1,3-Propanediol production from crude glycerol by *Clostridium butyricum* DSP1 in repeated batch

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Daria Szymanowska-Powałowska

Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Wojska Polskiego 48, 60-527 Poznan, Poland

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A B S T R A C T

Background: The production of biofuels from renewable energy sources is one of the most important issues in industrial biotechnology today. The process is known to generate various by-products, for example crude glycerol, which is obtained in the making of biodiesel from rapeseed oil. Crude glycerol may be utilized in many ways, including microbial conversion to 1,3-propanediol (1,3-PD), a raw material for the synthesis of polyesters and polyurethanes.
Results: The paper presents results of a study on the synthesis of 1,3-propanediol from crude glycerol by a repeated batch method with the use of *Clostridium butyricum* DSP1. Three cycles of fermentation medium replacement were carried out. The final concentration of 1,3-PD was 62 g/L and the maximum productivity, obtained during the second cycle, reached 1.68 g/L/h. Additionally, experiments conducted in parallel to the above involved using the entire quantity of the culture broth removed from the bioreactor to inoculate successive portions of fermentation media containing crude glycerol at concentrations of 80 g/L and 100 g/L. Under those conditions, the maximum 1,3-PD concentrations were 43.2 g/L and 54.2 g/L.
Conclusions: The experiments proved that by using a portion of metabolically active biomass as inoculum for another fermentation formula it is possible to eliminate the stage of inoculum growth and thereby reduce the length of the whole operation. Additionally, that strategy avoids the phase of microbial adaptation to a different source of carbon such as crude glycerol, which is more difficult to utilize, thus improving the kinetic parameters of 1,3-PD production.

1. Introduction

1,3-Propanediol (1,3-PD), a chemical compound belonging to the group of diols, serves as a substrate in many types of synthesis, especially polycondensation. It is a key monomer in the synthesis of polytrimethylene terephthalate (PTT), a polyester used in the production of textiles and carpet fibers [1]. 1,3-PD is a component in the manufacture of laminates, UV-cured coatings and antifreezes [2]. It is an ingredient of solvents, resins, detergents and cosmetics as well as an important material in the synthesis of polyurethanes and polycarbonates. Products obtained by 1,3-PD polymerization are characterized by good biodegradability, better specificity and higher industrial safety in addition to being cheaper than those based on 1,2-propanediol, ethylene glycol or butanediol. The chemical methods of 1,3-PD formulation that have been used to date include the

hydration of acrolein and the hydroformylation of ethylene oxide, both of which require high pressures and temperatures, and cause the release of environmentally toxic intermediate compounds [3]. Market demand and the drawbacks of chemical synthesis stimulated research into alternative ways of producing 1,3-PD, including microbiological methods [4,5]. It is significant that a substrate for technologies utilizing microorganisms may be crude glycerol, a byproduct of biodiesel production. The biotechnological methods that have been designed to obtain 1,3-PD by converting glycerol use the bacteria of the genera *Clostridium*, *Klebsiella*, *Citrobacter*, *Hafnia, Lactobacillus* [6,7,8]. In the process of selecting a 1,3-PD producer, consideration must be given not only to such vital features as resistance to osmotic, toxic, and mechanical stresses or maintenance of growth capability under the conditions of industrial-scale synthesis but also to an appropriate cultivation method [9,10]. The choice of a culturing protocol is mainly determined by the cost of fermentation medium (substrate included) and by process specifications, e.g. temperature, as well as technological parameters like productivity, efficacy, and selectivity. An interesting option in the wide range of methods available is repeated batch cultivation, whereby portions of the culture broth containing microbial biomass are removed at specified intervals from the bioreactor and replaced with fresh fermentation
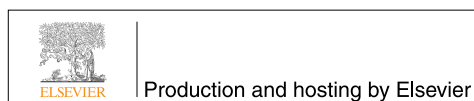
medium so that the volume of the bioreactor contents remains constant [11,12]. The aim of the study was to define the kinetic parameters of microbiological conversion of crude glycerol to 1,3-PD by a repeated batch method and to investigate the possibility of using the quantity of the culture broth containing microbial biomass that was removed from the bioreactor as inoculum for the next fermentation cycle, at a higher concentration of crude glycerol.

To the best of the author's knowledge, the present paper is the first report on the synthesis of 1,3-PD by *Clostridium butyricum* cultured in a repeated batch mode on a chemically defined medium containing crude glycerol. Repeated batch cultures were previously studied by Chatzifragkou et al. [12], the main difference being the use of rapeseed meal hydrolysate as a fermentation medium.

2. Materials and methods

2.1. Microorganism

In the process of converting crude glycerol to 1,3-PD the bacterial strain *C. butyricum* DSP1 was used. It was previously isolated from ruminal fluid and put in the collection of the Department of Biotechnology and Food Microbiology, Poznan University of Life Sciences, Poland, as well as deposited at the Polish Collection of Microorganisms (PCM) [13].

2.2. Culture medium

The strain was maintained in Reinforced Clostridial Medium (RCM, Oxoid, UK) in serum bottles, at 4°C. Pre-cultures of pure culture inoculum were cultivated in Hungate test tubes in an appropriate cultivation medium (37°C, 18 h). *Clostridium* bacteria were cultured in a chamber for cultivation of anaerobic microorganisms (Whitley MG500, Don Whitley Scientific, Shipley, UK), without pH regulation or stirring.

2.3. Fermentation medium

The composition of the first fermentation medium was (per liter of deionized water): 0.26 g K_2HPO_4; 0.02 g KH_2PO_4; 1.23 g $(NH_4)_2SO_4$; 0.1 g $MgSO_4 \times 7H_2O$; 0.01 g $CaCl_2 \times 2H_2O$; and 0.01 g $FeCl_2 \times 7H_2O$. The fermentation medium was supplemented with crude glycerol (Wratislavia-Bio, Wroclaw, Poland) at a concentration of 60.0–100.0 ± 1.0 g/L. The crude glycerol composition was (w/w) 85.6% glycerol, 6% NaCl, 8.0% water, and pH 6.5. The media were autoclaved (121°C, 20 min.).

2.4. Fermentation experiments

The batch and repeated batch experiments were performed in the 6.6 L reactor (Sartorius Stedim, Germany). The bioreactor was equipped with controls for temperature, pH, agitation speed and aeration rate. The pH was controlled at 7.0 by automatic addition of 1 M NaOH and all fermentation experiments were carried out at 37°C. Repeated batch fermentation was initiated as batch. When the substrate concentration inside the bioreactor decreased to less than 14 g/L, 30% of the culture broth was removed from the bioreactor and replaced with an equal volume of fresh sterile nutrient medium with the same composition as that used for the start-up of batch bioreactor cultivation. This first cycle of fermentation broth removal and subsequent replacement with fresh medium continued until the residual glycerol concentration inside the bioreactor fell again below 14 g/L, at which time another repeated batch cycle was started. The portion of the culture broth that was obtained from the bioreactor before the first repeated batch cycle was used as inoculum for batch fermentations where the initial concentrations of crude glycerol were 80 g/L and 100 g/L.

2.5. Analytical methods

The 1,3-PD, glycerol, and organic acids were assayed by high-performance liquid chromatography. Samples for chemical analysis were first centrifuged at 10,000 × g for 10 min at 4°C (Multifuge 3SR, Germany), filtered through a 0.22 μm membrane filter (Millex-GS, Millipore, USA), and then analyzed on an HPLC system (Agilent Technologies 1200 series An Agilent Technolgies 1200 series system, equipped with a refractive index detector, USA). Analyses were performed isocratically at a flow rate of 0.6 mL/min on an Aminex HPX-87H 300 × 7.8 column (Bio-Rad, CA, USA) at a constant temperature of 65°C. H_2SO_4 (0.5 mN) was the mobile phase. External standards were applied for identification and quantification of peak areas. Retention times (Rt) determined for the target compound were 17.17 min (1,3-PD), 13.03 min (glycerol), 20.57 min (butyric acid), 14.4 min (acetic acid), and 11.19 min (lactic acid). The cell concentration (g/L) was determined using a linear equation derived from the relationship of cell dry weight (90°C until constant weight) and the optical density (OD) at 600 nm (Analytik Jena Specord 50, Germany).

3. Results and discussion

Fig. 1 shows the batch kinetics of 1,3-PD synthesis from crude glycerol by *C. butyricum* DSP1. The greatest accumulation of biomass was observed for the first 15 h following inoculation, leading to a plateau of 1.0 g/L at the 17 h of the process whereas the synthesis of metabolites, including 1,3-PD, began at approx. 11 h. A complete utilization of the substrate (crude glycerol) occurred at 40 h and corresponded to a 1,3-PD concentration of 31.8 g/L. The volumetric productivity equaled 0.79 g/L/h. The kinetics of changes in the concentrations of the substrate, metabolites and biomass appeared to be typical of *C. butyricum* DSP1.

Fig. 2 presents the time course of *C. butyricum* DSP1 cultivation in the repeated batch mode, featuring three cycles of fermentation broth withdrawal and supplementation with fresh medium when the residual glycerol concentration diminished below 14–16 g/L. The first medium replacement was followed by an increased rate of microbial metabolism, particularly manifested by a reduction in the time needed for a decline of the initial substrate concentration to the desired residual value. That was accompanied by a 1.8-fold enhancement of productivity, from 0.61 g/L/h during the first stage of fermentation that may be classified as a batch operation to 1.12 g/L/h. After the second removal and replacement of the fermentation medium, the time required by the microorganisms to ferment glycerol to a concentration of 15 g/L was further reduced, resulting in a 1,3-PD productivity of 1.68 g/L/h. The 1,3-PD concentration was 45.7 g/L at 55 h. During the third cycle of fermentation medium removal and supplementation, a deceleration in the synthesis of 1,3-PD was noted leading to a longer time needed for the consumption of one-third of the substrate contained in the medium, which occurred at 18 h (Fig. 2). Consequently, the productivity diminished and the accumulation of biomass became less dynamic compared to the first and second cycles. Due to the slowdown in substrate utilization the next cycle was not performed and the substrate was allowed to be completely utilized. The overall concentration of 1,3-PD reached 62 g/L at 81 h. The basic parameters of the repeated batch experiment registered during particular cycles and those received during the batch operation are collected in Table 1. Finally, the concentrations of side metabolites such as butyric, acetic, and lactic acids proved typical of *C. butyricum* DSP1 and comparable to those observed for the batch cultivation, which did not expose the bacteria to environmental stress.

There are many reports on various methods of cultivating the *Clostridium* species for the synthesis of 1,3-PD from crude glycerol [4,12,13,14,15,16,17]. Table 2 shows the values of kinetic parameters
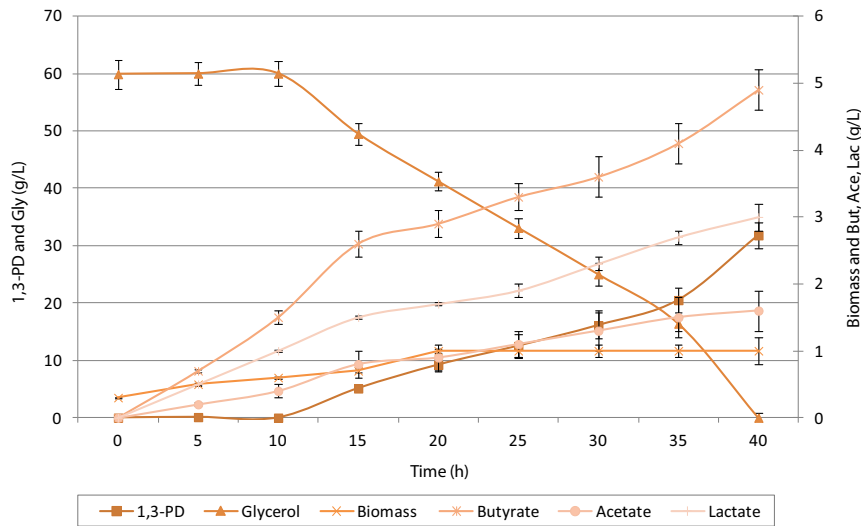
Fig. 1. *C. butyricum* DSP1 growth, glycerol consumption and metabolite (1,3-PD and organic acids) production during batch fermentation with crude glycerol as a carbon source.

obtained by other authors as against the results of the present work. The criterion for that selection was the use of crude glycerol as the exclusive source of carbon for different modes of cultivation.

The data demonstrate that the highest 1,3-PD concentration, 80.1 g/L, was achieved by means of fed batch cultivation [16]. It should be noted that an increasing volume of fermentation medium is a limitation of fed batch cultivation considering the capacity of the bioreactor. Also, a common problem is incomplete substrate fermentation and low productivity. One of the factors limiting the efficiency of microbial metabolism is the accumulation of metabolites in the fermentation medium. For comparison, the greatest 1,3-PD concentrations obtained by using continuous fermentation were 48.1 g/L (single-stage) and 43.5 g/L (two-stage). Although the maximum volumetric productivity was 3.4 g/L/h (two-stage), the post-fermentation sludge contained 9 g/L unfermented substrate [4,17]. Another method applied in biotechnological processes is repeated batch cultivation, which appears to be especially suited for anaerobic operations. Its chief merits include the use of one fermenter, no need to repeat the preparation and sterilization of new growth medium batches, no

limitations related to bioreactor capacity, elimination of osmotic pressure stress, and the possibility of receiving relatively high product concentrations. Despite the method's technological attractiveness, there are only two literature references to 1,3-PD synthesis via a repeated batch protocol: the papers of Kaur et al. [11] and Chatzifragkou et al. [12]. The latest work [12] investigated repeated batch fermentation of crude glycerol using *C. butyricum* VPI 1718, during which rapeseed meal hydrolysate was applied as a synthetic culture medium. In the fourth cycle of the repeated batch experiment the 1,3-PD concentration was 65.5 g/L, at an overall productivity of 1.15 g/L/h. The culture medium proposed by the authors was enzymatically converted into a generic fermentation feedstock, enriched in amino acids, peptides, and various micro-nutrients, using crude enzyme consortia produced via solid state fermentation by a fungal strain of *Aspergillus oryzae*. It was probably the rich composition of the fermentation medium (besides a different strain origin) that accounted for the higher kinetic parameters of repeated batch fermentation. Kaur et al. [11] studied the use of *Clostridium diolis* DSM15410 for the production of 1,3-PD from 98% pure glycerol.
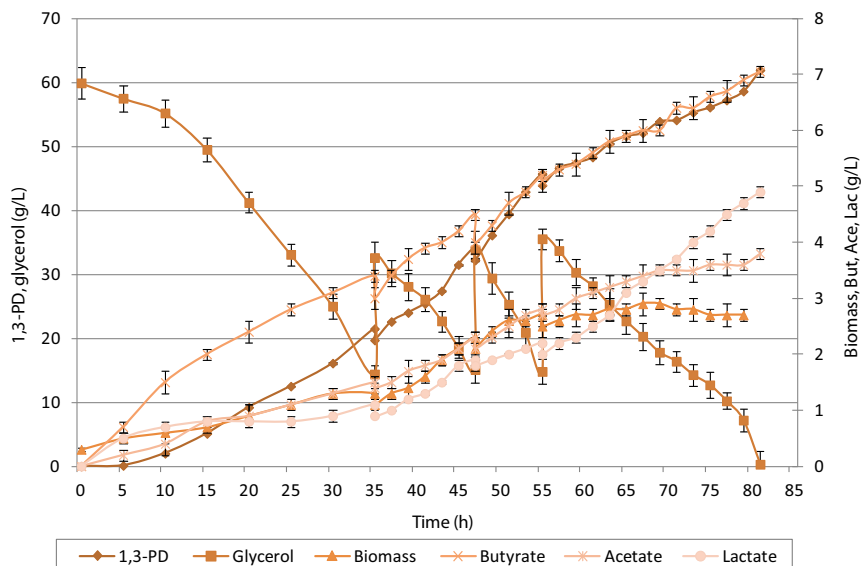

Fig. 2. *C. butyricum* DSP1 growth, glycerol consumption and metabolite (1,3-PD and organic acids) production during repeated batch fermentation with crude glycerol as a carbon source.

Table 1

Repeated batch fermentation parameters of *C butyricum* DSP1 on crude glycerol during different cultivation steps.

Cultivation step	1,3-PD (g/L)[a]	$Q_{1,3-PD}$ (g/L/h)[b]	Biomass (g/L)
After cycle 1 (batch)	21.5	0.61	1.3
Cycle 1	14.5	1.12	1.0
Cycle 2	13.5	1.58	0.6
Cycle 3	11.4	0.63	0.2

[a] 1,3-PD (g/L) — concentration of 1,3-propanediol.
[b] $Q_{1,3-PD}$ (g/L/h) — 1,3-propanediol productivity.

Their strategy involved four cycles of 20% culture broth replacement and resulted in the final 1,3-PD concentration of 67.8 g/L at the end of 65 h. The concentration achieved in the present work, using *C. butyricum* instead of *C. diolis*, was 9% lower. The average volumetric 1,3-PD productivity was 1.04 g/L/h for *C. diolis* and 0.76 g/L/h for *C. butyricum*. The basic difference between the two studies, apart from employing different species, was substrate purity. As shown in Fig. 2, the accumulation of biomass during cycles 2 and 3 was slower compared to cycle 1, in contrast with Kaur's [11] observation that a rise in the biomass paralleled an increase in the product. In the case of *C. butyricum*, the most rapid biomass accumulation occurred in the first stage of fermentation (batch) and after the first medium withdrawal and addition, reaching 2.3 g/L at 47 h. The biomass increase during the second and third cycles did not exceed 15%, which was probably caused by toxic factors contained in the crude glycerol introduced with subsequent batches of fresh medium that diminished the metabolic potency and growth ability of the microorganisms. Although crude glycerol is preferred as a substrate because of its lower price compared to pure glycerol, it carries impurities that may be responsible for toxic stress, such as sodium salts, heavy metal ions, soaps, methanol, and free fatty acids [18,19]. There have been reports of using high glycerol concentrations as a selection factor in pursuit of osmotolerant strains [20,21]. Ringel et al. [20] attempted to select strains resistant to high concentrations of pure and crude glycerol as well as 1,3-PD. All the obtained isolates maintained cell growth and 1,3-PD production up to 10% addition of glycerol to the medium, and two of them showed metabolic abilities at a glycerol concentration of 150 g/L. Other experiments indicated the possibility of modifying microorganisms to enable them to persist under adverse conditions. Abbad-Andaloussi et al. [22] examined *C. butyricum* DSM 5431 mutants, procured via chemical mutagenesis aimed at improving their resistance to osmotic stress, which was also a selection criterion. The results demonstrated that single mutations increased the resistance of *C. butyricum* cells to stress linked to high concentrations of glycerol and 1,3-PD. In batch fermentation, the wild-type strain produced more acetic acid than butyric acid, whereas *C. butyricum* DSM 5431 mutant generated the

same amounts of them. The ratio of acetic acid to butyric acid is known to be closely related to the yield of 1,3-PD production [23]. The total amount of the acids was higher in the case of the wild-type strain, which explains the lower yield of 1,3-PD synthesis from glycerol. In fed batch cultures, the mutants utilized 44% more glycerol and produced 50% more 1,3-PD than the wild-type strain. Petidemange et al. [24] isolated a strain termed *C. butyricum* E5 that resisted high concentrations of 1,3-PD and glycerol. While a glycerol concentration of 200 g/L totally inhibited the *C. butyricum* DSM 5431 strain, *C. butyricum* E5 was only 66% inhibited. The ability of *C. butyricum* DSP1 to adapt to high concentrations of crude glycerol is represented by results of the current study presented in Fig. 3b and Fig. 4b. The basis for the experiments was the application of culture broth removed from the bioreactor prior to the start of the first repeated batch cycle as inoculum for two different bioreactors containing fresh cultivation media where glycerol concentrations were 80 g/L and 100 g/L. Despite the high values of osmotic pressure in both systems, biomass accumulation and 1,3-PD synthesis were very dynamic, particularly in comparison with the conventionally inoculated batch fermentations serving as a control (Fig. 3a and Fig. 4a). In the experiments following the modified inoculation protocol the kinetic parameters of the processes were significantly different. In the system with the initial crude glycerol concentration of 80 g/L, the maximum biomass yield was 4.2 g/L, the final 1,3-PD concentration reached 43.2 g/L at 25 h, and the 1,3-PD productivity equaled 1.73 g/L/h. In the 100 g/L crude glycerol setup, the maximum yield of biomass was 3.6 g/L and the final 1,3-PD concentration was 54.2 g/L. Substrate fermentation terminated at 35 h allowing a 1,3-PD productivity of 1.55 g/L/h.

Results similar to those obtained in the present investigation for the control operations were reported by Chatzifragkou et al. [25], who achieved the maximum 1,3-PD concentration of 41.9 g/L at 52 h by employing *C. butyricum* VPI 1718 to convert 80.1 g/L crude glycerol. A procedure whereby a portion of one fermentation medium is used as inoculum for another microbial culture deserves attention especially in the industrial context by offering a cost-cutting solution to biomass accumulation and permitting the application of cascade fermentation strategies. In the present study, the addition of culture broth containing metabolically active biomass with crude glycerol of high osmotic pressure resulted in its rapid consumption. Interestingly, according to some literature data a 20 g/L glycerol concentration is considered optimal for the growth and metabolism of *Clostridium* bacteria [20,26,27,28,29]. Judging from the resistance of *C. butyricum* DSP1 to high osmotic pressures observed as early as the batch stage in this work (60 g/L crude glycerol), it is possible that the microorganisms activated an adaptation mechanism which made them very osmotolerant and so able to efficiently utilize a highly concentrated substrate. The vitality of *C. butyricum* DSP1 under those conditions was also proved by an intense growth of the bacteria

Table 2

1,3-Propanediol production by different *Clostridium* strains and fermentation strategies, cultivated on media composed of crude glycerol.

| Fermentation method	Strain	Crude glycerol purity (% w/w)	$C_{1,3-PD}$ (g/L)	$Y_{1,3-PD}$ ($g_{1,3-PD}/g_{Gly}$)	Q (g/L/h)	References
Batch	*C. butyricum* F2b	65.0	47.1	0.53	1.12	[17]
	C. butyricum CNCM1211	65.0	63.4	0.57	1.85	[14]
	C. butyricum DSP1	85.6	54.2	0.54	1.55	Present study
Fed batch	*C. butyricum* AKR102a	55.0	76.2	0.51	2.30	[37]
	C. butyricum VPI 1718	81.0	67.9	0.55	0.78	[25]
	Clostridium sp. IK124	ND	80.1	0.56	1.80	[16]
	C. butyricum DSP1	85.6	71.0	0.54	0.66	[9]
Continuous one-stage	*C. butyricum* F2b	65.0	48.1	0.55	0.96	[4]
Continuous two-stage	*C. butyricum* F2b	65.0	41–46	0.53	3.4	[4]
Continuous two-stage	*C. butyricum* F2b	65.0	43.5	0.49	1.33	[17]
Continuous	*C. butyricum* VPI 3266	65.0	31.5	0.50	3.15	[15]
Repeated batch	*C. butyricum* VPI 1718	81.0	65.5	0.52	1.15	[12]
Repeated batch	*C. diolis* DSM 15410	98.0	67.8	0.52	1.04	[11]
Repeated batch	*C. butyricum* DSP1	85.6	62.0	0.53	0.76	Present study
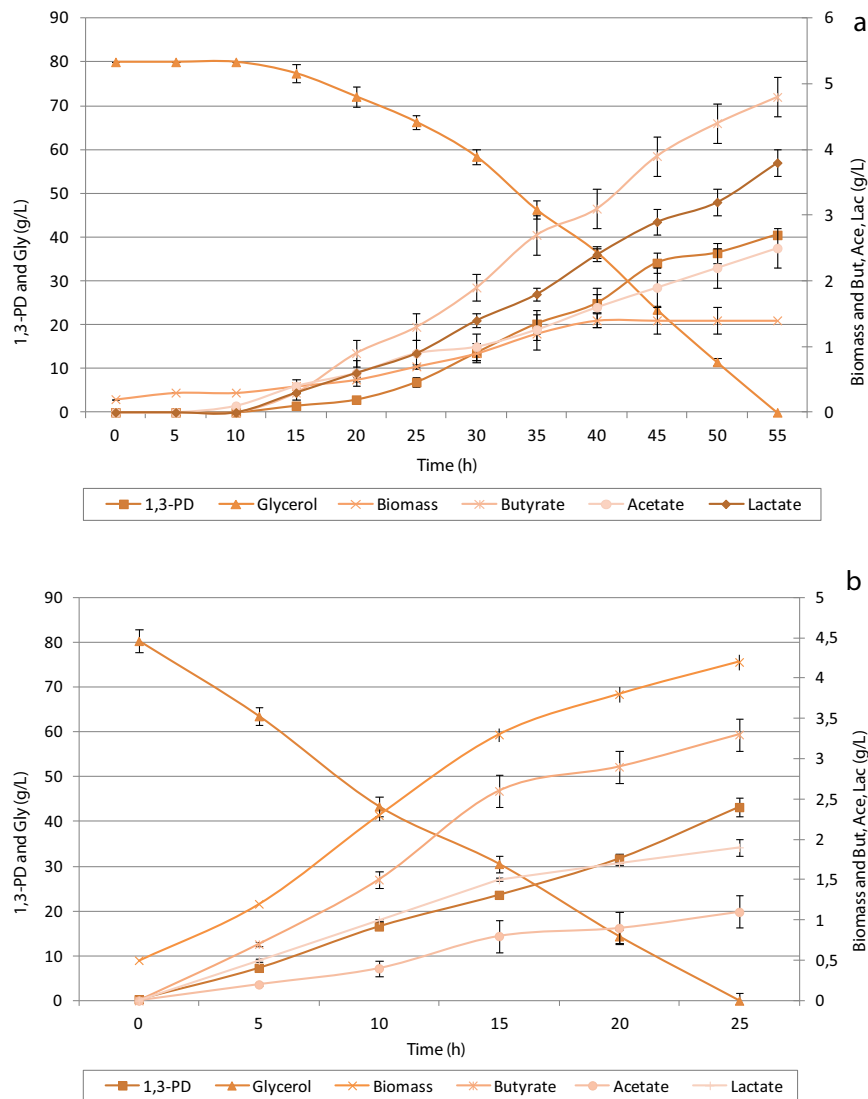
Fig. 3. *Clostridium butyricum* DSP 1 growth, glycerol consumption and metabolite (1,3-propanediol and organic acids) production during batch fermentation at an initial crude glycerol concentration of 80 g/L. Batch fermentation with conventional inoculation (control) (a) and modified inoculation (b).

leading to a substantial yield of biomass, an important factor accelerating biotechnological processes [30]. Another reason for notably good levels of fermentation parameters in an environment with a high concentration of crude glycerol was probably the fact that glycerol remained an exclusive source of carbon for the bacteria from inoculation to the end of the whole process. Nevertheless, crude glycerol conversion may still be inhibited due to such factors as a high concentration of the substrate (pure or crude glycerol), the accumulation of toxic products such as 1,3-PD, and the presence of organic acids. The harmful action of weak acids results not only from an increased concentration of hydrogen ions around cells, but also from the penetration of these acids inside the cell and their intracellular dissociation [31,32]. The so-released hydrogen ions have to be promptly removed from the cell by proton pumps, which requires large amounts of energy [31]. Biebl [33] noted that 19 g/L of butyric acid and 27 g/L of acetic acid inhibited the production of 1,3-PD by *C. butyricum*. Whereas the concentrations of organic acids formed during fermentation processes analyzed in the present study were not as high as those reported by Biebl (Fig. 3 and Fig. 4), the concentrations of lactic acid in the conventionally inoculated operations (from culture medium to fermentation medium) were greater (Fig. 3 and Fig. 4) despite a similar final 1,3-PD concentration (Fig. 3a and Fig. 3b). The difference was twofold in the experiment

with an initial crude glycerol concentration of 80 g/L (Fig. 3a and Fig. 3b). Moreover, an approximately threefold increase in the ratio of lactic acid to butyric acid occurred in the operation with an initial crude glycerol concentration of 100 g/L (Fig. 4a and Fig. 4b). A change in the metabolic pathway of *Clostridium* bacteria has been reported in the literature. Depending on the pH of a synthetic medium, a shift from acid to solvent formation was observed [34]. Although during 1,3-PD synthesis from glycerol by *C. butyricum* butyric, acetic and lactic acids as well as ethanol are produced, the main byproducts of a proper conversion of glycerol to 1,3-PD are butyrate and acetate. An increased content of lactic acid indicates that the process is blocked probably due to substrate excess, a high concentration of toxic carbon monoxide or stoppage at the stage of pyruvate generation. Chatzifragkou et al. [10] found an increase in the activity of lactate dehydrogenase in a 1 L bioreactor at a high substrate concentration in the absence of continuous N_2 sparging. The effect was more pronounced in large-scale fermentations than in small-scale processes and depended on the vessel geometry. Some studies have shown that nitrogen sparging throughout fermentation has a positive effect on the process carried out with *C. butyricum* as it influences bacteria metabolism because of the expulsion of dissolved CO_2 [35]. In the experiments of Chatzifragkou et al. [10] continuous sparging with N_2 allowed for an increased 1,3-PD yield and biomass formation that
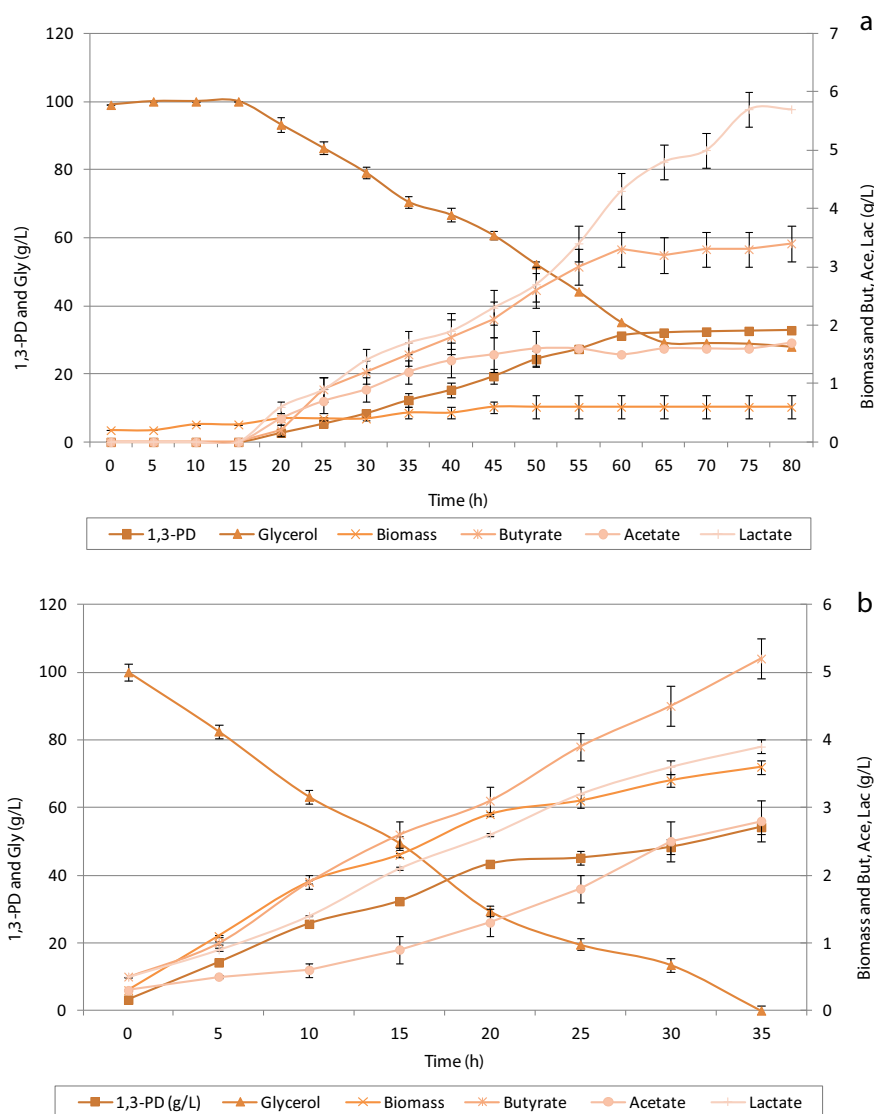
Fig. 4. *Clostridium butyricum* DSP 1 growth, glycerol consumption and metabolite (1,3-propanediol and organic acids) production during batch fermentation at an initial crude glycerol concentration of 100 g/L. Batch fermentation with conventional inoculation (control) (a) and with modified inoculation (b).

correlated with a decreased production of lactic acid. Metsoviti et al. [36] observed a totally different effect. Continuous sparging of the fermentation medium with nitrogen during fermentation induced by *K. oxytoca* produced a shift in the metabolism of glycerol towards ethanol whereas non-sparging favored 1,3-PD synthesis. A change in the quantity profile of organic acids may also point to the activation of adaptation mechanisms in the cells of *C. butyricum* DSP1.

4. Concluding remarks

Culturing method selection, inoculum preparation and growth as well as cultivation medium composition, including carbon source provision, are issues of great importance for biotechnological processes. This investigation demonstrated the possibility of using a repeated batch protocol for the microbiological conversion of crude glycerol to 1,3-PD. The results indicated that the method allows obtaining high concentrations of 1,3-PD from crude glycerol in a relatively short time. The experiments proved that by using a portion of metabolically active biomass as inoculum for another fermentation formula it is possible to eliminate the stage of inoculum growth and thereby reduce the length of the whole operation. Additionally, that strategy avoids the phase of microbial adaptation to a different source

of carbon such as crude glycerol, which is more difficult to utilize, thus improving the kinetic parameters of 1,3-PD production.

Conflict of interest

None.

Author contribution

Proposed the theoretical frame: DSP; Conceived and designed the experiments: DSP; Wrote the paper: DSP; Performed the experiments: DSP; Analyzed the data: DSP.

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