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Study of different co-fermentation strategies of hexoses and pentoses for butanol production

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ABSTRACT

Biobutanol has emerged as a promising biofuel due to its high energy density and compatibility in mixtures with gasoline. A fundamental step in biobutanol production is the acetone-butanol-ethanol (ABE) fermentation, a bioprocess widely used by anaerobic bacteria of the genus *Clostridium* that can assimilate various hexoses and pentoses. Although the simultaneous co-fermentation of hexoses and pentoses is a simple approach, the simultaneous assimilation of these sugars can lead to glucose's metabolic suppression of xylose. Thus, the present project aims to evaluate different strategies for the co-fermentation of glucose and xylose from lignocellulosic hydrolysates. Five strategies were evaluated, considering glucose and xylose as carbon sources, fermented singly, sequentially, or simultaneously. The glucose (40 g/L) and xylose (20 g/L) concentration was defined according to typical composition of lignocellulosic hydrolysates. The best strategy evaluated was to use glucose first, followed by xylose supplementation (S_5), which, in this context, acts as a stimulus to produce butanol. Butanol yield and concentration were 0.35 $g_{butanol}/g_{glucose\ consumed}$ and 11.97 g/L, respectively.

Keywords: Co-fermentation of glucose and xylose. ABE Fermentation. Butanol production.

1 INTRODUCTION

Fermentative processes for converting lignocellulosic waste into biofuels, chemicals, and materials are a sustainable option with low energy demand in the context of biorefineries. ABE (Acetone, Butanol, Ethanol) or IBE (Isopropanol, Butanol, Ethanol) fermentation is a promising option to exploit glucose and xylose, the two major sugars in lignocellulosic biomasses[1]. These fermentations are mediated by bacteria of the genus *Clostridium*, capable of metabolizing a variety of monosaccharides, including many hexoses and pentoses, providing a significant advantage over naturally ethanol-producing microorganisms, which can only synthesize a few hexoses[2].

Although *Clostridium* can metabolize glucose or xylose individually, xylose assimilation is inhibited when glucose is available in the reaction medium[3,4]. Therefore, studying strategies of hexoses and pentoses assimilation is essential for the efficient utilization of sugars in lignocellulosic biomass. Thus, this study aims to evaluate different co-fermentation strategies for glucose and xylose, adding these carbon sources individually, simultaneously, or sequentially.

2 MATERIAL & METHODS

Clostridium acetobutylicum ATCC 824 from ARS (Agricultural Research Services) Culture Collection, USA was used in fermentation assays. Activation of spores was made in RCM medium (Reinforced Clostridium Medium) with xylose or glucose as the carbon source at 37°C until density optical (D.O) of 2-2.5 (approximately 24 h). After the inoculation, 10% v/v of inoculum was resuspended in the medium with the following composition (g/L): carbon source (glucose (40) and/or xylose (20)); ammonium acetate (2.2); KH_2PO_4 (0.5); K_2HPO_4 (0.5); vitamins (para-amino-benzoic acid (0.1), thiamine (0.1) and biotin (0.01)) and mineral salts ($MgSO_4.7H_2O$ (0.2); $MnSO_4.H_2O$ (0.01); $FeSO_4.7H_2O$ (0.01); NaCl (0.01)[5]. The pH of the medium fermentation was adjusted to 6.5 with HCl or KOH, as needed. The solutions were autoclaved for 15 minutes at 120°C to sterilize the medium and then sealed to guarantee medium anaerobic and prevent contamination with the external environment. Fermentation was carried out statically in an anaerobic medium at 37 °C.

Different configurations of fermentation were carried out to determine the ideal strategy for metabolizing glucose and xylose, as follows: 1) Isolated fermentation of xylose (S_1) or glucose (S_2); 2) Co-fermentation of the glucose and xylose, where both sugars are fermented simultaneously (S_3); 3) Sequential batch: the first stage containing only xylose followed by glucose feeding (S_4), or the first stage containing only glucose followed by xylose feeding (S_5). The second carbon source was supplemented when the sugar concentration in the medium dropped below 10 g/L. The initial glucose and xylose concentrations were defined based on the expected levels in cellulosic and hemicellulosic hydrolysates.

Liquid samples were taken during fermentation and analyzed by HPLC for quantification of organic acids (acetic and butyric) and solvents (acetone, ethanol, butanol) produced, as well as the sugars (glucose and xylose) consumed. The chromatograph (Shimadzu LC-10AD) was equipped with a refractive index detector (Shimadzu RID-10A) and ionic exclusion column Aminex HPX-87H using 5 mM sulfuric acid at 0.6 mL/min and 35 °C.

To evaluate the fermentation parameters, the butanol yield and productivity were evaluated, as calculated by equations 1 and 2, respectively:

$$Yield\left(\frac{g\ butanol}{g\ sugar\ consumed}\right) = \frac{C_{butanol}}{C_{sugar,0} - C_{sugar,f}} \qquad (1)$$

where: $C_{butanol}$ is the concentration (g/L) of butanol obtained at the end of fermentation; $C_{sugar,0}$ is the concentration of sugars at the beginning of fermentation, and $C_{sugar,f}$, final is the concentration of sugars at the end of fermentation.

$$Productivity\left(\frac{g\ butanol}{L.h}\right) = \frac{C_{butanol}}{fermentation\ time} \qquad (2)$$

The fermentation time varied between the different strategies and was defined as the period until sugar consumption and butanol production reached a plateau.

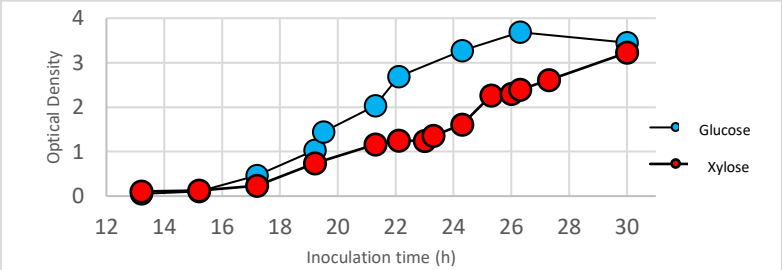
All the experiments were performed in duplicates. The results are reported as mean ± standard error. Tukey's test evaluated the statistical significance of the differences between groups, considering the confidence level of 90 % ($p < 0.10$)

3 RESULTS & DISCUSSION

Activation of Bacteria in RCM Medium With Different Sources of Carbon

The activation of *C.Acetobutylicum* spores was carried out in glucose or xylose to analyze their growth time in different carbon sources. Figure 1 shows cell growth using density optic as a function of inoculation time.

Figure 1 Growth kinetics on different carbon sources



The growth profile shows that *C. acetobutylicum* can grow in both carbon sources, but it grows more quickly on glucose. The exponential growth phase begins around 19 hours in the medium with glucose, while in the medium with xylose, it starts at 24 hours. Successful fermentations require exponential cell growth, characterized by intense metabolic activity and optical densities.

The results confirmed the bacteria's preference for glucose, which is expected since glucose is the most readily metabolizable carbon source by microorganisms.

Evaluation of Fermentation Strategies

Table 1 represents the main results of butanol concentration, productivity, and yield. The fermentation time in each test referred to the moment substrate consumption and product generation stagnated.

Table 1- Fermentation results for the different strategies evaluated

	S_1 20 g/L xyl.	S_2 40 g/L gluc.	S_3 20 g/L xyl + 40 g/L gluc.	S_4 20 g/L xyl sup. 40 g/L gluc.		S_5 40 g/L gluc. sup. 20 g/L xyl.	
	47 h	146 h	96 h	Before sup. 72 h	After sup. 168 h	Before sup. 146 h	After sup. 216 h
Glucose consumed (%)	0%	63%	95%	0%	55%	70%	100%
Xylose consumed (%)	44%	0%	15%	69%	0%	0%	0 %
Butanol (g/L)	3.57 ± 0.22^{a}	8.84 ± 0.31^{b}	8.47 ± 0.70^{b}	3.29 ± 0.49^{a}	9.15 ± 0.27^{b}	8.55 ± 0.42^{b}	11.97 ± 0.26^{c}
Butanol yield ($g_{butanol}/g_{sugars\ consumed}$)	0.43 ± 0.012^{d}	0.32 ± 0.017^{e}	0.21 ± 0.001^{f}	0.24 ± 0.017$^{f, g}$	0.28 ± 0.026$^{e, g}$	0.35 ± 0.018^{e}	0.34 ± 0.007^{e}
Substrate consumption rate (g/(L.h))	0.08 ± 0.00	0.17 ± 0.03	0.25 ± 0.01	0.17 ± 0.00	0.01 ± 0.02	0.16 ± 0.00	0.04 ± 0.00
Butanol productivity (g/(L.h))	0.076 ± 0.005^{h}	0.061 ± 0.002^{i}	0.088 ± 0.005^{h}	0.039 ± 3E-4	0.054 ± 0.002^{i}	0.059 ± 0.003^{i}	0.055 ± 0.001^{i}

The overwritten lowercases represent the Tukey test for multiple comparisons between rows ($p < 0.1$) of each assay

The xylose consumption rate in (S_1) was 2.1-fold lower than that of glucose (S_2), reiterating the microorganism's preference for the latter. Furthermore, in S_2, the butanol production was 2.48-fold higher. The butanol concentration was possibly low in S_1 due to the low initial xylose concentration, which was insufficient to sustain the acidogenic and solvatogenic phases. In this sense, the strategy S_3 was proposed to increase the initial substrate concentration, where glucose and xylose were available. In 96 hours of reaction, substrate consumption was 95% for glucose and 15% for xylose, indicating catabolic repression by glucose, where the cell suppresses pentose assimilation in favor of glucose presence, a more easily metabolizable carbon source[6]. Despite the low xylose consumption, the butanol production was satisfactory (8.47 g/L), reaching values statistically equal to S_2. Interestingly, the butanol productivity in S_3 was 1.4-fold greater than in S_2 and greater than the other conditions. This behavior is probably associated with the higher initial substrate concentration, which leads to a higher rate of cell growth, thus increasing the productivity of metabolites.

The S_4 strategy is proposed to exploit xylose more efficiently since this is the only carbon source up to the time of supplementation. In this assay, 69% of the xylose was consumed before supplementation, yielding 0.24 $g_{butanol}/g_{xylose\ consumed}$ and 3.3 g/L of butanol. After glucose supplementation, xylose consumption was completely inhibited, with 55% glucose utilization after an additional 96 h of fermentation. Incomplete glucose consumption is probably associated with cellular inhibition by the product (butanol). At the end of fermentation, a butanol concentration of 9.15 g/L was obtained. Narueworanon et al. (2020)[7] reported that 11 g/L of butanol had a toxic effect on *C. acetobutylicum*.

S_1 and S_4 (before supplementation) did not show a statistical difference ($p < 0.1$) in butanol concentration, which was already expected since the conditions were the same. The same behavior is observed in S_2, S_3, and S_5 (before supplementation), which also present equal initial conditions. S_3, despite containing xylose, does not show significant differences because the xylose was not metabolized to produce butanol.

S_1 presents significative differences regarding butanol yield due to the low concentration of xylose consumed. S_2, S_4 (after supplementation), and S_5 (before and after supplementation) did not show significant differences in butanol yield. It is observed that glucose supplementation (S_4) enhanced the butanol yield, increasing from 0.24 to 0.28 g/g. S_1 and S_3 did not show significant differences in butanol productivity, with values higher than in the other assays. This behavior is associated with fermentation stagnation in a short time, thus generating a higher productivity value.

Butanol production reached the maximum titer (11.97 g/L) in S_5 after xylose supplementation. After supplementation, glucose was completely consumed. As observed in S_3, the simultaneous presence of pentoses and hexoses can improve the fermentation, leading to higher butanol concentration and/or productivity. Certainly, the xylose was not consumed as the butanol level achieved was already toxic to bacteria. Xiao et al. (2011)[1] found that xylose uptake by *C. acetobutylicum* is a rate-limiting step highly sensitive to butanol.

4 CONCLUSION

ABE fermentation is a promising strategy for biofuel production from cellulosic and hemicellulosic hydrolysates since *C.acetobutylicum* demonstrated the ability to ferment C_5 and C_6 sugars. S_3 and S_5 were the best strategies for butanol production, reaching the higher suitable fermentation performance. For future studies, we will explore higher xylose concentrations (between 40 and 60 g/L) to investigate the potential for increased butanol production solely from this carbon source.

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