen gas (15-30 seconds, approximately), and autoclaved at 121 °C for 15 min, in order to eliminate any dissolved

oxygen before inoculation. After incubation and growth period, the submerged culture was stored in sterile and closed test tubes, being kept under refrigeration (4-10 °C).

To inoculum preparation of fermentation a stock culture was subjected to 80 °C for 5 minutes and cooled in an ice bath; after homogenization, 1.0 mL of the culture was transferred to 20 mL-closed flasks, containing 14 mL of RCM medium supplemented with 5.0 $g.L^{-1}$ of glucose, kept at 37 °C for 18-24 h. All procedures were performed under anaerobic conditions. All culture media were purged with N_2 gas and then autoclaved at 121 °C for 15 min, before inoculation.

Obtaining the green coconut shell hydrolyzate (GCSH)

The green coconut shell (GCS) used in this work was supplied by Aquacoco EPP (Maxaranguape, RN, Brazil). A crushed sample (50 kg, approximately) was collected on the spot, washed in running water and submitted to an air circulation oven (TE-394, Tecnal) at 60 °C during 48 h. Then, the dry material was processed in a knife mill (model TE-680, TECNAL, Brazil), sieved at 48 mesh and (now denoted GCSP) stored at environment temperature for future experiments, as shown in Figure 1.

The first step consisted of performing the alkaline pretreatment: 50 g (dry basis) of GCSP was mixed with a solution of 2.0 % ($w.v^{-1}$) NaOH in the proportion of 1:10 ($w.v^{-1}$) and placed into the autoclave (model AV 137, PHOENIX, Brazil) at 121 °C and 1 kgf during 30 min. The alkaline pretreatment has previously been used successfully to produce hydrolysates using coconut material (NOGUEIRA *et al*., 2018; OLIVEIRA *et al*., 2018; SOARES *et al*., 2017). It should be highlighted that alkaline pretreatment can remove mainly lignin, thus cellulose and hemicellulose would be converted to fermentable sugars during the hydrolysis. In addition, this pretreatment also favours the productivity of the cellulases. Finally, the material was washed, filtered and its solid fraction denoted GCSPT.

An enzymatic hydrolysis was performed in the second step. The enzymes used in the hydrolysis of GCSPT were cellulases from Cellic Ctec2, purchased from Sigma-Aldrich (MO, USA). The total cellulolytic activity was determined by the method proposed by Ghose (1987) and expressed in filter paper units (FPU), being approximately 180 $FPU.mL^{-1}$. An initial enzyme load of 10 FPU/g of GCSPT was used for a solid load of 5.0 % ($w.v^{-1}$) in sodium citrate buffer solution (50 mM, pH 4.8). The system was subjected to 50 °C and 150 rpm for 72 h. After this time, it was heated in boiling water bath for 5 min to denature the enzymes.

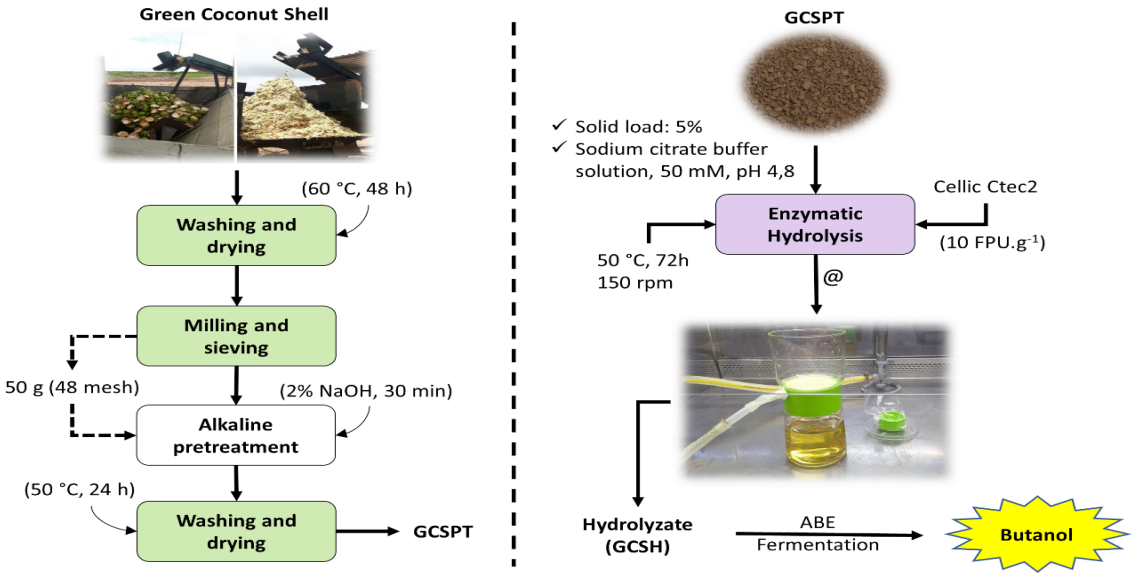

Figure 1. Illustration of the pretreatment and enzymatic hydrolysis steps to obtain the GCSH aiming the butanol production via ABE fermentation

Then, the hydrolyzate (GCSH) was centrifuged in SL-706 equipment (SOLAB, Brazil) at 2835 × g and filtered (0.22 µm) to be used as substrate in the fermentation.

Acetone-butanol-ethanol (ABE) fermentation

The fermentation experiments were performed in 20 mL-closed flasks under anaerobic conditions at 37 °C, without stirring or pH control, for 60 h. The medium was composed by GCSH, containing 19.4 $g.L^{-1}$ of reducing sugars (xylose and glucose, 1:4 ratio approximately), supplemented with 6.0 $g.L^{-1}$ tryptone, 2.0 $g.L^{-1}$ yeast extract, 2.2 $g.L^{-1}$ ammonium acetate, mineral solution (0.2 $g.L^{-1}$ $MgSO_4.7H_2O$; 0.01 $g.L^{-1}$ $MnSO_4.H_2O$; 0.01 $g.L^{-1}$ $FeSO_4$; 0.01 $g.L^{-1}$ NaCl) and phosphate buffer solution (0.50 $g.L^{-1}$ KH_2PO_4; 0.50 $g.L^{-1}$ K_2HPO_4), based on Qureshi and Blaschek (1999) with some modifications, and initial pH 6.0. Two conditions were tested: with addition of 0.2 $g.L^{-1}$ nickel powder, and without addition of nickel powder. For each condition tested, a control experiment (only with the presence of substrate) was used, as well as in the initial condition (time 0h, medium and inoculum), to discount amounts of sugars and/or products that could be present with the addition of inoculum at the beginning of the process. The amount of metal was chosen based on the study developed by Ketabchi *et al.* (2020b), which evaluated the effect of a catalyst on the yield of products from the ABE upgrade process, varying its load from 0.2 to 0.5 g, but maintaining other parameters such as reaction time, reagent ratio and temperature.

All media were purged with N_2 gas and then autoclaved at 121 °C for 15 min. A concentration of 1.0 $g.L^{-1}$ (dry basis) inoculum was added to the medium through of sterile syringe and needle. All experiments were performed in duplicate with a final volume of 15 mL. The equations (1) and (2) were used to evaluate the fermentation efficiency:

$$\text{Product yield } (\%) = \frac{\text{Concentration of product}}{\text{Concentration of consumed sugars}} \times 100 \quad (1)$$

$$\text{Productivity } (g.L^{-1}.h^{-1}) = \frac{\text{Concentration of product}}{\text{Fermentation time}} \quad (2)$$

In which the concentration was expressed in $g.L^{-1}$, and fermentation time in h.

Analytical methods and statistical analysis

Cell concentration was measured as optical density in a spectrophotometer (ThermoSpectronic Genesys 10UV/Vis, USA) at 600 nm. Then, the OD_{600} was converted to cell concentration (dry basis) by a calibration curve given by y (cells, $g.L^{-1}$) = 3.3514 x (absorbance), with determination coefficient (R^2) equal to 0.992. The inoculum volume of the fermentative medium was calculated by the dilution rule, Equation 3:

$$C_1.V_1 = C_2.V_2 \quad (3)$$

Where:
C_1 and C_2 = initial and final concentrations, $g.L^{-1}$, respectively; and
V_1 and V_2 = initial and final volumes, L, respectively.

The concentrations of sugars (xylose and glucose) and organic acids (acetic and butyric acids) were determined by high performance liquid chromatography (HPLC) using the Shim-pack SCR-101H column (Shimadzu, Japan), operating at a flow rate 0.6 mL min^{-1} at 50 °C and 5.0 mM sulfuric acid solution as the mobile phase. Solvent concentrations (acetone, butanol and ethanol) were also determined by HPLC using the Aminex HPX-87C column (Bio-Rad, CA) and Milli-Q water as the mobile phase, with the same flow rate and temperature mentioned above. In both columns a volume of 20 µL was injected to the sample previously filtered on 0.22 µm (Millipore) membranes. All components were detected by refractive index (RID-10A, Shimadzu) and the results expressed in $g.L^{-1}$ through previously constructed standard curves.

The results of the ABE fermentation were described in terms of mean and standard deviation. Comparison between assays was performed by analysis of variance (ANOVA) using the Statistica 7.0 software (StatSoft/USA), applying Tukey´s test at a confidence level of 95% ($p < 0.05$).

RESULTS AND DISCUSSION

In view of the structural modification and the sugar release potential, a pretreatment with 2.0% (w/v) NaOH was selected for the subsequent steps of this study. The chemical composition of GCSH in terms of levulinic acid, formic acid, HMF and FF has not been determined in this work. Although, Soares *et al*. (2017) evaluated levels of fermentation inhibitors in the liquid fraction of green coconut (CP) and its mesocarp (MP) hydrolysates, after saccharification using 15 FPU/g biomass (dry weight), and levulinic acid, FF and HMF were not detected. It is noteworthy that both (CP and MP) were obtained by alkaline pretreatment with 1 or 2% NaOH solution (at 25 °C for 1 h, in two rounds). Nogueira *et al.* (2019) used 2% NaOH (w/v) at 70 °C for pretreatment of the epicarp and mesocarp of green coconut, then determined the concentrations of glucose, xylose, acetic acid, and phenolic compounds in the liquor, which were 5.23, 3.56, 1.72, and 41.60 $g.L^{-1}$, respectively. In addition, due to the use of mild temperatures, traces of formic acid, HMF and furfural were not detected in this pretreatment. In this context, it is unlikely that the inhibitors generated during the pretreatment step used here have exerted negative influence on butanol production. However, these components must be observed in future studies.

The difference between the average values obtained for the analyzed components was significant ($p < 0.05$) for consumed sugars, and ethanol and butanol produced. Thus, indicating that the presence of nickel inhibited or delayed the metabolic pathway for converting sugars (xylose and glucose) into intermediate products. This factor had a negative influence on the production of butanol. However, the difference between the concentrations of organic acids present at the end of the fermentation was not significant ($p > 0.05$), reaching average concentrations of 1.9 $g.L^{-1}$ and 5.7 $g.L^{-1}$ and of acetic and butyric acids, respectively, as shown in Figure 2.

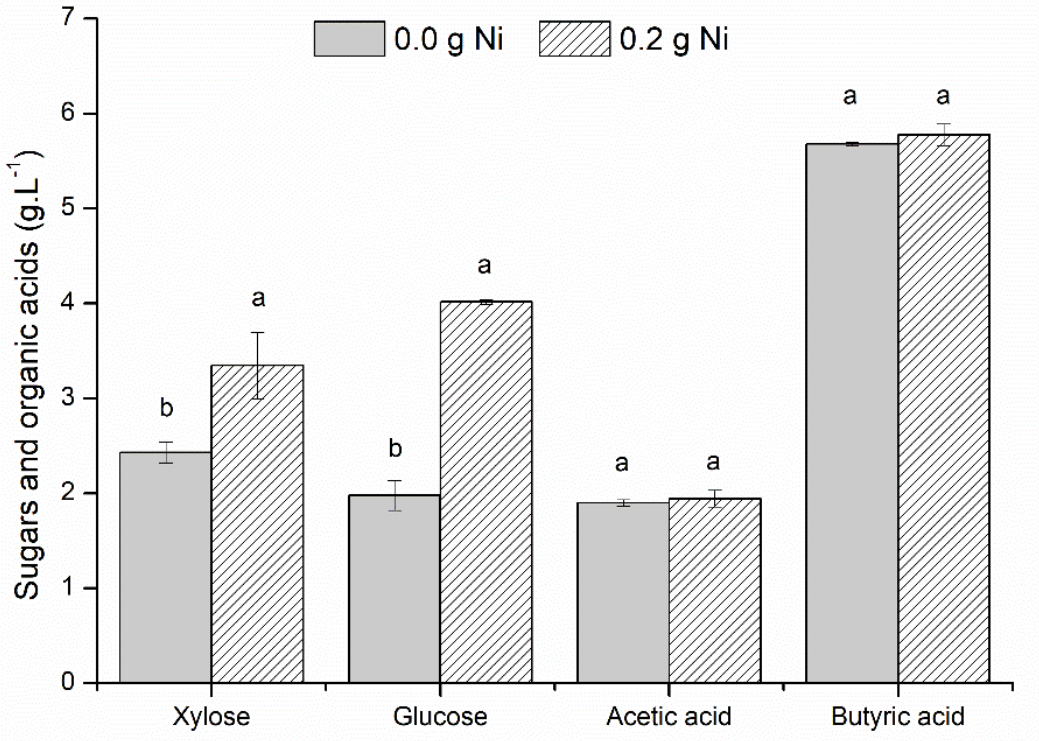

Figure 2. Concentration of sugars (glucose and xylose) and organic acids in GCSH with and without the addition of nickel after 60 h of fermentation. Results with the same letter for each type of sugar and organic acid show no significant differences between the treatments by Tukey test at 95% confidence level

In Figure 3 we can observe that the production of butanol was greater in GCSH without the addition of nickel. In this condition, an ABE concentration of 2.6 $g.L^{-1}$ was achieved, with 0.17 $g.L^{-1}$ of acetone, 2.1 $g.L^{-1}$ of butanol and 0.26 $g.L^{-1}$ of ethanol. Nickel (Ni^{2+}), like other transition metals, acts as an enzymatic cofactor in many metabolic processes, however when it comes to this element it can act by inhibiting the growth of microorganisms, either by inhibiting the synthesis of RNA and proteins, or by damaging heterochromatic regions of chromosomes (LI *et al*., 2009). Furthermore, as highlighted by Eitinger and Mardrand-Berthelot (2000) this element can also interfere with other trace elements such as Mg^{2+} and Fe^{2+} by compromising their functions. Moreover, Mg^{2+} and Fe^{2+} are known to be important, respectively, for the synthesis of ATP and ferrodoxin, a protein involved in electron transport. Furthermore, Mg^{2+} is important in cell membrane stabilization as a response to stress in the solvent production step by *C. beijerinckii*. Thus, these processes may have been compromised by the presence of nickel, leading to the formation of a smaller amount of butanol produced by *C. beijerinckii*.

The sugars consumed were 15 $g.L^{-1}$ in 60 h of fermentation, resulting in 0.14 g of butanol per gram of sugar consumed, and productivity of 0.04 $g.L^{-1}.h^{-1}$. In other process conditions, Magalhães (2018) evaluated the yield of butanol in the fermentation of sugarcane straw hydrolyzate, by different strains of the genus *Clostridium*, reaching 0.33 g of butanol per gram of sugar consumed, i.e., butanol yield of 33 %. However, specifically for the *C. beijerinckii* species its yield was lower (< 10 %) than that obtained in the present study (14 %).

In addition, Amiri and Karimi (2018) performed a review study on the different types of pretreatments used, on the ABE production and yield, obtained from various cellulosic hydrolyzates, whose results supported the data obtained in this study.

CONCLUSIONS

- The addition of nickel to the GCSH did not improve the conversion of sugars to butanol.

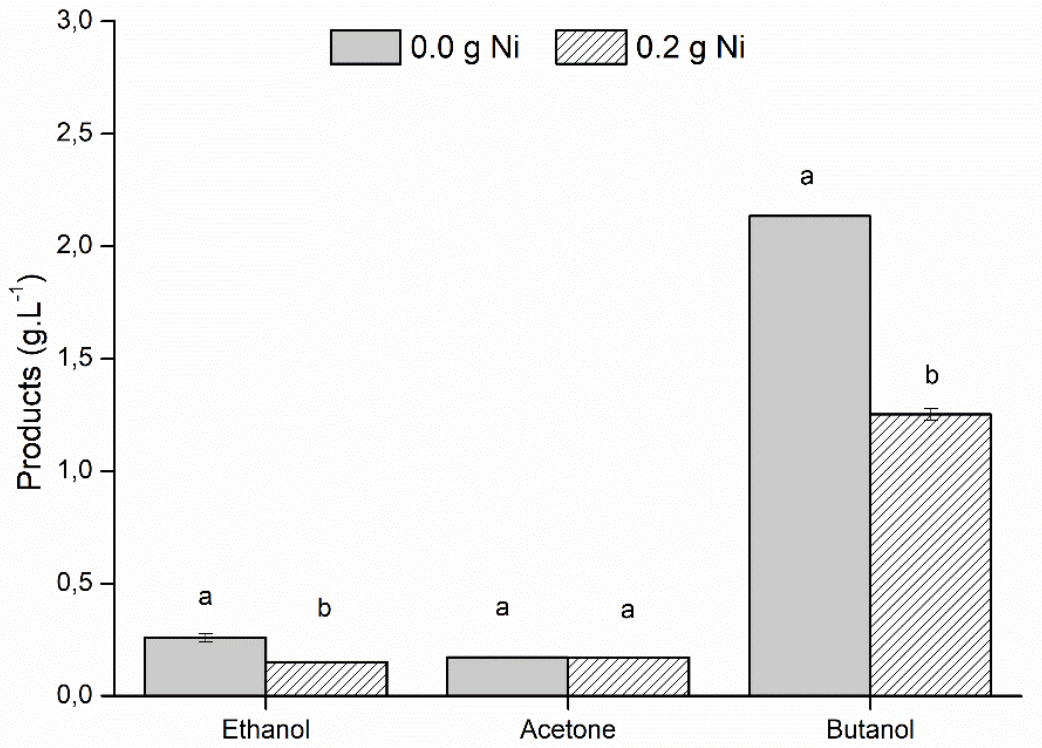

Figure 3. Concentration of the products acetone, butanol and ethanol in GCSH with and without the addition of nickel after 60 h of fermentation. Results with the same letter for each type of fermentation product show no significant differences between the treatments by Tukey test at 95% confidence level

- The final concentrations of organic acids, which are precursors in the biochemical pathway of ABE fermentation, were not influenced by the presence of nickel.
- Factors such as initial concentration of sugar, initial inoculum concentration, fermentation time, hydrogen gas (H_2) production, among other factors not included in this study merit attention to further elucidate the results.

AUTHORSHIP CONTRIBUTION STATEMENT

BEZERRA, P.K.S.B.: Conceptualization, Data curation, Formal Analysis, Methodology, Writing – original draft; **ARAÚJO, B.M.C.:** Methodology; **SILVA, O.L.:** Methodology; **AZEVEDO, B.:** Visualization; **MATIAS, S.C.B.M.:** Visualization; **SANTOS, E.S.:** Conceptualization, Formal Analysis, Project administration, Supervision, Writing – review & editing.

DECLARATION OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

AMIRI, H.; KARIMI, K. Pretreatment and hydrolysis of lignocellulosic wastes for butanol production: Challenges and perspectives. **Bioresource Technology**, v. 270, p. 702-721, 2018.

BURHANI, D.; TRIWAHYUNI, E.; SETIAWAN, R. Second-Generation Biobutanol: An Update. **Reaktor**, v. 19, n. 3, p. 101-110, 2019.

EITINGER, T.; MANDRAND-BERTHELOT, M-A. Nickel transport systems in microorganisms – Mini Review. **Archives of Microbiology**, v. 173, p. 1-9, 2000.

FAOSTAT - Food and Agriculture Organization of the United Nations. **World Production**. 2019. Available in: <http://faostat.fao.org/site/342/default.aspx> Access on 24 fev. 2021.

GHOSE, T. K. Measurement of cellulose activities. **Pure Applied Chemistry**, v. 59, n. 2, p. 257-268, 1987.

KETABCHI, E.; PASTOR-PÉREZ, L.; REINA, T. R.; ARELLANO-GARCÍA, H. Catalytic upgrading of acetone, butanol and ethanol (ABE): A step ahead for the production of added value chemicals in bio-refineries. **Renewable Energy**, v. 156, p. 1065-1075, 2020a.

KETABCHI, E.; PASTOR-PÉREZ, L.; ARELLANO-GARCIA, H.; REINA, T.R. Influence of Reaction Parameters on the Catalytic Upgrading of an Acetone, Butanol, and Ethanol (ABE) Mixture: Exploring New Routes for Modern Biorefineries. **Frontiers in Chemistry**, v.7, p. 906, 2020b.

LI, Y.; ZHANG, Z.; LEI, Z.; YANG, Y., UTSUMI, M., SUGIURA, N. Influence of metal addition on ethanol production with *Pichia stipitis* ATCC 58784. **Journal of Industrial Microbiology and Biotechnology**, v. 36, n. 4, p. 491-497, 2009.

MAGALHÃES, B.L. **Otimização da produção de butanol por cepas de *Clostridium spp.* utilizando hidrolisado lignocelulósico**. 2015. 111f. Dissertação de mestrado, Universidade Estadual de Campinas, Campinas, SP, 2015.

MAGALHÃES, B. L.; GRASSI, M. C. B.; PEREIRA, G. A.; BROCCHI, M. Improved n-butanol production from lignocellulosic hydrolysate by *Clostridium* strain screening and culture-medium optimization. **Biomass and Bioenergy**, v. 108, p. 157-166, 2018.

NOGUEIRA, C.C.; PADILHA, C.E.A.; LEITÃO, A.; ROCHA, L.S.; MACEDO, P.M.; MACEDO, G. R.; SANTOS, E. S. Enhancing enzymatic hydrolysis of green coconut fiber – Pretreatment assisted by tween 80 and water effect on the post-washing. **Industrial Crops and Products**, v. 112, p. 734-740, 2018.

NOGUEIRA, C.C.; PADILHA, C.E.A.; JESUS, A.A. SOUZA, D.F.S.; ASSIS, C.F.; SOUZA JUNIOR, F.C.; SANTOS, E. S. Pressurized pretreatment and simultaneous saccharification and fermentation with *in situ* detoxification to increase bioethanol production from green coconut fibers. **Industrial Crops and Products**, v. 130, p. 259-266, 2019.

QURESHI, N.; BLASCHEK, H. Butanol recovery from model solution/fermentation broth by pervaporation: evaluation of membrane performance. **Biomass and Bioenergy**, v. 17, n. 2, p. 175-184, 1999.

OLIVEIRA, S.D., PADILHA, C.E.A., ASEVEDO, E.A., PIMENTEL, V.C., ARAÚJO, F.R., MACEDO, G.R., SANTOS, E.S. Utilization of agroindustrial residues for producing cellulases by *Aspergillus fumigatus* on Semi-Solid Fermentation. **Journal of Environmental Chemical Engineering**, 6(1), p. 937-944, 2018.

PADILHA, C.E.A; NOGUEIRA, C.C.; SOUZA, D.F.S.; OLIVEIRA, J. A.; SANTOS, E. S. Valorization of green coconut fibre: Use of the black liquor of organolsolv pretreatment for ethanol production and the washing water for production of rhamnolipids by *Pseudomonas aeruginosa* ATCC 27583. **Industrial Crops and Products**, v. 140, p. 111604, 2019.

SÁ, L. R. V.; CAMMAROTA, M. C.; FERREIRA-LEITÃO, V.S. Produção de hidrogênio via fermentação anaeróbia – aspectos gerais e possibilidade de utilização de resíduos agroindustriais brasileiros. **Química Nova**, v. 37, n. 5, p. 857-867, 2014.

SOARES, J.; DEMEKE, M. M.; VAN DE VELDE, M.; FOULQUIÉ-MORENO, M. R.; KERSTENS, D.; SELS, B. F.; VERPLAETSE, A.; FERNANDES, A.A.R.; THEVELEIN, J.M.; FERNANDES, P. M. B. Fed-batch production of green coconut hydrolysates for high-gravity second-generation bioethanol fermentation with cellulosic yeast. **Bioresource technology**, 244, p. 234-242, 2017.

TRAN, P. D.; BARBER, J. Proton reduction to hydrogen in biological and chemical systems. **Physical Chemistry Chemical Physics**, 14(40), p. 13772-13784, 2012

TRCHOUNIAN, K.; MÜLLER, N.; SCHINK, B.; TRCHOUNIAN, A. Glycerol and mixture of carbon sources conversion to hydrogen by *Clostridium beijerinckii* DSM791 and effects of various heavy metals on hydrogenase activity. **International Journal of Hydrogen Ene**rgy, 42(12), p. 7875-7882, 2017.

ZETTY-ARENAS, A. M.; ALVES, R. F.; PORTELA, C. A. F.; MARIANO, A. P.; BASSO, T. O.; TOVAR, L. P.; MACIEL FILHO, R.; FREITAS, S. Towards enhanced n-butanol production from sugarcane bagasse hemicellulosic hydrolysate: Strain screening, and the effects of sugar concentration and butanol tolerance. **Biomass and Bioenergy**, v. 126, p. 190-198, 2019.

ZHEN, X.; WANG, Y.; LIU, D. Bio-butanol as a new generation of clean alternative fuel for SI (spark ignition) and CI (compression ignition) engines. **Renewable Energy**, v. 147, p. 2494-2521, 2020.