Production of Value-Added Bioproducts Using a Modified Continuous Biofilm Reactor by *Citrobacter Freundii* DSM 15979

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ABSTRACT

The present paper reports the ability of Citrobacter freundii, strain DSM 15979, to produce value-added chemicals from biodiesel derived glycerol in a mesophilic modified continuous biofilm reactor operating under mesophilic continuous conditions at a specified hydraulic retention time (HRT). Increasing initial glycerol concentrations (G_{in}) (10 to 144 g/L) were tested in order to evaluate their effects on simultaneous production of value-added products with immobilized whole cells. Initial glycerol concentration was found to be a significant independent variable for the productions of 1,3-PDO, 2,3-BD, ethanol, acetic, succinic and lactic acids under different organic loading rates (OLR). The major end-products in the metabolic pathway were found to be 1,3-PDO and 2,3-BD. They reached maximum values of 26.1 g/L and 18.8 g/L at 92 g/L of initial crude glycerol feeding, which represents an OLR of 11.5 g/L.h. These results suggest that the formation of biofilms were beneficial for the utilization of high substrate concentrations, thus, the employment of immobilized cells of *C. freundii* DSM 15979 in a modified continuous biofilms reactor is favorable for mixed glycerol fermentations.

Keywords: Glycerol fermentation, Biofilm, *Citrobacter freundii*

INTRODUCTION

Commodity chemicals are derived largely from raw materials based on fossil resources. The environmentally friendly biotechnological approaches potentially form same or functionally similar chemicals from sustainable materials. In addition, their effects on reduction in greenhouse gas emissions provide an additional advantage. Various industrial by-products and domestic waste materials can be used as raw materials to convert into value-added chemicals by means of fermentative pathways. Numerous studies reported the technical viability of valorization of biodiesel driven glycerol into 1,3-PDO, 2,3-BD, ethanol, butanol, H2, acetic acid, lactic acid and other organic acids (Fig. 1). The versatility of the final by-product of this conversion is due to the utilization of different metabolic pathways by numerous species including *Klebsiella pneumoniae* [1], *Citrobacter freundii* [2–6], *Pantoea agglomerans* [7], *Entrobacter aerogenes* [8], *Lactobacillus brevis* [9], *Lactobacillus diolivorans* [10], *Lactobacillus reuteri* [11], *Clostridium butyricum* [12], *Clostridium perfringens* [13], *Clostridium acetobutylicum* [14], *Clostridium paste-urianum* [15], and *Clostridium beijerinckii* [16]. The major end-products from the glycerol fermentation can be integrated to a wide variety of applications such as 1,3-PDO in the manufacturing of polyesters, polyethers, polyurethanes, polytrimethylene-terephthalate, textiles, carpets and plastics [17]; 2,3-BD in the manufacturing of printing inks, jet fuels, explosives, perfumes, foods, pharmaceuticals, and plasticizers [18], and acetic acid mainly as a solvent [19]. The beneficial properties of these chemicals characterize them as special commodity chemicals attributing important roles in industrial processes.

Glycerol can be metabolized by two main reactions including one reductive branch in which at first it is dehydrated to 3-hydroxypropionaldehyde and water, it is then reduced to 1,3-PDO under the utilization of the reducing power nicotinamide adenine dinucleotide (NADH) and an oxidative branch where the required reducing power is formed resulting in the formation of metabolites demonstrated in Fig. 1 [2, 20].
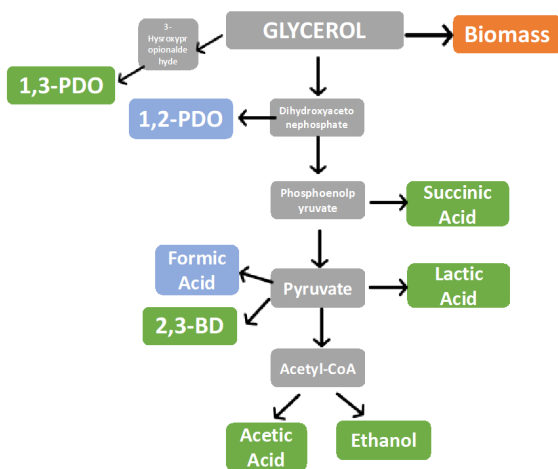
Figure 1. Main metabolites produced in the glycerol degradation pathways of Enterobacteriaceae, adapted from [2].

Several strategies have been employed for glycerol conversion with high yields of bioproducts [20] such as optimization of the fermentation medium [21] and operational parameters [22], employing different reactor configurations, applying fed-batch productions [23] to prevent the inhibitions of high initial substrate concentrations and end-products, and metabolic engineering [24]. Apart from these, one of the main approaches include increasing the final product concentrations with the help of high cell densities [25]. In this regard, immobilization is the key mode of action to enhance productivities. During this process microorganisms form biofilms without the involvement of chemicals and thick layers of cells are generated by either on attaching to materials or naturally on surfaces or they are entrapped [26]. In a study by Paranhos and Silva [27] a continuous anaerobic fluidized bed reactor (AFBR) was used to optimize the productions of H2, 1,3-PDO and propionic acid from crude glycerol with the formation of biofilms of mixed cultures indicating the effectiveness of immobilization on productivities [27]. Another report on the production similarly by the utilization of an anaerobic sludge also reported the ability of the microorganisms to stabilize under elevating OLR (25 to 62.5 g/L.d) with the help of granulation of cells [28].

As reported previously, several strains and isolates of *C. freundii* have been studied in the literature with different parameters. Whereas, the fluidized bed biofilm reactor was used for the first time in the literature with increasing crude glycerol concentrations in a chemostat providing the novelty of the work. The aim of the study was to investigate the effectiveness of the utilization of a modified fluidized bed biofilm reactor (MCBR) to produce value-added biochemicals from crude glycerol by *C. freundii* DSM 15979. Accordingly, the effects of glycerol concentration feed on the crude glycerol driven productions of 1,3-PDO, 2,3-BD, lactic and acetic acids and the influence of the OLR in the MCBR by *C. freundii* DSM 15979 was comparatively studied for the first time in the literature. The results of the present study

reports the advantages of the formation of biofilms to enhance the yields of final products at increasing OLRs.

MATERIAL AND METHODS

Experimental Set-up and Biofilm Reactor Conditions

A Pyrex® glass bioreactor (Corning Inc., USA) was employed for the fluidized bed biofilm reactor studies. The bioreactor had the following specifications: 15 cm height with an i.d. of 10 cm and 8 outlets for feed and gas connections. The total volume and working volume of the of the bioreactor were 1000 mL and 320 mL, respectively. Ceramic supports were added to the bioreactor as the immobilization material. The ceramic supports were decontaminated by distilled water followed by an overnight drying procedure at 105°C, prior to application.

The temperature in the bioreactor was kept at 30°C using a hot plate magnetic stirrer (MR Hei-Standard, Heidolph, Germany) and monitored by an external thermal probe (EKT Hei-Con, Heidolph, Germany). One peristaltic pump for inlet feeding and one peristaltic pump for effluent collection were employed during production (WM 400, Watson Marlow Fluid Technology Group, USA). The pump was calibrated using silicon tubing and distilled water using a mass based method. 10 different levels and 2 different directions in the pump were measured twice (60 to 120 seconds) with distilled water. Accordingly, mass of each level was recorded to calculate the calibration coefficient of the pump in regards to the set level. pH was monitored at each sampling point, and accordingly, adjusted to 6.5 to 7.0 with the manual addition of NaOH (2M) when necessary during fermentation. Samples were taken from the effluent to be analyzed for residual glycerol, 2,3-BD, ethanol, 1,3-PDO, acetic, lactic and succinic acids.

Substrate Preparation and Application

The substrate used in this study was crude glycerol obtained from biodiesel production from a factory in Torbalı (Izmir, Turkey) in 10 L plastic containers. The composition of crude glycerol used was provided from the company as 54.35% glycerol (w/v), 34.81% water, 6.52% fat and ester, < 1%methanol (ppm), 3.53% NaOH, and 0.64% NaCl.

Glycerol determination in biodiesel waste was done by High Pressure Liquid Chromatography (HPLC). It was found to be 50% (w/v), the rest of the content were soap, trace amount of mixed salts and caustic. The initial content of glycerol in biodiesel waste was 80%. This amount was diluted using distilled water for a final concentration of 50% (500 g/L) in order to keep the waste product liquified. The above mentioned solution was diluted to required concentration during fermentation. The fermentation medium was prepa-

red as 5.72 g K_2HPO_4, 1.5 g KH_2PO_4, 2.0 g $(NH_4)_2SO_4$, 1.0 g yeast extract, 0.24 g $MgSO_4 \cdot 7H_2O$, 0.5 mL $FeSO_4 \cdot 7H_2O$ solution and 1.0 mL trace element solution. The trace element solution consisted of (mg/L distilled water) 200 $CoCl_2 \cdot 2H_2O$, 100 $MnCl_2 \cdot 4H_2O$, 70 $ZnCl_2$, 60 H_3BO_3, 20 $CuCl_2 \cdot 2H_2O$, 35 $Na_2MoO_4 \cdot 2H_2O$, 25 $NiCl_2 \cdot 6H_2O$, and 0.9 ml HCl (37%).

The MCBR was fed with crude glycerol at the concentration determined previously by [7] for a concentration of between 11 to 144 g/L. The substrate was fed in incrementing concentrations as follows, initial glycerol concentrations of (G_{in}); 11, 23, 48, 72, 92, 109, 112, 144 g/L of crude glycerol corresponding to organic loading rates (OLR) of 1.4, 2.9, 6.0, 9.0, 11.5, 13.6, 14.0 and 18.0 g/L.h.

Inoculum Preparation and Biofilm Formation

The inoculum used in this study was Citrobacter *freundii*, strain DSM 15979. The microorganism was provided by Alma Mater Studiorum University in Bologna from the German Collection of Microorganisms and Cell Cultures (DSMZ). The microorganisms were sub-cultured from agar cultures in Tryptic Soy Broth (TSB) that was used as growth medium, to be activated. They were then incubated at 30°C during at least 10 h.

Prior to incubation and fermentation, the bioreactor filled with immobilization materials was autoclaved (121°C at 1 atm for 30 min) for sterilization. The reactor was allowed to cool down and then inoculated with a stock culture of C. *freundii* DSM 15979 (10% (v/v)) together with sterile fermentation medium. The bioreactor was washed with N_2 to maintain anaerobic conditions. The bacteria was inoculated to the immobilized bioreactor under sterile conditions for 3 days with a dilution rate of 0.04 h^{-1} using a peristaltic pump (Watson Marlow 400). The continuous fermentation was initialized at the 72th h of the fermentation following a visible formation of the biofilm on the immobilization material as shown in Fig. 2. Glycerol concentration, thus OLR, in the fermentation medium was changed after at least 6 cycles (=~every 2 days) maintaining a hydraulic retention time (HRT) of 8 h (Dilution rate: 0.125 1/h). The reported results belong to the final three obtained values for each OLR, thus, the analysis were performed in triplicates and the measurements were reported as mean values with standard deviations.

Sampling and Analysis

10 mL of samples were collected from the bioreactor at each sampling point and 1.5 mL of the sample was centrifuged at 7379 x g for 15 min to be analyzed using a microcentrifuge (Beckman Coulter™ Microfuge® 18 Centrifuge with 24 x 0.22 L angle rotor). The supernatant was filtered through 0.22 μm syringe filters. Glycerol as the substrate, and 1,3-PDO, 2,3-BD, lactic acid, acetic acid, succinic acid and ethanol as the products were measured using HPLC (Agilent, USA) with a Phenomenex Rezex RHM Monosaccharide (H+) 300 x 7.8 mm ion exchange column, using a Thermo Surveyor Refractive Index Detector. The column temperature was 65°C and the detector temperature was 40°C and the injection volume is 20 μL. A solution of 5 mM H_2SO_4 was used as mobile phase at a flow rate of 0.6 mL/min [29].

Phosphate buffer solution (pH 7.1) was used to rinse the immobilization materials at the end of fermentation in order to remove the attached microorganisms from the surface of the materials. An additional 10 min sonication was employed in order to further remove remaining biomass on the surfaces followed by a final washing with the phosphate buffer solution was applied to calculate the immobilized to suspended cells percentile. The gravimetric method was used for total suspended solids (TSS) measurements [30]. Filter papers with a diameter of 0.45 μm were dried in an oven for 24 h at 105°C. Following, they were placed into a desiccator to cool to room temperature and filter papers were weighed to record their tares that were used for calculation. After that, the sample that was nearly 5 mL were filtered by the filter paper in the Erlenmeyer flask. The filtered paper was placed into an oven for 24 h at 105°C. Then, they were transferred to the desiccator and left to cool down for 30 min and weighed. All results of weighing were calculated

0th h
Bioreactor setup

48th h
Biofilm formation

72th h
Fermentation initiated
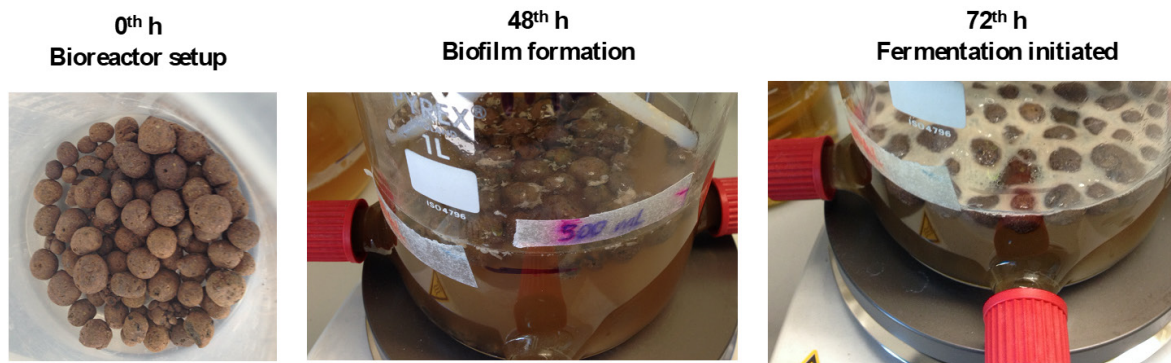
Figure 2. The stages of the ceramic immobilization material at the bioreactor setup (0th h), during biofilm formation (48th h) and at the initiation of fermentation (72th h).
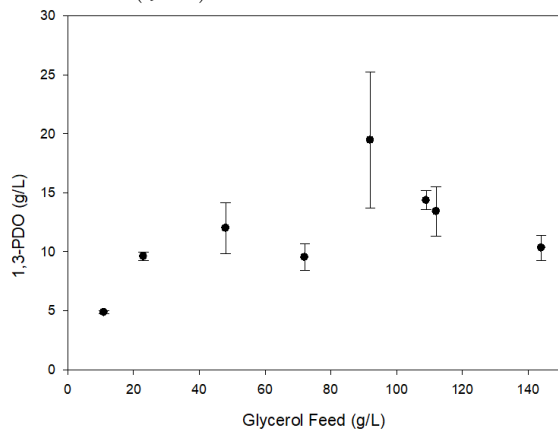
according to the Equation (1) [30]:

$$T_{SS}\ (mg/L) = \begin{pmatrix} Final\ weigh\ of\ filter\ paper\ (g) \\ -Tare\ of\ filter\ paper(g) \end{pmatrix} \quad (1)$$

$x1000000 / Volume\ of\ sample$

Calculations

Equation 2 was used to calculate the yields for the end product concentrations, and Equation 3 was used to calculate the productivities of the end products [31].

$$Y = C_x / S_i \quad (2)$$

$$Q = C_x / (t_f - t_i) \quad (3)$$

Where;

Y: the end product yield ($mol_{end\ product}/mol_{consumedglycerol}$); C_x: the compound concentration (g/L); S_i: the initial substrate concentration (g/L); Q: the productivity (g/L.h); t_f: the final fermentation time (h); t_i: the hydraulic retention time (h).

Statistical analysis

All data obtained from the MCBR were compared with a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks (Sigma Plot, USA). The results were evaluated with a 95% confidence interval.
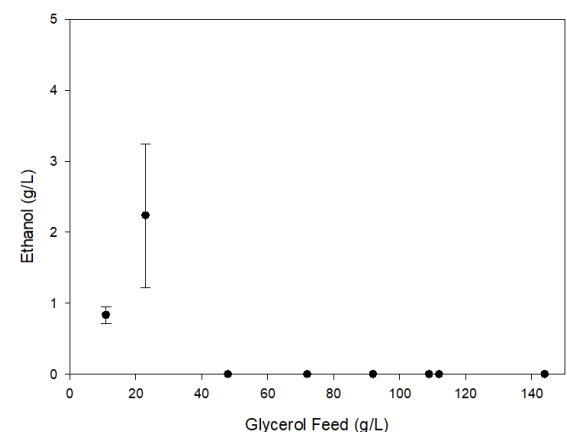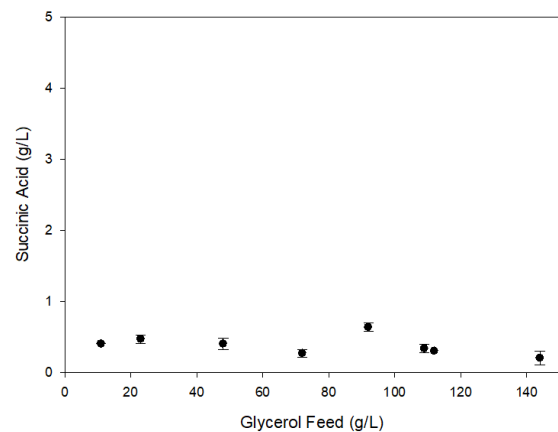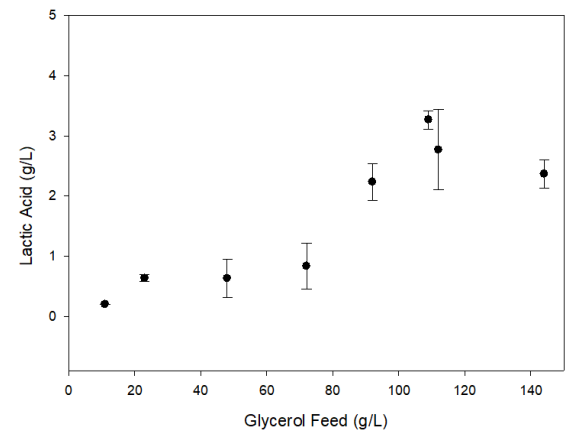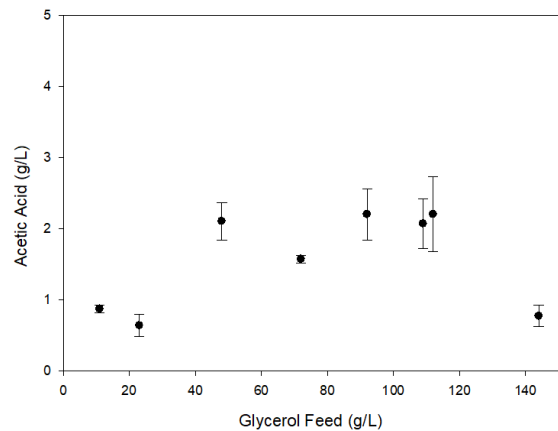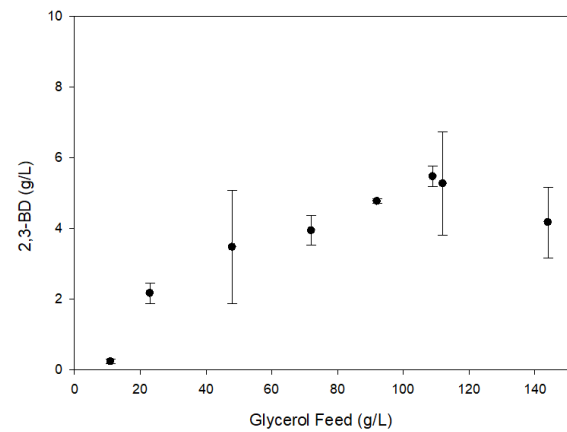

Figure 3. By-product concentrations at elevating glycerol feed (11 to 144 g/L).

RESULTS AND DISCUSSION

The biofilm reactor employed in the current study resulted in several by-products including organic acids, 1,3-PDO, and 2,3-BD. As depicted in Fig. 3, the final product with the highest concentration was 1,3-PDO reaching a maximum value of 26.1 g/L at an OLR of 11.5 g/L.h. 2,3-BD reached the second highest concentration followed by lactic acid, ethanol, acetic acid, and succinic acid; the highest concentrations were reported as 18.8, 3.5, 3.4, 3.0, and 0.8 g/L, respectively. Throughout the study the highest ethanol yield was reported to reach 0.2 mol/mol. However, when the glycerol feed was increased over 48 g/L, no ethanol production was observed. Similarly, according to Vivek et al. (2017), when the initial glycerol concentration was increased up to 60 g/L, no ethanol was reported [9].

In the present study, increased OLR negatively affected glycerol utilization (Table 1). The consumption rate was inversely proportional to the increasing OLR values except for the slight remission at 6.0 g/L.h. This situation can be explained by the acclimatation of the microorganisms following 6 cycles of the same condition, due to a sharp increase in the substrate concentration by 108%. Previous reports in the literature stated the inhibitory effects of high concentrations of the substrate on wild type microorganisms [20]. In order to overcome this issue and enhance the titers of 1,3-PDO and other metabolites, strategies such as acclimation of the microorganisms with the formation of biofilms [32], immobilization on inert materials , entrapment of cells in gels [33] or fed-batch mode of processing have been employed [5, 34, 35]. Similarly to the reported literature, at least 6 cycles of OLR was also successfully applied to the biofilm of C. *freundii* DSM 15979 cells in the present study to enhance the productivities (Table 1). The represented calculations were average of triplicate data during the experiments. ANOVA on ranks (Sigma Plot, USA) showed that there was a statistically significant difference in molar yields of the products based
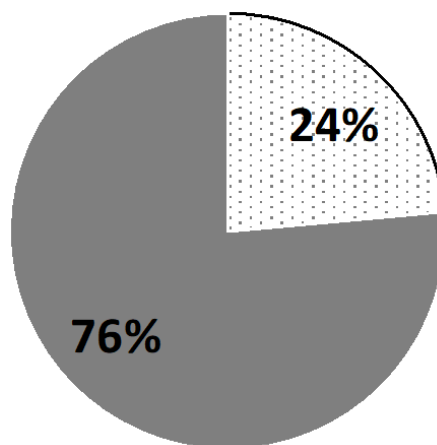
Figure 4. Immobilized (solid colored) vs suspended (dot illustrated) cell ratios as calculated at the end of the study.

on the elevating initial glycerol concentrations ($p < 0.05$). Aquino de Souza et al. (2014) reported that the entrapped cells of K. *pneumoniae* BLh-1 reached the maximum productions of 1,3-PDO, 2,3-BD and ethanol by 22.22 g/L, 9.98 g/L and 1.42 g/L, respectively when 65 g/L of crude glycerol was fed to the system. In the present study, C. *freundii* DSM 15979 reported the maximum 1,3-PDO production when 92 g/L of crude glycerol was fed to the biofilm reactor together with the highest 2,3-BD titer (18.8 g/L), however, glycerol concentrations higher than 23 g/L resulted in no production of ethanol (Fig. 3). As shown in Fig. 4, the results of the TSS measurements also proved that 76% of the microorganisms were successfully immobilized on the ceramic immobilization material in the form of a biofilm.

A previous study by the research group reported the production of various metabolites from crude glycerol by suspended systems [7]. Accordingly, glycerol consumption by C. *freundii* DSM 15979 was reported to be sharply declined (as low as 4% consumption at Gin 140 g/L) with high glycerol loading as opposed to immobilized cultures reported in the present study. These results indicate the improved resistance of whole cells with the formation of biofilms.

Table 1. MCBR performance (Y in $mol_{end\ product}/molc_{onsumedglycerol}$ and Q in g/L.h) with incrementing glycerol feed.

Monitoring Time (h)	Glycerol initial (g/L)	OLR (g/L.h)	Glycerol Removal (%)	Y1,3-PDO	Y2,3-BD	Y$_{lactic}$ acid	Y$_{aetic}$ acid	Y$_{succinic}$ acid	Y$_{ethanol}$	Q1,3-PDO	pH
72	11	1.4	100	0.54±0.01	0.02±0.01	0.02±0.00	0.12±0.01	0.03±0.00	0.15±0.02	0.60±0.00	6.28±0.35
120	23	2.9	99	0.51±0.02	0.10±0.01	0.03±0.00	0.04±0.01	0.02±0.00	0.20±0.09	1.17±0.06	6.43±0.43
168	48	6.0	51	0.78±0.27	0.23±0.23	0.03±0.02	0.17±0.09	0.02±0.01	0	1.27±0.55	6.37±0.15
216	72	9.0	69	0.18±0.02	0.16±0.05	0.0±0.06	0.0±0.08	0.0±0.01	0	1.17±0.12	6.54±0.07
264	92	11.5	70	0.56±0.28	0.31±0.10	0.14±0.05	0.22±0.10	0.03±0.01	0	2.47±0.72	6.01±0.17
288	109	13.6	64	0.25±0.02	0.08±0.01	0.05±0.22	0.05±0.01	0	0	1.80±0.10	6.18±0.16
384	112	14.0	42	0.42±0.22	0.14±0.08	0.07±0.04	0.10±0.07	0.01±0.00	0	1.70±0.26	6.56±0.47
432	144	18.0	20	0.44±0.09	0.15±0.02	0.09±0.02	0.04±0.01	0.01±0.00	0	1.30±0.10	6.27±0.16

Comparison of 1,3-PDO, 2,3-BD and organic acid production

The 1,3-PDO and 2,3-BD molar yields achieved in this study are the maximum ones obtained via crude glycerol fermentation compared to the other reported studies by wild type C. *freundii* strains that formed biofilms (Table 2). The literature reports depicted the feasibility of the valorization of crude glycerol by various strains reaching up to 170 g/L of feed [3, 5].

As shown in Table 1, as the initial substrate concentration was increased, the consumption of glycerol was declined also causing a decrease in the productions of end-products suggesting an exertion of inhibition. A similar case was reported by Metsoviti et al. [5]. The group employed a fed-batch strategy in order to prevent this issue. Accordingly, they successfully improved the yields reaching a maximum 1,3-PDO concentration of 68.1 g/L.

Previous studies has suggested that under conditions where the energy is sufficient, microorganisms prefer the production pathways of products that are less toxic and that energy needs do not direct metabolism [36]. Accordingly, as also reported in Fig. 1, amongst all intermediate and end-products of glycerol fermentation, lactic acid, ethanol, butanol and H2 are the most important competitors of 1,3-PDO due to the requirement of reduced NADH during their production. Lower concentrations of these products are desired to enhance the yields of the target end-product. In the present study, as reported in Table 1, succinic acid was not capable of competing with 1,3-PDO for reducing equivalents, due to its low yields. In addition, although lactic acid commonly achieved lower yields (<0.09 mol/mol), it reached a yield of 0.14 mol/mol at an initial glycerol concentration of 92 g/L. A study by Maina et al. [6] also reported that C. *freundii* VK-19 was capable of producing lactic acid as high as 21.7 g/L, yielding 0.17 mol/mol. Furthermore, similar to the present study, succinic acid productions were reported to be negligible (<0.04 mol/mol). In another study by mixed cultures of Clostridia and Enterobacteria, considerable amounts of lactic acid production were observed during glycerol fermentation [28]. These results in the literature were in agreement with our study where increasing OLR up to a specific concentration (62.5 g/L) increased the 1,3-PDO yields and then decreased the yields due to the possible undesirable presence of inhibitory substances in the raw substrate. These results suggest that lactic acid production should also be minimized to enhance overall yields of 1,3-PDO or 2,3-BD.

One of the main challenges of the utilization of crude glycerol is the choice of microorganisms due to the inhibitory components of the raw substrate [28]. The growth associated production of all products reported in Fig. 1 requires a primary metabolism. In this context, utilizing tolerant species or improving the cell yields are of great importance for higher concentrations of end-products. As shown in Table 2, the 1,3-PDO productivities were significantly improved with the employment of immobilized and continuous systems [9, 37]. Compared with the previously reported literature by C. *freundii*, the highest molar yield was achieved in the present study.

Table 2. Comparison of the operating conditions, 1,3-PDO yields and productivities in various studies using crude or pure glycerol.

Reactor Type*	Glycerol (g/L)**	Mode of action	Inoculated Microorganisms	1,3-PDO Yield (mol/mol)	1,3-PDO Productivity (g/L.h)	Reference
CSTR	25 PG	Batch	Klebsiella pneumonia CGMCC 1.6366	0.52	1.65	[38]
CSTR	20-80 CG	Fed-batch	C. butyricum VPI 1718	0.67	0.78	[39]
PBR	40 CG 40 Glu	Continuous	L. brevis N1E9.3-3	0.53	0.49	[9]
CSTR	40-170 CG	Fed-batch	C. freundii FMCC-B 294 (VK-19)	0.40	0.79	[5]
CSTR	15-50 PG	Batch	C. freundii AD119	0.72	0.96	[2]
PBR	36.8 PG	Continuous	C. freundii DSM 30040	0.57	8.2	[37]
Shaked cultures	50 CG 1.5 Glu	Batch	C. freundii	0.69	-	[40]
MCPR	11-144 CG	Continuous	C. freundii DSM 15979	0.78	2.47	This study

*CSTR: Continuous stirred tank reactor; PBR: Packed bed reactor; MCPR: Modified continous biofilm reactor

**PG: Pure Glycerol, CG: Crude Glycerol, Glu: Glucose

CONCLUSION

An adequate approach to enhance the valorization of glycerol into several value-added products was performed with the aid of a modified continuous biofilm reactor (MCBR). This study depicted a novel approach to obtain the multi end-products including 1,3-PDO, 2,3-BD and acetic acid out of crude glycerol in a continuous mode of operation. The glycerol removal was realized between 20 to 100%. 1,3-PDO was obtained in the highest yield of 0.78 mol/mol. The continuous MCBR showed good stability under elevating crude glycerol concentrations showing its feasibility to high OLR. The outcomes of this study may be used as a know-how to scale-up the process in non-sterile conditions for integrated systems to industrial scale biodiesel plants. Furthermore, the production of the valuable biomonomer 1,3-PDO, which has a very important place in the textile sector, can be a very profitable return to the manufacturer.

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