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EVALUATION OF DIFFERENT NUTRIENT MEDIA ON *Clostridium acetobutylicum* ATCC 824 GROWTH

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Research on *Clostridium* bacteria is pivotal for both hydrogen production and the synthesis of high-value industrial compounds. However, comparative analyses of different strains, particularly within biorefinery contexts, remain underexplored. The composition of lignocellulosic materials and the specific nutritional requirements of these strains significantly influence fermentation yields, necessitating precise nutritional supplementation. This approach enhances yield, reduces process costs, valorizes waste, and supports agro-industrial sustainability. Preliminary experiments with synthetic culture media indicated that the CH medium extended the lag phase to 24 h, with a subsequent pH decrease and gas production of 13.7 mL at 48 h. Conversely, the RCM medium exhibited a shorter lag phase of 15 h, with a pH drop at 15 h and gas production of 27.4 mL at 20 h and 75 mL at 48 h. These results underscore the effects of catabolic repression and highlight the criticality of substrate selection.

Keywords: Bioprocessing. Circular bioeconomy. Lignocellulosic materials. Biorefinery.

1 INTRODUCTION

Bacteria of the genus *Clostridium* are gram-positive, sporulating anaerobic microorganisms capable of utilizing both simple and complex carbohydrates to produce a variety of compounds, organic acids, solvents, and biofuels[2]. These are raw materials for various industry segments, such as food, pharmaceutical, chemical, and biofuels[8]. Several anaerobic microorganisms are natural producers of these compounds, with the genus Clostridium being widely studied.

Although *Clostridium* strains with product-producing capacity and added value have been identified, comparative studies between these strains are still scarce, especially in biorefineries. In these facilities, obtaining high-value-added products is based on lignocellulosic materials (LM) that go through different stages, including pretreatment, saccharification, removal of inhibitory compounds and fermentation. Pretreatment is a critical step that influences the generation of inhibitory compounds that can impair the performance of subsequent steps. The composition of LMs can vary significantly according to harvest, season and origin, directly impacting the profile of available sugars. In addition to fermentable sugars, strains require adequate nutritional loads for their full development and, consequently, for the fermentation process to achieve high yields[4]. Therefore, nutritional supplementation is an essential practice in producing biofuels and chemicals from bacteria of the genus *Clostridium*.

A limited amount of microorganisms have an intrinsic capacity to use pentoses and hexoses as fermentable sugars[3]. However, even those who possess this ability may have low efficiency and productivity, due to several phenomena, such as catabolic carbohydrate repression and limited efficiency in xylose uptake[3].

To ensure high yields in the fermentation process, the strains of the genus *Clostridium* require an adequate nutritional load. A viable option to supplement nutrients is using agro-industrial residues, which are rich in compounds of interest in high concentrations. In this context, this work aims to improve the production of compounds of industrial interest by using nutrients and strains selected in lignocellulosic residues (pretreated). In addition to maximizing the production of desired metabolites, the strategy aims to reduce process costs, add value to waste, and contribute to the sustainability of the agro-industrial sector. The aim of this work is to compare the growth of *Clostridium acetobutylicum* ATCC 824 in different synthetic nutrient media, assessing the impact on the production of industrial compounds and fermentation. The research seeks to understand the functioning of this strain to optimize the use of LM as a substrate in the future, enhancing yield, reducing costs, and promoting agro-industrial sustainability. Preliminary results using synthetic culture media will be presented below.

2 MATERIAL & METHODS

The microorganism used in this work was *Clostridium acetobutylicum* ATCC 824 from the André Tosello Foundation Tropical Culture Collection - Campinas, SP. Preliminary assays were performed on 100 mL serum flasks with 60 mL of useful volume. They were closed with rubber stoppers, fixed with an aluminum seal, and purged with nitrogen for 3 min to maintain anaerobic conditions[5]. The composition of the culture medium is described in the items below, according to the test performed. All bottles will be kept at 37 °C and 150 rpm, and the fermentation will be carried out for 48 h. For kinetic study in different culture media nutritional supplementation strategies will be applied to the selected microorganism. In this stage, two synthetic media were selected: i) adapted CH medium (pH 7.0) described by Fonseca *et al.* (2016) compound (g/L) by glucose (20), xylose (5), yeast extract (1), Na_2HPO_4 (5), KH_2PO_4 (1), NaCl (1), $MgSO_4.7H_2O$ (0.1), $FeSO_4$ (0.025), and a solution of trace elements (2 mL/L) containing H_3BO_3 (2.86 g/L), $MnSO_4.4H_2O$ (2.03 g/L) and $FeCl_3$ (0.1 g/L) and ii) RCM (*Reinforced Clostridial Medium)* composed

(g/L): Casein enzymatic hydrolysate (10), Beef Extract (10), Yeast Extract (3), Starch (1), Sodium Chloride(5), Glucose (5), L-Cysteine Hydrochloride (0.5), and Sodium Acetate (3) (KASVI, Spain). The experiments were conducted in triplicate. Gas production was measured every 4 h during the first 24 h, with the sampling interval increasing to every 24 h until reaching 48 h, using a syringe. Additionally, bacterial growth (OD600) and pH were monitored throughout the experiment.

3 RESULTS & DISCUSSION

Studies have shown that microorganisms prefer to use glucose as a carbon source due to its abundance as a carbohydrate monomer[3]. Although *Clostridium* strains can metabolize the major C5 and C6 sugars released during saccharification, studies suggest that these strains selectively metabolize glucose, indicating that xylose is a secondary carbon source regulated by catabolic carbon repression[7]. However, the strain *C. acetobutylicum* ATCC 824 showed a growth delay in a media with high glucose concentration using the CH medium (Figure 1).

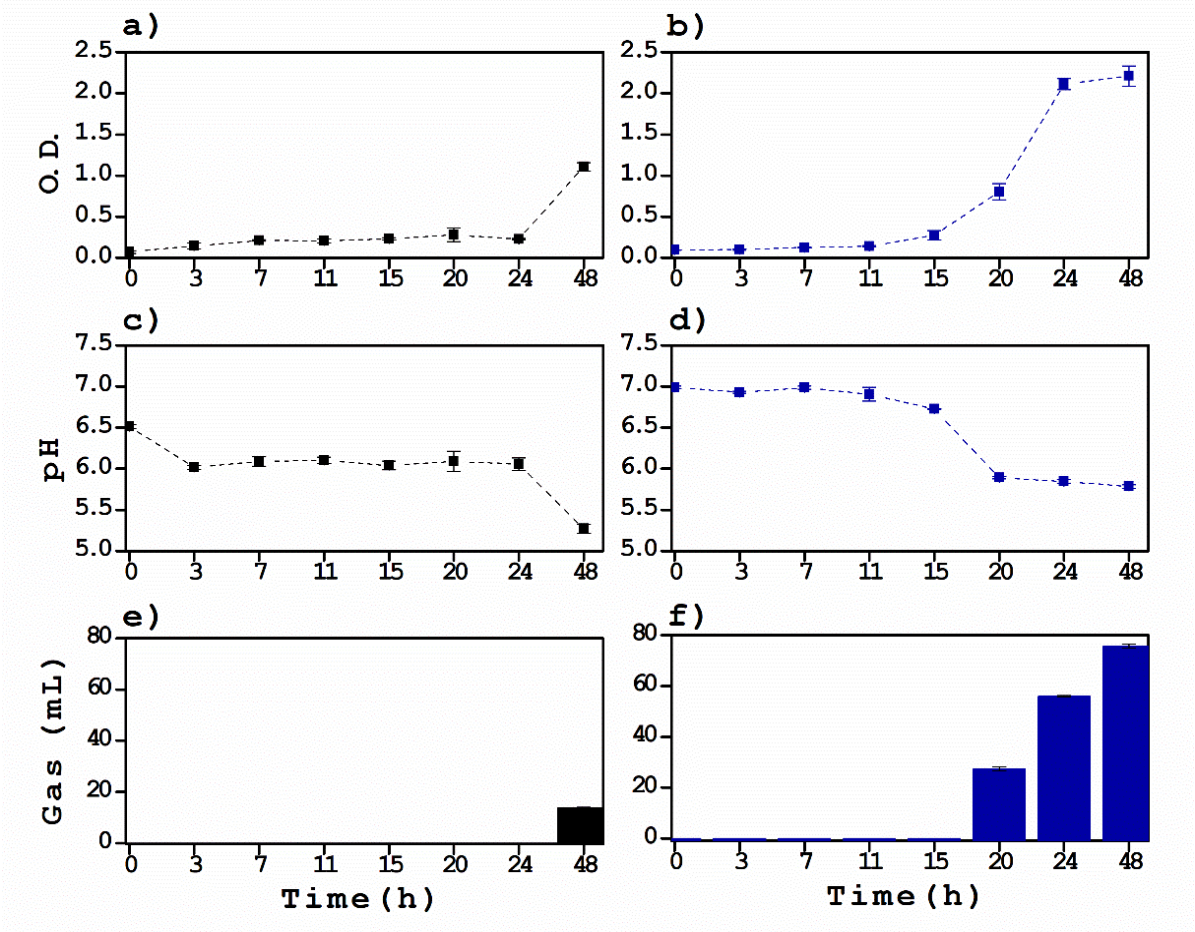

Figure 1 Growth kinetics of *C. acetobutylicum* ATCC 824 using CH medium (■) and RCM medium (■).

As observed in Figure 1a and 1b, the CH medium (supplemented with glucose) elongated the Lag phase up to 24 h. Meanwhile, for the RCM medium (supplemented with 5 g/L of glucose the Lag phase was only up to 15 h. This profile can also be observed by the decrease in pH, where for the CH medium (Figure 1c), the pH decreased after this period. For the RCM medium (Figure 1d), the pH decreased in 15 h and stabilized after 20 h. It is important to highlight that the RCM medium is a complete medium, composed of a variety of essential nutrients that promote the growth of this strain.

Meat peptone and yeast extract provide rich sources of nitrogen, essential vitamins, and amino acids. Starch contributes to the detoxification of harmful metabolites, while glucose acts as a fermentable carbohydrate. Sodium chloride ensures osmotic balance, and sodium acetate serves as a buffer. L-cysteine hydrochloride acts as a reducing agent, creating an anaerobic environment and maintaining a low redox potential[9]. This effect is enhanced by the low level of agar, which reduces oxygen permeability in the medium. These components, together, increase the efficiency of the RCM medium in promoting a faster reduction of the Lag phase and stabilization of pH levels, highlighting its more complete nutritional profile compared to the CH medium.

The gas production profile shows that only in 48 h was a gas production of 13.7 mL for the CH medium (Figure 1e). Meanwhile, for the RCM medium (Figure 1f), the gas production was 27.4 mL and 75 mL at 20 and 48 h, respectively. According to Espindola *et al.* (2017), *Clostridium* strains produce hydrogen and CO_2 in the proportion of 48% and 23%, respectively. According to Kandia *et al.* (2018), when comparing the kinetic profile of *C. acetobutylicum* in a medium containing glucose or cellobiose, it became evident that cultures cannot co-use the substrates simultaneously. According to the authors, these results confirm the phenomenon of catabolic repression, where some substrates repress the expression of genes encoding catabolic and/or protein-transporting enzymes. However, this mechanism is different for all microorganisms[3].This factor directly relates to the substrate (LMs) choice and the microorganism used.

4 CONCLUSION

The knowledge of new strategies for adding carbon sources allowed the proposal of future stages of optimization of the production of value-added products using different substrates and the scaling of the process.

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