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RESEARCH PAPER

Optimized 1,3-propanediol production from crude glycerol using mixed cultures in batch and continuous reactors

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Abstract
The production of 1,3-propanediol from crude glycerol and mixed anaerobic sludge was investigated in batch experiments and continuous reactors. Using a 2^3 complete factorial design, the effects of the concentration of glycerol (22–30 g L^{-1}), KH_2PO_4 (1.50–2.00 g L^{-1}), and vitamin B12 (7–8 mg L^{-1}) were examined in batch reactors. As an evaluated response, the highest 1,3-PD yields occurred for high concentrations of vitamin B12 and low levels of KH_2PO_4, reaching 0.57 g g^{-1} glycerol consumed. The variable glycerol concentration was not significant in the studied range. In addition, the condition that provided the best 1,3-PD yield was applied to an anaerobic fluidized bed reactor fed with crude glycerol (26.0 g L^{-1}), which was monitored as the hydraulic retention time (HRT) decreased from 36 to 12 h. The greatest 1,3-PD yield, of 0.31 g g^{-1} glycerol, was obtained with an HRT of 28 h.

Keywords 1,3-Propanediol · Design of experiments · AFBR · Vitamin B12 · Hydraulic retention time

Introduction

The demand for alternative energy sources is growing across the world. Biomass, which can be used to produce renewable biofuels, is one of the most promising sources. Brazil is somewhat of a pioneer in the use of biofuels as clean energy sources. The Brazilian government resolved to replace gasoline with fuel alcohol in 1973, through the National Alcohol Program ("Pro-Álcool"), and by the mid-80s, around 95% of the automobiles produced in Brazil had been modified for the combustion of ethanol. This paved the way for "Flex" vehicles. These flexible-fuel vehicles, which were introduced in 2003, now represent more than 80% of the cars sold in the country [1].

Another biomass-generated biofuel is biodiesel, which is produced through the transesterification of vegetable oils or animal fats. The reaction is carried out using alcohols such as ethanol or methanol and is usually catalyzed by NaOH or KOH. Glycerol is generated as a byproduct of this process, with a yield of approximately 1–1.4 kg kg^{-1} of biodiesel produced [2]. The global production of biodiesel has increased considerably in the past decades—from 500×10^8 in 2004 to 7.5×10^9 gallons $year^{-1}$ in 2013—and it is expected that it will continue to grow [2]. In Brazil, data from the National Agency for Petroleum, Natural Gas and Biofuels (ANP) indicate that the annual production of biodiesel in 2017 was 4.29×10^6 m^3, while that of glycerol was 3.74×10^5 m^3 [3].

This increased biodiesel production means that excess amounts of glycerol are generated. Furthermore, the biodiesel production process introduces impurities, such as salts, methanol, and fatty acids. Consequently, glycerol derived from this process has such a low market value that it has largely become a waste product. Moreover, its high chemical oxygen demand (COD) necessitates its adequate treatment or disposal [4]. New uses for glycerol are needed to stabilize the price and supply, and to avoid the accumulation of a material that may have an environmentally negative impact [5].

Fermentation of glycerol can produce highly valued and widely used products, including 1,3-propanediol (1,3-PD) [6–8], dihydroxyacetone [9], succinic acid [10], propionic acid [11, 12], ethanol [13, 14], citric acid [15], and hydrogen [16, 17]. The process involves two parallel metabolic routes: oxidative and reductive. In the oxidative route, glycerol is

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converted to dihydroxyacetone, and following phosphorylating, the product undergoes glycolysis to form acetate, butyrate, propionate, ethanol, H_2, and other metabolites. In the reductive route, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA), which goes on to generate 1,3-PD [1]. Most notably, 1,3-PD is used in cosmetics, lubricants, and medicines. Furthermore, it is a potentially important chemical intermediate in the manufacture of certain polymers (polyesters, polyethers, polyurethanes, etc.), and in the synthesis of heterocyclic compounds [18]. This product is highly specific to glycerol fermentation and cannot be obtained by any other anaerobic conversion [19].

Among the bacteria capable of converting glycerol to 1,3-PD are some species of *Lactobacillus, Pantoea agglomerans* (formerly *Enterobacter agglomerans*), *Citrobacter freundii, Klebsiella pneumoniae, Clostridium pasteurianum*, and *Clostridium butyricum* [8, 20–22]. Recent studies involving pure cultures in 1,3-PD production, such as da Silva et al. [8] and Tee et al. [20], reached 1,3-PD yields of 0.37 and 0.51, from 25 to 40 g L^{-1} glycerol, respectively. As an alternative to pure cultures, 1,3-PD can be produced using mixed anaerobic cultures [4, 5, 23, 24]. Comparing with pure cultures, microbial consortium is able to metabolize unpurified substrates, such as crude glycerol [25]. However, without the nutritional support necessary to the development of the 1,3-PD producing bacteria, the conversion of high glycerol concentrations becomes impaired, affecting the 1,3-PD yield [25]. Using inoculum from organic farm soil, Kanjilal et al. [26] studied the 1,3-PD production from pure and crude glycerol, and observed a decrease in yields by increasing the substrate concentration from 20 to 30 g L^{-1}. A microbial consortium DL38 from marine sludge was screened which displayed high tolerance to crude glycerol and high production of 1,3-PD. A yield of 0.52 g g^{-1} and a final concentration of 81.40 g L^{-1} were obtained in batch fermentation, according to Jiang et al. [27].

In continuous operation, microbial consortium shows a significant improvement in raw material utilization and robustness against environmental fluctuations, which has potential application in industrial production of biochemical [25]. Different configurations of bench-scale reactors have been investigated in the continuous production of 1,3-PD. Varrone et al. [28] reached yields of 0.52 and 0.46 g g^{-1} applying anaerobic sludge and activated sludge, respectively, in continuous stirred-tank reactor (CSTR). Gallardo et al. [24] used an expanded granular sludge blanket (EGSB) reactor inoculated with granular anaerobic sludge and reached 0.43 g g^{-1}, from 25 g L^{-1} of crude glycerol. Anaerobic fluidized bed reactors (AFBRs) retain large amounts of biomass through adherence to a support material. AFBRs have good stability in continuous operations with high and low hydraulic retention times (HRTs) [29], and represent a good alternative for the fermentation of glycerol using mixed anaerobic cultures. Several authors have demonstrated the potential of AFBR in the continuous fermentation of wastewater [30–33], including glycerol. Recently, Nazareth et al. [34] showed the use of an AFBR system in the continuous production of propionic acid from crude glycerol. Despite the advantages of AFBR, there are no studies in the literature known to the authors regarding the production of 1,3-PD from glycerol in this type of reactor configuration. Nevertheless, studying the behavior of the system and identifying the optimal range of operation have great importance in maximizing 1,3-PD yield.

In this work, a 2^3 complete factorial design was used to determine the individual and interactive effects of three components of the nutrient medium (glycerol, KH_2PO_4, and vitamin B12) on the 1,3-PD yield from mixed cultures. In addition, continuous 1,3-PD production was evaluated in an AFBR operated with 26.0 g L^{-1} of crude glycerol at HRTs between 36 and 12 h, in the best condition determined by the experimental design.

Materials and methods

Fermentation medium and inoculum

The batch experiments and AFBR operation were performed with a single carbon source, crude glycerol, provided by Bio-Brotas Oleoquímica (Brotas, São Paulo, Brazil). The residue consisted of approximately 84% glycerol, with impurities of salts (~13%), methanol (~1%), water (~2%), and fatty acids (<0.05%). The nutrient medium from Barbirato et al. [35] was adapted for culturing the microorganisms. It was constituted by: 3.4 g L^{-1} $K_2HPO_4{\cdot}3H_2O$; 2.0 g L^{-1} $(NH_4)_2SO_4$; 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$; 0.02 g L^{-1} $CaCl_2{\cdot}2H_2O$; 0.005 g L^{-1} $FeSO_4{\cdot}7H_2O$; 0.5 g L^{-1} yeast extract. In addition, it was supplemented with the trace element solution SL7 at a concentration of 2 mL L^{-1}, as described by Biebl and Pfennig [36]. KH_2PO_4 (1.5–2.0 g L^{-1}) and vitamin B12 (7.0–8.0 mg L^{-1}) were dosed according to the experimental design.

The inoculum was obtained from sludge from an upflow anaerobic sludge blanket (UASB) reactor used for the treatment of effluent from a poultry slaughterhouse (Avícola Dacar, Tietê, São Paulo, Brazil). To inhibit the development of methanogenic archaea, the sludge was subjected to a thermal pretreatment, in accordance with Kim et al. [37].

Experimental design of the batch reactors

A 2^3 complete factorial design was performed to verify the significance of the variables tested in the yield of 1,3-PD, produced from crude glycerol and mixed cultures. The ranges and levels of the independent variables—glycerol

Table 1 Experimental range and levels of the independent variables

Independent variables	Symbol code	Range and levels		
		−1	0	+1
Glycerol (g L^{-1})	X_1	22.00	26.00	30.00
KH_2PO_4 (g L^{-1})	X_2	1.50	1.75	2.00
Vitamin B12 (mg L^{-1})	X_3	7.00	7.50	8.00

(X_1), KH_2PO_4 (X_2), and vitamin B12 (X_3)—are shown in Table 1.

The response surface for the significant variables was generated according to the first-order polynomial equation, described by the following equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ij} x_i x_j, \quad (1)$$

where Y represents the predicted response, β_0 is a constant, β_i is the linear coefficient, β_{ij} is the coefficient of the interaction parameter, and x_i and x_j are the coded independent variables X_i and X_j ($i, j = 1, 2, 3$). The tested variables were coded according to the following equation:

$$x_i = \left(\frac{X_i - X_0}{\Delta X_i} \right), \quad (2)$$

where x_i is the coded value and X_i is the real value of the independent variable, X_0 is the real value of the center point, and ΔX_i is the value of the difference between the points.

The response surface and the analysis of the parameters [F test and analysis of variance (ANOVA)] were performed using Statistica 10.0 software (StatSoft Inc. 2010, USA). ANOVA was performed to test the significance of the factors (glycerol, KH_2PO_4, and vitamin B12) in the fit of the linear model. The parameters with p values less than 5% ($p < 0.05$) were considered significant for the maximization of 1,3-PD yield.

The experiments were conducted in batch reactors that consisted of 2-L Duran® flasks with 1 L of useful volume, into which 10% (100 mL) of inoculum was added. Crude glycerol was used as the sole source of carbon (22–30 g L^{-1}). The nutrient medium used was previously described, and KH_2PO_4 (1.50–2.00 g L^{-1}) and vitamin B12 (7–8 mg L^{-1}) were added as additional nutrients for the development of the 1,3-PD producing bacteria at the concentrations given in Table 1. N_2 was purged inside the flasks to ensure anaerobic conditions, and the pH was adjusted to 6.72 ± 0.02 with the aid of 30% HCl and/or 6 M NaOH. The flasks were kept in a temperature-controlled incubator at 37 °C under agitation at 200 rpm for 168 h.

Continuous anaerobic fluidized bed reactor (AFBR) operation conditions

The AFBR used in this study was very similar to that used by Nazareth et al. [34]. The reactor was built in transparent acrylic, and had an internal diameter of 3.6 cm, a height of 151.5 cm, and a total volume of 1542.1 cm^3. The temperature was maintained at 30 ± 1 °C with the aid of a thermostatic bath and reactor jacket. A support material was used to immobilize the biomass in the form of expanded clay particles (2.8–3.35 mm) with an apparent density of 1.06 g cm^{-3} and a porosity of 23% [38].

The operating conditions of the AFBR are given in Table 2. The concentration of glycerol applied to the reactor was based on the results obtained by the factorial design, as well as the adjustment of the nutritional medium in relation to the KH_2PO_4 and vitamin B12 concentration. To promote microbial adhesion to the support and microbial adaptation, the AFBR-containing expanded clay particles were inoculated in the batch mode by recycling the pretreated sludge (7%) with a fixed glycerol concentration (26.0 g L^{-1}) for 15 days. N_2 gas was sprayed into the fermentation medium to ensure anaerobic conditions and HCl 30% v/v was added to maintain the affluent pH adjust to 6.70 ± 0.20. For the reactor operation, the upward velocity was 1.3 times greater than the minimum fluidization velocity used. Thereafter, the reactor was transferred to the continuous mode of operation with the initial HRT of 36 h and fixed glycerol concentration of 26.0 g L^{-1}. The next phase was begun when the steady state was reached, based on a glycerol consumption efficiency and 1,3-PD production with a variation of less than 10% for 15 days. The reactor was operated for 53 days. The biogas composition and the soluble metabolites were monitored as a function of time. Only the results obtained in the steady state were reported.

Analytical methods

The chemical oxygen demand (COD), volatile suspense solids (VSS), and pH were analyzed in accordance with standard methods [39]. The volumetric production of biogas was measured according to the method proposed by Walker et al. [40]. The biogas content was determined

Table 2 Operating conditions of the AFBR

Phase	Glycerol (g L^{-1})	HRT (h)	OLR (kg m^3 day^{-1})
1	26.0	36	17.6
2		28	22.7
3		20	31.7
4		12	52.9

using a gas chromatograph (GC-2010, Shimadzu, Japan), described by Amorim et al. [38]. The glycerol concentration was determined according to the spectrophotometric method described by Bondioli and Della Bella [41].

1,3-PD and other aqueous products were measured by gas chromatography (GC-17A, Shimadzu, Japan), using the headspace method, with an automatic sampler injection sampler system. The chromatograph was equipped with a flame ionization detector (FID) and DB-WAX capillary column, 30 m × 0.25 mm × 0.25 µm, with a flow rate of 1.56 mL min^{-1}. The injector and detector temperatures were 250 °C and 280 °C, respectively. From an initial temperature of 35 °C, the temperature ramp used was: 2 °C min^{-1} until 42 °C; 20 °C min^{-1} until 75 °C; 35 °C min^{-1} until 120 °C; 10 °C min^{-1} until 170 °C. The temperature was held at 120 °C for 1 min and at 170 °C for 2 min. The flow rates of the carrier gas (H_2), auxiliary gas (N_2), and flame gas (synthetic air) were kept constant at 50, 35, and 500 mL min^{-1}, respectively.

Results and discussion

Screening of nutrient medium components in batch assays

A 2^3 complete factorial design was performed to verify the statistical significance of certain components of the nutrient medium, with the aim of maximizing the 1,3-PD yield. Table 3 shows that the 1,3-PD yield varied from 0.031 to 0.571 g g^{-1} glycerol consumed, reaching the maximum value with 30.0 g L^{-1} of glycerol, 1.50 g L^{-1} of KH_2PO_4, and 8.0 mg L^{-1} of B12. Results of the dependent variable were used to determine regression coefficients for 1,3-PD yield (Table 4), to calculate ANOVA (Table 5), and to construct three-dimensional response surface for significant results (Fig. 1).

According to Table 4, the variables KH_2PO_4 and vitamin B12 were considered significant for 1,3-PD yield ($p < 0.05$). Some studies show that high concentrations of glycerol favor 1,3-PD production [7, 42, 43]; however, as the current study

Table 3 Screening design using three independent variables

Essay	Glycerol (g L^{-1}) X_1	KH_2PO_4 (g L^{-1}) X_2	Vitamin B12 (mg L^{-1}) X_3	1,3-PD yield (g g glycerol consumed^{-1}) Experimental
2^3 factorial design				
1	22.0 (−1)	1.50 (−1)	7.0 (−1)	0.040
2	30.0 (+1)	1.50 (−1)	7.0 (−1)	0.031
3	22.0 (−1)	2.00 (+1)	7.0 (−1)	0.058
4	30.0 (+1)	2.00 (+1)	7.0 (−1)	0.037
5	22.0 (−1)	1.50 (−1)	8.0 (+1)	0.531
6	30.0 (+1)	1.50 (−1)	8.0 (+1)	0.571
7	22.0 (−1)	2.00 (+1)	8.0 (+1)	0.094
8	30.0 (+1)	2.00 (+1)	8.0 (+1)	0.031
Central points				
9	26.0 (0)	1.75 (0)	7.5 (0)	0.042
10	26.0 (0)	1.75 (0)	7.5 (0)	0.044

Table 4 Regression coefficients for 1,3-PD yield

Factors	Effects	Coefficient	Error	t (3)	p values (Prob > F)
Average	0.147913	0.147913	0.030790	4.80393	0.017170*
x_1 (L)	−0.013178	−0.006589	0.034424	−0.19140	0.860433
x_2 (L)	−0.238021	−0.119010	0.034424	−3.45717	0.040724*
x_3 (L)	0.265325	0.132662	0.034424	3.85375	0.030862*
x_1x_2	−0.028688	−0.014344	0.034424	−0.41668	0.704932
x_1x_3	0.001666	0.000833	0.034424	0.02419	0.982219
x_2x_3	−0.250416	−0.125208	0.034424	−3.63721	0.035812*

$R^2 = 0.9306$, R^2 adj = 0.7920, $p < 0.05$

*p values less than 0.05 indicates that model terms are significant

Table 5 Variance analysis of 1,3-PD yield

Source	Sum of squares	DF	Mean squares	*F* value	Probe *F*
Model	0.379518	3	0.126506	26.69	4.76
Residual	0.028441	6	0.004740		
Total	0.407958	9			

covered a narrow range of high glycerol concentrations (22–30 g L^{-1}), this variable did not show a significant effect on the response ($p = 0.86 > 0.05$).

Through multiple regression analysis, the first-order polynomial model was established to predict the yield of 1,3-PD. Non-significant terms (based on p value < 0.05) were neglected and the model Eq. (3) was reduced from the coded and significant variables:

$$Y = 0.1479 - 0.1190\,x_2 + 0.1327\,x_3 - 0.1252\,x_2x_3, \quad (3)$$

where Y represents the predicted value for 1,3-PD yield, and x_2 and x_3 are the coded values for the independent variables KH_2PO_4, and B12, respectively.

The statistical significance of Eq. (3) was confirmed using F tests and ANOVA for the model (Table 5). The calculated F value was much higher than the critical value, indicating the high statistical significance of the predicted model with regard to the 1,3-PD yield. The fit of the model was also expressed as the coefficient of determination R^2, where the closer the R^2 value is to 1, the better the model fits the experimental data [42]. The fit had an R^2 value of 0.9306, which indicated that 93.06% of the variability in the response be explained by the model. The adjusted coefficient of determination (R^2 adj = 0.7920) was satisfactory to confirm the significance of the model.

The three-dimensional response surface for the 1,3-PD yield, based on the linear model described by Eq. (3) and the significant variables, is shown in Fig. 1. Higher yields of 1,3-PD correlated with higher concentrations of vitamin B12 and lower levels of KH_2PO_4.

Kaur et al. [44] also evaluated the statistical significance of nutrients, glycerol, $(NH_4)_2SO_4$, K_2HPO_4, and KH_2PO_4 in maximizing the 1,3-PD production in batch reactors with *Clostridium diolis*. They verified that, in addition to glycerol, the phosphate sources (K_2HPO_4 and KH_2PO_4) were significant for the response, which shows the agreement to the results of the present study. In fact, phosphate sources are important for the maintenance of the buffer capacity of the fermentative medium, contributing to the growth and bioenergy of the bacterial cell [44]. In addition, these substances act as a source of phosphorus, which is essential in the synthesis of DNA, RNA, and phospholipids (plasma membrane constituents), and in the conversion of ADP to ATP, and vice versa, during oxidative phosphorylation [45, 46].

The effect of vitamin B12 on 1,3-PD production from glycerol has been verified previously using *Lactobacillus*

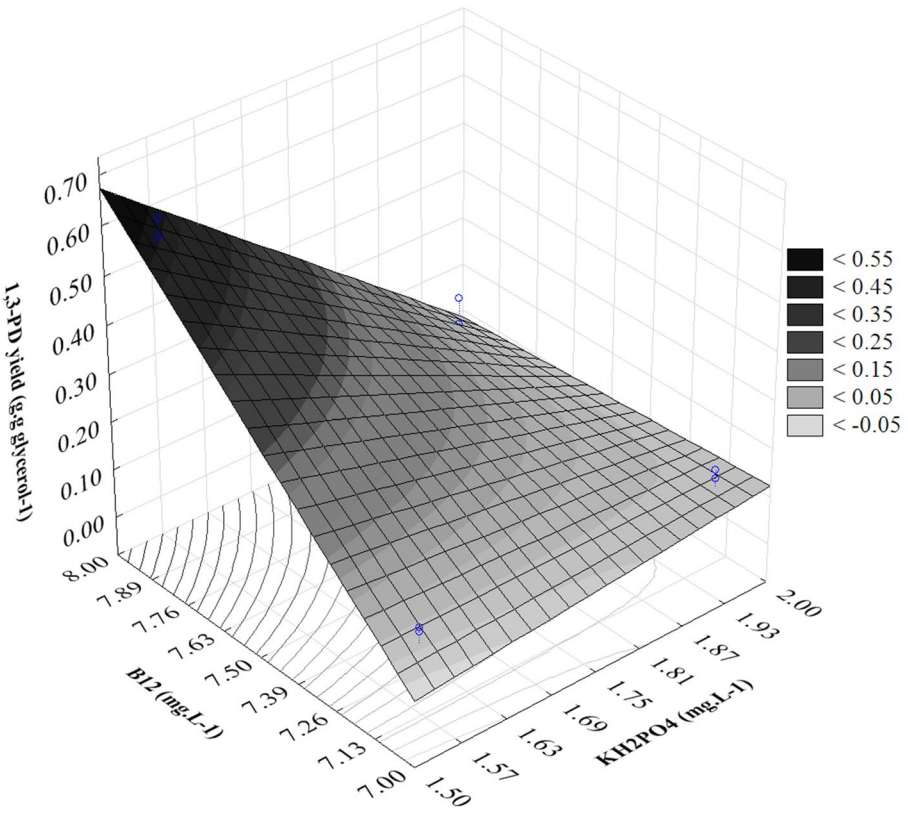

Fig. 1 Response surface for 1,3-PD yield as a function of KH_2PO_4 and B12

diolivorans [22] and *Halanaerobium saccharolyticum* subsp. *Saccharolyticum* [6]. In both studies, the addition of vitamin B12 to the fermentative medium containing glycerol significantly increased 1,3-PD production. Thus, despite the limited range in the present study, the fact that the current results are in agreement with these previous studies emphasizes the significance of vitamin B12 for maximizing the 1,3-PD yield from glycerol. According to Biebl et al. [18], most of the 1,3-PD producing microorganisms are vitamin B12-dependent, since the enzyme *glycerol dehydratase* that is included in the first stage of 1,3-PD production requires vitamin B12 as a coenzyme for active functioning.

1,3-Propanediol (1,3-PD) production in the AFBR

The factorial design allowed some of the concentrations of the components in the nutrient medium to be adjusted to maximize 1,3-PD production in an anaerobic reactor. Therefore, the AFBR was operated continuously, and the nutritional medium applied consisted of: 26.0 g L^{-1} crude glycerol; 1.50 g L^{-1} KH_2PO_4; 7.8 mg L^{-1} B12, in addition to the nutrients described in the "Fermentation medium and inoculum".

Figure 2 shows the variation of glycerol conversion, and the concentrations of glycerol in the affluent and glycerol effluent. The concentration of residual glycerol remained high throughout the reactor operation, which can be attributed to the high organic loading applied to the system. The highest glycerol conversion in the reactor was observed with an HRT of 36 h, and the lowest was observed with an HRT of 12 h. Gonen et al. [47] also observed reductions in glycerol consumption from 100 to 75% by decreasing the HRT from 8 to 2 h in an anaerobic packed-bed reactor (APBR), which was immobilized with *Clostridium beijerinckii* NRRL B-593 and fed with 45 g L^{-1} of glycerol.

Figure 3 shows the concentration of 1,3-PD produced in the AFBR reactor as a function of HRT, and Table 6 shows the 1,3-PD yields, substrate conversion efficiency, pH, and the byproduct concentrations formed in the different phases of operation. The AFBR produced similar concentrations of 1,3-PD with HRTs of 28 h and 20 h, reaching the maximum with a HRT of 20 h (1310.0 mg L^{-1}). However, a lower 1,3-PD production occurred with an HRT of 12 h, as a consequence of the low conversion efficiency.

The decreased glycerol conversion and 1,3-PD production with an HRT of 12 h can be attributed to an inhibitory effect by the substrate, also leading to "feedback inhibition" by the product. According to Edwards [48], an excessive amount of substrate may unbalance the metabolism of the cell, causing overproduction of a product from one metabolic pathway, and blocking a related second pathway. On the other hand, when the concentration of this product rises, the tendency is that this metabolite acts as an allosteric inhibitor, decreasing the velocity of the pathway and its own production, causing the so-called feedback inhibition.

In this study, the high glycerol concentration applied to the reactor (26.0 g L^{-1}) disadvantaged the substrate consumption, which remained at around 20% in the first three phases of operation. In addition to this, the 1,3-PD production was high in all the phases of reactor operation (Table 6),

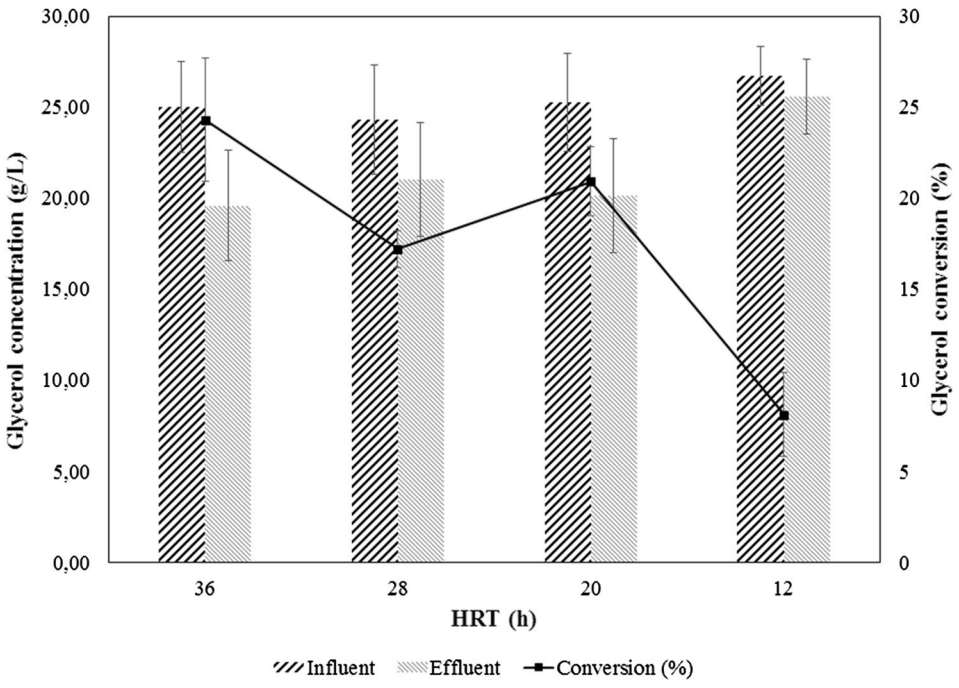

Fig. 2 Glycerol concentrations and conversion during continuous AFBR operation

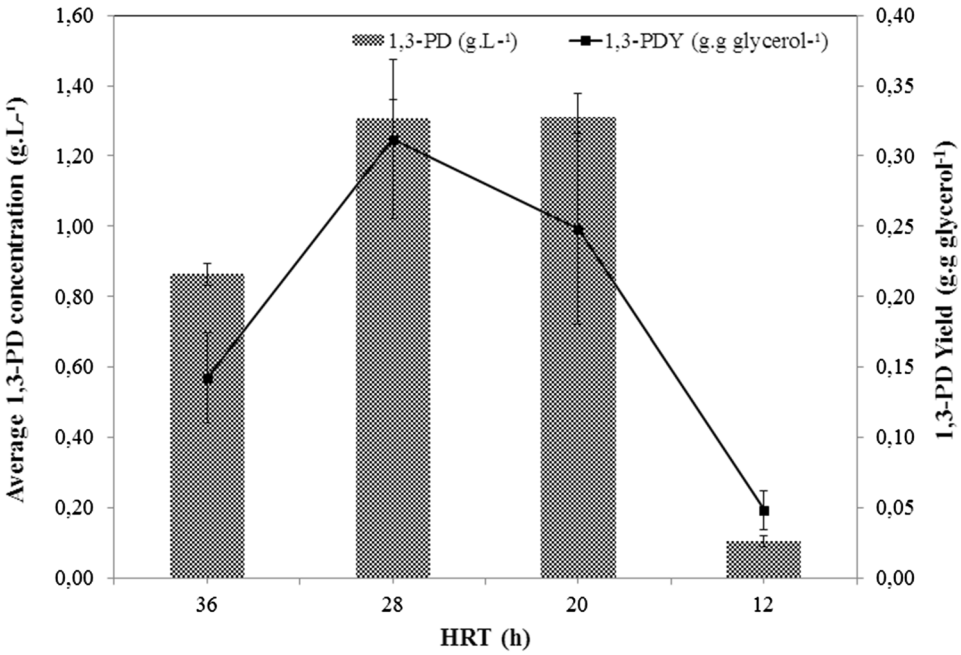

Fig. 3 Average 1,3-PD concentration and yield in the AFBR

Table 6 1,3-PD yield and byproducts at different HRTs

HRT (h)	1,3-PD yield (g g glycerol consumed^{-1})	Glycerol consumption (%)	1,3-PD (mg L^{-1})	EtOH (mg L^{-1})	HAc (mg L^{-1})	HPr (mg L^{-1})	pH
36	0.14 ± 0.03	24.3 ± 3.4	863.0	365.4	19.5	9.5	4.54
28	0.31 ± 0.06	17.2 ± 1.0	1305.0	13.5	58.4	34.5	4.02
20	0.25 ± 0.07	20.9 ± 1.9	1310.0	16.2	47.5	22.6	4.55
12	0.05 ± 0.01	8.1 ± 2.3	104.0	1.1	0.8	5.7	4.83

EtOH ethanol, *HAc* acetic acid, *HPr* propionic acid

and there were only small amounts of metabolites from the oxidative path, such as ethanol, acetic acid, and propionic acid.

The variation of the 1,3-PD yield during the operation of the AFBR is shown in Fig. 3 as a function of the HRT. The 1,3-PD yield in the reactor reached a maximum of 0.31 ± 0.06 g g^{-1} glycerol consumed with an HRT of 28 h, and the HRT of 12 h gave the lowest 1,3-PD yield obtained.

Cheng et al. [49] were pioneers in reporting 1,3-PD production, using *K. pneumoniae* on a pilot scale. In a 5 L fermenter operated in the batch-fed mode, the authors achieved a 1,3-PD yield of 0.36 g g^{-1} glycerol consumed from 50 g L^{-1} glycerol, by varying the incubation time from 12 to 48 h. Gonen et al. [50] reported a 1,3-PD yield of 0.49 g g^{-1} glycerol consumed with an HRT of 8 h, in an APBR, containing *K. pneumoniae* immobilized on polyurethane foam, which was fed with 45 g L^{-1} glycerol.

Gallardo et al. [24] evaluated different pretreatments of sludge in 1,3-PD production in an expanded granular sludge blanket (EGSB) reactor which was fed with 25 g L^{-1} of glycerol. The HRT was varied between 24 and 3 h. The authors determined the maximum 1,3-PD yield (0.43 g g^{-1} glycerol consumed) in with an HRT of 12 h for the reactor with untreated sludge. However, in the reactors inoculated with fragmented sludge and thermally pretreated sludge—similar to the present study—the authors obtained 0.38 and 0.39 g g glycerol consumed in, respectively, with an HRT of 6 h.

Table 6 shows the concentrations of the main byproducts produced. Butyric acid was produced in negligible concentrations. CH_4 was not detected in any phase of reactor operation, emphasizing the efficiency of the pretreatment used and indicating that the operating conditions were unfavorable for the development of methanogenic archaea. H_2 was also not detected in any phase of reactor operation, indicating the complete favoring of the reductive path of glycerol fermentation.

H_2 and 1,3-PD are produced by competing metabolic pathways in terms of reducing equivalents. The cells produce reduced metabolites, such as 1,3-PD, H_2, acetate,

Table 7 Different systems used for 1,3-PD production in recent studies

Reactor	Glycerol (g L^{-1})	Inoculum	1,3-PD (g L^{-1})	1,3-PD yield (g g $glycerol^{-1}$)	References
Batch systems					
Batch	Crude (25 g L^{-1})	*C. pasteurianum*	9.3	0.37	[53]
Batch	Pure (40 g L^{-1})	*K. pneumoniae*	20.4	0.51	[8]
Batch	Pure (40 g L^{-1})	*C. butyricum*	19.6	0.50	[20]
Batch	Pure (20/30 g L^{-1})	Microbial consortium from organic farm soil	–	0.52/0.08	[26]
	Crude (20/30 g L^{-1})		–	0.71/0.19	
Batch	Crude (200 g L^{-1})	Microbial consortium DL38 from marine sludge	81.4	0.52	[27]
Batch	Crude (30 g L^{-1})	Anaerobic sludge	16.4	0.57	This study
Continuous systems					
CSTR	Crude (34.9 g L^{-1})	Microbial consortium from organic farm soil	–	0.49	[26]
CSTR	Crude (10–13 g L^{-1})	Anaerobic sludge	4.89–6.45	0.52	[28]
		Activated sludge	2.70–4.40	0.46	
EGSB	Crude (25 g L^{-1})	Anaerobic granular sludge	10.7	0.43	[24]
UASB	Pure (25 g L^{-1})	Anaerobic granular sludge	4.63	0.17	[4]
	Crude (25 g L^{-1})		4.22	0.25	
AFBR	Crude (5 g L^{-1})	Anaerobic sludge	2.14	0.43	[34]
AFBR	Crude (26 g L^{-1})	Anaerobic sludge	1.31	0.31	This study

CSTR continuous stirred-tank reactor, *EGSB* expanded granular sludge blanket reactor, *UASB* upflow anaerobic sludge blanket reactor

butyrate, and ethanol, to maintain the balance of electrons. The reaction mechanism includes recycling of NADH and NAD+ to maintain redox balance [51]. For example, in *Clostridium* sp., the reducing equivalents generated in the conversion of pyruvate to acetyl-CoA are used to reduce ferredoxin, which is reoxidized through the H_2 production by the enzyme hydrogenase [24]. If little or no H_2 is formed, the reducing equivalents must be available for the production of other reduced compounds, such as 1,3-PD. Similarly, in the absence of 1,3-PD, the electrons are transferred to the formation of compounds such as H_2, ethanol, acetate, and butyrate provided from oxidative pathway [6, 24, 51].

The theoretical maximum 1,3-PD yield in co-production with acetate is 0.70 mol mol^{-1} glycerol (0.59 g g^{-1} glycerol) when no H_2 is produced. Practical maximum yields of 0.56 and 0.57 g g^{-1} glycerol have been previously reported by Saint-Amans et al. [52] using a pure culture of *C. butyricum*, and by Selembo et al. [5] from mixed wheat soil cultures, respectively. In the current work, the maximum yield obtained was 0.57 g g^{-1} glycerol in the batch process, and 0.31 g g^{-1} glycerol during continuous fermentation in the AFBR reactor. The yields obtained in this study are comparable with others reported recently in the literature (Table 7), although the 1,3-PD yield in the continuous system is considered low. This discrepancy may be a consequence of low substrate conversion during all the phases of the reactor.

The significant favoring of the reductive pathway of glycerol fermentation can be attributed to the addition of vitamin B12 in the fermentation medium, since 1,3-PD was formed in most phases of the reactor operation, and the products from the oxidative pathway—H_2, acetate, ethanol—were produced in reduced concentrations. This corroborates the work by Huang et al. [54], in which it was found that adding vitamin B12 to a fermentative medium containing pure glycerol as the sole carbon source increased the 1,3-PD production with *K. pneumoniae*. In a similar way, Kivisto et al. [6] showed that the addition of vitamin B12 enhanced the useful reactions of glycerol, and decreased the yields of H_2, CO_2, and acetate, via *H. saccharolyticum* subsp. *Saccharolyticum*, in addition to inducing the production of 1,3-PD from glycerol. Recently, Vivek et al. [55] observed that the addition of Co^{+2} and vitamin B12 in the fermentation medium maximized the production of 1,3-PD by *Lactobacillus brevis* N1E9.3.3.

Conclusions

A complete factorial design showed that the variables KH_2PO_4 and vitamin B12 exerted a significant influence on 1,3-PD yield from crude glycerol using mixed cultures. The maximum 1,3-PD yield (0.57 g g^{-1} glycerol consumed) was obtained with 30 g L^{-1} glycerol, 1.50 g L^{-1} KH_2PO_4, and 8 mg L^{-1} B12 in batch tests. Based on factorial design, the AFBR was operated continuously with 26.0 g L^{-1} crude glycerol; 1.50 g L^{-1} KH_2PO_4; 7.8 mg L^{-1} B12. The continuous reactor showed a low capacity to consume the substrate concentration applied to the system. Nevertheless,

satisfactory 1,3-PD yields were obtained as a result of the favoring of this reductive pathway by mixed cultures supplemented with vitamin B12.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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