I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM XV SHEB XV ENZITEC FLORIANÓPOLIS 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
BIOPROCESS ENGINEERING

EVALUATION OF SUBSTRATES FOR PROPIONIC ACID FERMENTATION

Isabella M. T. S. Santos[1]*, Ricardo G. R. Brenes[1], Ninoska Bojorge[2], Nei Pereira Junior[1]

[1] *School of Chemistry, Federal University of Rio de Janeiro, Av. Athos da Silveira Ramos, 149, Ilha do Fundão 21941-972, RJ, Brazil.*
[2] *Department of Chemical and Petroleum Engineering, Fluminense Federal University, R. Passos da Pátria 156, Niterói 24210-140, RJ, Brazil.*
** Corresponding author's email address: isabellatenorio@eq.ufrj.br*

ABSTRACT

Xylose, glucose and glycerol were used as substrates to evaluate the ability of three different strains of propionic acid bacteria (*Acidipropionibacterium acidipropionici*, *Propionibacterium jensenii*, and *Propionibacterium thoenii*) to produce propionic acid (PA) and cell growth. pH has been found to have a significant impact on propionic acid production and cells. *Acidipropionibacterium acidipropionici* DSM 4900 was the most efficient strain in PA production as it completely consumed xylose and glycerol. After 69h of reaction, 13.2 g/L of PA were produced, with a productivity of 0.19 g/L.h and a fermentation efficiency of 72.9%.

Keywords: organic acid. bioreactor. fermentation. carbon source. hemicellulosic fraction.

1 INTRODUCTION

Commercial interest in propionic acid is driven by its diverse applications, traditionally obtained from petroleum derivatives[1]. However, there is growing interest in the use of renewable sources and by-products for the fermentative production of propionic acid. Nevertheless, the proposed methods have not yet successfully competed against chemical synthesis due to three main reasons: low yield, formation of acetic acid and slow or inhibit cell growth caused by acid production[2]. The most studied carbon sources, are glucose, lactose, and lactic acid leads to high acetic acid formation, which had a detrimental effect on the separation and purification process of propionic acid. This aim of this study is to evaluate propionic acid production of three different strains using xylose as the main substrate.

2 MATERIAL & METHODS

Acidipropionibacterium acidipropionici DSM 4900, *Propionibacterium jensenii* DSM 20274, and *Propionibacterium thoenii* DSM 20270 strains were obtained from the Leibniz Institute DSMZ (Braunschweig, Germany) culture collection. These strains were preserved at -80ºC in a glucose medium containing 20 g/L glucose, 10 g/L yeast extract, 5 g/L tryptic soy broth, and 0.05 g/L manganese (II) sulfate, and cryopreserved in 20% (v/v) glycerol. Inoculums were grown in 500 mL flasks with a nutrient medium and incubated at 30°C and 70 rpm for 72 hours. The cells were harvested by centrifugation and resuspended in the fermentation broth.

Propionic acid fermentations with the three strains were conducted in 500 mL flasks containing 300 mL of fermentation broth, incubated at 30°C and 70 rpm under relatively anaerobic conditions. All fermentation experiments were run in duplicate, and the optical density of the cultures was monitored at 600 nm and converted to dry cell weight. Culturing with *A. acidipropionici* was performed in a 1L STR bioreactor, with pH and temperature maintained at 6.0 and 37°C, respectively.

The concentrations of organic acids and substrates such as glucose, xylose, and glycerol were measured using two HPLC systems equipped with a refractive index detector and a Hi-plex H column heated to 60ºC. The mobile phase consisted of 5 mM H_2SO_4, with a flow rate of 0.6 mL/min.

3 RESULTS & DISCUSSION

Experiments were conducted with *A. acidipropionici* DSM 4900, *P. jensenii* DSM 20274, and *P. thoenii* DSM 20270 strains in chemically defined media using xylose, glucose, and glycerol as carbon sources (Table 1). The analysis included substrate consumption, cell growth, propionic acid production, and key response variables ($Y_{P/S}$, $Y_{X/S}$, and Q_P). These assays were essential to determine the most suitable strategy for acid production and the comprehensive utilization of carbon sources.

Given the prevalence of xylose in the hemicellulosic fraction[3], this pentose was chosen as a factor for selecting the carbon source in the synthetic media used in the experiments, aiming to provide a basis for future use of lignocellulosic raw feedstocks. The concentrations of xylose used in the experiments ranged from 10.5 to 29.4 g/L, corresponding to the initial concentration values reported in the literature. Co-fermentation of carbon sources such as xylose, glucose[4,5], and glycerol[6,7,8] were also tested to compare with literature findings (Table 1).

Table 1 Identification of the most efficient strain for propionic acid production in chemically defined media using xylose, glucose and glycerol as carbon sources.

Fermentation settings	Strain	Medium	Substrate	[S] (g/L)	PSR (%)	$[PA]_{max}$ (g/L)	$Y_{AP/S}$ (g/g)	$Y_{X/S}$ (g/g)	Q_{AP} (g·L/h)

Time: 46h Temperature: 30ºC pH 7 Agitation: 70 rpm Volume: 300 mL	*A. acidipropionici*	1	Glucose Xylose	12.8 10.5	99.1	4.9	0.1	0.32	0.048
		2	Xylose	23.1	79.6	5.9	0.24	0.21	0.098
		3	Xylose Glycerol	27.8 5.5	71.8	7	0.2	0.2	0.1
		4	Xylose	20.8	89.4	6.1	0.25	0.22	0.1
Time: 46h Temperature: 30ºC pH 7 Agitation: 70 rpm Volume: 300 mL	*P. thoenii*	1	Glucose Xylose	17.2 12.9	99.3	9.6	0.26	0.29	0.17
		2	Xylose	29	20	3.2	0.28	0.1	0.035
		3	Xylose Glycerol	29.4 5.7	21.3	5.2	0.47	0.4	0.076
Time: 68h Temperature: 30ºC pH 7 Agitation: 70 rpm Volume: 300 mL	*P. jensenii*	1	Xylose	26.6	10.9	2.7	0.45	0.85	0.019
		2	Glucose	28.4	63.7	8.6	0.38	0.76	0.1
		3	Xylose Glycerol	26.5 5.2	9.8	1.2	0.19	0.76	0.007
		4	Glucose	29.9	76.6	7.1	0.23	0.16	0.076

[S]: substrate concentration; PSR: percentage of substrate reduction; [PA]: propionic acid concentration; $Y_{AP/S}$: propionic acid yield; $Y_{X/S}$: cellular biomass yield; Q_{AP}: propionic acid volumetric production rate.

The strain *A. acidipropionici* demonstrated the highest performance in propionic acid production, productivity, and fermentation efficiency using xylose and glycerol as carbon sources, achieving a maximum of 7 g/L of propionic acid. Glycerol proved to be a promising substrate for continuous fermentation, potentially making the process economically feasible by using inexpensive industrial residues[9]. The surplus of crude glycerol from biodiesel production makes it a valuable substrate for industrial applications. Additionally, *Propionibacterium* can utilize nitrogen sources like peptone, corn steep, and yeast extract to boost propionic acid production[14], with studies showing enhanced yields and productivity (5 to 10 g/L) using corn steep as an agro-industrial effluent[12].

A batch of *A. acidipropionici* was carried out under optimized culture conditions with automatic pH control at 6 (Figure 1). The pH control strategy allowed for the complete consumption of xylose and glycerol by cellular biomass, leading to maximal propionic acid (PA) production of 13.2 g/L at 69h and a maximum dry cell weight (DCW) of 15.8 g/L. Some authors revealed that propionic bacteria growth is greatly inhibited in pH below 5.0; pH of 6.0 -7.0 is reported as optimal for PA production[10,11].

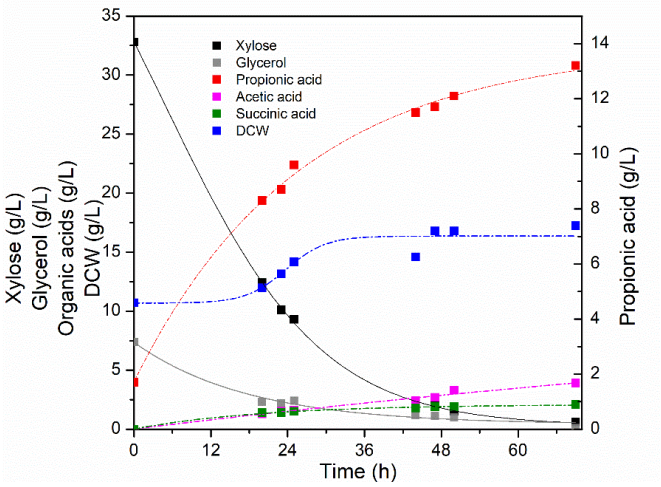

Figure 1 Production of organic acids in bioreactor with xylose and glycerol as substrates. The experiment was carried out in bioreactor at 70 rpm, 37°C by *A. acidipropionici*. The cell concentration was 11 g/L, and the parameters obtained are on Table 2.

Kinetics of cell growth and propionic acid production were studied (Figure 1; Table 2) starting with a cell concentration of 11 g/L, over 69 hours at 30°C and pH 6. The high initial cell concentration led to higher propionic acid yields. Xylose and glycerol were completely consumed, with organic acid production increasing over time, while cell growth plateaued after 36 hours. Propionic acid production continued even after cell growth ceased. Glycerol, yielding only propionic acid, produced 0.8 g of propionic acid per gram, compared to 0.49 g from xylose and 0.62 g from glucose. Additionally, glycerol acted as a compatible solute, mitigating osmotic pressure and preventing xylose's repressive effects on cells.

Table 2 Response variables of the experiment performed in Figure 1.

Medium	Organic acid	Time (h)	Organic acid produced	Q_P (g/L.h)	$Y_{OAc./TS}$ (g/g)	$Y_{OAc/Xil}$ (g/g)	PSR (%)	EF (%)

Glucose: Xylose (4:1)	Propionic acid	69	13.2	0.191	0.3	0.36	98.17	72.9
	Acetic acid	69	3.9	0.057	01	0.12	98.17	-
	Succinic acid	69	2.1	0.030	0.05	0.07	98.17	-

$Y_{OAc./TS}$: yield factor (organic acid/total substrate); Q_P: volumetric productivity; PSR: percentage of substrate reduction; EF: efficiency of fermentation.

At the beginning of fermentation, propionic acid production was 4 g/L, with 1 g/L attributed to the pre-inoculum and the rest due to interference from the nitrogen source in the fermentation medium. HPLC chromatograms showed peaks at the same retention time as propionic acid (16.74 min). However, subtracting the initial PA value from the final concentration could underestimate the cells' production capacity.

Primary sugars found in hemicellulose (glucose, xylose, and arabinose) were evaluated for propionic acid production by *P. acidipropionici* ATCC 4875[3], achieving maximum cell densities of 7.6, 6.3, and 5.0 g/L, respectively, and final propionic acid concentrations of 13.4, 13.3, and 13.8 g/L[15]. These results indicate the effectiveness of these substrates for propionic acid production. Additionally, *P. acidipropionici* DSMZ 4900, when cultivated in a medium containing glycerol and other nutrients, produced 8.72 g/L of propionic acid with a productivity of 0.17 g/L.h[16]. Higher values were achieved in the present study, likely due to co-fermentation with xylose and the use of corn steep liquor as a nitrogen source.

The fermentative production of propionic acid from sweet sorghum bagasse hydrolysate (SSB), a source of fermentable sugars (primarily glucose, but also xylose and arabinose), was compared to production from glucose. The SSB hydrolysate yielded 9.9 g/L of propionate with a yield and productivity of 0.51 g/g and 0.080 g/L.h, respectively, surpassing the performance of glucose, which yielded 8.5 g/L of propionate with a yield and productivity of 0.44 g/g and 0.070 g/L.h, respectively. Furthermore, both the cited study and our present work identified the production of acetic acid, with similar concentrations observed in both instances (3.35 g/L with SSB and 3.41 g/L with glucose)[6].

In contrast to fermentation in bioreactors there was no automatic pH control for PA bioproduction in shake flasks (Table 1). Thus, the fermentation processes were inhibited by the formation of by-product which is important for the transport of nutrients in and out of cells. Inhibition by propionic acid is caused by the disruption of the pH gradient, which is important for nutrient transport into and out of cells. The cytoplasmic membrane prevents ionizing compounds from entering bacterial cells[12]. Therefore, only the undissociated propionic acid penetrates the cell interior, releasing a proton H^+. As a result, the pH gradient across the cell membrane is disrupted. An extra ATP molecule is consumed to re-establish it, decreasing the number of free ATP molecules available for cellular metabolism, and compromising nutrient metabolism and transport[13].

4 CONCLUSION

Acidipropionibacterium acidipropionici DSMZ 4900 was identified as the strain with the highest propionic acid fermentation activity. It demonstrated the ability to consume xylose, glucose, and glycerol, either as sole substrates or in combination, showcasing significant metabolic versatility. An effective fermentation process was developed using a xylose molar ratio of 4:1, with complete consumption of both substrates. This indicates that both C5 carbon sources from lignocellulosic biomass and glycerol, a biodiesel byproduct, can be used to produce propionic acid. Utilizing these residues aligns with the biorefinery concept, potentially reducing costs and enhancing the environmental sustainability of propionic acid production.

REFERENCES

1 CALVO-FLORES, F. G., MARTIN-MARTINEZ, F. J. 2022. Front. Chem. 10. 973417.
2 AMMAR, E. M., PHILIPPIDIS, G.P. Appl. Microbiol. Biotechnol. 2021. 105 (16-17). 6199-6213.
3 JONES, R. W., KRULL, L. H., BLESSIN, C. W., INGLETT, G. E. 1979. Cereal Chem. 56. 441–442.
4 CARRONDO, M. J., CRESPO, J. P., MOURA, M. 1988. Appl. Biochem. Biotechnol. 17. 295–312.
5 WEI, P., LIN, M., WANG, Z., FU, H., YANG, H., JIANG, W., YANG, S.-T. 2016. Bioresour. Technol. 219. 91–97.
6 AMMAR, E.M., MARTIN, J., BRABO-CATALA, L., PHILIPPIDIS, G.P. 2020. Appl. Microbiol. Biotechnol. 104 (22). 9619–9629.
7 LIU, Y., ZHANG, Y.-G., ZHANG, R.-B., ZHANG, F., ZHU, J. 2011. Curr. Microbiol. 62 (1). 152–158.
8 WANG, Z., YANG, S.-T. 2013. Bioresour. Technol. 137. 116–123.
9 AHMADI, N., KHOSRAVI-DARANI, K., MORTAZAVIAN, A. M. 2017. Elect. Jour. of Biotec. 28. 67-75.
10 GONZALEZ-GARCIA, R. A., MCCUBBIN, T., NAVONE, L., STOWERS, C., NIELSEN, L. K., MARCELLIN, E. 2017. Fermentation. 3. 1–20.
11 KOŚMIDER, A., DROŻDŻYŃSKA, A., BLASZKA, K., LEJA, K., CZACZYK, K. 2010. Pol. Jour. of Envir. Stud. 19. 1249–1253.
12 RANAEI, V., PILEVAR, Z., KHANEGHAH, A. M., HOSSEINI, H. 2020. Food Technol. Biotechnol. 58 (2). 115-127.
13 TELES, J. C., STOLLE, E. M., KOLODA, S. A., BARANA, A. C. 2019. Braz. Arch. Biol. Technol. 62.
14 FENG, X., CHEN, F., XU, H., WU, B., LI, H., LI, S. Bioresour. Technol. 2011. 102 (10). 6141-6.
15 LIU, Z., MA, C., GAO, C., XU, P. 2012. Bior. Tech. 114. 711-714.
16 CAVERO-OLGUIN, V. H., RAHIMPOUR, F., DISHISHA, T., ALVAREZ-ALIAGA, M. T., HATTI-KAUL, R. 2021. Proc. Bioch. 110. 223-230.

ACKNOWLEDGEMENTS

Thanks to Sinochem Holdings Corporation for financial support.